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Research Article

Free Zn^{2+} enhances inhibitory effects of EGCG on the growth of PC-3 cells

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Epigallocatechin-3-gallate (EGCG), a major component of green tea, has both preventive and therapeutic beneficial actions in prostate cancer. In the present study, we compared the growth inhibitory effects and the antioxidant and ability to modify cell membrane permeation of zinc-EGCG complex and Zn^{2+}/EGCG mixture on androgen-insensitive prostate cancer (PC-3) cells. It was noted that free Zn^{2+} enhanced the growth inhibitory effects of EGCG on PC-3 cells at 160 μmol/L concentration, whereas zinc-EGCG complex was ineffective. EGCG showed potent free radical scavenging ability in the presence of Zn^{2+}. EGCG in the presence of Zn^{2+} was more effective than EGCG alone in enhancing the permeability of the cell membrane, whereas zinc-EGCG complex had no effect on PC-3 cell membrane permeability. These results indicate that though Zn^{2+} enhanced the action of EGCG on PC-3 cells, zinc-EGCG complex is highly unlikely to be formed in the presence of Zn^{2+} and EGCG to explain the potentiating action of Zn^{2+} on the growth inhibitory property of EGCG on PC-3 cells.

Keywords: Complex / Epigallocatechin gallate / PC-3 cells / Prostate cancer / Zn^{2+}

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1 Introduction

Epigallocatechin gallate (EGCG), the most abundant catechin in green tea, showed anti-inflammatory and anti-cancer activity both *in vitro* and *in vivo*, suggesting that it is the potential functional component responsible in the prevention of prostate cancer [1 – 3]. Previous studies showed that EGCG suppresses the growth of prostate cancer cells by apoptosis [4 – 7]. EGCG decreases the activity of 5a-reductases, an important enzyme involved in the proliferation and development of prostate cancer cells [8]. Zinc is an essential trace element and plays an important role in the prevention of initiation and development of prostate cancer [9]. Hence, it is likely that an imbalance in the metabolism of Zn^{2+} could play an important role in the pathogenesis of prostate cancer. EGCG modulates absorption and metabolism of Zn^{2+}, and thus, a better understanding of the interaction between EGCG and Zn^{2+} might lead to measures to prevent and treat prostate cancer.

EGCG chelates metal ions, especially transitional metal ions, an important factor that affects the bioactivity of EGCG. Recent studies showed that EGCG-metal ion complex have a role in the initiation and development of cancer [10 – 13]. Kagaya *et al*. [14] suggested that the biological behavior of EGCG against human cancer cells is as a result of the formation of complexes of EGCG with metal ions. But, it is not known how exactly zinc-EGCG complex influences the growth of prostate cancer cells.

EGCG has two rings known as B and D that have the same structure that could potentially participate in the formation of complexes with metal ions such as the OH groups on the D-ring that can occupy the first coordination sphere around the metal ions [15]. Esparza *et al*. [16] reported that catechin was able to form a 1:1 complex with either Zn^{2+}or Cu^{2+} that was monitored through the linearization algorithms as competitive with pre-concentration of the metal ions on the mercury electrode. In view of this, in the present study, we prepared zinc-EGCG complex and identified whether OH groups of EGCG have been changed by employing FT-IR (Fourier transformation infra red) and

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Abbreviations: CLI, chemiluminescence intensity; **EGCG**, Epigallocatechin-3-gallate; **FT-IR**, Fourier transformation infra red spectroscopy; **MTT**, 3-[4, 5-dimethylithiazol-2-yl]-2, 5-diphenyl tetrazolium bromide; **PC-3 cells**, androgen-insensitive prostate cancer cells

UV-*vis* spectroscopy methods. We also studied the effects of EGCG, zinc-EGCG complex and Zn^{2+}/EGCG mixture on the growth of PC-3 cells. In addition, the effects of EGCG, zinc-EGCG complex and EGCG in the presence of Zn^{2+} on prostate cancer cells were also investigated for their antioxidant ability and ability to permeate the cytoplasmic membrane.

2 Materials and methods

2.1 Materials

Purified preparation of EGCG was purchased from Sigma (St. Louis, MO, USA). Human androgen-insensitive prostate cancer cells (PC-3) was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. MTT (3-[4, 5-dimethylithiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) was purchased from JINMEI BIOTECH (Shanghai, China). All other chemicals were extra-pure grade or analytical grade.

