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Research Article
Enhanced Bioavailability of Epigallocatechin Gallate (EGCG) after Esterification and Complexation with Fish Oil

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Abstract

Background and Objective: Green tea is rich in epigallocatechin gallate (EGCG), which is responsible for various biological functions but its bioavailability is limited. The objective of the present study was to check the beneficial efficacy of EGCG esterified with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil (ester derivatives) and EGCG-Fish oil complex on C2BBe1 cells. **Materials and methods:** The EGCG and DHA/EPA (fish oil) esterification/complexation was confirmed by various techniques including Thin-Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR) and Gas Chromatography-Mass spectrometry (GC-MS) as well as antioxidant and antiglycation activity was checked using 3 different experimental samples (EGCG, EGCG-ester and EGCG-Fish oil complex). **Results:** The antioxidant activity (TEAC, DPPH scavenging activity) of EGCG-ester (DHA/EPA) was significantly higher ($p<0.05$) than the EGCG-fish oil complex and EGCG alone group. In the cell model, both EGCG-ester (DHA/EPA) and EGCG-Fish oil complex did not show any significant changes in viability (cytotoxicity) with no morphological changes and thus implication its safety. The best EGCG permeability coefficient (cell uptake) was observed in EGCG-ester (DHA/EPA) group at 24 hrs (18.73%) and 48 hrs (20.74%) as compared to the EGCG-Fish oil complex and EGCG alone. Also, EGCG-ester (DHA/EPA) ester showed good anti-glycation activity by significantly inhibiting ($p<0.05$) the advanced glycation end products (AGEs) production (BSA-MGO, BSA-Fructose) in C2BBe1 cells as compared to the EGCG-Fish oil complex group. **Conclusion:** Overall, current study indicated that EGCG-ester (DHA/EPA) showed potent antioxidant activity by improving EGCG bioavailability (cellular uptake) and followed by the improved anti-glycation property through inhibiting AGEs production.

Key words: EGCG, EPA, DHA, esterification, bioavailability

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Green tea is the second most consumed beverage in the world due to its numerous beneficial properties. Many researchers have indicated that green tea is rich in catechins especially epigallocatechin gallate (EGCG), which is responsible for major biological functions including antioxidant, anti-inflammatory, anti-diabetic, anti-obesity, anti-hypertensive and anti-microbial properties[1,2]. However, the biological function of green tea is much limited due to the low bioavailability of EGCG (active phytocomponent)[3,4]. The major reason for the low bioavailability of EGCG including high sensitivity towards pH, light, temperature and ionic strength with high polarity nature (hydrophilicity) and its high reactivity property (especially with digestive enzymes) which makes EGCG more unstable and easily oxidizable[5,6]. The Pharmacokinetics studies of EGCG in animal models revealed that only less than 5% of tea catechins (EGCG) could reach the systemic circulation. Likewise, in human also less than 2% of EGCG was detected in blood after consumption of green tea (rich in EGCG) with 3 to 4.5 hrs of half-life and thus both the animal and human studies indicate that EGCG shows lower bioavailability and bioaccessibility[3,7,8]. Therefore, many researchers started to focus on improving the bioavailability of EGCG by complexation or esterification of EGCG with various components like piperine, curcumin, ascorbic acid, proteins (albumin, lactoglobulin) by changing the physical and chemical properties of EGCG[9-11]. Moreover, recently our team also conducted a study by combining EGCG with royal jelly proteins (Major royal jelly protein-MRJP) and the EGCG-MRJP complex showed better bioavailability of EGCG with increased EGCG cellular uptake in the cell model[3].

Fish oil is primarily composed of omega-3-fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are considered as major ingredients responsible for various health benefits[12]. Ample amounts of studies indicated that EPA and DHA show an array of biological functions including antioxidant, anti-inflammatory, anticancer, antidiabetic and antihyperlipidemic properties[2,13]. However, the stability of DHA and EPA is lowered due to low water solubility (lipophilicity), increased light and pH sensitivity and high oxidation susceptibility (autoxidation-lipid peroxidation) which results in lower bioavailability in human[14,15] as similar to EGCG. Hence, the author hypothesizes that a combination of both EGCG (water-soluble) and EPA/DHA (lipid-soluble) would be a better idea to make both nutraceuticals for improved bioavailability (both protect each other). For the same reason, few scholars esterified EGCG with various fatty acids (fatty acid esters-acyl donor) and found better bioavailability[16,17]. However, the above-mentioned studies lack to check the other beneficial efficiency of those esterified compounds as well as not compared the effect of esterified EGCG with EGCG complex. Hence, this novel study was designed to evaluate the beneficial efficacy of esterified EGCG with fish oil (DHA/EPA) by assessing the esterification efficiency and permeability ability (bioavailability of EGCG) as well as its various biological function by exploring the oxidative capacity and anti-glycation activity on C2BBe1 cells by comparing with EGCG and EGCG-Fish oil complex.

