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Effects of in vitro simulated digestion on the antioxidant activity of different *Camellia sinensis* (L.) Kuntze leaves extracts

Paolo Governa[1] · Fabrizio Manetti[1] · Elisabetta Miraldi[2] · Marco Biagi[2]

Abstract

The stability of tea phenolic compounds is influenced by pH value and digestive processes. However, the complex mixture of constituents in tea may modulate the stability of these compounds during digestion. In this study, tea infusions obtained from green, black, and Oolong tea leaves were exposed to in vitro simulated gastrointestinal digestion, and the stability of (+)-catechin, caffeine, (−)-epicatechin, epigallocatechin-3-gallate (EGCG), and gallic acid was compared to that of isolated compounds. Changes in antioxidant activity were also evaluated by means of DPPH assay and in a H_2O_2-induced in vitro oxidative stress model, using Caco-2 cells. The stability of teas antioxidant constituents was different when using teas extract, compared to the reference compound alone, with the total phenolic content being more stable in extracts containing them in higher amount. EGCG degradation correlated well with changes in the DPPH inhibition assay, confirming its pivotal role in the antioxidant activity of tea. Differently, the antioxidant effect in the in vitro cell-based model was much more related to the initial total phenolic content of the extracts, with green tea being more effective than black tea and Oolong tea. Moreover, the antioxidant activity of teas was strongly affected by gastrointestinal digestion. Taken together, these findings suggest a protective role of teas phytocomplex against gastrointestinal digestion of antioxidant constituents. In conclusion, the effect of gastrointestinal digestion on the antioxidant activity of tea should be taken into account, as this may be different from one extract to another and information on the stability of active constituents cannot be extrapolated from data obtained using single compounds.

Keywords *Camellia sinensis* (L.) Kuntze · Epigallocatechin-3-gallate · Simulated gastrointestinal digestion · Antioxidant activity · DPPH · Caco-2

Introduction

Oxidative stress is a major concern of modern medicine, and has been reported to cause harmful effects on cellular structures in human tissues, such as proteins, lipids, and nucleic acids [1–6]. These phenomena can lead to the onset of several diseases, including cancer, stroke, and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases and multiple sclerosis [7–10].

Camellia sinensis (L.) Kuntze leaves have been used for centuries to produce tea. Once harvested, differences in the manufacturing process of *C. sinensis* leaves lead to different kind of tea, with green, black, and Oolong tea being the most used worldwide [11]. Green tea is produced by using freshly harvested leaves, which are immediately steamed or pan-fried to prevent polyphenols oxidation, thus inhibiting fermentation. Differently, black and Oolong tea are typically produced using fully and semi-fermented withered rolled leaves, respectively [12, 13].

Tea is one of the most popular beverage rich in antioxidants and its bioactivity, together with that of its main constituents, has been observed in several in vitro and in vivo models [14–16].

The chemical composition of *C. sinensis* leaves has been deeply investigated, and is known to be influenced by leaf processing steps [17]. Among the bioactive components of tea, flavan-3-ols (catechins), a group of phenolic compounds,

✉ Paolo Governa
   paolo.governa@unisi.it

1   Department of Biotechnology, Chemistry and Pharmacy –
   Department of Excellence 2018-2022, University of Siena,
   Via Aldo Moro 2, 53100 Siena, Italy

2   Department of Physical Sciences, Earth and Environment,
   University of Siena, Strada Laterina 8, 53100 Siena, Italy

are the most abundant [18–20]. They can be found as flavonol monomers (i.e. catechin, epicatechin) and as flavonol gallates (i.e., epigallocatechin gallate) and their content varies during the fermentation process, due to oxidation or condensation reactions [21, 22]. Epigallocatechin-3-gallate (EGCG) is the most abundant flavan-3-ol in most kind of teas. In addition, the presence of other polyphenols, such as gallic acid, chlorogenic acid, ellagic acid, and flavonols has been reported, with gallic acid being the most common. Theaflavins are present in smaller amount in fermented teas [23]. Caffeine is another characteristic component of tea and is present in considerable amount, almost regardless of the kind of tea [24–26].

