Published in final edited form as:

J Basic Clin Physiol Pharmacol. 2012 ; 23(4): 139–146. doi:10.1515/jbcpp-2012-0021.

Combinatorial cytotoxic effects of *Curcuma longa* and *Zingiber officinale* on the PC-3M prostate cancer cell line

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Abstract

Background—Many plant-derived products exhibit potent chemopreventive activity against animal tumor models as well as rodent and human cancer cell lines. They have low side effects and toxicity and presumably modulate the factors that are critical for cell proliferation, differentiation, senescence and apoptosis. The present study investigates the effects of some medicinal plant extracts from generally recognized as safe plants that may be useful in the prevention and treatment of cancer.

Methods—Clonogenic assays using logarithmically-growing cells were performed to test the effect. The cytotoxic effects of *Curcuma longa* and *Zingiber officinale* were studied using sulforhodamine B assay, tetrazolium dye assay, colony morphology and microscopic analysis.

Results—Out of the 13 lyophilized plant-derived extracts evaluated for growth-inhibitory effects on the PC-3M prostate cancer cell line, two extracts derived from *C. longa* and *Z. officinale* showed significant inhibitory effects on colony-forming ability. The individual and augmentative effects of these two extracts were tested for their narrow range effective lower concentration on PC-3M in clonogenic assays. At relatively lower concentrations, *C. longa* showed significant inhibition of colony formation in clonogenic assays; whereas at same concentrations *Z. officinale* showed only moderate inhibitory effects. However, when both the agents were tested together at

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

Employment or leadership: None declared.

Honorarium: None declared.

the same concentrations, the combined effects were much more significant than their individual ones. On normal prostate epithelial cells both *C. longa* and *Z. officinale* had similar effects but at a lower magnitude. These observations were confirmed by several cytotoxicity assays involving the morphological appearance of the colonies, microscopic observations, per cent inhibition in comparison to control by sulforhodamine B and tetrazolium dye assay.

Conclusions—From these observations, it was concluded that the combined effects of *C. longa* and *Z. officinale* are much greater than their individual effects, suggesting the role of multiple components and their synergistic mode of actions to elicit stronger beneficial effects.

Keywords

chemoprevention; clonogenic assays; *Curcuma longa*; PC-3M; sulforhodamine B/tetrazolium dye (SRB/ MTT) assays; *Zingiber officinale*

Introduction

Chemoprevention can play an integral role in the overall strategy towards reducing the incidence of cancer and has been successfully achieved in numerous in vitro and in vivo studies, as well as being validated in several human intervention trials (1 – 3). Many of the phytochemicals present in plants have been identified as potential chemopreventive agents, and consuming relatively large amounts of vegetables and fruits can prevent the development of cancer (4 – 9). An inverse relationship has been suggested between the consumption of fruits and vegetables and the incidence of cancer (10 – 13). Black raspberries, an abundant source of flavonoids, vitamins, minerals and phytosterols, have been shown to be a potent chemopreventive agent against esophageal cancer (14, 15). Many plant-derived products exhibit potent chemopreventive activity against animal tumor models as well as rodent and human cancer cell lines (16 – 22). These chemopreventive agents have low side effects and toxicity and are involved in scavenging, blocking or suppressing activity, thereby impeding the initiation, promotion and progression stages of cancer development. They are implicated in the neutralization of carcinogenic agents and their effects on cells, presumably by modulating the factors that are critical for cell proliferation, differentiation, senescence and apoptosis. Further, they also possess potent anti-invasive and anti-metastatic activities (23).

Prostate cancer is the second leading cause of cancer death in American men. The American Cancer Society estimates that 28,170 men in the USA will die of prostate cancer in 2012 and that about one man in 36 will die of prostate cancer (24). Accordingly, we have attempted to test some medicinal plant extracts from Generally Recognized as Safe (GRAS) plants that may be useful in the prevention and treatment of cancer using a highly metastatic (PC-3M) prostate cancer cell line as model system (see Figure 1). We recently reported that *Curcuma longa* extract not only inhibited the colony-forming ability of PC-3M cells but also up-regulated cell cycle genes *P57^{KIP2}* and *Rad9* and reduced the migration and invasive ability of prostate cancer cells (25). Using proteomic profiling, we further reported that this extract induced 29 differentially-expressed proteins, among which 13 were down-regulated and 16 up-regulated (26). The ultimate goal of the present work will be to extrapolate the experimental findings in the development of anticancer strategies using plant products for

therapeutic intervention against prostate cancer that will provide the basis for subsequent clinical trials using prostate cancer patients.