MATERIALS AND METHODS

Samples/chemicals: EGCG (98% HPLC grade) was purchased from Hunan Sunfull Biotech Co., Ltd. (Hunan, China). Fish oil (as a capsule) rich in DHA/EPA was provided by Herbalife Nutrition Corp (CA, USA). All the experiments were carried out at Chung Shan Medical University at School of Nutrition, Taichung, Taiwan from 2018-2019.

Separation/isolation of DHA and EPA from fish oil: In short, the Free Fatty Acids (FFA) were separated from the fish oil capsule by saponification procedure and followed by urea complexation procedure to purify only DHA and EPA from other FFAs as indicated by Zhong and Shahidi[16]. Then the presence of DHA and EPA in FFA was confirmed by TLC (supplement data).

Esterification/complexation of EGCG with FFA (DHA/EPA): Esterification of EGCG with isolated FFA rich in DHA/EPA based on the method described by Sekhon-Loodu and Rupasinghe[18] using Lipase B (Novozym 435) to trigger acylation. The efficiency of esterification and complexation is confirmed by checking the levels of EPA, DHA and EGCG using various techniques like NMR spectroscopy and GC-MS as mentioned by Zhong and Shahidi[16].

Antioxidant indexes

TEAC, DPPH scavenging activities: Trolox equivalent antioxidant capacity (TEAC) or total antioxidant capacity of different samples (EGCG, EGCG-esters and EGCG-Fish oil) was determined using the Arnao method[19] and was expressed as μg Trolox eq mg^{-1}. The DPPH scavenging activity was examined by the method of Shimada and his colleagues[20] using the below formula:

$$\frac{\text{Blank absorbance} - \text{Sample absorbance}}{\text{Blank absorbance}} \times 100\ (\%)$$

DPPH scavenging activity is expressed in percentage and also different standards are used for comparison.

Cell line: For the current cell study, the human colon epithelial adenocarcinoma C2BBe1 cells (clonal of Caco2 cells: CRL-2102) were bought from ATCC (Tainan, Taiwan) and cultured with Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with 1% L glutamine, 1% penicillin-streptomycin, 10% Fetal Bovine Serum (FBS), 1.5 g L^{-1} sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA). The C2BBe1 cells were maintained in a humidified atmosphere (5% CO_2) at 37°C using a CO_2 incubator.

Cell viability (Cytotoxicity) and morphology analysis: The C2BBel cells viability of various experimental samples (EGCG, EGCG-Esters and EGCG-Fish oil) at different duration (24 and 48 hrs) were checked using 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay as mentioned by Han and his coworkers[3]. Then, the morphological changes in C2BBel cells were also checked after the addition of various experimental samples (EGCG, EGCG-Esters and EGCG-Fish oil) under a compound microscope (10X) by comparing with control cells for any morphological changes or abnormalities.

EGCG cell uptake or permeability ability: To assess the EGCG cellular uptake (permeability coefficient), the C2BBel cells were grown and treated with various experimental samples (100 µg mL^{-1} of EGCG, EGCG-Esters and EGCG-Fish oil) for 24 and 48 hrs under 37°C. Then the cells were dissolved in PBS solution (Sigma-Aldrich, St. Louis, MO, USA) and lysed with sonicator and centrifuged (5000 rpm for 10 min) and the resultant supernatant was filtered and EGCG levels were quantified using HPLC and the permeability coefficient were calculated using the formula as indicated by our previous study[3].

Antiglycation activity: Antiglycation activity (AGEs inhibition activity) of various experimental samples (100 µg mL^{-1} of EGCG, EGCG-Esters and EGCG-Fish oil) in C2BBel cells were calculated using BSA-MGO and BSA-Fructose model (protein glycation model) by the methods mentioned by Wang and others[21] and Shen and others[22]. Aminoguanidine (AGEs trapper) was used as a positive control. The AGEs inhibition was calculated using the below formula and expressed in percentage.

$$\text{AGEs inhibition (\%)} = \frac{\text{1-fluorescent intensity with inhibitor}}{\text{Fluorescent intensity without inhibitor}} \times 100$$

Statistical data: All the cell line studies are conducted in triplicate (n = 3) and all the data are exemplified as the Mean±Standard Deviation (SD). All the data were analyzed using one-way ANOVA followed by Tukey's multi-range test using SPSS software (IBM Corp., USA). A p-value less than 0.05 is considered statistically significant.

RESULTS

Confirmation of esterification (EGCG-Ester): To confirm the esterification (efficiency) of EGCG with free fatty acids like DHA and EPA, we used ^{13}CNMR and GC-MS techniques. The NMR spectrum including EGCG ester (EGCG-DHA/EPA) was shown in Fig. 1. The LC-MS spectrum of all the fatty acids in Fish oil was shown in Fig. 2a. Whereas Fig. 2b represents the LS-MS spectrum including EGCG (alone), standards of DHA and EPA, EGCG-Ester and EGCG-Fish oil complex. Both, NMR and LC-MS spectrum shows the esterification of EGCG with DHA and EPA. Moreover, TLC was used to confirm the presence of DHA and EPA in free fatty acid (from fish oil) before esterification (Fig. S1).

