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Phytochemical characterization and biological activities of green tea (*Camellia sinensis)* produced in the Azores, Portugal

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ABSTRACT

Background: Green tea is not only one of the most widely consumed beverages worldwide, but is also known for its health promoting and therapeutic effects. Green tea is cultivated in areas with high humidity and acidic soils in China, Indonesia and Japan. Those places have well-marked dry and rainy seasons. In opposite, Azores have a climate with constant average annual rainfall and, unlike eastern regions, relatively constant air humidity throughout the year. While a brand implemented on the Portuguese market, the quality of green tea produced in Azores must be guaranteed. Quality control measures based on phytochemical determination of the chemical composition and biological activities are needed in order to address whenever climate changes interferes significantly with composition and biological effects.

Purpose: Make the phytochemical characterization of various extracts of green tea leaves coming from Azores and evaluate the anti-cancer activities of the extracts in order to compare the obtained results with those of teas coming from eastern regions.

Methods: Phytochemical characterization (catechins, oxyaromatic acids, flavonols, alkaloids and theanine) and total catechins contents (TCC) was performed by using HPLC-DAD analysis, in infusions (5–7 min and 30 min), maceration and methanolic extracts of *Camellia sinensis* samples coming from Azores, Portugal. The antioxidant activity of extracts was measured by the DPPH assay and the total phenolics contents (TPC) were estimated using the Folin-Ciocalteu colorimetric method. The cytotoxic activity towards drug sensitive and multidrug-resistant leukemia cell lines was determined by the resazurin assay.

Results: The TCC was higher in methanolic extracts and lower in maceration, as epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) concentrations were significantly higher in methanolic extracts and were only residual in maceration extracts. Maceration extracts showed the highest content of gallic acid, indicating that methanol extracts contained more flavonols of higher molecular weight and/or that maceration may lead to the degalloylation of catechins. The amount of *o*-caffeoylquinic acid extracted was significantly higher in methanolic samples. Short-term extraction at high temperatures resulted in high amounts of neochlorogenic acid. The contents of glycosylated quercitin-3-D-galactoside and kaempferol-3-glucoside were small in maceration samples and high in methanolic samples. Caffeine was easily extracted by methanol (99%) compared with water, while extraction of the amino-acid L-theanine was impossible with methanol. TPC values correlated linearly with DPPH• IC_{50}, with infusion samples showing the best antioxidant capacities. The aqueous and the methanol/water extracts were active in multidrug-resistant and drug sensitive cancer cells.

Abbreviations: BHT, butylated hydroxytoluene; C, (+)-catechin; DPPH•, 1.1-diphenyl-2-picrylhydrazyl; EC, (-)-epicatechin; ECG, (-)-epicatechin gallate; EGC, (-)-epigallocatechin; EGCG, (-)-epigallocatechin gallate; GA, gallic acid; GAE, GA equivalent; HPLC-DAD, high-performance liquid chromatography with diode-array detection; SPME-GC, solid phase microextraction-gas chromatography; IC_{50}, half maximal inhibitory concentration; K-3G, kaempferol-3-glucoside; NCA, neochlorogenic acid; *o*-CQA, *o*-caffeoylquinic acid; Q-3 Gal, quercitin-3-D-galactoside; SD, standard deviation; TCC, total catechins content; TPC, total phenolics content.

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Conclusion: Despite these climatic differences, the phytochemical characterization revealed that tea coming from the Azores compares well with those from China and Japan. In addition, tea can be produced without fungicides and pesticides to control pests that appear during rainy seasons. This allows the production of high-quality bioorganic tea. Aqueous and methanol/water extracts of the Azorean tea showed to be useful targeting sensitive and drug-resistant tumor cells.

Introduction

The health benefits of consuming green tea in prevention of lifestyle-related diseases are well reported (Sueoka et al., 2001; Naveed et al., 2018), including prevention of several types of cancer (Yang et al., 2007; Kavanagh et al., 2001; Geybels et al., 2013; Yang et al., 2011) cardiovascular diseases (Yang and Zhang, 2019; Yin et al., 2017; Peng et al., 2014) and arthritis (Haqqi et al., 1999). Prevention effects in diabetes (Yang et al., 2014; Jiao et al., 2015) and in other metabolic diseases (Osada et al., 2001; Basu et al., 2013, 2010) have been also reported.

Green tea (*Camellia sinensis* (L.) Kuntze (Theaceae)) is produced in São Miguel Island Azores, 37° 46′ 49.48″ N 25° 29′ 49.369″ W, Portugal since the 19th century. The tea culture was introduced in Azores around 1820 with seeds brought from Rio de Janeiro. In 1883, professional tea cultivation was implemented by local associations of farmers, who succeeded in bringing more seeds from China (Macau), as well as Chinese technicians, who taught traditional techniques of tea manufacturing (de Melo, 2012). The regional variations of tea quality can be attributed to genetic diversity and its interaction with the environment (Sabhapondit et al., 2012). Pedoclimatic (soil type, sun exposure, rainfall) or agronomic (culture in greenhouses or fields, biological culture, hydroponic culture) factors influence the contents of catechins (Manach et al., 2004). São Miguel has a temperate climate that is mostly free of rainy and dry seasons. As common plant pests such as mosquitoes, tea fly, red spider and others do not appear under these specific climatic conditions in the Azores, *C. sinensis* is cultivated in Azores without herbicides, fungicides or pesticides. Furthermore, only vegetable manure is used (https://gorreana.pt/pt/). Therefore, it is pertinent to consider that the unique quality of *C. sinensis* planted in the Azores may be characterized by a specific profile of secondary metabolites concerning type and quantity of substances.