Materials and methods

Cell culture

The effects of plant extracts were studied on the PC-3M cell line and normal prostate epithelial cells (RWPE-1), both obtained from American Type Culture Collection. The PC-3M cells were grown in 25 cm² flasks/60×15 mm tissue culture dishes containing RPMI 1640 (GIBCO/Invitrogen, NY, USA) with 10 % fetal bovine serum (GIBCO/Invitrogen, NY, USA) and antibiotics. The prostate epithelial cells were grown in 25 cm² flasks/60 × 15 mm tissue culture dishes containing keratinocyte serum free medium (GIBCO/Invitrogen, NY, USA) supplemented with bovine pituitary extract (0.05 mg/mL) and epidermal growth factor (5 ng/mL). The cells were maintained in a humidified, 95 % air and 5 % CO₂ atmosphere incubator at 37 ° C.

Plant extracts

We have evaluated the extracts of 13 plant-derived products for growth inhibitory effects. The products studied were derived from *Zingiber officinale* (ginger in English and adrak in Hindi), *Curcuma longa* (turmeric in English and haldi in Hindi), *Tinospora cordifolia* (heart-leaved moonseed in English and giloe or gurcha in Hindi), *Piper longum* (long pepper in English and pipili in Hindi), *Piper betle* (betel in English and paan in Hindi), *Murraya koenigii* (curry tree in English and karipatta in Hindi), *Trigonella foenum* (fenugreek in English and methi in Hindi) *Trachyspermum copticum* (bishop ' s weed in English and ajwain in Hindi), *Nigella sativa* (nutmeg flower in English and kalonji or mangrail in Hindi), *Linum usitatissimum* (common flax or linseed in English and tisi in Hindi) (four different fractions).

The extracts were prepared at Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Mumbai, India. In brief, the dried and powdered plant materials (100 g) were extracted with isopropyl alcohol:acetone:water:chloroform: methanol in a ratio of 4:4:6:3:3 for 48 h at room temperature in the dark. The extracts were filtered and the residues left were re-extracted with the same solvents. The filtrates were combined and concentrated under reduced pressure at low temperature (40 – 45 ° C) in a rotary vacuum evaporator. The yield (Y) of the product extracted and the known active principles (AP) are given in brackets:

- *Z. officinale* (Y: 10 %: AP: gingerols and shaogols).
- *C. longa* (Y: 10 %: AP: curcuminoids).
- *T. cordifolia* (Y: 10 %: AP: tinosporoid).
- *P. longum* (Y: 10%: AP: piperine and piperlongumin).
- *P. betle* (Y: 3.47 %: AP: steroids, cardiac glycosides, flavonoids, tannins and phenolic compounds).
- *M. koenigii* (Y: 8.2%: AP: carbazole alkaloids).

- *T. foenum* (Y: 16.2 %: AP: diosgenin).
- *T. copticum* (Y: 15.5 %: AP: thymol).
- *N. sativa* (Y: 4%: AP: nigellone alkaloid).
- *L. usitatissimum* (four different fractions; Y: 4 % – 10 %: AP: carbohydrates, proteins and phenolic compounds).

The dried extracts were stored at 4 ° C until further analysis.

Clonogenic assays

Clonogenic assays using logarithmically-growing cells were performed as described earlier (20, 21, 27 – 29). In brief, approximately 1000 cells obtained from sub-confluent culture flasks were seeded per 60 mm tissue culture dish in 5 mL of medium (five dishes per point). Twenty-four hours after seeding the cells, test compounds at selected concentrations were added to the medium. Control cultures received solvent in the place of the test chemical. Dimethyl sulfoxide (DMSO) solubilized compounds at a final concentration of 0.4 % in the culture medium volume/volume. Preliminary experiments have shown that this has no effect on cell survival. Dishes were returned to the incubator for up to 7 – 8 days and the surviving cells were allowed to form colonies. When the colonies were discrete and well defined, the dishes were washed with phosphate buffered saline (PBS) solution, fixed with methanol, stained with Giemsa and allowed to dry. The colonies per dish were counted using Computer based Quantity One BIO-RAD version 4.6.3. Windows and Macintosh Software. The assays were repeated two times and depicted for one assay. The results were the same in other assays.