Antioxidant status: The antioxidant activity of different samples (EGCG, EGCG-Esters and EGCG-Fish oil) on C2BBel cells were determined by TEAC, DPPH scavenging activity (Table 1). EGCG-ester (EGCG-DHA/EPA) group showed better TEAC (17.97±1.14) and DPPH (96.7%) scavenging activity as compared to EGCG or EGCG-fish oil complex group.

Table 1: Trolox equivalent antioxidant capacity (TEAC) or total antioxidant capacity, DPPH scavenging activity of different samples (EGCG, EGCG-Esters and EGCG-fish oil) on C2BBel cells

Samples	TEAC (µg trolox eq mg^{-1})	DPPH scavenging activity (inhibition %)
EGCG	15.65±1.15^{b}	95.5±10.15^{b}
EGCG-ester	17.97±1.14^{a}	96.7±09.15^{a}
EGCG-fish oil complex	13.49±1.10^{c}	94.8±10.15^{c}

Data are expressed as the Mean±Standard deviation (n = 3). Data within the same column sharing different superscript letters (a, b, c) were statistical differences ($p<0.05$)

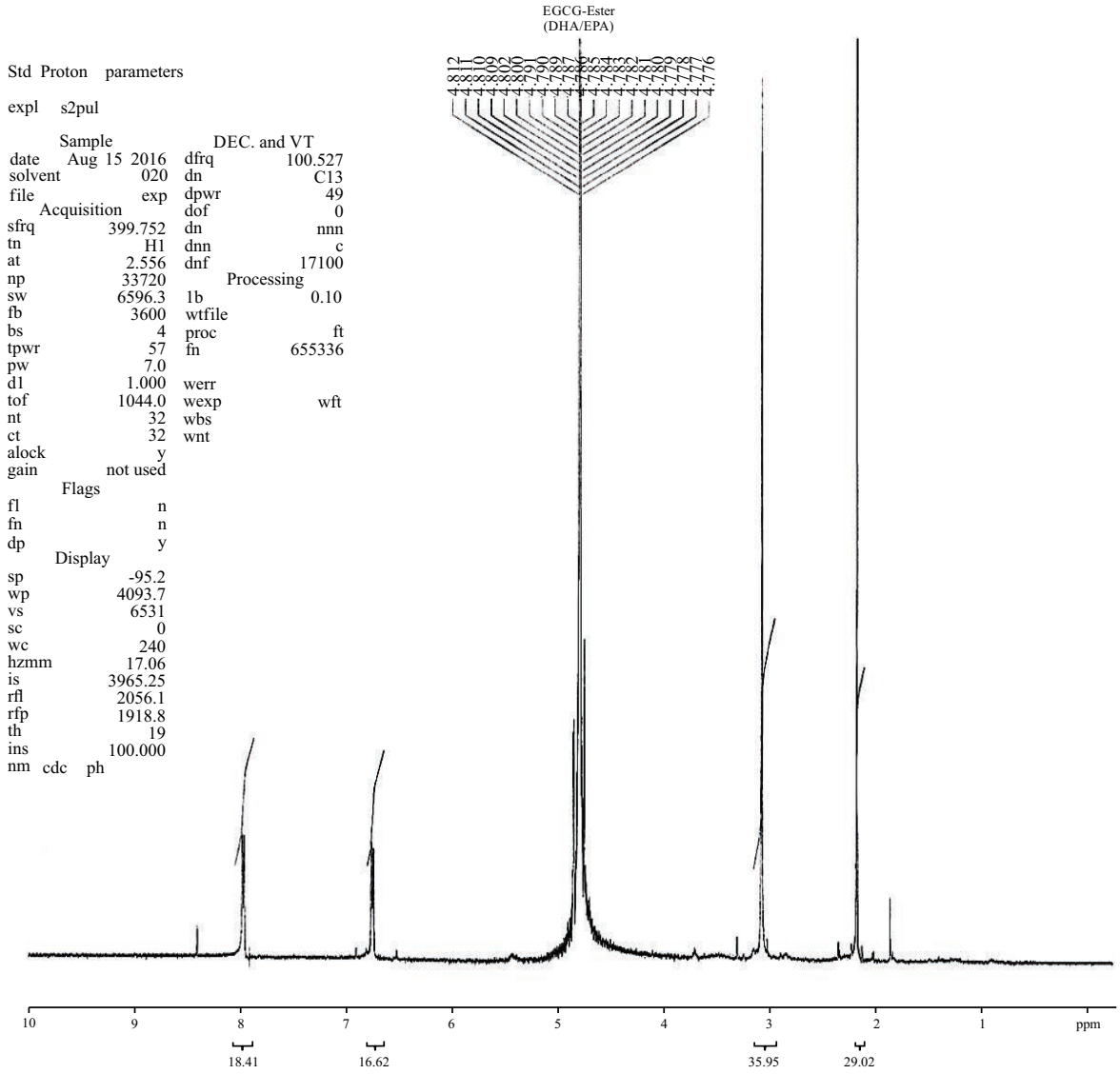

Fig. 1: ^{13}CNMR spectrum, showing EGCG-Ester(rich in DHA and EPA)