For this reason, we analysed several extracts (infusions, maceration and methanolic) of green tea cultivated in the Azores in the present investigation. The chemical analysis included the quantification of total phenolics, five flavanols, two flavonols, two alkaloids, three oxyaromatic acids and the amino-acid L-theanine (Fig. 1). The quantitative and qualitative compositions of the extracts were determined by HPLC-DAD. Radical scavenging activities were also measured. In addition, resazurin reduction assay was performed, in order to evaluate the cytotoxic activity of the green tea extracts towards sensitive and multidrug-resistant leukemia cell lines.

Materials and methods

Solvents, reagents and standards

Certified commercial samples of green tea leaves were purchased on the Portuguese market. Samples of different batches were blended for extraction. All solvents and reagents were of the highest purity. Methanol (LC-MS) and formic acid (p.a.) were obtained from Fluka Analytical (Munich, Germany), acetonitrile (LC-MS) from Sigma-Aldrich (Steinheim, Germany). The Folin-Ciocalteu reagent was from Merck (Darmstadt, Germany). Butylated hydroxytoluene (BHT) and DPPH$^{\bullet}$ (1.1-diphenyl-2-picrylhydrazyl) were purchased from Sigma-Aldrich Reference standards of L-theanine (>99%), caffeine (>99%) and (-)-epigallocatechin gallate (EGCG > 99%). were purchased from Sigma-Aldrich. Water processed by a Milli-Q purification system (Simplicity-UV. Millipore Corp. City France) was used for sample preparation and analysis.

All standards were prepared as stock solutions in methanol or water (theanine). Working standards were made by diluting stock solutions to yield concentrations ranging between 5 μg/ml and 10 mg/ml. Stock/working solutions of the standards were stored in darkness at −18 °C.

Extraction methods

It has been reported that alkaloid and catechin contents in green tea vary with different plucking periods (Lee et al., 2014). In order to diminish the differences in chemical composition, the material used for extraction was gathered after blending tea samples from different batches in accordance to the corresponding guideline of European Medicines Agency (EMA, 2006).

Several extraction methods were adopted, *i.e.* **A1** for hot infusion (5–7 min); **A2** for hot infusion (30 min); **A3** for maceration (48 h); and **A4** for methanolic extraction. All powdered extracts were kept frozen (−18 °C) until further use.

Extraction method **A1**. Hot tea infusions were prepared for samples by pouring purified water at 90 °C on 1.5 g green tea and brewed for 5–7 min. according to the recommendations of the manufacturer. This procedure allows to have a quantification of the amount of catechins and theanine existing in a normal cup of beverage. The infusions were then filtered through Whatman membrane filters 0.45 μm, lyophilized and stored. The pH of the extracted aqueous solutions was less than 5.0.

Extraction method **A2**. Hot tea infusion was prepared by pouring purified water at 80 °C on 1.5 g green tea and brewed for 30 min as suggested by Vuong et al. (2011). They were then filtered through Whatman membrane filters 0.45 μm, lyophilized and stored.

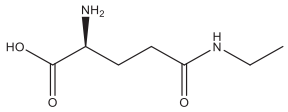
Extraction method **A3**. Green tea (1.5 g) placed in a 250 ml Schott flask, and 100 ml water were added. The flask was covered, and the plant was macerated for 48 h at room temperature in the dark. This allows to study the effect of long-time term extraction on the chemical composition. The water fraction was removed by filtration through Whatman membrane filters mixed cellulose ester 0.45 μm, lyophilized and stored. The pH was kept below 5, because the pH affects the catechin contents due to decomposition (Komes et al., 2010). At pH 6–7, the epi-structured catechins can be partially epimerized to non-epi-structured catechins and both groups may be degraded at pH > 9.

Extraction method **A4**. Methanolic extracts were prepared with dried plant material (1.5 g) that was thoroughly mixed with methanol (99%), (3 × 50 ml) for 30 min. Collected extracts were filtered and centrifuged (3000 rpm, 15 min) and the solvent was evaporated. Although a polar solvent, methanol has a lower dielectric constant than water. Thus, it is expected to be more efficient to extract high molecular weight polyphenols or substances that are not present as halo-hydrates. Some authors found that methanol was less efficient for flavonol extraction than water and used methanol mixtures (50:50 or 70:30) as extraction solvent (Xu and Chang, 2007).