Sulforhodamine B assay

Sulforhodamine B (SRB) assays using logarithmically-growing cells were performed according to Houghton et al. (30) with modifications. In brief, 3×10^5 cells obtained from sub-confluent culture flasks were seeded per 60 mm tissue culture dish in 5 mL of medium (five dishes per point). Twenty-four hours after seeding the cells, test compounds were added to the medium at a concentration of 5 µg/mL. Control cultures received solvent in the place of the test chemical. DMSO solubilized the compounds. Dishes were further incubated for 48 h and were then washed with PBS solution, fixed with icecold trichloroacetic acid (TCA), stained with sulforhodamine and allowed to dry. The optical density of the solubilized bound SRB in Tris Base was determined spectrophotometrically, with the absorbance at 492 nm being measured. The optical density of SRB in each tissue culture dish is directly proportional to the cell number and utilized for calculations using GraphPad PRISM Software (La Jolla, CA, USA). The assay was repeated two times and depicted for one assay. The results were the same in both assays. To determine the morphological appearance of the colonies and for photomicrography, approximately 1000 cells obtained from sub-confluent culture flasks were seeded per 60 mm tissue culture dish in 5 mL of medium (five dishes per point) and the cells surviving after treatment were allowed to form colonies for up to 7 – 8 days. The rest of the procedure was the same as described above.

MTT cell viability assay

The tetrazolium dye or MTT assay was performed as described by Wilson (31) with modifications. In brief, 3×10^5 cells obtained from sub-confluent culture flasks were seeded per 60 mm tissue culture dish in 5 mL of medium (five dishes per point). Twenty-four hours after seeding the cells, test compounds were added to the medium at a concentration of 5 $\mu\text{g/mL}$. Control cultures received solvent in the place of the test chemical. DMSO solubilized the compounds. The dishes were further incubated for 24 h. After this time the dishes were given a media change with 1 mL medium and 100 μL MTT (100 mg MTT/20 mL PBS) was added for each dish and incubated at 37 °C for 3 h. After that, one volume of stop mix solution was added and the dish rocked for about 2 h, centrifuged and the optical density of the solubilized formazan determined spectrophotometrically by measuring the absorbance at 550 nm. The optical density of formazan in each tissue culture dish is directly proportional to the cell viability can be and utilized for calculations. The assays were repeated two times and depicted for one assay. The results were the same in the other assay.

Statistical analysis

The results were expressed as mean $\pm$ standard deviation and the significance was evaluated using the Student's t-test (GraphPad Software, CA, USA). Results giving $p < 0.05$ were considered significant. The final values were expressed as either percent relative plating efficiency or per cent decrease/inhibition compared to solvent controls.

Results

1. *Curcuma longa* and *Z. officinale* extracts showed the most significant inhibitory effects on the growth of prostate cancer cells out of the 13 herbal extracts tested.

From the 13 lyophilized plant extracts evaluated for growth-inhibitory effects, two extracts derived from *C. longa* and *Z. officinale* showed significant inhibitory effects on the colony-forming ability of the highly metastatic PC-3M prostate cancer cell line at 20 and 40 $\mu\text{g/mL}$ concentrations (Figure 2). Clonogenic assays were used to examine the individual and combinatorial effects of the two extracts that showed inhibitory effects PC-3M cells on a narrow range effective lower concentration on PC-3M and normal prostate epithelial cells. Figures 3 and 4 show the results of both the individual and combined effects of these extracts on PC-3M prostate cancer and normal prostate epithelial cells, respectively. At relatively lower concentrations only *C. longa* showed significant inhibition of colony formation in clonogenic assays, especially at 5 $\mu\text{g/mL}$ and higher concentrations tested. The LD_{50} value was 4.6 $\mu\text{g/mL}$. At same concentrations, *Z. officinale* showed only moderate inhibitory effects (Figure 3). When both the agents were added together at the same concentrations, however, the cytotoxic effects were much more enhanced than when they were used individually. The combinatorial study showed an LD_{50} of 2.7 $\mu\text{g/mL}$, which was much lower than *C. longa* alone (Figure 3). These experiments indicate that at 2.7 $\mu\text{g/mL}$ of *C. longa* and *Z. officinale* are much less effective on PC-3M cells individually than when combined, suggesting the role of multiple components and their synergistic modes

of action elicit stronger beneficial effects. On normal prostate epithelial cells, both *C. longa* and *Z. officinale* showed similar effects but their magnitude was less (Figure 4), indicating differential effects on normal and cancer cells.

