Chemopreventive potential of the tannase-mediated biotransformation of green tea

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A B S T R A C T

Green tea (Camellia sinensis) is one of the most widely consumed beverages in the world. The cancer chemopreventive qualities of green tea have been well documented. Epigallocatechin gallate (EGCG) is often described as the most potently chemopreventive green tea catechin; however, the low bioavailability of EGCG is a limiting factor for its biological effect. Thus, the aim of this work was to test the chemopreventive potential of green tea extract and EGCG after tannase-mediated hydrolysis. The results showed that the biotransformed compounds retained most of the beneficial properties of the original compounds, and some beneficial properties were improved in the biotransformed compounds. Biotransformation of EGCG decreased its toxicity without affecting its antiproliferative effects. Furthermore, human cells gene expression profiling showed that the biotransformed compounds modulated the expression of several genes related to carcinogenesis. These results demonstrate the benefits of the biotechnological modification of natural food molecules, allowing the improvement of the nutraceutical potential of a beverage as green tea.

1. Introduction

Green tea is one of the most widely consumed beverages in the world. Epidemiologic research has revealed that individuals who drink large quantities of green tea are less likely to develop cancer (Kato et al., 1990; Yu et al., 1995). Recently, a relationship between the consumption of green tea and a reduced risk of type 2 diabetes was also reported (Iso, Date, Wakai, Fukui, & Tamakoshi, 2006). Green tea contains many compounds considered to have beneficial health effects, such as polyphenolic flavonoids, of which epigallocatechin gallate (EGCG) is the major constituent. The cancer chemopreventive function of green tea catechins has been well documented, and in particular, EGCG has been shown to have anticarcinogenic activity in vitro (Banerjee, Manna, Saha, & Panda, 2005; Cooper, Morre, & Morre, 2005; Maeta, Nomuro, Takatsume, Izawa, & Inoue, 2006). EGCG, often described as the major biologically active component in green tea, is one of the most potent catechins capable of inhibiting cell proliferation and inducing apoptosis in cancer cells (Shimizu, Adachi, Masuda, Kozawa, & Moriwaki, 2011).

Phenolic compounds also contribute to the chemopreventive activity of tea through antioxidant activity mediated by their redox properties, which allows them to act as reducing agents, singlet-oxygen quenchers and metallic-ion chelators (Atoui, Mansouri, Boskou, & Kefalas, 2005). Polyphenols are reducing agents and are considered the most common antioxidants in our diet, however, the chemical structure of these compounds may affect their biological properties such as bioavailability, antioxidant activity and interactions with specific cell receptors and enzymes (Scalbert & Williamson, 2000).

Despite the proven antioxidant capacity of tea polyphenols, many clinical studies and animal models have shown that these compounds, and especially their polymers, esters, and glycosides, are abundant but are not always absorbed upon oral administration. The functional effect of the compound depends not only on the amount ingested, but also on its bioavailability (Holst & Williamson, 2008). Studies have shown that the enzymatic hydrolysis of polyphenols results in not only increased absorption, but also increased antioxidant activity when compared to the original unmodified compounds. This is especially true of the conversion of the most abundant polyphenol of green tea, epigallocatechin gallate, into epigallocatechin, which possesses higher antioxidant activity (Battestin & Macedo, 2007).

Considering the broad variation in the amounts of available polyphenolic antioxidants via diet, in combination with reduced bioavailability, strategies to enrich the diets with phenolic antioxidants needed to be develop with consistent phytochemical profile for enhanced health benefits (Vattem & Shetty, 2003). As an alternative, bioprocesses, such as fermentation or enzymatic hydrolysis of plant sources and their products, can release the phenolic glycosides or other conjugates and consequently, enhance the functional activity of these antioxidants.
Tannin acylhydrolases, commonly referred to as tannases (E.C. 3.1.1.20), are inducible enzymes produced by fungi, yeast and bacteria. Tannases have mostly been characterised by their activity on complex polyphenolics and are able to hydrolyse the ester bond (galloyl ester of an alcohol moiety) and the depside bond (galloyl ester of gallic acid) of substrates such as tannic acid, epicatechin gallate, epigallocatechin gallate, and chlorogenic acid (Garcia-Conesa, Ostergaard, Kauppinen, & Williamson, 2001).

In this study, the activity of tannase on the extracts of green tea was investigated. The aim of this work was to evaluate the chemo-preventive potential of green tea extract and EGCG after an enzymatic reaction, catalysed by the tannase, produced by Paecilomyces variotii (Battestin & Macedo, 2007).