High-performance liquid chromatography with diode-array detection (HPLC-DAD)

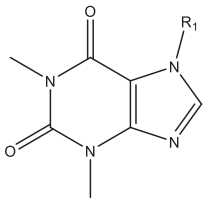
An analytical HPLC unit (Thermo Scientific Dionex Ultimate 3000) equipped with a C18 column (15.0 × 0.46 cm; particle size) from Supelco was employed. The solvent system used was a gradient of water–formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15% B at 2 min, 25% B at 8 min, 30% B at 15 min, 35% B at 21 min, 45% B at 23 min, 45% B at 25 min, 50% B

Aminoacids



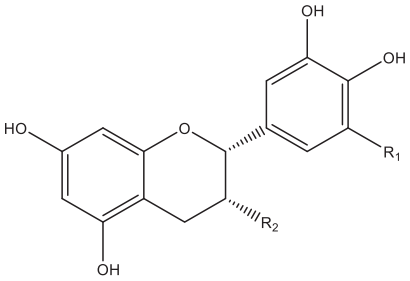
L-Theanine

Alkaloids

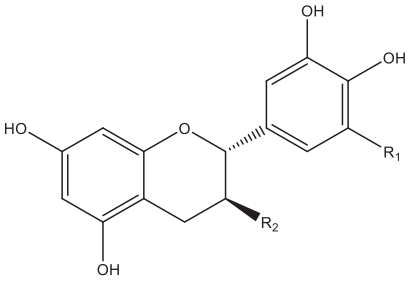


Theophiline	R_1	= H
Caffeine	R_1	= -CH3

Catechins



(+)-Catechin (C)	R_1 =	- H
	R_2 =	- OH



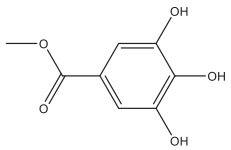
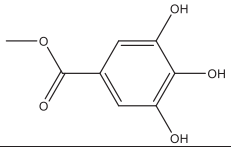
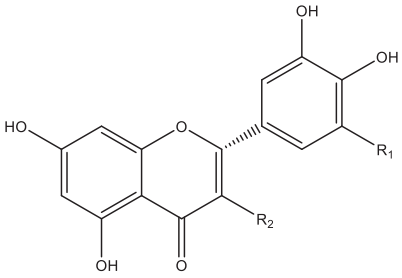
(-)-Epicatechin (EC)	R_1 =	- H
	R_2 =	- OH
(-)-Epigallocatechin (EGC)	R_1 =	- OH
	R_2 =	- OH
(-)-Epicatechin gallate (ECG)	R_1 =	-H
	R_2 =	
(-)-Epigallocatechin gallate (EGCG)	R_1 =	- OH
	R_2 =	

Fig. 1. Chemical structures secondary metabolites identified in extracts of green tea cultivated in the Azores.

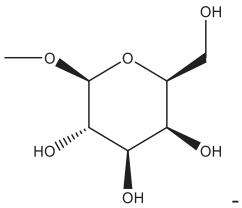
Flavonols



Quercitin-3-D-galactoside (Q-3Gal)

R_1 = H

R_2 =

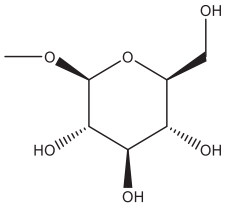


R_3 = - OH

Kaempferol-3-glucoside (K-3G)

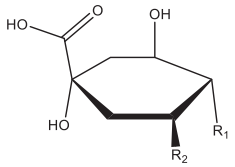
R_1 = = H

R_2 =



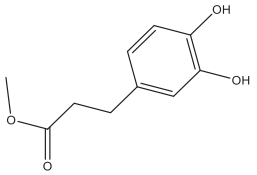
R_3 = -H

Oxyaromatic acids



o-Caffeoylquinic acid (*o*-CQA)

R_1 =

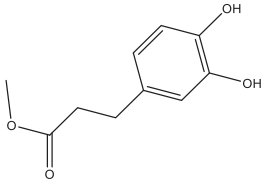


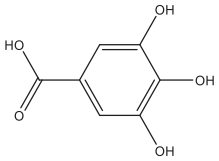
R_2 = -OH

Neochlorogenic acid (NCA)

R_1 = - OH

R_2 =





Gallic acid (GA)

Fig. 1. Continued

at 26 min, 55% B at 28 min, 70% B at 30 min, 75% B at 34 min and 80% B at 36 min, at a solvent flow rate of 0.9 ml/min. Spectral data from all peaks were accumulated in the range 200–400 nm, and chromatograms were recorded at 280, 320 and 350 nm. Chromatographic data were processed with a Chromeleon™ 7.2 Chromatography Data System Software. The compounds in each sample were identified and quantified by comparing their retention times and peak area in the chromatograms relative to external standards. The quantification of theanine was performed according to Song et al. (2012) at 200 nm.