The effect of gastrointestinal digestion on the content of different tea polyphenols has been reported recently [27]. Moreover, the bioaccessibility and bioavailability of tea catechins are modulated by the co-administration of food and common beverage additives, such as citric acid, ascorbic acid, milk, and citrus juice [28, 29]. In addition, tea infusions and EGCG were found to modulate the stability during digestion of other food ingredients, such as soybean emulsion and starch [30–33]. In vitro simulated digestion was reported to influence the antimicrobial activity of green tea [34]. The changes in the secretion of cholecystokinin and glucagon-like peptide induced by green tea in vitro was also found to be modulated by simulated digestion [35]. Nevertheless, information regarding the effect of digestion on the bioactivity of tea, such as the antioxidant activity, is scarce. In 2015, Jilani and co-authors investigated the effect of biosorption of green and black tea polyphenols into *Saccharomyces cerevisiae* on their antioxidant activity before and after digestion, reporting an important loss of total phenolic content (TPC) and flavan-3-ols content [36]. The antioxidant activity, measured using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and expressed as Trolox equivalent antioxidant capacity, was reduced for green tea, and increased for black tea after in vitro simulated gastrointestinal digestion. The same research group used a similar approach to evaluate the effect of biosorption into *S. cerevisiae* on the antiproliferative activity of digested green and black tea in Caco-2 cells. Among the observed effects, a reduction of the production of reactive oxygen species was highlighted, even if no comparison between pre- and post-digested teas was performed [37]. Finally, in 2021, Chen and co-workers showed a decrease in the antioxidant capacity and TPC of nine commercially available tea juices after in vitro simulated digestion, using cell-free methods [38].

The complexity of tea composition may influence the stability of its constituents upon gastrointestinal digestion, thus affecting the antioxidant activity, and this effect can differ based on the kind of tea used.

To verify this hypothesis, we performed in vitro simulated gastrointestinal digestion on different *C. sinensis* leaves infusions and we evaluated the influence of digestion on the antioxidant activity of tea using a validated cell-free method and an in vitro model of intestinal epithelial cells.

Materials and methods

Sample preparation and chemical analysis

Green, black, and Oolong tea leaves were purchased from Erbamea (San Giustino, Perugia, Italy). Leaves (3 g) were extracted with 30 mL of ultrapure double-distilled water (ddH$_2$O) (Drug Extract Ratio, DER, 1:10) at 90 °C for 10 min. The extraction time was prolonged to 10 min to maximize the TPC and the extraction of flavan-3-ols [39]. Green tea extract (GTE), black tea extract (BTE), and Oolong tea extract (OTE) were then filtered, and their chemical profile was analyzed by means of HPLC–DAD, using a Shimadzu Prominence LC 2030 3D instrument. A Bondpak® C18 column, 10 μm, 125 Å, 3.9 mm × 300 mm (Waters Corporation, Milford, MA), was used as stationary phase. The mobile phase was composed of water with 0.1% V/V formic acid (A) and acetonitrile with 0.1% V/V formic acid (B), using the following gradient phases: from 10 to 25% in 15 min, from 25 to 35% in 3 min, and from 35 to 50% in 7 min. The flux was set to 0.8 mL/min and the injected volume was 10 μL. Absorbance was recorded at 280 nm and calibration curves using gallic acid, (+)-catechin, caffeine, (−)-epicatechin, and EGCG as reference standards (Sigma-Aldrich, Milan, Italy), ranging from 0.008 to 0.5 mg/mL (R^2 > 0.99), were used to quantify the amount of teas constituents.

The TPC was evaluated with the Folin-Ciocalteu method, as previously described [40].

In vitro simulated digestion

In vitro simulated digestion was carried out as previously described [41], with some modifications. Briefly, 1 mL of GTE, BTE, or OTE was diluted in 19 mL of simulated gastric juice, containing pepsin from porcine gastric mucosa (300 UI/mL, Sigma-Aldrich) and NaCl (10 mg/mL). The pH of the solution was adjusted to 1.7 using HCl. Gallic acid, (+)-catechin, caffeine, (−)-epicatechin, and EGCG were independently diluted in the same solution to the final concentration of 1 mg/mL. Samples were incubated for 2 h at 37 °C with shaking. Then, pancreatin from porcine pancreas (activity equivalent to 4 × U.S.P., 10 mg/mL, Sigma-Aldrich) and bile salts mixture (20 mg/mL, Sigma-Aldrich) were added, and the pH was increased to 7.0 by adding NaHCO$_3$ (15 mg/mL, Sodalco S.p.A., Corsico, Italy) to simulate the intestinal environment. Intestinal digestion was carried out for 2 h at 37 °C with shaking. Samples were then filtered and immediately used for further analysis.

The relative gastrointestinal stability was calculated as the % recovery of the sample after digestion, compared to the initial amount of sample used.