2. Materials and methods

2.1. Materials

Green tea was purchased from local markets (Chá Leão®). Epigallocatechin gallate (EGCG, 95%), epicatechin gallate (EGG, 98%), 2,2'-azobis(2-methylpropionamide) (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sulforhodamine B sodium salt (SRB), trichloroacetic acid, T1503 Trizma® base, thiazolyl blue tetrazolium bromide, agarose (LMP) and Triton X-100 were purchased from Sigma-Aldrich. All other chemicals were purchased in the grade commercially available. Fluorescein was purchased from ECIBRA, and Trolox® (97%) was purchased from ACROS Organics. Cell culture reagents were purchased from Invitrogen®.

2.2. Enzyme

Tannase was isolated from P. variotii using a previously published procedure (Battestin & Macedo, 2007). A 250-ml conical flask containing 5 g of wheat bran, 5 g of coffee husk, 10 ml of distilled water and 10% tannic acid (w/w) (Ajinomoto OmniChem Division, Wetteren, Belgium) was used for the fermentation process. The culture medium (pH 5.7) was sterilised at 120 °C for 20 min. After sterilisation, the flasks were inoculated with 2.5 ml (5.0 × 10⁵ spores/ml) of the pre-inoculum suspension and incubated at 30 °C for 120 h. After fermentation, 80 ml of 20 mM acetate buffer, pH 5.0 was added, and samples were shaken at 200 rpm for 1 h. The solution was filtered and centrifuged at 9650g, for 30 min, at 4 °C (Beckman J2-21 centrifuge, Beckman-Coulter Inc., Fullerton, CA, USA). The supernatant was then treated with solid ammonium sulphate (80% saturation) and incubated overnight at 4 °C. The precipitate was collected by centrifugation (9650g for 30 min), resuspended in distilled water and dialysed against distilled water. The dialysed preparation was freeze-dried and used as crude tannase.

2.3. Sample preparation

2.3.1. LC–MS analysis

Samples were prepared, and the enzymatic reaction, catalysed by the P. variotii tannase, was characterised by LC–MS analysis as described by Battestin, Macedo, and Freitas (2008) and Macedo, Battestin, Ribeiro, and Macedo (2011).

2.3.2. Preparation of tea extract for antioxidant and cellular assays

In 125-ml Erlenmeyer flasks, 50 ml of distilled water and 250 mg of each sample of tea were combined. The extraction of compounds from green tea was performed in a water bath, at 100 °C, for 30 min. The samples were filtered on filter paper, and the extracts were freeze-dried. The resulting powder, referred to as dried tea extract, was used for antioxidant assays (Cao, Sofic, & Prior, 1996).

2.3.3. Polyphenol commercial standards

As an identified representative polyphenol from green tea, standard commercial epigallocatechin gallate (EGCG, 95%) was used as a control. This sample was tested and treated with tannase using the same procedures as were employed for the tea extract.

2.4. Enzymatic biotransformation

The extracts obtained from the green tea and the commercial control samples were used as substrates for enzymatic hydrolysis by tannase isolated from P. variotii (Battestin et al., 2008). The dried tea extract (5 mg) was dissolved in 1 ml of phosphate buffer (pH 7.4, 75 mM) and incubated with 5 mg of tannase, at 40 °C, for 30 min. The hydrolysis process was stopped by placing the reaction in an ice bath for 15 min. The biotransformed tea was used for the antioxidant assay after suitable dilution with the same phosphate buffer (pH 7.4, 75 mM) for ORAC and with a 70% methanol solution for DPPH. For the cellular assays, the samples were diluted with DMEM.

2.5. ORAC assay

ORAC assays were performed using fluorescein (FL) as the fluorescent probe, as described by Macedo et al. (2011). The automated ORAC assay was carried out on a NovoStar Microplate reader (BMG LABTECH, Germany) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The measurements were made in a COSTAR 96 plate. The reaction was performed at 37 °C, the reaction was started by thermal decomposition of AAPH in a 75 mM phosphate buffer (pH 7.4) due to the sensitivity of FL to pH. The measurements were performed in triplicate. ORAC values were defined as the difference between the area under the FL decay curve and the blank (net AUC). Regression equations between net AUC and antioxidant concentration were calculated for all of the samples. A tannase control was performed, and the ORAC value obtained was subtracted from the samples treated with the enzyme. ORAC-FL values were expressed as μmol of Trolox equivalent/mg of tea extract (Cao & Ito, 2004).