Diphenyl-1-picrylhydrazyl (DPPH•) assay

The antiradical activity of the extracts was determined spectrophotometrically in a microplate reader by monitoring the decrease of DPPH• (2.2-diphenyl-1-picrylhydrazyl) concentration at 515 nm at room temperature. A dilution series (12 different concentrations) was prepared in a 96-well plate. The reaction mixtures in the sample wells consisted of 25 µl extract and 200 µl of 150 µM DPPH• (dissolved in methanol). Four experiments were performed with three parallel measurements each. BHT (buthylated hydroxytoluene) was used for comparison. Inhibition

of free radical by DPPH• in percent (%*I*) was calculated as follows:

$$\%I = 100 \times \frac{(A_{Control} - A_{Sample})}{A_{Control}}$$

where $A_{Control}$ is the absorbance of the control reaction and A_{Sample} is the absorbance of the test compound. The antiradical activity was expressed in terms of IC_{50} that was obtained from linear analysis of the plot of the percentage of DPPH• scavenging *vs*. the extract concentration.

Total phenolics content (TPC)

Total phenolics were estimated using the Folin-Ciocalteu colorimetric method as previously described (Luthria et al., 2006) with small modifications. Briefly, 300 µl extract (1 mg/ml) were diluted in a 10.0 ml volumetric flask, to which 1.0 ml undiluted Folin-Ciocalteu reagent was added. Immediately 5.0 ml 20% (w/v) Na_2CO_3 were added. After 30 min incubation at room temperature, the absorbance was measured at 735 nm and compared to a pre-prepared gallic acid calibration curve. Determinations were performed in triplicate. Results were expressed as milligram of gallic acid equivalents (GAE) per gram of extract.

Cytotoxic activity against cancer cells

Green tea was extracted with H_2O, 70% MeOH:30% H_2O or 50% CH_2Cl_2: 50% MeOH. The cytotoxicity of tea extracts was tested against sensitive CCRF-CEM and multidrug-resistant CEM/ADR5000 leukemia cells as previously reported by us (Adem et al., 2019; Saeed et al., 2019; Mbaveng et al., 2019; Hegazy et al., 2019). A total of 1×10^4 cells per well were added into the 96-well-plates in a total volume of 100 µl. Various concentrations of tea extracts were placed in an additional 100 µl of culture medium, 20 µl of 0.01% w/v resazurin (Sigma-Aldrich) was added to each well after 72 h incubation, and the plates were incubated at 37 °C for 4 h. Fluorescence was detected on an Infinite M2000 Proplate reader (Tecan, Crailsheim, Germany) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each assay was done three times, with six replicates each. IC_{50} values are the concentrations required to inhibit 50% of cell proliferation and were calculated from a calibration curve by linear regression using Microsoft Excel.

Statistical analysis

A completely randomized design was used with three replications. Statistical analysis was performed using SPSS v. 24 (IBM Corp. Armonk. NY. USA). Data of all analysis were expressed as mean ± standard deviation. Analysis of Variance (ANOVA) followed by Tukey's HSD post-hoc test for multiple comparisons was used to assess the statistical differences among means ($p \leq 0.05$). The results from hole-plate diffusion and time-kill assays are represented as mean standard deviation (SD) of three replicate measurements. Graphic representations were made with SigmaPlot for Windows v.10.

Results and discussion

Phytochemical fingerprinting and marker compound analysis

Chromatographic methods are the methods of choice for the analysis of complex multi-component mixtures in tea and are widely used for identification and to make quantitative analysis of tea bioactive compounds (Zuo et al., 2002). In this study, we identified and quantified the phytochemical compounds of green tea by using HPLC-DAD. In total, 12 catechins can be identified by chromatography, although only 8 catechins are present in tea in significant quantities (Yashin et al., 2015). However, most published studies focus specifically on epigallocatechin gallate and epigallocatechin that constitute 70% of the total catechin amount in tea. In this work, the following compounds were detected: (1) five flavan-3-ols, namely (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG) and (+)-catechin (C); (2) two flavonols, *i.e.* quercetin-3-D-galactoside (Q-3 Gal) and kaempferol-3-glucoside (K-3 G); (3) the oxyaromatic acids *o*-caffeoylquinic (*o*-CQA), neochlorogenic (NCA) and gallic (GA); (4) two alkaloids: caffeine and theophiline and (5) the amino-acid L-theanine. The chemical structures are shown in Fig. 1. Representative chromatograms of the extracts are depicted in Fig. 2. It was clearly evident that differences in the phytochemical profiles were dependent on the extraction method.

Contents of catechins, flavonols and oxyaromatic acids: Table 1 shows the results for the catechin contents in the studied samples in comparison to the results published in literature for several tea grades, extraction solvents (water/methanol), extraction times (from 5 min to 30 min) and temperatures (from room temperature to boiling water). The total catechin content (TCC) of commercial green tea samples varied in a range of 21.4 to 228.2 mg/g dry tea for aqueous extractions and of 32.2 to 141.2 mg/g dry tea for methanol extracts (Uchenna et al., 2010). There was a trend that extractions with methanol/water mixtures revealed higher catechin amounts than extraction with absolute methanol. Interestingly, concentration range was larger in aqueous extracts than in the corresponding methanol extracts. The TCC values determined in our samples varied between 35.8 and 163.9 mg/g dry tea for aqueous extractions with maceration giving the lowest value, and the TCC was 13.7 mg/g dry tea for methanolic extractions.