Antioxidant activity measured using a DPPH test

The radical scavenging activity of the pre- and post-digestion samples was measured by means of the DPPH assay, as previously reported [42]. Briefly, 10 μL of different concentrations (0.16–10 mg/mL) of the samples were added to 190 μL of freshly prepared methanolic DPPH solution (0.1 mM) and incubated for 30 min at rt in the dark with shaking. Then, absorbance was recorded at 517 nm using a Victor® Nivo™ plate reader (PerkinElmer, Waltham, MA). ddH$_2$O was used as the blank control. The antiradical activity of the samples was calculated according to the following formula:

$$\text{Antiradical activity \%} = \left(\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}\right) / \text{Abs}_{\text{blank}} \times 100.$$

Data were plotted using Microsoft Excel and the IC$_{50}$ (μg/mL) was determined for each sample.

Cell culture

Intestinal epithelial cells (Caco-2), a kind gift from Prof. Monica Montopoli (University of Padua, Italy) were cultured in 25 cm^2 flasks (Sarstedt, Milan, Italy) in high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 1% glutamine (Sigma-Aldrich) and 1% penicillin/streptomycin solution (Sigma-Aldrich). EDTA-trypsin (Sigma-Aldrich) solution was used for detaching cells from flasks, and cell counting was performed using a hemocytometer by Trypan Blue staining. Cell passage number was kept between 30 and 40.

Determination of antioxidant activity in H$_2$O$_2$-stimulated Caco-2 cells

Caco-2 cells (1×10^4) were seeded into 96-well plates and allowed to grow to confluence (70–80%). Cells were then pre-treated overnight with pre- and post-digestion GTE, BTE, and OTE (diluted 1:1000, according to previously performed cell viability assays) and oxidative stress was induced by administering 5 mM hydrogen peroxide (H$_2$O$_2$) for 6 h. Medium was then removed, and cells were washed three times with phosphate buffered saline (PBS). Then, 100 μL of Cell Counting Kit (CCK-8, Sigma-Aldrich) solution (1:100 in RPMI without phenol red) were added to each well and incubated for 1 h at 37 °C. Absorbance was recorded at 450 nm using a Victor® Nivo™ plate reader. Treatments were performed in six replicates, in three independent experiments, and cell viability was calculated by normalizing the absorbance of the test wells to the untreated control.

Measurement of trans-epithelial electric resistance

The efficiency of the intestinal barrier functions was evaluated by measuring trans-epithelial electric resistance (TEER) using a voltmeter [43]. Caco-2 cells (8×10^5) were placed in transparent polyester membrane cell culture inserts with 0.4 μm pore size (Sarstedt), and cultured in 24-well plates, as previously described [44]. Culture medium was replaced every other day. The integrity of the cell monolayers was monitored by measuring the TEER of the monolayer from day 14th to day 21st after seeding. When a stable value was reached, a 12 h pre-treatment was done by adding pre- and post-digested samples (diluted 1:1000) in the apical chamber in appropriate wells and TEER was measured after 0 and 12 h. Then, 0.5 mM H$_2$O$_2$ was added to the basal chamber and TEER was measured after 0, 4, 8, and 24 h. TEER measurements were performed in HBSS with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 mM D-glucose (pH = 7.4), after an equilibration period at rt, using a Millicell® ERS meter, (Millipore Corporation, Bedford, MA) connected to a pair of chopstick electrodes. Only cells with TEER value between 360 and 500 Ω cm^2 were used for the experiments [45, 46]. Treatments were performed in duplicate in three independent experiments and TEER was expressed as percentage of resistance, normalized to initial value.

DPPH-HPLC-DAD analysis

The DPPH-HPLC-DAD analysis was performed as described elsewhere [47]. Briefly, 100 μL of each sample were added to 300 μL of 10 mM DPPH solution and incubated for 30 min at rt in the dark, with shaking. Samples were then filtered and analyzed by means of HPLC-DAD, using the same chromatographic conditions described in the "*Sample preparation and chemical analysis*" paragraph.

Statistical analysis

The statistical differences between the biological results were determined by the analysis of the variance (ANOVA). Pearson linear correlation test was used to analyze the correlation between TPC and DPPH. Values are expressed as mean values ± standard deviation, and the differences between the means were considered statistically significant at $p < 0.05$. Graphs and calculations were performed using GraphPad Prism.

Results and discussion

Chemical analysis

Gallic acid (retention time (RT) = 4.5 min), (+)-catechin (RT = 7.2 min), caffeine (RT = 8.3 min), and EGCG (RT = 10.4 min) were identified in each sample by comparison of their RT and UV spectra with those of reference standards. (−)-Epicatechin (RT = 9.0 min) was quantifiable only in GTE.