2.6. DPPH assay

The potential antioxidant activity of the tea extract was assessed based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, as described by Macedo et al. (2011). The measurements were performed in triplicate, and anti-radical activity was calculated using the linear regression equation determined by plotting the anti-radical activity of Trolox solutions of known concentrations. Antiradical activity was expressed as μmol of Trolox equivalent/mg of tea extract.

2.7. Cell culture and maintenance

The human gastric adenocarcinoma cell line PG100, the human colon adenocarcinoma grade II cell line HT29 and the mouse leukemic monocyte/macrophage cell line RAW264.7 were purchased from the Rio de Janeiro Cell Bank, RJ, Brazil. The cells were cultured in DMEM supplemented with 5% fetal bovine serum (Invitrogen®). The cells were maintained at 37 °C and 5% CO₂–95% humidified air.
2.8. Cytotoxicity assay

Ninety-six-well culture dishes were inoculated with RAW264.7 cells at a density of $10 \times 10^4$ cells per well. After incubation at 37 °C, in an atmosphere of 5% CO₂ and 100% relative humidity for 24 h, the adherent cells were washed once with PBS (phosphate-buffered saline). Cells were then incubated in media containing various concentrations of unmodified or biotransformed green tea extract or EGCG (25–150 μg/ml) to observe the toxic effects of the extracts on the tested cells. Untreated cells were used as positive controls. After incubation for 24 h, the cultures were assayed for cellular viability using the MTT method (Mosmann, 1983) with modifications described at Madeira, Macedo, and Macedo (2001). The plates were centrifuged for 10 min at 500g and 4 °C. After removing the medium, 10 μl of MTT solution and 90 μl of PBS were added to each well of an ELISA plate, and the plate was incubated at 37 °C for 3 h. The formazan was then dissolved by adding 100 μl of 10% SDS in 0.01 M HCl to each well, and the samples were incubated for 18 h. Finally, an ELISA plate reader was used to measure the absorbances of each well at 540 nm.

2.9. Cell proliferation assay

Ninety-six-well culture dishes were inoculated with two human cell lines, PG100 and HT29, at a density of $5 \times 10^4$ cells per well. Following incubation for 24 h, the adherent cells were washed once with PBS (phosphate-buffered solution). Cells were then incubated in DMEM containing 50–250 μg/ml of unmodified or biotransformed green tea extract or EGCG. Positive and negative controls were also performed. After incubation at 37 °C in an atmosphere of 5% CO₂ and 100% relative humidity for 48 h, the cultures were assayed to detect the effects of the tested compounds on cellular proliferation.

Cellular proliferation was measured using the sulforhodamine B (SRB) assay, which has been described in detail by Monks et al., 1991. Briefly, adherent cell cultures were fixed in situ by adding 50 μl of cold 50% (w/vol) trichloroacetic acid (TCA) (final concentration, 10% TCA) and incubating the samples for 60 min at 4 °C. The supernatant was then discarded, and the plates were washed five times with deionised water and dried. One hundred microlitres of SRB solution (0.4% w/vol in 1% acetic acid) was added to each microtiter well, and the cultures were incubated for 10 min at room temperature. Unbound SRB was removed by washing the samples five times with 1% acetic acid. The plates were then air-dried.

Bound stain was solubilised with Tris buffer, and the optical densities were read at a single wavelength of 515 nm using an automated spectrophotometric plate reader.

2.10. Determination of genotoxicity – comet assay

The comet assay was used to detect DNA damage (strand breaks and alkali-labile sites) at the individual cell level. PG100 gastric cells were used for this test. Ninety-six-well culture dishes were inoculated with PG100 cells at a density of $1 \times 10^5$ cells per ml. Following incubation for 24 h, the cells were then incubated in DMEM containing 100 or 250 μg/ml of unmodified or biotransformed green tea extract or EGCG. After 24 h of incubation, the comet assay was performed on the exposed cells. The cell positive control was the cells non-treated with the tea samples.