2. There is morphology-based corroboration of the augmentative cytotoxic effects of *C. longa* and *Z. officinale* on prostate cancer cells.

In order to corroborate these results and see their validity, several cytotoxicity assays were carried out for the individual and combined effects of *C. longa* and *Z. officinale* at a concentration of 5 µg/mL on PC-3M prostate cancer and normal prostate epithelial cells. Figure 5 shows the individual and combined effects of *C. longa* and *Z. officinale* on the morphological appearance of colonies of PC-3M and prostate epithelial cells. Figure 6 gives the microscopic observations and Figure 7 the percent inhibition in comparison to control by SRB assay. In all three assays the results showed more or less the same trend: that at 5 µg/mL *C. longa* showed significant inhibition whereas same concentrations *Z. officinale* showed only moderate inhibitory effects. However, when both the agents were tested together at the same concentrations the effects were much more significant and the magnitude of the effect was less on normal prostate epithelial cells compared to PC-3M cells.

3. *C. longa* and *Z. officinale* have effects on the cell viability of prostate cancer cells.

Table 1 shows the MTT cell viability assay results of the individual and combined effects of *C. longa* and *Z. officinale* on PC-3M and normal prostate epithelial cells. These results clearly corroborate the above observations.

Discussion

Recent research provides evidence that many dietary compounds that are consumed on a daily basis possess cancer-preventive properties. Animal and epidemiological investigations have revealed that regular consumption of fruits and vegetables can reduce the risk of developing certain cancers. These dietary fruits and vegetables have been found to possess several components that can induce multiple cellular effects through complementary and overlapping mechanisms. The principle constituents and micronutrients present in different vegetables and fruits with chemopreventive properties include genistein, resveratrol, diallyl sulfide, S-allyl cysteine, allicin, lycopene, capsaicin, curcumin, 6-gingerol, ellagic acid, ursolic acid, silymarin, anethol, catechins, eugenol, etc., and in many cases either a single or purified agent is used to study chemopreventive activity. Accordingly there is a lacuna in studies utilizing several comprehensive herbal extracts in toto and also to select the most effective agent from them using rodent and human cancer cell line model systems, especially prostate cancer cell lines. By virtue of its diagnosis in the elderly population with a relatively slower rate of growth and progression, prostate cancer is an ideal cancer for chemoprevention. Even a reasonable delay in the development of cancer through nutritional intervention could result in improved overall survival of prostate cancer patients (32 – 35). From this point of view, we selected 13 plant extracts from GRAS in toto that are in common use as dietary supplements or traditional medicine to test their growth - inhibitory effects on prostate cancer cell lines.

Herbal medicine utilizing GRAS plants remains one of the common forms of therapy available for much of the world's population. According to the World Health Organization, about three-quarters of the world's population currently uses herbs and other forms of traditional medicine to treat diseases. Traditional medicine is widely used in India and China and other places. Even in the USA, the use of plants and phytomedicine has increased dramatically in the past two decades. A National Centre for Complementary and Alternative Medicine has been established. The herbal products have been classified under 'dietary supplements' and are included with vitamins, minerals, amino acids and 'other products intended to supplement the diet'. However, the scientific basis for the bioactivity and the underlying molecular mechanisms for the activities of most of these products are at present either unknown or incomplete. The doses selected in this study were well standardized, but for human consumption, tolerance dose levels should be taken in to consideration.