The chemical composition of the samples is reported in Table 1. BTE and OTE contain comparable amount of gallic acid and (+)-catechin, whereas, ECGC was significantly higher in BTE. The amount of gallic acid and (+)-catechin was lower in GTE, compared to the other two samples. This is partially consistent with the results of Sun and co-authors, who observed lower amount of gallic acid and higher levels of (+)-catechin in green tea, in comparison to black and Oolong teas [48]. On the contrary, a significantly higher content of EGCG was found in GTE, compared to the other samples. (−)-epicatechin was quantifiable only in GTE, whereas, caffeine content was comparable in all the samples. The higher amount of EGCG in GTE, compared to BTE, is consistent with the data reported by Tenore and co-authors, who, on the contrary, observed higher amount of (+)-catechin in green tea, compared to black tea [49]. However, in a more recent work, the same research group reported lower amount of (+)-catechin in green tea, compared to black tea, which is coherent with our data [27].

Several other flavanols could be found in tea extracts, and their amount and number may vary depending on the extraction method used. In this work, we focused on the main antioxidant constituent (i.e., EGCG), which is present in all the studied extracts, and its main parent compounds and metabolites. As caffeine is also present in high amount in all the samples, and it belongs to a different class of polyphenols, we decided to include it in this study.
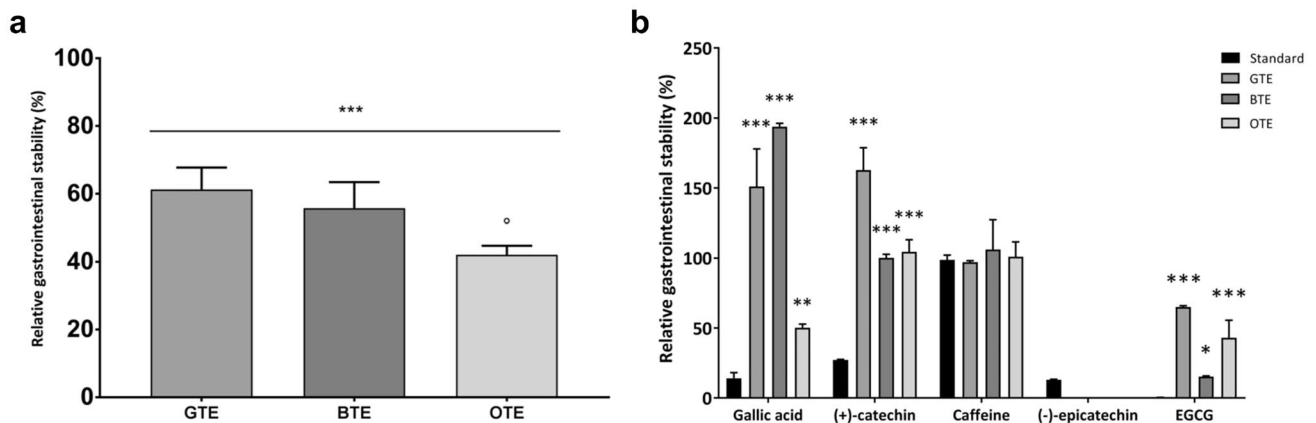
Stability of tea constituents under in vitro simulated digestion

Stability of tea constituents resulted to be significantly influenced by in vitro simulated digestion (Table 2). Indeed, the recovery of flavan-3-ols, tested as single reference standards, after the digestion process was very low, with EGCG being the most affected among the three flavan-3-ols tested. These data are consistent with that obtained by Krook and Hagerman, who observed that, despite being stable at acidic pH similar to that of the stomach, 90% of EGCG was decomposed after intestinal digestion [50]. Similarly, Yoshino and co-authors found that 80% of EGCG was already decomposed after 5 min in authentic intestinal juice [51]. In our work, we employed longer incubation time than that used in these two studies (i.e., 2 h of intestinal digestion phase, compared to 1 h and 5 min, respectively), which explains a much more evident reduction of EGCG. Interestingly, (−)-epicatechin was reported to be more stable than EGCG at pH = 7 [52], with a recovery of 90% after 17 h. We found that, although being higher than that of EGCG, the recovery of (+)-catechin and (−)-epicatechin after the whole gastrointestinal digestion process were 27.29% and 13.13%, respectively. Gallic acid recovery was also low and comparable to that of (−)-epicatechin. Caffeine was completely recovered, suggesting a very high stability to gastrointestinal digestion.