In vitro studies (Cell model)

Cell viability (Cytotoxicity) and morphology analysis: The C2BBe1 cells viability efficacy (cytotoxicity) of various experimental samples like EGCG, EGCG-Ester and EGCG-Fish oil complex were checked by MTT assay and was shown in Fig. 3. The C2BBe1 cells treated with 100, 200 and 500 µg mL^{-1} of EGCG, EGCG-Ester and EGCG-Fish oil complex did not show any significant difference between each group at 24 and 48 hrs. Thus, indicating no cytotoxicity activity in any of the experimental drugs (EGCG, EGCG-Ester and EGCG-Fish oil complex). Moreover, C2BBe1 cells treated with different experimental samples (EGCG, EGCG-Ester and EGCG-Fish oil complex), did not show any morphological changes under the microscope (Fig. S2).

EGCG cell uptake or permeability ability: The cellular uptake (permeability efficiency) of EGCG of different samples (EGCG, EGCG-Ester and EGCG-Fish oil complex) on C2BBe1 cells at a different time interval (2, 24 and 48 hrs) was shown in Fig. 4. The EGCG cell uptake (apical transmembrane permeability) was calculated based on the permeability coefficient. At 2, 24 and 48 hrs, the EGCG-ester group (100 µg mL^{-1} showed significantly higher levels ($p<0.05$) of EGCG uptake than EGCG alone or EGCG-Fish oil group. Based on the above results it's clear that esterified EGCG (EGCG-DHA/EPA) showed the best EGCG uptake ability.

Antiglycation activity: The antiglycation activity (AGEs inhibition activity) of various experimental samples