We conclude that the high variability in catechin contents largely depended on extraction temperatures, times, and solvents. While the EGCG composition of green tea from Azores was significantly lower than of teas from China and Japan, the EGC contents were higher. Baptista et al. (1998) measured the catechin levels of hot water extracts of green tea from Azores by HPLC/SPME-GC. With an extraction time of 20 min, these authors measured four-fold higher TCC in Azorean tea samples than we did in the present study with an extraction time of 5–7 min. The total contents of epicatechin derivatives and EGCG from Azorean green tea accounted for 74.5 and 47.9% (w/w) of the TCC, respectively, and an EGCG:caffeine ratio was four-fold higher compared with teas from other origins (Baptista et al., 1998). In the present investigation, the EGCG:caffeine ratio was about two-fold higher, if the mg of catechins/g of dry extract were considered. The results obtained in this study indicated that methanol better extracted the high molecular weight flavanols (ECG and EGCG) and the two flavonols, quercetin-3-D-galactoside (Q-3Gal) and kaempferol-3-glucoside (K-3G).

Determination of catechin, flavonol and oxyaromatic acid contents: The contents of phytochemicals have been expressed in mg/g extract to allow direct comparison with commercial lyophilized tea preparations. The chatechin quantities as well as the TPC values are shown in Table 2 and Fig. 3. Catechins, flavonols and oxyaromatic acids are the major flavonoids in green teas, and 90% of all phenolic compounds are catechins (Rafael et al., 2011). As expected, catechins were also prevalent among the phenolics in the Azorian green teas, and the ratio of TCC/TPC ascend to 90%, except for maceration extraction. In Fig. 3, the three first points are for the aqueous extracts arranged from lower to higher extraction times (**A1, A2** and **A3**, respectively), The 4th position showed the result for the methanolic extraction (**A4**). Fig. 3(a) refers to TCC. The TCC of **A3** was significantly lower than those of **A1** and **A2**, suggesting that the high extraction time for maceration did not increase but decrease TCC. A closer view on the contents of individual catechins (Fig. 3b and c) showed that the catechin amount in **A3** was similar to that in **A1** and **A2,** but contained lower amounts in the remaining catechins.

Extraction is a process mainly governed by diffusion and solubility. Longer extraction times increase the permeability of cell walls in green tea leaves, and higher temperatures enhance solubility constants. This enables more tea catechins and other substances to move into the solution. Yet, the stability of the catechins may be affected, if tea is brewed for too long because there may be an increased probability