The TPC recovery was also significantly reduced by the gastrointestinal digestion, with GTE showing the highest stability (Fig. 1a). Indeed, the relative gastrointestinal stability of GTE, BTE, and OTE was $61.29\% \pm 6.45\%$, $55.77\% \pm 7.69\%$ and $42.11\% \pm 2.63\%$, respectively, with

Table 2 Relative gastrointestinal stability of reference standards. Values (%) are expressed as mean ± standard deviation

Chemical classification	Sample compound	Gastrointestinal stability
Flavan-3-ols	(+)-catechin	27.29 ± 0.49
	(−)-epicatechin	13.13 ± 0.24
	EGCG	0.25 ± 0.09
Phenolic acids	Gallic acid	14.16 ± 3.98
Xanthines	Caffeine	98.77 ± 4.87

Table 1 Chemical characterization of the extracts

Sample	TPC	Gallic acid	(+)-catechin	Caffeine	(−)-epicatechin	EGCG
GTE	6.23 ± 0.11[a]	0.25 ± 0.01[a]	0.28 ± 0.01[a]	1.68 ± 0.01[a]	1.20 ± 0.01[a]	2.30 ± 0.04[a]
BTE	5.21 ± 0.29[b]	0.57 ± 0.02[b]	0.47 ± 0.02[a]	1.70 ± 0.03[a]	n.d.[b]	1.56 ± 0.04[b]
OTE	3.84 ± 0.53[c]	0.51 ± 0.02[a,b]	0.37 ± 0.01[a]	1.58 ± 0.20[a]	n.d.[b]	0.22 ± 0.01[c]

Values (mg/mL) are expressed as mean ± standard deviation

Significant differences ($p < 0.05$, two-way ANOVA, followed by Tukey's post-hoc) are indicated within each compound using lowercase letter notation

Fig. 1 a Relative gastrointestinal stability of GTE, BTE, and OTE TPC. ***$p < 0.001$ vs. reference standard; ° $p < 0.05$ vs. GTE; one-way ANOVA followed by Dunnett's post-hoc. **b** Relative gastrointestinal stability of GTE, BTE, and OTE main constituents. *$p < 0.05$ vs. reference standard; **$p < 0.01$ vs. reference standard; ***$p < 0.001$ vs. reference standard; two-way ANOVA followed by Sídàk's post-hoc

OTE recovery being significantly lower in comparison to GTE. Jilani and co-authors reported that the TPC of green and black teas after digestion was 85% and 96% of the initial value, respectively [36]. While the higher reduction of TPC observed by us could be related to differences in the applied model of simulated digestion, the increased stability of black tea TPC, compared to green tea is not consistent with our results. This could be due to differences in the chemical composition of BTE, compared to the black tea used by these authors, which is possibly related to the use of black teas from different sources. Indeed, Annunziata and co-authors obtained a higher duodenal bioaccessibility of green tea TPC (24%), compared to black tea TPC (13%), using HPLC-DAD for quantification [27].

Interestingly, teas phytocomplex protected flavan-3-ols and gallic acid from the degradation (Fig. 1b). Consistently with the study of Tenore and co-workers, the stability of (+)-catechin, (−)-epicatechin, and EGCG was higher in GTE, compared to BTE [49]. The higher percentage recovery of flavan-3-ols in GTE and BTE obtained in our work could be due to differences in the simulated digestion model used and in the chemical composition of the samples. Indeed, (+)-catechin recovery after simulated digestion was approximately 100% in BTE and OTE and approximately 160% in GTE. (−)-Epicatechin was not found in BTE and OTE, thus, its stability to digestion was not quantified in these extracts. However, (−)-epicatechin completely disappeared after simulated digestion of GTE. A possible explanation of the increased recovery of (+)-catechin in digested GTE may be related to the partial degradation of flavan-3-ols oligomers and conversion of (−)-epicatechin to catechin, which may occur in aqueous solution as a consequence of temperature and pH changes [53, 54]. The gastrointestinal stability of EGCG was dependent on the

tea sample, suggesting that different phytocomplexes may specifically influence its recovery after digestion. Indeed, EGCG recovery was approximately 65%, 15%, and 42% in GTE, BTE, and OTE, respectively, with the recovery from BTE being significantly lower compared to GTE and OTE. Interestingly, the relative recovery of EGCG contained in the test teas was always significantly higher compared to the reference standard alone. Similarly, gallic acid stability to digestion was different for each sample, with BTE resulting in the highest recovery and OTE in the lowest. The increased recovery of gallic acid in teas, compared to the reference standard alone, may result from the hydrolysis of the galloyl moiety from galloyl catechins such as catechin-gallate, gallocatechin-gallate, epicatechin-3-gallate, and EGCG [48, 50]. Consistently with this explanation, in fact, the recovery of gallic acid was higher in BTE, and was accompanied by a lower recovery of EGCG. Caffeine recovery was around 100% in each tea sample, similarly to its reference standard. Interestingly, caffeine was reported to modulate the oral bioavailability of EGCG in humans: beverages containing higher amount of EGCG than caffeine showed higher EGCG bioavailability compared to those containing higher amount of caffeine than EGCG [55]. These observations are consistent with our results in which GTE, which contained more EGCG than caffeine, showed a higher gastrointestinal stability of EGCG in comparison to BTE, which contained similar amount of EGCG and caffeine, and to OTE, which contained more caffeine than EGCG.