To detect DNA damage, the alkaline comet assay was performed on the cell suspensions using a modified version of the method described by Singh, Mccoy, Tice, and Schneider (1998). Briefly, 20 μl of the cell suspension was mixed with molten 0.5% low-melting-point agarose (Promega Co., Madison, WI, USA) and spread on agarose-prec Coated microscope slides. The slides were immersed overnight in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, 2% sodium salt N-lauryl sarcosine, pH 10, with 1% Triton X-100 and 10% dimethyl sulphoxide; all from Sigma–Aldrich) at 4 °C. After incubation, the slides were washed in cold PBS (Invitrogen Life Technologies) for 30 min. Subsequently, the cells were exposed to alkaline buffer (1 mM EDTA and 300 mM NaOH, pH 13.4) at 4 °C, for 40 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was then conducted in the same solution at 4 °C for 20 min at 25 V and 300 mA.

After electrophoresis, the slides were neutralised (0.4 M Tris, pH 7.5), stained with 40 μl EtBr (20 mg/ml) and analysed with a fluorescence microscope (Eclipse E400; Nikon, Melville, NY, USA), using the Komet 5.5 image analysis system (Kinetix Imaging, Nottingham, UK). One hundred randomly selected cells (50 from each of two replicate slides) were evaluated from each sample, and the mean olive Tail moment was determined. Tail moment (TM) is defined as the product of the fraction of the total DNA in the tail and the mean distance of migration in the tail and is calculated by multiplying tail intensity/sum comet intensity by the tail’s centre of gravity peak position. A higher percentage of tail DNA signifies a higher level of DNA damage.

2.11. Cancer-related gene expression analysis

Ninety-six-well culture dishes were inoculated with PG100 cells at a density of $10 \times 10^4$ cells per well. Four replicate wells were inoculated for each sample tested. After incubation at 37 °C, in an atmosphere of 5% CO₂ and 100% relative humidity for 24 h, cells were incubated in media containing pre-defined concentrations (from 50 to 250 μg/ml) of unmodified or biotransformed green tea extract or EGCG. Positive controls (untreated cells) were also performed. After incubation for 48 h, the cultures were assayed for cancer-related gene expression.

The cells were collected, and total RNA was isolated using an RNeasy® tissue kit (Qiagen). Single-stranded cDNA was synthesised using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s protocol. The Human Cancer PathwayFinder™ RT² Profiler™ PCR Array (SA Biosciences, Frederick, MD, USA) was used to evaluate cancer-related gene expression. This kit provides expression profiles of 84 genes representative of the six biological pathways involved in transformation and tumourigenesis. Quantitative PCR was performed using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and threshold cycle numbers were determined using RQ Study Software (Applied Biosystems). Reactions were performed in duplicate, and the threshold cycle numbers were averaged. The 50-μl reaction mixtures contained 25 μl of Platinum® SYBR Green Quantitative PCR SuperMix-UDG (Invitrogen™ Life Technologies, Alameda, CA, USA) and 100 ng of cDNA. The reactions were cycled with preliminary UDG treatment for 2 min at 50 °C and denatured for 2 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing for 15 s, and primer extension at 72 °C for 15 s. This was followed by a melting point analysis of the double-stranded amplicons consisting of 40 cycles with a 1 °C decrement (15 s each), beginning at 95 °C. The data were analysed using web-based PCR data analysis (SA Biosciences) and normalised against the expression of each tested gene in control cells.

2.12. Calculations and statistics

Values are expressed as arithmetic means. The statistical significance of the differences between the groups was analysed using the Tukey test. Differences were considered significant when $P < 0.05$. 
3. Results and discussion

3.1. Antioxidant activity

Various methods have been developed to characterise the total antioxidant capacity of biological fluids and natural products. One such method, the semi-automated ORAC protocol developed by Cao et al. (1996), has been extensively utilised in the field of antioxidant activity and oxidative stress.

Table 1 describes the antioxidant capacities of the samples (EGCG and green tea extract) before and after tannase treatment, as determined by the ORAC-FL and DPPH methods. For the ORAC assays, the linearity between the net AUC and the sample concentrations was determined for all compounds (Table 1). For each sample, the solutions with concentrations within the linearity range resulted in the same ORAC-FL values.

As described previously by Macedo et al., 2011, the results of the ORAC analyses (Table 1) indicate that the antioxidant capacity of the tea increased after tannase treatment. The tannase hydrolysed the substrates contained in the tea on the same way it did to the pure EGCG standard, and the products of hydrolysis apparently contributed to the observed increase in the tea’s antioxidant capacity. The antioxidant capacity of the green tea sample increased by 55% after enzymatic treatment. Similarly, biotransformation increased the antioxidant activity of the commercial EGCG by 46%. These results indicate that the tannase from P. variotii was able to hydrolyse the ester bonds of natural substrates. Epigallocatechin and gallic acid can be formed by the degalloylation of the gallate (epigallocatechin gallate) present in the tea extract (Fig. 1). According to Battestin et al. (2008) and Macedo et al. (2011), tannase can completely hydrolyse the epigallocatechin gallate in green tea to epigallocatechin and gallic acid by increasing the antioxidant activity of tea.