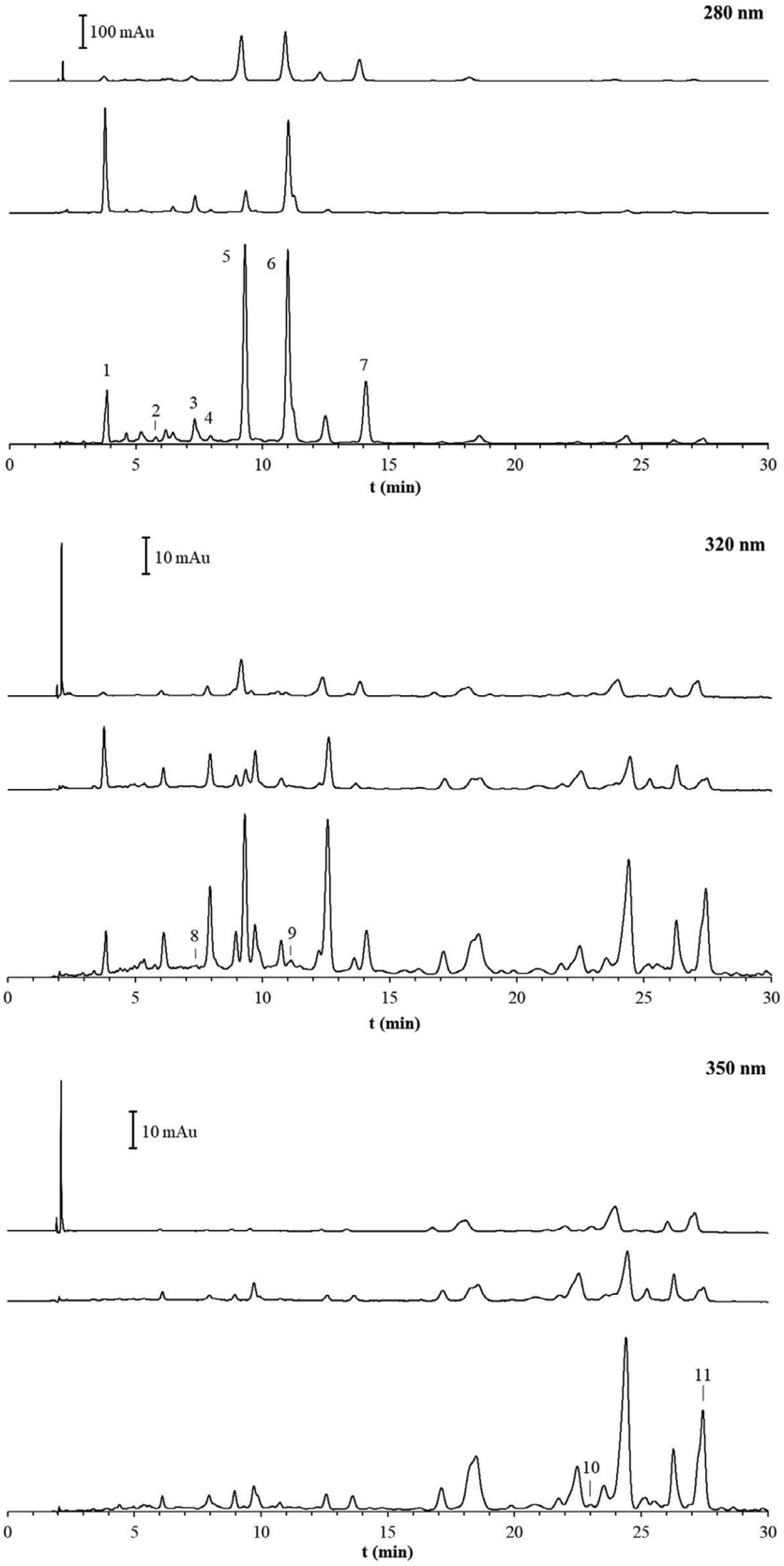


Fig. 2. Chromatograms of different extracts of Azores green tea obtained at different wavelengths. At each wavelength, the top chromatogram corresponds to the methanolic extract, the middle one to the maceration and the one in the bottom to the infusion.

Table 1
Content of catechins in green tea extracts as determined by HPLC and expressed in mg/g (dry tea), infusions (time, min).

Tea grade (Extraction time)	Catechins average (% of dry weight)							Ref
	EGCG	EGC	ECG	EC	C	TCC	Solvent	
Average (68 different tea grades)	71.1	20.6	14.9	8.1	0.6	12% (mean) 33% (max)		Wu et al., 2000
Green tea from China (mean value for 15 teas)	134.0	4.4	29.0	5.5	0.2	18% (13%–22%)		Lin et al., 1998
Green tea from Japan (mean value for 13 teas)	131.4	8.8	19.5	8.7	0.2	17.8% (9%–24%)		Lin et al., 1998
Azores (5–7 min)	66.4 ± 1.9	46.9 ± 6.3	23.0 ± 9.7	25.9 ± 6.4	0.9 ± 0.3	163.9	Hot water	This work
Azores (30 min)	23.5 ± 1.7	20.3 ± 0.9	10.8 ± 0.7	21.7 ± 3.7	1.3 ± 0.3	77.6		This work
China (5 min)	43.1	15.2	9.0	8.7	0.7	76.6		Wu et al., 2000
China (10 min)	52.2	23.3	14.3	10.1	1.0	100.9		Wu et al., 2000
Jasmine (5 min)	22.5	6.9	10.4	8.5	1.6	49.9		Wu et al., 2000
Lung Ching (5 min)	31.0	10.2	7.8	6.3	0.6	55.9		Wu et al., 2000
Xian-Zhe-Zhu-Jian	24.47	26.29	2.54	2.54	1.00	56.8		Lin et al., 2008
E-Mei-Shan (China)	14.74	37.20	3.14	3.70	1.10	59.8		Lin et al., 2008
Lu-Shan (China)	30.64	40.11	8.68	3.93	1.70	85.1		Lin et al., 2008
Longjing (Taiwan)	31.17	67.31	5.18	4.14	0.98	108.8		Lin et al., 2008
Decoct (Japan)	21.30	82.44	3.64	6.05	0.96	114.4		Lin et al., 2008
Azores (20 min)	97.7	31.3	38.5	14.6	2.51	184.6		Baptista et al., 2014
Azores (24 h)	5.1 ± 0.5	5.7 ± 0.6	0.65 ± 0.06	24.1 ± 2.3	0.24 ± 0.05	35.8	Maceration	This work
Meifoo Me	52.7	27.7	21.8	10.3	–		30% methanol	Zuo et al., 2002
Shanghai	51.1	30.8	11.3	7.25	–			Zuo et al., 2002
Hongzhou	62.4	37.6	16.3	6.6	–			Zuo et al., 2002
Jasmine	54.2	27.6	15.8	6.9	–			Zuo et al., 2002
Xian-Zhe-Zhu-Jian (China)	29.38	20.88	9.98	1.94	0.50		75% methanol	Lin et al., 2008
E-Mei-Shan (China)	14.90	28.02	5.51	2.49	0.49			Lin et al., 2008
Lu-Shan (China)	47.34	77.58	11.16	6.59	2.41			Lin et al., 2008
Longjing (Taiwan)	43.48	18.81	11.95	2.91	1.82			Lin et al., 2008
Decoct (Japan)	35.76	83.07	6.79	5.93	–			Lin et al., 2008
Azores (5–7 min)	4.33 ± 0.38	1.87 ± 0.15	5.43 ± 0.37	2.04 ± 0.15	–	13.7	99% methanol	This work

Table 2
Content of catechins in green tea extracts from the Azores as determined by HPLC and expressed as mg/g. A1, infusion (5–7 min); A2, infusion (30 min); A3, maceration (48 h); A4, methanolic extract; TCC, total catechins; TPC, total phenolic content.