2.2 Preparation of zinc-EGCG complex and Zn^{2+}/EGCG mixture

Excessive Zn^{2+} was added to the saturated solution of EGCG (pH 4.0). NaCl was added to the saturated solution of EGCG with Zn^{2+} and zinc-EGCG complex was precipitated immediately. The precipitation was harvested by centrifuging at 10 000 rpm for 5 min. Finally, the precipitation was dissolved in double distilled water and the solution was dried by lyophilization (Savant Novalyphe-NL500, USA). Zinc-EGCG complex was identified by FT-IR (Thermo Nicolet AVATAR 370) and UV-*vis* spectroscopy (HP 8453, Hewlett-Packard, USA).

Our previous studies showed that inhibitory effects of EGCG in the presence of Zn^{2+} on the growth of prostate cancer cells depended on their concentrations and added orders, and the best ratio of EGCG with Zn^{2+} was 1:1 [17]. In the present paper, Zn^{2+}/EGCG mixture was prepared by mixing EGCG solution with $ZnSO_4$ solution at equal molar concentration.

2.3 Cell culture

PC-3 cell was cultured in F-12 medium (GIBCO, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin. The cells were cultured in a 5% CO_2 humidified atmosphere at 37°C.

2.4 Cell viability by MTT assay

Three different concentrations (40, 80 and 160 μmol/L) of EGCG, Zn^{2+}, and zinc-EGCG complex were used to investigate the bioactivity of EGCG. EGCG, $ZnSO_4$ (Zn^{2+}) and zinc-EGCG complex were dissolved in double-distilled water prior to use in the present study. The cells were seeded at 1×10^5 cells/well in 96-well plates and incubated for an additional 24 h prior to treatment. After treatment with EGCG, Zn^{2+} and zinc-EGCG complex at various concentrations, the cells were incubated for further 12 h. Twenty microliters of 5 mg/mL MTT was then added to each well from a stock solution and cultured for 4 h. The reaction was stopped by addition of 150 μL of DMSO. The absorbance in each well was then measured with a microplate reader (Thermal Labsystem, Finland) at 490 nm. All experiments were repeated at least three times to know the reproducibility of the results obtained.

2.5 Measurement of antioxidant ability of zinc-EGCG complex and EGCG in the presence of Zn^{2+} by chemiluminescence

$FeSO_4$, H_2O_2 and yeast were dissolved in citric acid buffers (pH 5.6). Zero point two milliliters H_2O_2 (5%), 0.2 mL yeast solution (75 mg/mL), 0.4 mL samples and 0.4 mL $FeSO_4$ (0.8 mmol/L) were mixed according to the order in tube. The chemiluminescence intensity (CLI) was measured by Luminescence Measurement (Lumat LB 9507, Germany). CLI was simultaneously recorded once per 3 s (citric acid buffers replaced the sample in the control). Each experiment was repeated at least four times. The scavenging rate was obtained according to the following formula:

$$\text{Scavenging rate} = [\text{CLI (control)} - \text{CLI (sample)}] \times 100\%/\text{CLI (control)}$$

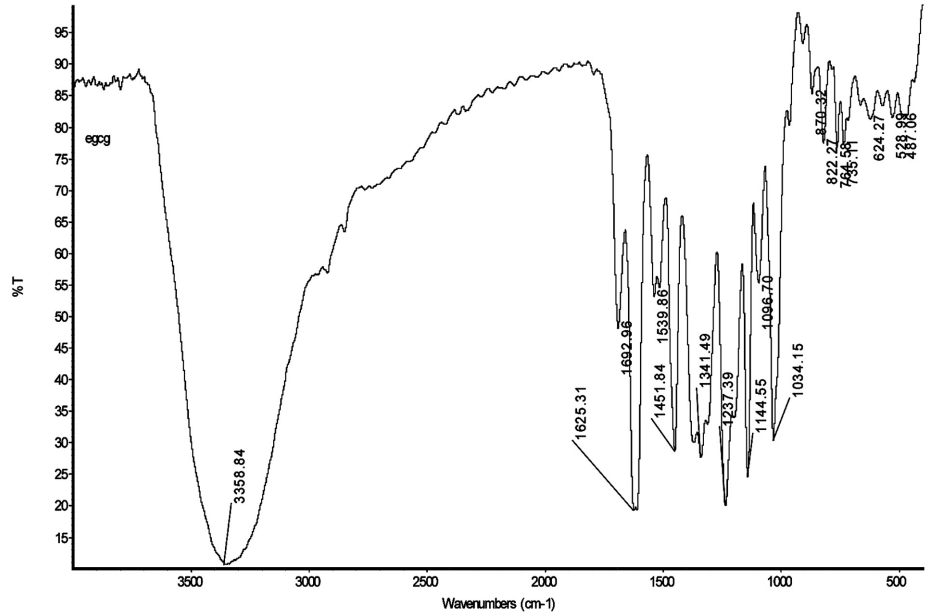
2.6 Determination of EGCG content by HPLC