Table 1 also describes the antioxidant capacity of the EGCG and green tea extract before and after tannase treatment, as determined by the DPPH method. The DPPH assay has been used many times before to demonstrate the high antioxidant potential of green tea. Komes, Horzˇic, Belšcˇak, Ganic´, and Vulic ´ (2010) used DPPH, among other methodologies, to relate the elevated antioxidant capacity of green tea samples to their EGCG concentrations. The results in Table 1 indicate a trend toward increased radical-scavenging capacity after enzymatic hydrolysis. This trend was similar to the one observed in ORAC assays, supporting the results obtained by enzymatic treatment of the extracts.

Catechins (including epicatechins) with three hydroxyl groups in the B ring are known as gallatecinches, and those esterified to gallic acid at the 3-OH group in the C ring are known as catechin gallates (Fig. 1). With antioxidant activity governed broadly by the rule that the structures with the most hydroxyl groups exert the greatest antioxidant activity, the catechin-gallate esters reflect the contribution of gallic acid (Rice-Evans, Miller, & Paganga, 1996). Potential structure–activity relationships have been suggested by findings that the o-dihydroxy groups in the B-ring and the hydroxyl group in the C-ring are associated with the antioxidant properties of the flavonoids (Faria, Oliveira, Gameria, Santos-Budaga, & De Freitas, 2005).

3.2. Cytotoxicity and cell proliferation assay

The effects of EGCG on cellular growth have been extensively studied using MTT assays. The authors of some studies have interpreted the results of MTT assays to indicate that EGCG exerts anti-proliferative activity (Uesato et al., 2001); however, other authors have described these effects as a potentially dose-dependent toxic effect of this compound (Schmidt et al., 2005). In order to distinguish between cytotoxic and anti-proliferative effects and to compare the effects of compounds before and after biotransformation, we used the MTT assay to evaluate cytotoxic effects and the SRB assay to study anti-proliferative activity.

MTT assays were performed to assess the cytotoxicity of green tea extract and EGCG before and after biotransformation on the RAW 264.7 cells (Fig. 2a and b). The data in Fig. 2a reveal a trend toward a dose-dependent effect, with a small decrease in absorbance when higher concentrations of either unmodified or biotransformed green tea extract were used. At each concentration, no significant differences could be observed between the samples treated with tannase and the untreated samples. In contrast, biotransformation of EGCG eliminated the dose-dependent effect, as the absorbances remained constant for every concentration, with a small decrease compared to the positive control (Fig. 2b). In general, the absorbances of all samples tested varied less than 15% from those of the positive control. Changes of this magnitude are not indicative of cytotoxicity, but may instead indicate a decrease in cellular metabolism.

The results of the SRB assay are shown in Table 2. The rates of cellular proliferation in treated cultures are normalised to those of positive control cells. In agreement with previously published studies (Hwang et al., 2006; Jung et al., 2001; Yang, Liao, Kim, Yurkow, & Yang, 1998), the green tea extract demonstrated antiproliferative activity in HT29 cells; however, this antiproliferative activity was not observed in PG100 cells. Independent of the particular response of each cell line, the biotransformation of the green tea extract resulted in a higher degree of inhibition of cellular growth at almost every concentration tested in both cell lines.

Unmodified EGCG demonstrated a strong cytotoxic antiproliferative effect at a range of concentrations in PG100 cells. Interestingly, biotransformation of EGCG inhibited this cytotoxic effect without significantly affecting its antiproliferative activity. This finding points to potentially interesting avenues for future studies of cancer chemoprevention.

Table 1
Troxol equivalents and linearity ranges for the ORAC assay (net AUC vs. concentration) performed on the control and biotransformed samples and Troxol equivalents of the control and biotransformed samples by DPPH.