Sample	EGCG	EC	EGC	ECG	C	TPC[1]	TCC	%TCC/TPC
A1	105.13 ± 7.16	41.15 ± 0.96	21.50 ± 0.60	36.56 ± 1.82	1.37 ± 0.16	221.18	205.7	93.0
A2	60.04 ± 4.42	55.45 ± 9.60	27.66 ± 1.80	51.79 ± 2.26	3.41 ± 0.70	209.2	198.4	94.8
A3	9.28 ± 1.42	31.51 ± 2.85	8.84 ± 1.00	1.10 ± 0.06	0.29 ± 0.03	79.83	51.0	63.9
A4	195.03 ± 7.77	37.18 ± 0.64	33.83 ± 0.02	99.09 ± 2.00	–	378.68	365.1	96.4

Values expressed as mean ± standard deviation obtained from three measurements per replicate.
[1] Sum of TCC with the oxyaromatic acids.

of epimerization, oxidation and degradation, especially under higher extraction temperatures (Chen et al., 2001). At high temperatures, the EGCG and ECG amounts diminished, if the extraction time was raised from 5 to 7 to 30 min (Fig. 3c). By contrast, increasing extraction time yielded higher quantities of catechins, epimerized catechins and EGC (Fig. 3b). Increasing extraction times led to smaller extracted amounts of catechins even at room temperature, particularly concerning degalloylated catechins (Fig. 3c). By contrast, the GA contents were higher upon maceration than in the remaining samples (Fig. 3d). This may be explained by the occurrence of hydrolysis during maceration. In fact, EGC and GA may be generated by degalloylation of EGCG by enzymatic catalysis as previously described (Battestin et al., 2008).

Aqueous and methanolic extracts are expected to differ in composition, namely concerning total phenols and secondary metabolites with free radical scavenging activities. Therefore, the change of solvents may impart different increments on the solubility of the individual catechins due to their different structure and molecular weight. The TCC in methanolic extract was the highest one. Methanol seemed to easier extract higher molecular weight catechins (EGCG and ECG) (Fig. 3c) than lower weight ones (Fig. 3b). Catechin itself appeared as residual in the methanolic **A4** extract. This sample also contained the lowest gallic acid content.

Table 3 and Fig. 4 show the contents of *o*–caffeoylquinic acid (*o*-CQA), GA, quercetin-3-D-galactoside (Q-3Gal), K-3G and neochlorogenic acid (NCA) in green tea determined by HPLC and expressed as mg/g extract. The *o*-CQA amount was significantly higher in the methanolic sample, while there was not a significant difference between the values in the water extracts (Fig. 4a). By contrast, the quantity of NCA decreased in a linear manner depending on the extraction time, and the maceration extract showed the lowest value. Also, short-term and high temperature extraction led to higher NCA amounts. The contents of glycosylated Q-3Gal and K-3G were small in the maceration sample, which could be due to the hydrolysis of the compounds in water solutions. Flavonols were more effectively extracted with methanol as solvent (Fig. 4b).

L-theanine contents: L-theanine, a typical amino-acid of green tea has been widely studied due to its beneficial properties for human health, since it interacts with diverse physiological and biochemical pathways, *e.g.* it elicits significant effects on the general state of mental alertness by increasing α-wave brain activity and the reduction of anxiety (Kelly et al., 2008). It was also linked to the inhibition of the *in vivo* and *ex vivo* growth of human solid or leukemia cell lines of diverse tumor types by the induction of apoptosis (Calgarotto et al., 2018). Table 4 lists the amounts of L-theanine, caffeine and theophylline obtained by our measurements. Theanine was undetectable in methanolic extracts being higher in maceration.

Alkaloid contents: Green and black teas are another well-known source of xanthines (Lin et al., 1998)). Among xanthines, caffeine is the