In order to determine the intracellular EGCG content in PC-3 cells, cells were seeded onto 25 cm^2 plates at a density of 1×10^5 cells/mL for 24 h and treated with a high concentration of EGCG (640 μmol/L), zinc-EGCG complex and Zn^{2+}/EGCG mixture for 1 h. Subsequently, the cells were harvested and washed with phosphate buffers (pH 5.0) twice, dissolved in phosphate buffers (pH 5.0), broken by Ultrasonic Cell Disruption System (JY92- , China) and centrifuged at 10 000 rpm for 15 min. EGCG present in the supernatant was determined by HPLC (LC-2010A, Shimadzu, Japan). The samples were analyzed on an ODS column (4.6 mm × 250 mm). A gradient elution was carried out using the following solvent systems: mobile phase: solvent A, ACN/acetic acid/distilled water (3:0.5:96.5); solvent B, ACN/acetic acid/distilled water (30:0.5:69.5). The linear gradient elution system was: from 100% A to 100% B in 45 min, holding at 100% B for 10 min and returning to 100% A after 5 min. The flow rate was 1.0 mL/min, and effluent was monitored at 280 nm.

3 Results

3.1 Identification of zinc-EGCG complex

FT-IR spectra of EGCG and zinc-EGCG complex shown in Fig. 1 revealed that the stretching vibration absorption peak of associating hydroxyl ($\nu_{O\text{-}H}$) of zinc-EGCG complex had the distinct shift compared with EGCG (From 3358.84 cm^{-1} of EGCG to 3363.35 cm^{-1} and 3480.31 cm^{-1} of zinc-EGCG complex) that supports the contention that zinc-EGCG complex was formed and OH groups of EGCG participated in a reaction with Zn^{2+}. From the UV-*vis* absorption spectra of EGCG, zinc-EGCG complex and Zn^{2+}/EGCG mixtures given in Fig. 2, it is evident that the absorbance of EGCG in the presence of Zn^{2+} was almost the same as that of EGCG at 274 nm. However, zinc-EGCG complex had a lower value than that of EGCG at 272 nm with the formation of a broad shoulder at 320 – 330 nm. These results suggest that Zn^{2+} reacted with EGCG to form a complex but did not change the main configuration of EGCG. Because it is difficult to confirm the configuration of the complex of Zn^{2+} with individual polyphenols based on the past reports [13, 15, 16], we propose that the most possible and theoretical



(a)