<table>
<thead>
<tr>
<th>Samples</th>
<th>ORAC</th>
<th>DPPH</th>
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<tbody>
<tr>
<td></td>
<td>Troxol equivalents (µmol/g dry sample)</td>
<td>Sample concentration range (mg/ml)</td>
</tr>
<tr>
<td><strong>Tea extract samples</strong></td>
<td></td>
<td></td>
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<tr>
<td>Green tea</td>
<td>4704 ± 138&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2–0.06</td>
</tr>
<tr>
<td>Green tea biotransformed</td>
<td>7356 ± 616&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2–0.06</td>
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<tr>
<td><strong>Commercial standard</strong></td>
<td></td>
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<tr>
<td>EGCG</td>
<td>11,735 ± 886&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2–0.02</td>
</tr>
<tr>
<td>EGCG biotransformed</td>
<td>17,182 ± 514&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2–0.01</td>
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</table>

<sup>a</sup>Results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different, with p < 0.05.
3.3. Determination of genotoxicity – comet assay

Studies by Morley et al. (2005) and Malhomme de la Roche et al. (2010) investigated whether ingestion of green tea by healthy human volunteers afforded any genotoxic protection to their circulating peripheral leukocytes upon experimental exposure to various amounts of UVR radiation. Both studies used the comet assay to determine the genotoxic protection potential of green tea on human cells and demonstrated that up to 90 min following green tea ingestion, there was a significant decrease (p < 0.05) in DNA damage (detected by alkaline single cell gel electrophoresis (the comet assay)) in peripheral leukocytes when they were subsequently exposed to 12 min of UVA/VIS irradiation.

In the present work, the comet assay was performed on cells treated with biotransformed or unmodified green tea extracts. These experiments demonstrated significantly reduced Tail Moment (TM) values when compared to positive control cells, demonstrating that green tea extract provided protection against DNA damage (Table 3). The TM data obtained for these samples were statistically smaller than the cell control. This is a clear indication of DNA damage protection capacity of the tested samples. The TM values appeared to be negatively correlated with the concentration of green tea extract and were slightly higher in samples treated with biotransformed extract than in samples treated with unmodified extract; however, TM values of all treated cell cultures were statistically similar and significantly smaller than those of control cell cultures.

Analysis of the TM values of samples treated with EGCG (Table 3) demonstrated that except for one condition (treatment with 250 μg/ml unmodified EGCG), all samples were statistically similar to the positive control sample. These results show that the tested concentrations of EGCG were not genotoxic, meaning that they did not induce any significant DNA damage in the tested cells. Biotransformation of EGCG with tannase did not alter these results.

3.4. Cancer-related gene expression analysis

In summary, our data show that unmodified and biotransformed green tea extracts and EGCG were neither cytotoxic nor genotoxic. Furthermore, we observed that the antioxidant and anti-proliferative capacities of these compounds were significantly increased by enzymatic intervention. Due to the potential cancer chemopreventive mechanisms of green tea and EGCG include protection (Malhomme de la Roche et al., 2010; Morley et al., 2005), inhibition of inflammatory processes, decreased angiogenesis, and antiproliferative/pro apoptotic effects (Shimizu et al., 2011; Yang & Wang, 2011), we used the Human Cancer Pathway Finder Array to evaluate the effects of unmodified and biotransformed green tea extract and EGCG on the expression profiles of 84 genes representative of the six biological pathways involved in transformation and tumorigenesis. Treatment with either unmodified or biotransformed green tea extract significantly changed the expression of 14% of the tested genes (12/84), whereas treatment with either unmodified or biotransformed EGCG altered the pattern of expression of 17% (14/84) of the genes. The statistically significant and biologically relevant results are shown in Table 4. The gene expression values presented were obtained by normalising expression levels to those observed in the control cells.

In relation to apoptosis and cell cycle control, our data showed that APAF1 (apoptotic peptidase activating factor 1), CASP8 (caspase 8, apoptosis-related cysteine peptidase), CDKN1A (cyclindependent kinase inhibitor 1A), and FAS (TGF receptor superfamily member 6) were up regulated by biotransformed green tea extract, unmodified EGCG and biotransformed EGCG. We also observed a down regulation of CDK2 and 4 (Cyclin-dependent kinase 2 and 4), bcl2 (B-cell CLL/lymphoma 2), bcl2L1 (BCL2-like-1), E2F1 (E2F transcription factor 1), and c-myc (V-myc myelocytomatosis viral oncogene homologue) (Table 4).