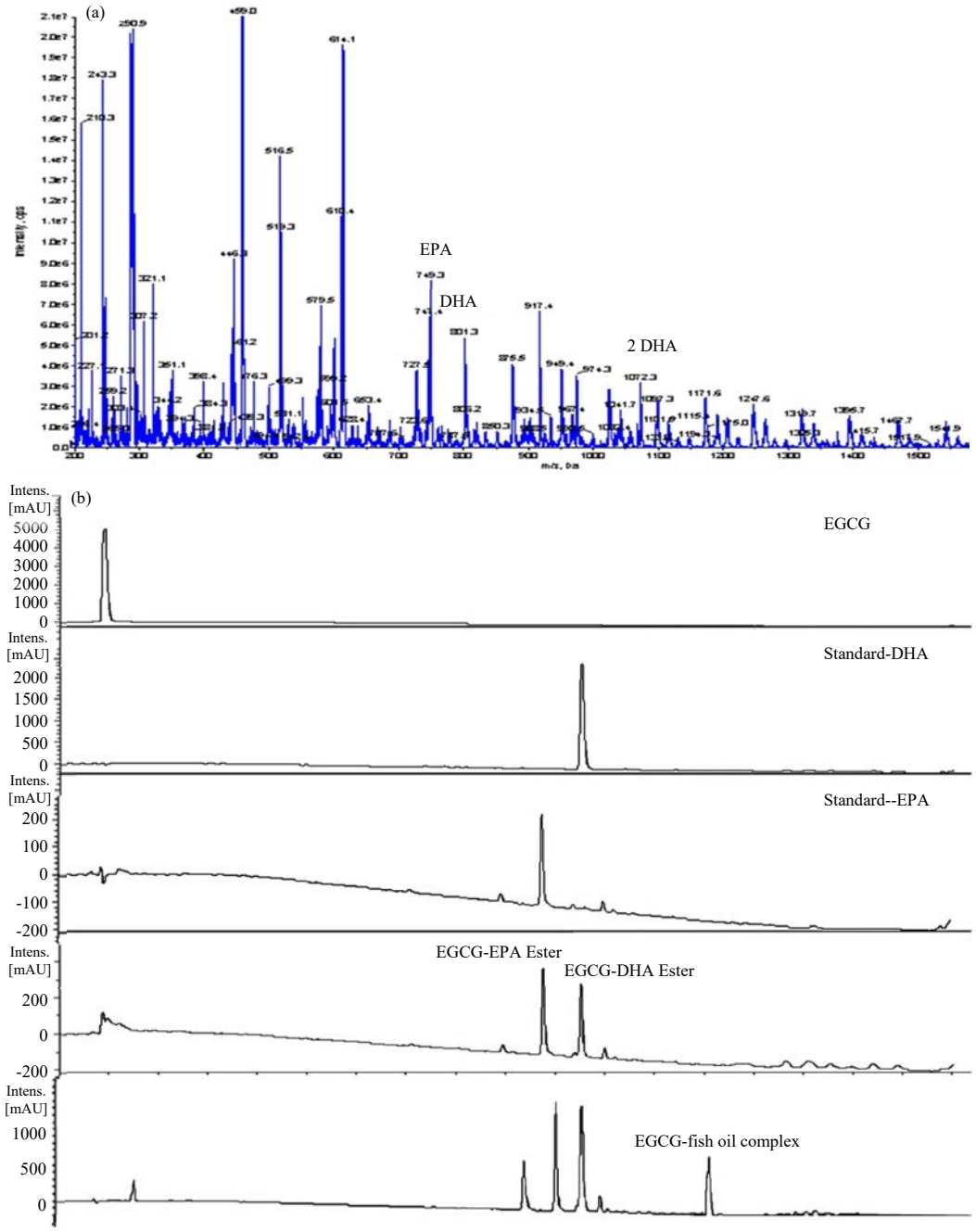

Fig. 2(a-b): LC-MS spectrum of all the fatty acids in Fish oil (a) and chromatograms of EGCG, DHA, EPA, EGCG-EPA Ester, EGCG-DHA Ester, and EGCG-Fish oil complex at 210 nm (b)

(100 μg mL^{-1} of EGCG, EGCG-Esters and EGCG-Fish oil) on C2BBel cells were assessed by BSA-MGO (Fig. 5a) and BSA-Fructose (Fig. 5b) models. The C2BBel cells treated with MGO and fructose trigger BSA protein glycation but the addition of samples like EGCG, EGCG-Esters and EGCG-Fish oil lower the AGEs production by trapping MGO and fructose. However, EGCG display potent AGEs inhibition activity in both MGO and fructose models than EGCG-Esters and EGCG-Fish oil complex groups. Since EGCG alone group has any free hydroxyl group which might effectively trap MGO and form adduct and thus lower AGES production. Nevertheless, the esterified EGCG and EGCG-fish oil complex lacks many free hydroxyl groups and hence showed lower AGEs inhibitor activity than the EGCG

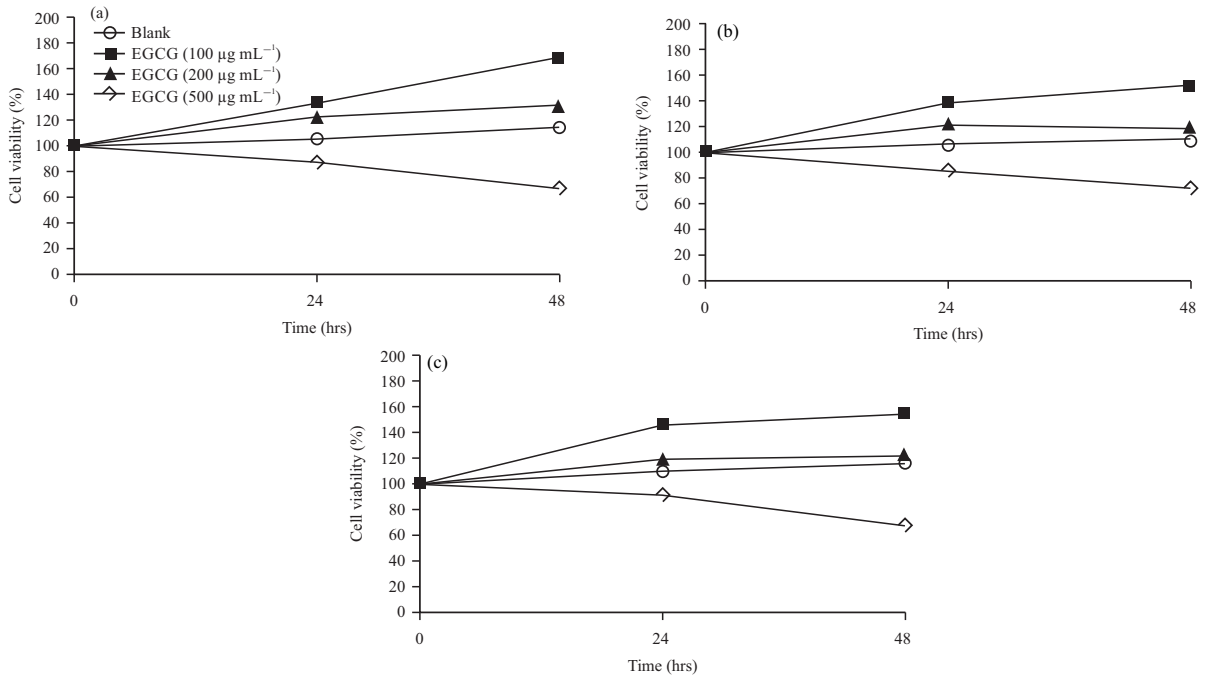

Fig. 3(a-c): Viability efficacy of various experimental samples (a) EGCG, (b) EGCG-Ester and (c) EGCG-Fish oil complex (3C) on C2BBe1 cells