One limitation of this study is represented by the in vitro simulated digestion model used. Indeed, differences in the results obtained using variations of this method are common across different laboratories, highlighting the need for a unified method. An attempt to standardize the in vitro static digestion method is being carried out by the INFOGEST

COST action, which is continuously updating the guidelines for setting up a standardized static digestion model suitable for foods analysis [56, 57]. Although this method was not used in the present work, our results are consistent with those obtained by other research groups, suggesting the reliability of our approach.

The antioxidant activity of teas is reduced by digestion

The DPPH assay was used as a fast and simple method to evaluate the effect of gastrointestinal digestion on the antioxidant activity of teas. The radical scavenging potential of pre-digestion samples (expressed as IC_{50} values in Table 3) was less than 600 µg/mL, with GTE being the most active. Simulated digestion significantly increased the IC_{50} values of GTE, BTE, and OTE by 2.3-, 9.6-, and 5.5-fold, respectively, thus suggesting a negative effect of digestion on antioxidant potential. When comparing the antioxidant activity of green, black, and white teas using the DPPH assay, Annunziata and co-authors found that in vitro simulated digestion had a stronger negative effect on black tea radical scavenging activity, compared to green tea, which is consistent with our results [27].

The Pearson's linear correlation test between the TPC and DPPH of the samples after gastrointestinal digestion showed a good, though not statistically significant, correlation ($r = 0.99$, $p = 0.062$). Similarly, a strong correlation ($r = 0.99$, $p = 0.071$) was found between the DPPH/TPC ratio of the undigested and digested samples. The high p values obtained despite the good r values are likely dependent on the sample size used ($N = 3$).

By combining pre-column DPPH assay with HPLC–DAD analysis, it is possible to observe which of the identified teas constituent is predominantly involved in the radical scavenging activity of the samples. Indeed, upon reaction with DPPH, the peak area of the anti-radical compounds should decrease or disappear in the HPLC-DAD chromatogram [47, 58, 59]. Figure 2 shows the complete disappearance of peaks area corresponding to gallic acid, (+)-catechin, (−)-epicatechin, and EGCG, thus suggesting their pivotal role for the

antioxidant activity of teas. Differently, caffeine peak was smaller but still present in the post-DPPH, compared to the pre-DPPH chromatogram (approximately −25% of the initial peak area).

In GTE, only EGCG recovery was significantly lower after simulated digestion, compared to the other tested constituents. This result, combined with the increased level of gallic acid and (+)-catechin, may account for the relatively small increase in DPPH IC_{50}. EGCG stability in BTE was the most affected by simulated digestion among the three different samples. This is consistent with the increase in DPPH IC_{50}, which is the highest, compared to the other teas. OTE can be distinguished from the other teas, because of the strong reduction of gallic acid levels after simulated digestion. EGCG, however, was more stable in OTE, compared to BTE, thus explaining why the DPPH IC_{50} was less affected, in terms of fold changes, compared to BTE, but more affected compared to GTE.

Caco-2 cells have been used as a stable and reliable model of intestinal epithelium [60, 61]. 5 mM H_2O_2 induced a significant oxidative damage to Caco-2 cells, reducing cell viability by approximately 20% compared to the untreated control after 6 h. The pre-treatment with GTE, BTE, and OTE significantly protected Caco-2 cells from the cytotoxic effect of H_2O_2, with GTE being the most effective extract. The antioxidant activity, in terms of Caco-2 protection, of GTE after in vitro digestion was slightly reduced compared to the pre-digestion sample, although the difference was not statistically significant. On the contrary, BTE and OTE activity was significantly reduced after simulated digestion, with OTE completely losing its protective effect (Fig. 3a). Consistently, the prolonged stimulation with lower concentration of H_2O_2 (0.5 mM) caused a time-dependent alteration of the intestinal barrier integrity, with TEER values being reduced by approximately 45% after 24 h. GTE, BTE, and OTE significantly reduced the oxidative damage provoked by H_2O_2, with similar effectiveness compared to the cell viability test. The protection from the H_2O_2-impaired intestinal barrier integrity of each sample was reduced after simulated digestion, even if statistical significance was obtained only for OTE (Fig. 3b).