Recently, we reported that *C. longa* extract showed not only inhibition of the colony-forming ability of PC-3M cells but also up-regulated the cell cycle genes *P57^{KIP2}* and *Rad9* and reduced the migration and invasive ability of prostate cancer cells (25). Using proteomic profiling, we have further reported that this extract induced 29 differentially-expressed proteins, among which 13 were down-regulated and 16 up-regulated (26). In the present study, first screening showed that two plant extracts, namely *C. longa* and *Z. officinale*, have potential activity against PC-3M cell line. Second screening for selection at lower concentrations showed that the *C. longa* extract is more potent than that from *Z. officinale*. However, the combined effects of both these extracts are much stronger than their individual effects. It is reasonable to expect that appropriate combinations of chemopreventive agents might provide greater efficacy than the administration of individual agents. For example, a combination of curcumin and phenethyl isothiocyanate at lower doses inhibited the growth of PC-3M prostate tumor xenografts more effectively than the administration of phenethyl isothiocyanate or curcumin alone at a higher dose (36). Similarly, a combination of piroxicam and difluoromethylornithine produced synergistic inhibitory effects on colon cancer development in male F344 rats (37). Likewise, a combination TPA and all- *trans*-retinoic acid showed synergistic inhibitory effects on the growth of LNCaP cells in athymic nude mice (38) and a combination of docosahexaenoic acid and 1,4-phenylenebis(methylene) selenocyanate suppressed colon cancer cell growth and induced apoptosis more effectively than high doses of individual agents (39). Despite some of these examples, by and large the utility of combined herbal medications and cytotoxic chemotherapeutics has been largely unexplored. It is unlikely that chemoprevention can be achieved by a single agent. Accordingly, there is need to develop a mixed cocktail approach with herbal ingredients that act in a concerted way and produce multiple cellular effects with further enhancement of the efficacy in a positive manner for the effective management of cancer. Studies are in progress from this point of view.

Acknowledgments

This study was supported by grants from the National Institutes Health: 1R01MH085259, 1R37DA025576, 5R01DA021537, and 1R01DA027049 to Prof. Madhavan Nair. The authors would like to thank Prof. Lidia Kos and Mr. Mario Perez, Biological Sciences, Florida International University, Miami, USA for photomicrographs.

Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Research funding: None declared.

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Figure 1.

The representative pictures of the respective herb/part/seed/stem from which the extract was made.

1. *Piper betle*; 2. *Murraya koenigii*; 3. *Trigonella foenum*; 4. *Zingiber officinale*; 5. *Curcuma longa*; 6. *Tinospora cordifolia*; 7. *Piper longum*; 8. *Trachyspermum copticum*; 9. *Nigella sativa*; 10. *Linum usitatissimum* (four different fractions).

Figure 2.

Effect of plant extracts on the highly metastatic PC-3M prostate cancer cell line.

1. Control; 2. *Zingiber officinale*; 3. *Curcuma longa*; 4. *Tinospora cordifolia*; 5. *Piper longum*; 6. *Piper betle*; 7. *Murraya koenigii*; 8. *Trigonella foenum*; 9. *Trachyspermum copticum*; 10. *Nigella sativa*; 11 – 14. *Linum usitatissimum* (four different fractions).

B

Inhibition of colony formation						
Treatment, $\mu\text{g/mL}$	<i>Curcuma longa</i>		<i>Zingiber officinale</i>		<i>Curcuma longa</i> + <i>Zingiber officinale</i>	
	APE $\pm$ SD	% Inhibition	APE $\pm$ SD	% Inhibition	APE $\pm$ SD	% Inhibition
DMSO	631.0 $\pm$ 22.19	-	631.0 $\pm$ 22.19	-	631.0 $\pm$ 22.19	-
0.625	593.0 $\pm$ 26.88	6	627.0 $\pm$ 26.6	1	589.4 $\pm$ 20.42	7
1.25	558.6 $\pm$ 32.80*	11	620.8 $\pm$ 11.95	2	545.8 $\pm$ 15.30***	14
2.5	487.0 $\pm$ 37.37***	23	611.4 $\pm$ 8.36	3	343.0 $\pm$ 24.87***	46
5.0	298.8 $\pm$ 44.24***	53	567.6 $\pm$ 22.15*	10	99.4 $\pm$ 9.39***	84
7.5	148.0 $\pm$ 14.0***	77	538.4 $\pm$ 29.48**	15	23.2 $\pm$ 2.04***	96
10	141.6 $\pm$ 6.62***	78	506.0 $\pm$ 8.25***	20	9.4 $\pm$ 1.02***	99

APE, absolute plating efficiency.

Figure 3.