Data are expressed as the Mean±Standard deviation (n = 3)

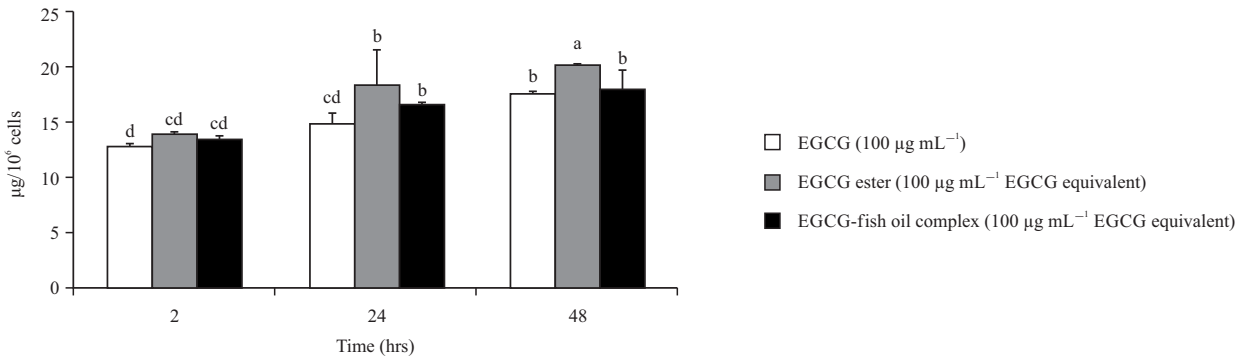

Fig. 4: Cellular uptake (permeability efficiency) of EGCG on C2BBe1 cells at a different time interval (2, 24 and 48 hrs)

Values are expressed as the Mean±Standard Deviation (SD). Different letters show the significantly different (p<0.05)

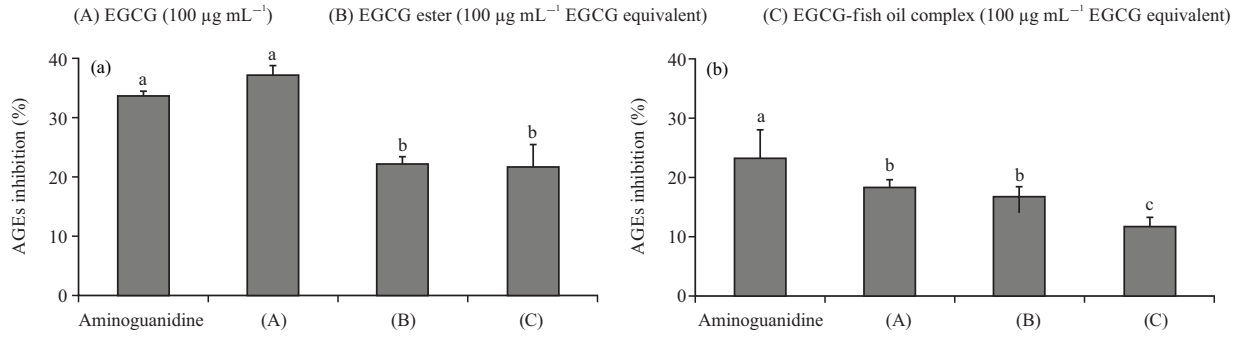

Fig. 5(a-b): Antiglycation activity (AGEs inhibitory activity)of various experimental samples like EGCG, EGCG-Ester and EGCG-Fish oil complex on C2BBe1 cells using (a) BSA-MGO model and (b) BSA-Fructose

Data are expressed as the Mean±Standard deviation (n = 3). Different letters show the significantly different (p<0.05). Aminoguanidine-positive control

group. Meanwhile, EGCG-ester showed better antiglycation activity than EGCG-Fish oil complex and thus hinting that EGCG-ester has few free OH groups that might influence the MGO trapping property, which results in good antiglycation activity.