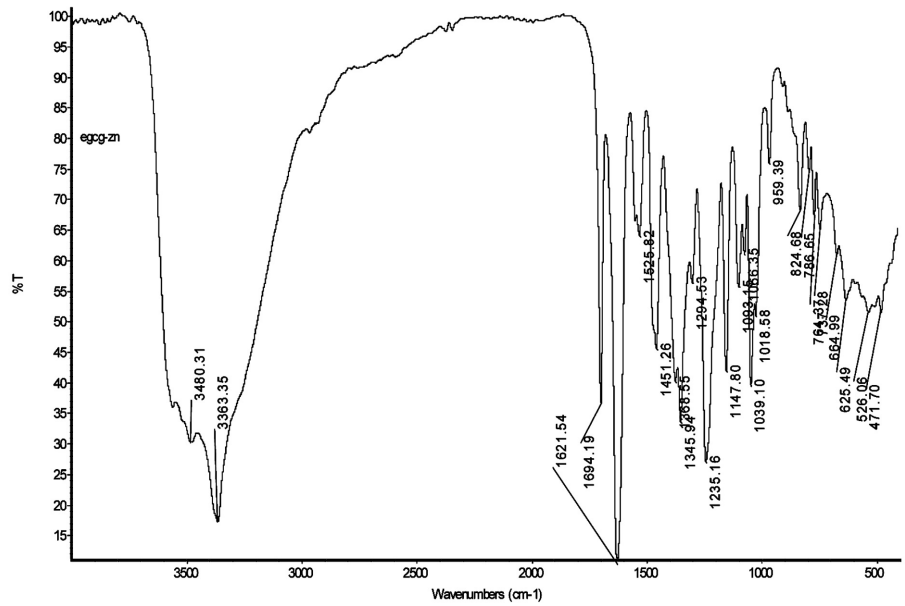


Figure 1. Fourier tranform-ion cyclotron resonance absorption spectra of (a) EGCG alone and (b) zinc-EGCG complex. The stretching vibration absorption peak of associating hydroxyl ($\nu_{O\text{-}H}$) of zinc-EGCG complex had the distinct shift compared with EGCG alone (From 3358.84 $cm^{-1}$of EGCG to 3363.35 cm^{-1} and 3480.31 cm^{-1} of zinc-EGCG complex).

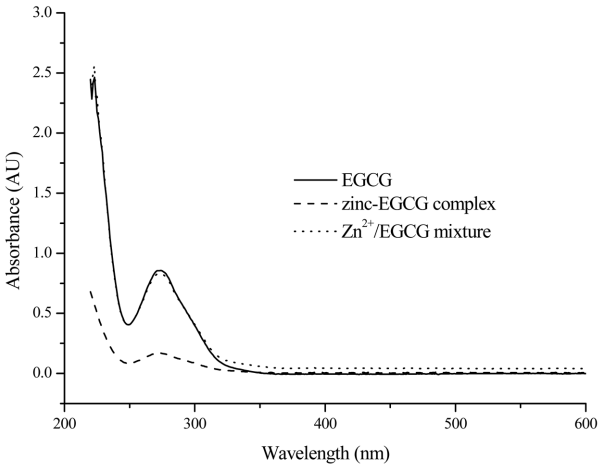


Figure 2. UV-*vis* absorption spectra of EGCG alone, zinc-EGCG complex and Zn^{2+}/EGCG mixture at a uniform concentration of 80 μmol/L.

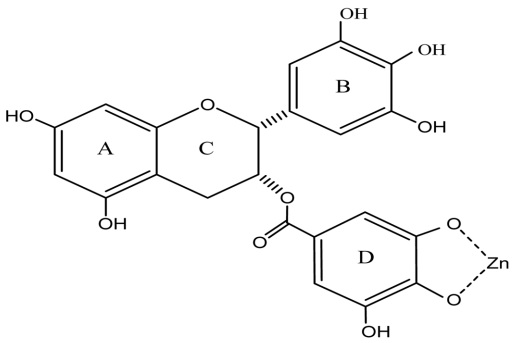


Figure 3. A possible coordination scheme proposed for zinc-EGCG complex. The D-ring OH groups were occupied by Zn^{2+} at molar ratio of 1:1.

coordination mode for EGCG in zinc-EGCG complex could be as shown in Fig. 3.

3.2 Effects of zinc-EGCG complex and Zn^{2+}/EGCG mixture on the viability of PC-3 cells

PC-3 cells were treated with 40, 80, 160 μmol/L of Zn^{2+}, EGCG, zinc-EGCG complex or Zn^{2+}/EGCG mixture for 12 h. MTT assay showed that all samples inhibited the growth of PC-3 cells in a dose-dependent fashion with maximum loss of cell viability at 160 μmol/L (Fig. 4). Zn^{2+}/EGCG mixture, when used at 160 μmol/L, produced significant inhibition of PC-3 cells growth compared with EGCG. Zn^{2+} alone decreased the viability of PC-3 cells to a significant degree compared with Zn^{2+}/EGCG mixture. These results suggest that EGCG protected PC-3 cells from damages induced by Zn^{2+}. Based on these results, it can be inferred that when a solution containing Zn^{2+}/EGCG was employed Zn^{2+} reacts with EGCG to form a complex as a result of which the effects of Zn^{2+} on the cells were decreased.