APAF1, CASP8 and CDKN1 are closely related to the caspase enzyme family. Some of these genes encode members of the caspase family of proteases, whereas others encode proteins responsible for caspase activation. In either case, these proteins contribute to the initiation of the caspase cascade that commits the cell to apoptosis (Gramantieri et al., 2005; Jones et al., 2011; Yang, Zhang, Peteklow, Ramoni, & Tsao, 2006). The protein encoded by the FAS gene is a member of the TNF-receptor superfamily. This superfamily includes FAS, CD40, CD27, and RANK. FAS contains a death domain, and the interaction of this receptor with its ligand allows the formation of a death-inducing signalling complex that includes Fas-associated death domain protein (FADD), caspase 8, and caspase 10. The autophagy process processing of the caspases in this complex triggers a downstream caspase cascade and leads to apoptosis (Idriss & Naismith, 2000). In addition, EGCG has been shown to cause G0/G1 cell cycle arrest and apoptosis of human epidermoid carcinoma cells (Ahmad, Feyes, Nieminen, Agarwal, & Mukhtar, 1997; Ahmad, Gupta, & Mukhtar, 2000). Furthermore, EGCG treatment of human epidermoid carcinoma cells resulted in induction of cyclin kinase inhibitors such as CDKN1, which through downregulation of cyclins D1 and D2 and cyclin-dependent kinases (cdk2, cdk4, and cdk6) causes G0/G1 cell cycle arrest, ultimately culminating in apoptotic cell death (Ahmad et al., 2000).

In agreement with these data, we demonstrate that all tested compounds decreased up regulation of CDKN1A and down regulation of cdk2 and cdk4.

Analysis of genes encoding members of the BCL-2 family showed that, although treatment with unmodified EGCG resulted in increased expression of the BCL2 (B-cell CLL/lymphoma 2) gene, treatment with biotransformed EGCG or biotransformed green tea extract suppressed the expression of this gene. In contrast, the only significant effect on the expression of the BCL2L1 (BCL2-like 1) gene was the suppression of its expression by the biotransformed green tea extract. These results showed the superiority of the biotransformed samples in down-regulating the expression of these genes, reducing the generation of BCL-2 proteins, which function in inhibiting apoptosis.

Leone et al. (2003) showed that green tea catechins are very potent inhibitors of the antiapoptotic Bcl-2 family proteins Bcl-xL and Bcl-2, suggesting a strong link between the anticancer activities of these tea polyphenols and their inhibition of a crucial antiapoptotic pathway. As this pathway has been implicated in the development of many human malignancies, the reduction of the expression of these genes is considered a pro-apoptotic function (Yang & Wang, 2011).

In addition, EGCG has been shown to induce apoptosis in S180 cells by altering the G2/M phase of the cell cycle through down-regulation of the oncogenes c-myc and bcl-2 (Manna, Banerjee, Mukherjee, Das, & Panda, 2006). Subsequently, Thyagarajan, Zhu, and Silva (2007) showed that EGCG suppressed the expression of the oncogene c-myc in breast cancer cells. Our findings demonstrate that all tested compounds significantly down regulated the expression of c-myc.

A key regulator of the G1/S phase transition in the cell cycle is the retinoblastoma (pRb) tumour suppressor protein (Nevins, Leone, DeGregori, & Jakoi, 1997). Members of the retinoblastoma family suppress cell growth, at least in part, by inhibiting E2F-dependent transcription of genes whose products are required for DNA synthesis and/or cell cycle progression (Nevins et al., 1997; Parreño, Garriga, Limón, Albrecht, & Graña, 2001). It has been shown that the anti proliferative and apoptotic effects of EGCG may be attributed to the down regulation of pRb and E2F1 proteins, which leads to the inhibition of cell cycle progression,
causing cell cycle arrest and subsequent apoptotic cell death (Ahmed, John, Vijayasarathy, Robin, & Raza, 2002). Although we did not detect down regulation of pRb expression, our data show a significant decrease in E2F1 expression under all tested conditions, suggesting that the inhibition of proliferation we observed could be partially related to this pathway.