DISCUSSION

The present study was framed to check the beneficial efficacy of esterified EGCG with fish oil (holistic effect) by assessing the esterification efficiency and permeability ability (bioavailability of EGCG). Followed by exploring the oxidative capacity and anti-glycation activity on C2BBe1 cells by comparing with EGCG and EGCG-Fish oil complex. The major reason for this esterification is to improve EGCG bioavailability and thereby its impact on various biological functions. The esterification/complexation of EGCG with fish oil (DHA/EPA) was confirmed by the results of the TLC, ^{13}CNMR and GC-MS technique. During the esterification process, the hydroxyl group of EGCG will bind to the acryl group of different fatty acids (acylation reaction) like DHA and EPA and finally form an EGCG-ester (EGCG-DHA or EPA). Also, during this study by NMR analysis, few free hydroxyl groups in EGCG-ester (data not shown) were confirmed and thus retaining its antioxidant activity. NMR and MS spectrum results were similar to the outcome of Zhong and Shahidi[16] studies, where different EGCG ester derivative is observed in both NMR and GC-MS spectrum.

The antioxidant status of different samples (EGCG, EGCG-Esters and EGCG-Fish oil) on C2BBel cells were quantified by TEAC and DPPH scavenging activity. Both the TEAC and DPPH scavenging activity was significantly higher in EGCG-ester (DHA/EPA) group as compared with EGCG and EGCG-fish oil complex group. Similarly, Zhong and others[4]demonstrated that EGCG-fatty acid ester showed potent antioxidant activity due to increased lipophilicity (improve bio accessibility)and by facilitating the hydrogen atom donating property (due to increased acylation process). Moreover, oxidative stability index (OSI) was higher in EGCG-ester (9.87 hrs) than EGCG-Fish oil complex (8.2 hrs). In addition as mentioned previously that still, the EGCG-ester has few free hydroxyl groups, which might also contribute to better antioxidant activity with improved bioavailability.

For the current cell line study, we preferred human colon epithelial adenocarcinoma C2BBe1 cells (clonal of Caco2 cells) to check the EGCG bioavailability (cellular uptake) as C2BBe1 colon cells mimic the human intestinal environment[23].Before, check the EGCG bioavailability, the author would first like to check the cytotoxicity or proliferation property of different samples (different concentrations at various time intervals) on C2BBe1 cells. The outcome of the MTT assay showed that C2BBe1 cells cultured with different concentrations of EGCG, EGCG-Ester and EGCG-Fish oil complex did not infer any significant changes in the cell number (viability) or proliferation rate at 24 and 48 hrs. Hence, showcasing that all the samples (EGCG, EGCG-Ester and EGCG-Fish oil complex) are safe, even at higher concentrations. Furthermore, C2BBe1 cells treated with EGCG, EGCG-Ester and EGCG-Fish oil complex, did not show any morphological changes. Based on the above results, the author has confirmed that none of the experimental samples could induce toxicity or cell death, even at higher concentrations. Previously, Mori and his colleagues[24] also indicated that EGCG- fatty acid ester derivatives did not show any difference in cell viability using MTT assay and thus concluded that EGCG and its fatty acid derivatives are safe.

Aforementioned that EGCG is highly hydrophilic in nature and sensitive towards pH, light, temperature and its high reactivity property (especially with digestive enzymes) which makes EGCG more unstable and easily oxidizable molecule. Hence, EGCG bioavailability is significantly hampered to overcome this issue, many researchers started to esterify EGCG with the different molecules to improve the bioavailability[3,5,6]. The EGCG cellular uptake efficiency of each sample was calculated based on the permeability coefficient as mentioned before. The EGCG cellular uptake (permeability coefficient) was higher in the EGCG-ester group as compared to EGCG alone or EGCG-Fish oil complex group. The author speculates that during esterification (EGCG-ester), process the lipophilicity and steric property of EGCG-ester were considerably increased. That might enhance the EGCG-ester stability and superior cellular affinity (plasma membrane) and thus increase the EGCG permeability or cell uptake. Zhong and his colleagues[4] also demonstrated that EGCG ester derivatives (lipophilized EGCG) showed greater cellular uptake or absorption than parent EGCG.

AGEs are the end products produced by the non-enzymatic reaction between reducing sugars and amino groups of proteins, nucleic acids, lipids (Amadori rearrangement). AGEs were considered as a pathogenic factor linking Diabetic Mellitus and Cardiovascular Disease as they trigger inflammation and oxidative stress[25,26]. BSA-MGO and fructose model tests are the standard method to check the anti-glycation or anti-AGEs activity of any drug[27]. For the current study, the anti-glycation activity of EGCG, EGCG-ester and EGCG-Fish oil complex was examined by including a standard aminoguanidine (trap MGO) in C2BBc1 cells. C2BBel cells treated with EGCG, EGCG-Esters and EGCG-Fish oil slightly lowered the AGEs production by trapping MGO and fructose (avoid MGO-BSA glycation). However, EGCG display greater AGEs inhibition activity in both MGO and fructose models than