3.3 Comparison of antioxidant ability of zinc-EGCG complex and Zn^{2+}/EGCG mixture

EGCG is known to have anti-oxidant activity that can be used as an important criterion for evaluating its bioactivity. Furthermore, the ability of EGCG to modulate the redox status of both normal and tumor cells might be an important mechanism by which it prevents cancer. In the present study, it was noted that the free radical scavenging ability of EGCG, zinc-EGCG complex and EGCG in the presence of Zn^{2+} increased with increasing concentrations (see Fig. 5).

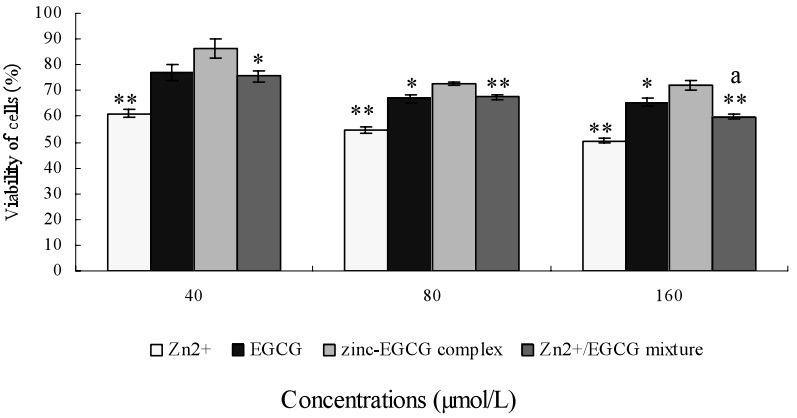


Figure 4. Effects of EGCG, zinc-EGCG complex and Zn^{2+}/EGCG mixture on viability of PC-3 cell at different concentrations. The Zn^{2+}/EGCG mixture was prepared by mixing EGCG with Zn^{2+} at equal molar concentration. The ultimate concentrations of EGCG and Zn^{2+} in the mixture were 40, 80, 160 μmol/L. Cells were seeded on a 96-well plate for 24 h and then incubated with different concentrations of EGCG, zinc-EGCG complex or Zn^{2+}/EGCG mixture for 12 h. Twenty microliters of 5 mg/mL MTT was then added to each well from a stock solution and cultured for 4 h. The reaction was stopped by addition of 150 μL of DMSO. The absorbance in each well was then measured with a microplate reader (Thermal Labsystem, Finland) at 490 nm. The results were means ± SE of three experiments. *$p < 0.05$ compared to zinc-EGCG complex, **$p < 0.01$ compared to zinc-EGCG complex, $^{a}p < 0.05$ compared to EGCG.

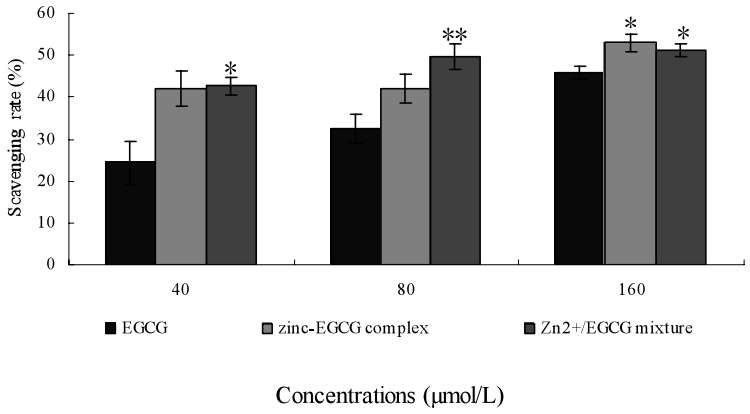


Figure 5. Comparison of antioxidant activities of EGCG alone, zinc-EGCG complex or Zn^{2+}/EGCG mixture at different concentrations by chemiluminescence. The concentrations of EGCG and zinc-EGCG complex were 40, 80, 160 μmol/L. The ultimate concentrations of EGCG and Zn^{2+} in the mixture were 40, 80, 160 μmol/L. The results were means ± SE of four experiments. *$p < 0.05$ compared to EGCG, **$p < 0.01$ compared to EGCG.