Contrarily to the DPPH assay, the results obtained in the cell-based experiments suggest that the reduction of Caco-2 protection from the oxidative damage is not directly dependent on the degree of stability of teas main constituents alone, but rather depends on the initial amount of antioxidant constituents (i.e., TPC) in the samples. Indeed, in the cell viability assay, the post-digestion antioxidant activity was reduced by 7%, 8%, and 10% in GTE, BTE, and OTE, respectively, compared to the pre-digestion samples. Moreover, in the TEER experiment, the post-digestion protection of the intestinal barrier integrity was reduced by 14%, 7%, and 23% in GTE, BTE, and OTE, respectively, compared to

Table 3 IC_{50} values (µg/mL) resulting from DPPH test performed before and after in vitro simulated digestion

Sample	Pre-digestion	Post-digestion
GTE	74.5 ± 8.4^a	$176.0 \pm 14.1^{a\,*}$
BTE	122.8 ± 12.3^a	$1176.2 \pm 151.1^{b\,***}$
OTE	597.1 ± 56.3^b	$3277.2 \pm 19.7^{c\,***}$

Significant differences ($p < 0.05$, two-way ANOVA, followed by Sídàk's post-hoc) are indicated within each digestion phase using lowercase letter notation. $*p < 0.05$ vs. pre-digested; $***p < 0.001$ vs. pre-digested, two-way ANOVA, followed by Tukey's post-hoc
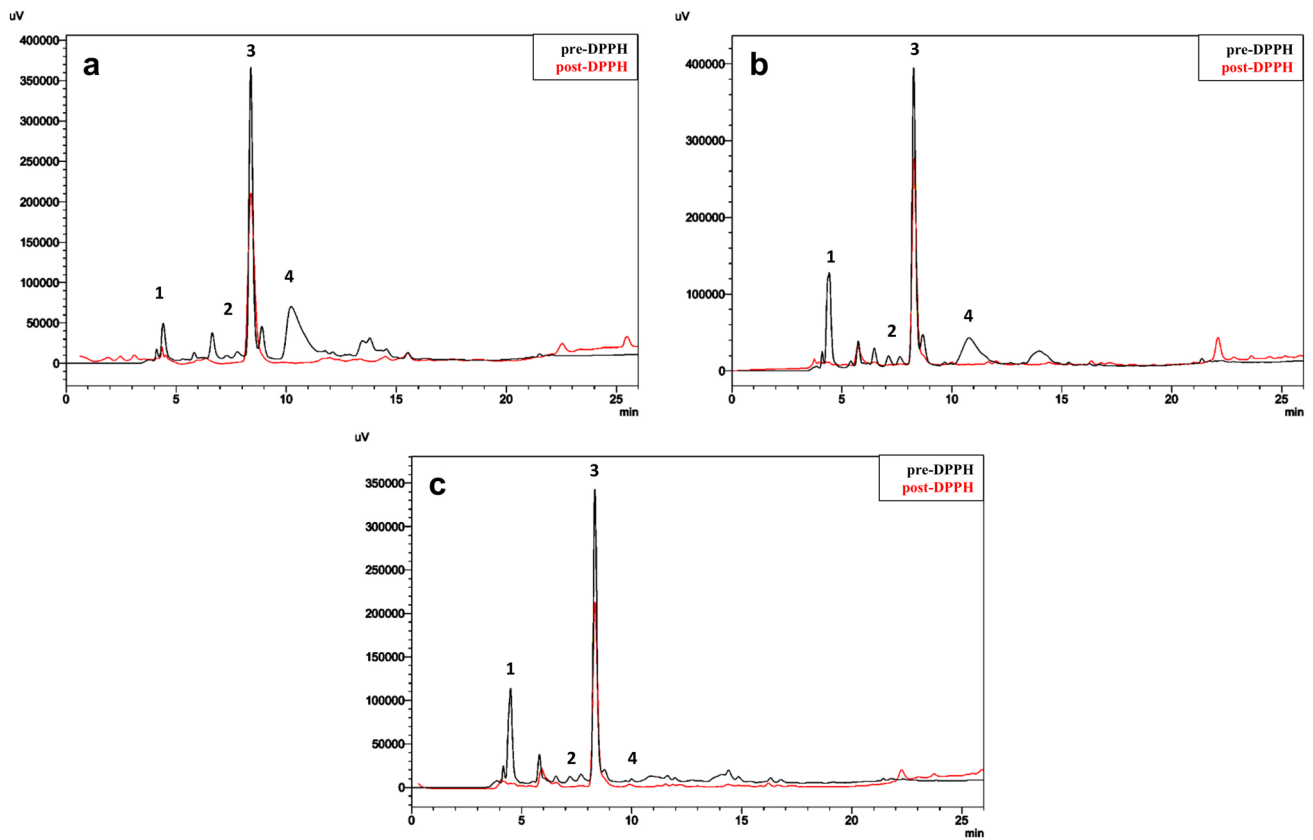
Fig. 2 HPLC-DAD chromatograms of **a** GTE, **b** BTE, and **c** OTE, before (black) and after (red) DPPH addition, showing the almost complete oxidation of flavan-3-ols and gallic acid. Caffeine is still present in significant amount. 1: gallic acid; 2: (+)-catechin; 3: caffeine; 4: EGCG
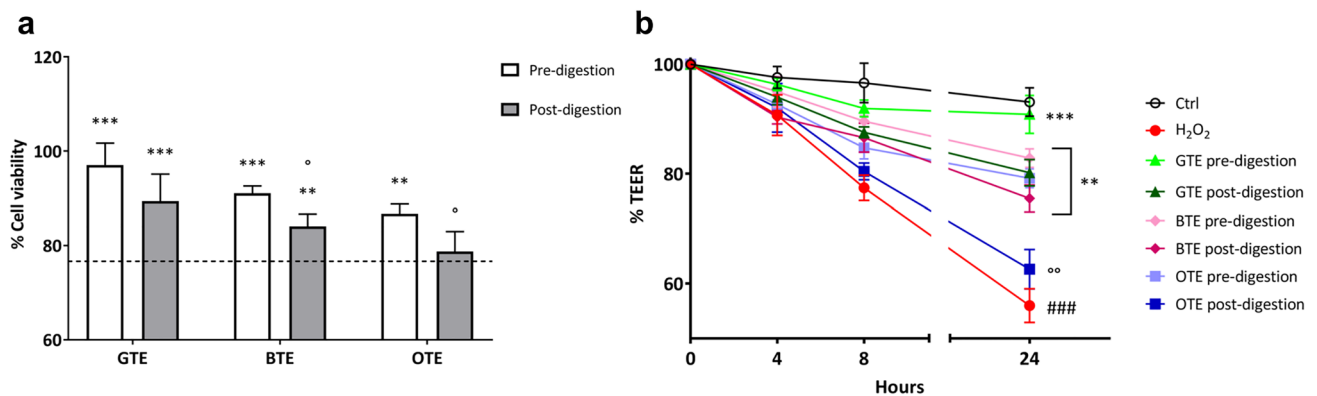
Fig. 3 Effect of simulated digestion on the antioxidant activity of GTE, BTE, and OTE in Caco-2 cells. **a**: Protection from H_2O_2-impaired cell viability. Data are expressed as percentage of cell viability compared to untreated cells. The effect of H_2O_2 is represented by the dashed line. **b**: Protection of the intestinal barrier function observed by transepithelial electrical resistance measurement. $^{###}p < 0.001$ vs. untreated control; $^{**}p < 0.01$ vs. stimulus; $^{***}p < 0.001$ vs. stimulus; $^{°}p < 0.05$ vs. pre-digestion sample; $^{°°}p < 0.01$ vs. pre-digestion sample; two-way ANOVA followed by Dunnett's post-hoc