EGCG-Esters and EGCG-Fish oil complex groups. Since EGCG alone group has many free hydroxyl groups which might effectively trap MGO and form adduct and thus lower AGES production. But, the esterified EGCG and EGCG-fish oil complex has limited free hydroxyl groups. Thus, demonstrating lower AGEs inhibitor activity than the EGCG group. Nevertheless, EGCG-ester showed better antiglycation activity than EGCG-Fish oil complex and thus hinting that EGCG-ester has few free OH groups (contribute to antioxidant activity) that might influence the MGO trapping property, which results in good antiglycation activity. In agreement with our results, Wang and his co-workers[28] hinted that the EGCG-fatty acid ester derivatives especially EGCG-DHA/EPA showed higher antioxidant and MGO trapping activity due to higher lipophilicity, improved bioavailability and stability makes EGCG-esters a better anti-glycation agent than EGCG-Fish oil complex. In addition, the EGCG-ester displays better α-glucosidase activity than EGCG or EGCG-Fish oil complex and thus conferring its antiglycation property. The major strength of this study was to compare the bioavailability efficiency and beneficial effect (antioxidant and antiglycation activities) of EGCG-ester with EGCG-fish oil complex and parent EGCG. Because of limitations, current study lacks the detailed structural, physical and chemical properties elucidation of various EGCG ester derivatives. Hence, extensive pharmacokinetic and dynamic studies should be conducted with different EGCG ester derivatives.

CONCLUSION

Present study demonstrated the beneficial efficacy of EGCG esters and EGCG-Fish oil complex through improving antioxidant capacity (better TEAC and DPPH scavenging activity), followed by increased EGCG permeability (bioavailability of EGCG) and anti-glycation activity (MGO and Fructose induced AGEs inhibitory activity) on C2BBe1 cells. Overall, EGCG-ester showed superior antioxidant and antiglycation activity owing to enhance lipophilicity, steric effect and electron/nucleophilic donor capacity. Further, pharmacokinetic and dynamics studies are needed to elucidate the structural modification undergone during esterification/complexation. Also, animal studies (toxicity and dose fixation) and human trials are needed to check the real bioavailability efficacy of EGCG esters.

SIGNIFICANCE STATEMENT

Current study indicates that EGCG-ester showed superior EGCG bioavailability, antioxidant and antiglycation activity than EGCG-fish oil complex and EGCG. This novel combination of EGCG with fish oil (esterification/complexation) would significantly improve various beneficial effect of EGCG due to high lipophilicity and increase electron donor capacity. Hence, EGCG-fatty acid ester would be commercially developed in large scale and might be used to improve overall health status. However, further animal and human trial are need to confirm its beneficial efficacy.

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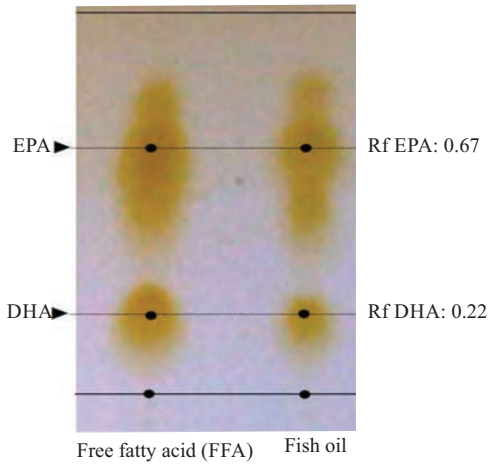

Fig. S1: Thin Layer Chromatography (TLC) of fish oil and its isolates (free fatty acids- EPA/DHA)

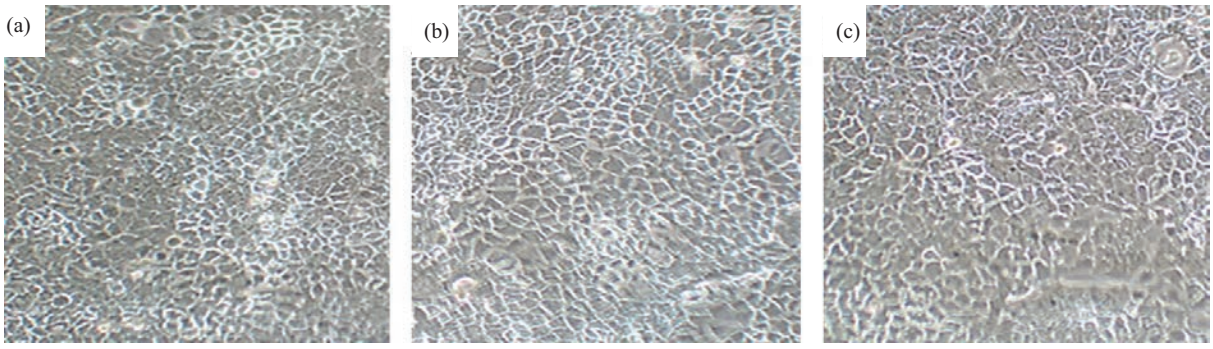

Fig. S2(a-c): Morphological changes on C2BBe1 cells treated with EGCG (A), EGCG ester (B), EGCG-Fish oil complex (C)