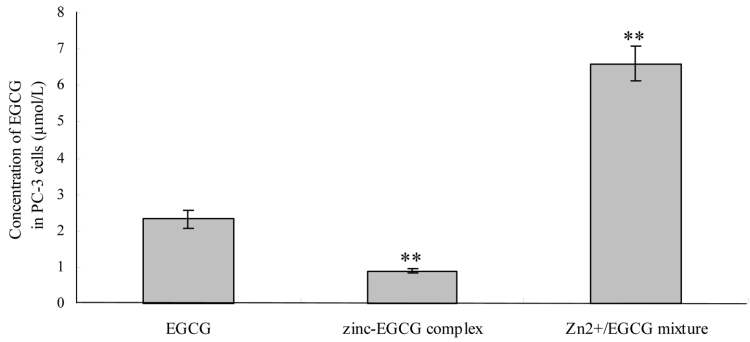


Figure 6. Concentration of EGCG in PC-3 cells treated with high concentration (640 μmol/L) of EGCG, zinc-EGCG complex and Zn^{2+}/EGCG mixture for 1 h. The cells were harvested and washed with phosphate buffers twice, dissolved in phosphate buffers, broken by Ultrasonic Cell Disruption System and centrifuged at 10 000 rpm for 15 min. Finally, EGCG of the supernatant was determined by HPLC. The results were means ± SE of three experiments. *$p < 0.05$ compared to EGCG, **$p < 0.01$ compared to EGCG.

It was noted that the free radical scavenging rate of Zn^{2+}/EGCG mixture and zinc-EGCG complex was higher compared with EGCG alone.

3.4 Influx of zinc-EGCG complex and EGCG in the presence of Zn^{2+} in PC-3 cells

It is not clear whether EGCG need to be inside the cells to exert its bioactivity or could bring about its actions by remaining outside. Experiments performed to verify this possibility, revealed that EGCG could permeate cytoplasmic membrane and exerts its bioactivity inside the cells. Furthermore, concentrations of EGCG inside PC-3 cells were significantly different in different treatments. EGCG content in cells treated with Zn^{2+}/EGCG mixture was the highest whereas with zinc-EGCG complex was the lowest (Fig. 6). These results suggest that permeability of EGCG was hampered when zinc forms a complex with EGCG. On the other hand, EGCG permeated more easily in the presence of Zn^{2+}. These results imply that Zn^{2+} enhanced the influx of EGCG.

4 Discussion

EGCG is one of the major bioactive components in green tea and had been studied intensively as an anti-carcinogenic and anti-angiogenic agent [18, 19]. Its chemoprotective actions have been well documented both *in vitro* and several animal tumor models. EGCG suppressed the growth of cancer cells by inducing apoptosis at physiologically attainable concentrations with little or no action on normal cells [20]. The antioxidant ability and biological behavior of EGCG is influenced by metal ions, especially transition metal ions (*e.g.* iron and copper ion) [16, 21 – 24]. Although, Kagaya *et al.* [14] reported that 0.02 mmol/L EGCG did not have any hepatocyte cytoprotective activity *in vitro*; it (EGCG) protected against bromobenzene-induced cytotoxicity when used at 0.02 mmol/L EGCG and 0.02 mmol/L zinc. These results suggested that formation of zinc-EGCG complex is necessary to bring about the cytoprotective activity of EGCG. Yu *et al*. [25] showed that EGCG and Cd^{2+} significantly inhibited the growth of prostate cancer cells that could be mediated through the suppression of absorption of Zn^{2+} and Cd^{2+}. More particularly, it was reported that both EGCG and Cd^{2+} need to be present together to show the inhibitory action of EGCG suggesting that formation of a complex between EGCG and metal ions is an essential requirement, though the ratio between EGCG and cation in the complex formed or prepared is not always the same. Esparza *et al.* [16] reported that not all metals bind equally strong with EGCG. Zn^{2+} showed a sluggish kinetics but, at the same time, had the highest conditional formation constants. EGCG has two rings, B and D, which have exactly the same local structure and could potentially participate in metal complexation. The OH groups of the D-ring could

occupy the first coordination sphere around the metal ion, whereas the OH groups of the B-ring may have a secondary effect on the formation of a complex [15]. Thermo-gravimetric and atomic absorption spectrophotometric studies indicated that 3-OH-4-oxo and 3′-OH-4′-OH moieties are the most likely binding sites of flavonoids for metals [16]. As evident from our results, OH groups of EGCG were changed and this could be attributed to the reaction of OH groups with Zn^{2+} in zinc-EGCG complex. On the other hand, zinc-EGCG complex did not change the main configuration of EGCG except for OH groups as revealed from the UV-*vis* absorption spectra. Studies performed with EGCG in the presence of metal ions (EGCG complexed with metal ions) reported similar spectroscopic shifts [14, 26].