Individual and combined effects of *Curcuma longa* and *Zingiber officinale* on PC-3M prostate cancer cells. A. Inhibition of colony formation by *C. longa* and *Z. officinale* both individually and in combination on highly metastatic PC-3M prostate cancer cell line showing the LD₅₀ values. B. Values for absolute plating efficiency and percent inhibition are shown in the table. *p 0.01, **p 0.001 and ***p 0.0001 when compared with DMSO control.

B

Inhibition of colony formation						
Treatment, $\mu\text{g/mL}$	<i>Curcuma longa</i>		<i>Zingiber officinale</i>		<i>C. longa</i> + <i>Z. officinale</i>	
	APE $\pm$ SD	% Inhibition	APE $\pm$ SD	% Inhibition	APE $\pm$ SD	% Inhibition
DMSO	656.20 $\pm$ 52.3	-	656.20 $\pm$ 52.3	-	656.20 $\pm$ 52.3	-
0.625	635 $\pm$ 46.37	3	652.2 $\pm$ 47.75	1	616 $\pm$ 8	6
1.25	625 $\pm$ 29.67	5	635.8 $\pm$ 15.75	3	591 $\pm$ 48.13	10
2.5	593.2 $\pm$ 44.45	10	594.6 $\pm$ 35.88	9	463.2 $\pm$ 72.38*	29
5.0	540.2 $\pm$ 41.03*	18	588.4 $\pm$ 20.16	10	293.2 $\pm$ 50.65***	55
7.5	296.0 $\pm$ 7.24***	55	569.2 $\pm$ 32.92	13	82.8 $\pm$ 3.06***	87
10	191.6 $\pm$ 37.79***	71	536 $\pm$ 27.88*	18	58.8 $\pm$ 6.79***	99

APE, absolute plating efficiency.

Figure 4.

Individual and combined effects of *Curcuma longa* and *Zingiber officinale* on normal prostate epithelial cells. A. Inhibition of colony formation by *C. longa* and *Z. officinale* both individually and in combination on normal prostate epithelial cells showing the LD₅₀ values. B. Values for absolute plating efficiency and percent inhibition are shown in the table. *p 0.01 and ***p 0.0001 when compared with DMSO control.

Figure 5.

Individual and combined effects of *Curcuma longa* and *Zingiber officinale* on the morphological appearance of the PC-3M and normal prostate epithelial cell colonies. 1. PC-3M cells; 2. normal prostate epithelial cells.

A. DMSO Control; B. *C. longa*- treated; C. *Z. officinale*- treated; D. *C. longa* + *Z. officinale*- treated.

Figure 6.

Individual and combined effects of *Curcuma longa* and *Zingiber officinale* on microscopic observations of PC-3M and normal prostate epithelial cells ($\times 40$). 1. PC-3M cells; 2. normal prostate epithelial cells. A) DMSO control; (B) *C. longa*-treated; (C) *Z. officinale*-treated; (D) *C. longa* + *Z. officinale*-treated.

Figure 7.

Individual and combined effects of *Curcuma longa* and *Zingiber officinale* on SRB activity of PC-3M and normal prostate epithelial cells.

(A) PC-3M cells; (B) normal prostate epithelial cells. C.l: *C. longa*-treated; Z.o: *Z. officinale*-treated; C.l + Z.o: *C. longa* + *Z. officinale*-treated. The values represent the mean and standard deviation of five independent values. *p 0.0001; **p 0.004; ***p 0.155 when compared with DMSO control.

Table 1

MTT assay showing the inhibition of cell viability by *Curcuma longa* and *Zingiber officinale* both individually and in combination on PC-3M and normal prostate epithelial cells.

Treatment	PC-3M, OD at 550 nm	PC-3M, % inhibition	Prostate epithelial cells, OD at 550 nm	Prostate epithelial cells, % inhibition
MTT cell viability assay				
DMSO	0.662±0.04	–	0.584±0.007	–
<i>C. longa</i>	0.328±0.023 *	49.55	0.471±0.034 *	19.35
<i>Z. officinale</i>	0.551±0.028 **	16.76	0.516±0.012 *	11.6
<i>C. longa</i> + <i>Z. officinale</i>	0.114±0.013 *	82.77	0.282±0.014 *	51.71

The values represent the mean and standard deviation of five independent values.

*
p 0.0001;

**
p 0.0009.

OD, optical density.