the pre-digestion samples. To the best of our knowledge, this is the first study evaluating the effects of in vitro simulated digestion on the antioxidant activity of different teas, using the H_2O_2-impaired Caco-2 cell viability model. Using the

same model, Song and Gao reported the protective effect of Fuzhuan brick-tea, without performing gastrointestinal digestion, and found that this protective effect was related to a reduction of lipid peroxidation and to the increase of

glutathione and antioxidant enzymes [15]. However, information on the chemical composition of the used tea was not reported, making their results difficult to compare with ours.

Conclusion

Phenolic compounds are a major class of phytochemicals with known antioxidant properties. In this study, the effect of in vitro simulated gastrointestinal digestion on the antioxidant effect of three different *C. sinensis* extracts was evaluated. The stability of teas main constituents was influenced by the initial extract composition, with TPC being more stable when present in higher amount. EGCG degradation correlated well with changes in the DPPH inhibition assay, thus confirming its pivotal role in the antioxidant activity of tea. Differently, the antioxidant effect in the in vitro cell-based model was much more related to the initial TPC content of the extracts, with green tea being more effective than black tea and Oolong tea. Overall, gastrointestinal digestion strongly affected the biological effectiveness of teas. Interestingly, the stability of teas antioxidant constituents was different when using teas extract, compared to the reference compound alone, thus suggesting a protective role of the phytocomplex, which could lead to increased biological effectiveness. In conclusion, the effect of gastrointestinal digestion should be considered when evaluating the biological properties of herbal extracts, as this may be different from one extract to another and information on the stability of active constituents during the digestion process cannot be extrapolated from data obtained using single compounds.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00217-021-03864-1.

Declarations

Conflict of interest The authors report no conflict of interest.

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