Zn^{2+} is an effective inhibitor of numerous proteases, such as prostate specific antigen, a marker of prostate cancer. Zn^{2+} in human prostate might inhibit the invasion and metastasis of prostate cancer cells by regulating the proteolytic activity of prostate specific antigen [17]. Zinc is reported as an anti-oxidant and suppressor of reactive oxygen species-induced apoptosis as well as of oxidative damage, though this has been disputed [27]. Based on our results, we presumed that Zn^{2+}/EGCG mixture reduced the inhibitory activity of Zn^{2+} on the growth of PC-3 cells because EGCG and Zn^{2+} partly formed complex or EGCG protected PC-3 cells from the Zn^{2+} induced damage. We suggest that free Zn^{2+} enhanced the inhibitory effects of EGCG on PC-3 cells, while Zn^{2+}/EGCG mixture was found to be more effective than zinc-EGCG complex since, low concentrations (40 μmol/L and 80 μmol/L) of Zn^{2+}/EGCG was ineffective whereas high concentrations (160 μmol/L) were effective in inhibiting the growth of PC-3 cells. This also indicates that D-ring OH groups of EGCG in Zn^{2+}/EGCG mixture could participate in reacting with Zn^{2+}. It is likely that D-ring OH groups of zinc-EGCG complex reacted with Zn^{2+} completely, such that the bioactivity of EGCG in the presence of Zn^{2+} was higher compared with zinc-EGCG complex. Previously, the transition metals in the complex with a bioflavonoid were thought to acquire additional superoxide dismuting centers and therefore might become more effective free radical scavengers than their parent bioflavonoid [28]. By comparing antioxidant ability of zinc-EGCG complex and Zn^{2+}/EGCG mixture, it is evident that free Zn^{2+} rendered EGCG a more effective free radical scavenger than ECGG alone. The differences in antioxidant activity of zinc-EGCG complex and Zn^{2+}/EGCG mixture suggests that Zn^{2+} did not form a complex fully with EGCG in the latter. Therefore, we suggest that it might be due to a decrease in the number of active OH groups in zinc-EGCG complex.

Yu *et al*. [29] reported that it is not EGCG, but its oxide and complex that formed with Cu^{2+}, were the bioactive components responsible for cytotoxicity. The results of the present study showed that Zn^{2+} enhanced the permeation ability of EGCG to cytoplasmic membrane, but zinc-EGCG complex was more difficult to permeate compared with EGCG alone. Tang *et al*. [30] identified at least two zinc transporters in human mesenchymal stem cells and osteoblastic cells as the ubiquitous zinc transporter, ZIP1, and LIV-1, which are expressed by breast cancer cells. These zinc transporters are present both in the plasma membrane and cytoplasm. Over-expression of ZIP1 in RWPE2 (prostate cancer cell) resulted in an elevation of intracellular zinc concentration and suppression of RWPE2 cell growth due to the increased apoptosis [9]. Hence, it can be postulated that Zn^{2+} might regulate membrane protein (such as ZIP1) expression and augment the influx of EGCG.

In the present study, it was noted that EGCG, zinc-EGCG complex and Zn^{2+}/EGCG mixture inhibited the growth of PC-3 cells in a dose dependent fashion. These findings imply that Zn^{2+}, when complexed with EGCG, could not enhance the inhibition of EGCG against PC-3 cells. It was speculated that Zn^{2+} and EGCG might bind to receptors located on the cytoplasmic membrane first, and then is delivered into the cells. Recent studies reported that EGCG inhibited cell growth by inducing the myosin II regulatory light chain phosphorylation and the 67 kDa laminin receptor (67LR), which was identified as a cell surface EGCG receptor that mediated its anticancer action. EGCG could disrupt the contractile ring by decreasing the myosin II regulatory light chain phosphorylation resulting in the inhibition of cell proliferation [31 – 34]. Accordingly, Zn^{2+} that disassociates from the Zn^{2+}/EGCG mixture might regulate and enhance the effect of EGCG on PC-3 cells by binding to a 67 kDa laminin receptor on the cell surface, that also could contribute to an increase in the intracellular EGCG content. Further studies are necessary to identify how Zn^{2+} regulates the binding of EGCG to the 67 kDa laminin receptor.

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The authors have declared no conflict of interest.

5 References

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