In some cases, the modulation of the expression of genes of interest caused by unmodified EGCG was more pronounced than that achieved by the biotransformed compound; however, cell culture assays often ignore the bioavailability of the compounds in vivo. The literature shows that the systemic bioavailability of EGCG is a limiting factor for its effectiveness in cancer chemoprevention in internal organs (Yang & Wang, 2011). Orally ingested EGCG has limited systemic bioavailability, with most of it passing through the colon; and the absorbed EGCG is excreted mostly through the bile into the intestine (Yang & Wang, 2011). Studies

![Degalloylation of EGCG by the tannase from Paecilomyces variotii.](image1)

![Cytotoxicity by MTT assay in RAW 246.7 cells for various concentrations of the (a) Green tea extract and (b) EGCG, before and after tannase biotransformation.](image2)

**Table 2**

<table>
<thead>
<tr>
<th>Samples</th>
<th>PG 100</th>
<th></th>
<th>HT 29</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Samples concentration (µg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>100%</td>
<td>100%</td>
<td>97%</td>
<td>109%</td>
</tr>
<tr>
<td>200</td>
<td>100%</td>
<td>100%</td>
<td>97%</td>
<td>109%</td>
</tr>
<tr>
<td>150</td>
<td>100%</td>
<td>100%</td>
<td>97%</td>
<td>109%</td>
</tr>
<tr>
<td>100</td>
<td>100%</td>
<td>100%</td>
<td>97%</td>
<td>109%</td>
</tr>
<tr>
<td>50</td>
<td>100%</td>
<td>100%</td>
<td>97%</td>
<td>109%</td>
</tr>
<tr>
<td>Samples concentration (µg/ml)</td>
<td>250</td>
<td>200</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>Green tea</td>
<td>100%</td>
<td>100%</td>
<td>97%</td>
<td>109%</td>
</tr>
<tr>
<td>Green tea – biotransformed</td>
<td>69%</td>
<td>68%</td>
<td>85%</td>
<td>73%</td>
</tr>
<tr>
<td>EGCG</td>
<td>–45%</td>
<td>–50%</td>
<td>–48%</td>
<td>–53%</td>
</tr>
<tr>
<td>EGCG – biotransformed</td>
<td>77%</td>
<td>86%</td>
<td>86%</td>
<td>79%</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Samples</th>
<th>DNA damage in PG 100 cells (TM ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell positive control</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>Green tea (without tannase)</td>
<td>2.66 ± 0.20</td>
</tr>
<tr>
<td>Green tea biotransformed</td>
<td></td>
</tr>
<tr>
<td>Epigallocatechin gallate (without tannase)</td>
<td>1.08 ± 0.24</td>
</tr>
<tr>
<td>Epigallocatechin gallate biotransformed</td>
<td>1.86 ± 0.03</td>
</tr>
<tr>
<td>Epigallocatechin gallate biotransformed</td>
<td>1.29 ± 0.12</td>
</tr>
</tbody>
</table>

The values represent the mean ± standard deviation for TM of 100 cells in arbitrary units.

* p < 0.05, compared to the control group.
have shown that the serum levels of EGCG, EGC, ECG, and EC in rats 8 h after oral administration of green tea were 0.061, 0.440, 0.018 and 2.6 μM, respectively, demonstrating that the hydrolyzed forms of EGCG are more efficiently absorbed and present at higher concentrations in the serum (Lubet et al., 2007). Based on these findings, although biotransformed EGCG causes less up-regulation of apoptosis-related genes in vitro than unmodified EGCG, the biotransformed compound may be more effective in vivo.

4. Conclusion

Here, we have shown that the biotransformation of green tea extract and EGCG did not alter the beneficial properties of the original compounds (low genotoxicity, anti-proliferative activity, and up-regulation of pro-apoptotic genes) and improved their bioavailability.

The biotransformation of both green tea extract and EGCG significantly increased their antioxidant potential, as shown by the ORAC and DPPH assays. ORAC assays demonstrated that the antioxidant capacity of green tea extract increased by 55% after enzymatic treatment, and that of EGCG increased by 46%. MITT and SRB assays demonstrated that biotransformation did not render the compounds cytotoxic; instead, biotransformation reduced the toxicity of the EGCG sample without altering its anti-proliferative effects on the HT29 and PG100 cell lines. Furthermore, biotransformation increased the anti proliferative capacity of the green tea extract.

In relation to apoptosis and cell cycle control, our data showed that either native and biotransformed green tea and/or EGCG upregulated the expression of APAF1, CASP8, CDKN1A and FAS; on the other hand we observed a down regulation of CDK2 and 4, bcl2, bcl2L1, E2F1, and c-myc.

Importantly, this study has demonstrated the usefulness of the nutrigenomics perspective and tools in evaluating the benefits of biotechnological modifications of natural food molecules. Using this perspective, we have identified methods to improve the nutraceutical potential of one of the most widely consumed beverages – green tea.

Acknowledgements

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References