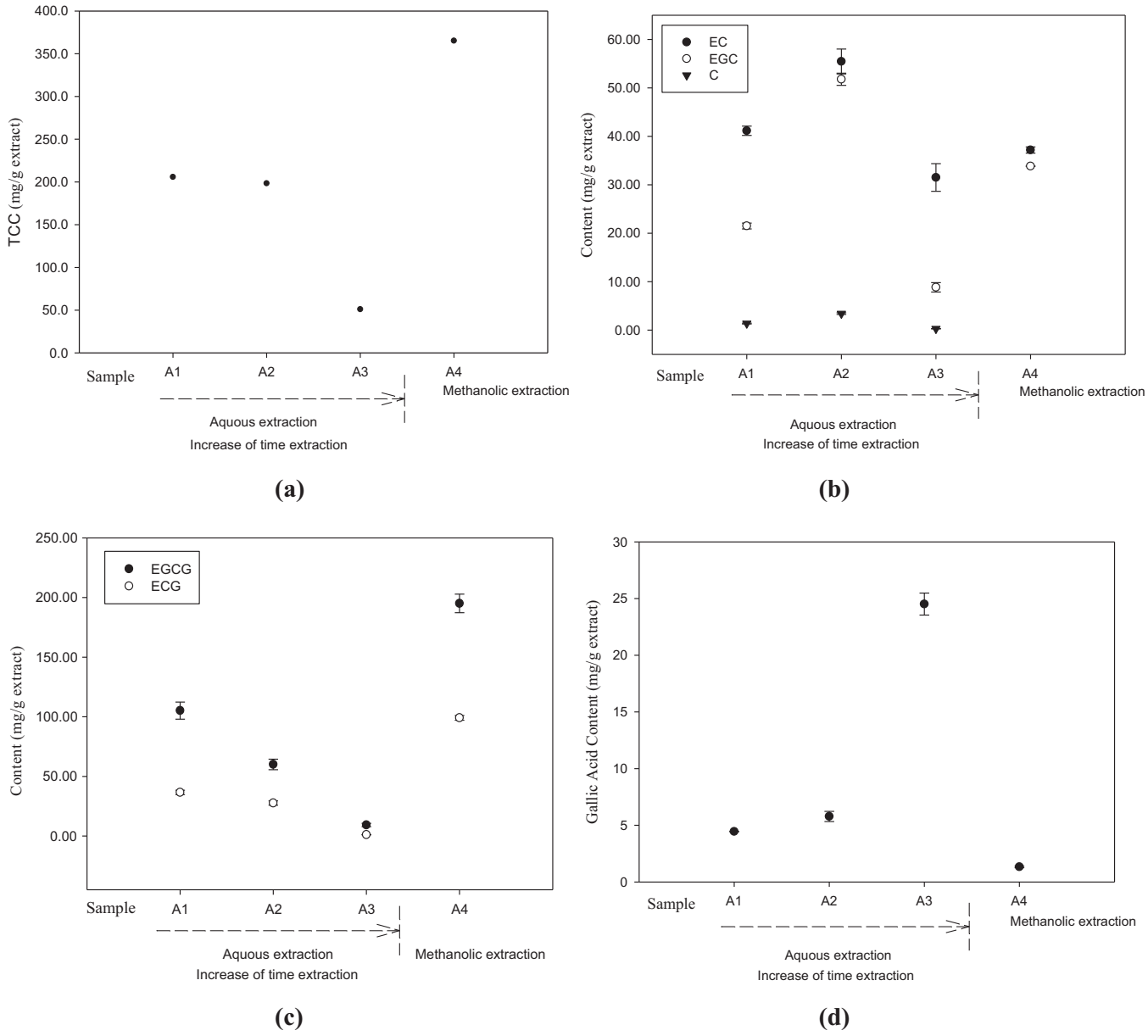


Fig. 3. (a) Total catechins content; (b) amounts of non galloylated catechins; (c) amounts of galloylated catechins and (d) amount of gallic acid present in green tea samples from the Azores. **A1**, infusion (5–7 min); **A2**, infusion (30 min); **A3**, maceration (48 h); **A4**, methanolic extract.

Table 3
Content of *o*-caffeoylquinic acid (*o*-CQA), gallic acid (GA), quercetin-3-D-galactoside (Q-3Gal), kaempferol-3-glucoside (K-3G) and neochlorogenic acid (NCA) in green tea extracts as determined by HPLC and expressed in mg/g. A1, infusion (5–7 min); A2, infusion (30 min); A3, maceration (48 h); A4, methanolic extract.

Sample	*o*-CQA	GA[a]	Q-3Gal	K-3G	NCA
A1	2.73 ± 0.09	4.45 ± 0.01	1.97 ± 0.09	2.00 ± 0.06	4.32 ± 0.14
A2	2.82 ± 0.22	5.78 ± 0.46	2.20 ± 0.17	2.50 ± 0.07	2.25 ± 0.18
A3	2.22 ± 0.07	24.51 ± 0.97	0.63 ± 0.03	1.00 ± 0.05	0.45 ± 0.20
A4	3.95 ± 0.04	1.33 ± 0.07	2.73 ± 0.03	4.51 ± 0.16	1.02 ± 0.83

Values expressed as mean ± standard deviation obtained from three measurements per replicate.
[1a] The graphic representation of GA is in Fig. 2d.

most abundant one, as can be seen in Table 4 and Fig. 5a. Numerous biological effects have been attributed to caffeine, *e.g.* it exerted effects on cognitive and physical function through adenosine A_1 and A_{2a} receptor blockade in the central nervous system and peripheral tissues (McLellan et al., 2016)). Methanolic extracts yielded significantly higher xanthine contents. The caffeine amounts extracted by water did not differ significantly in samples **A2** and **A3**, being higher than in **A1**. The caffeine content was highest in methanol extracts (Fig. 5a). The theophylline content was significantly lower in maceration, and was not significantly different in the other extractions (Fig. 5b).

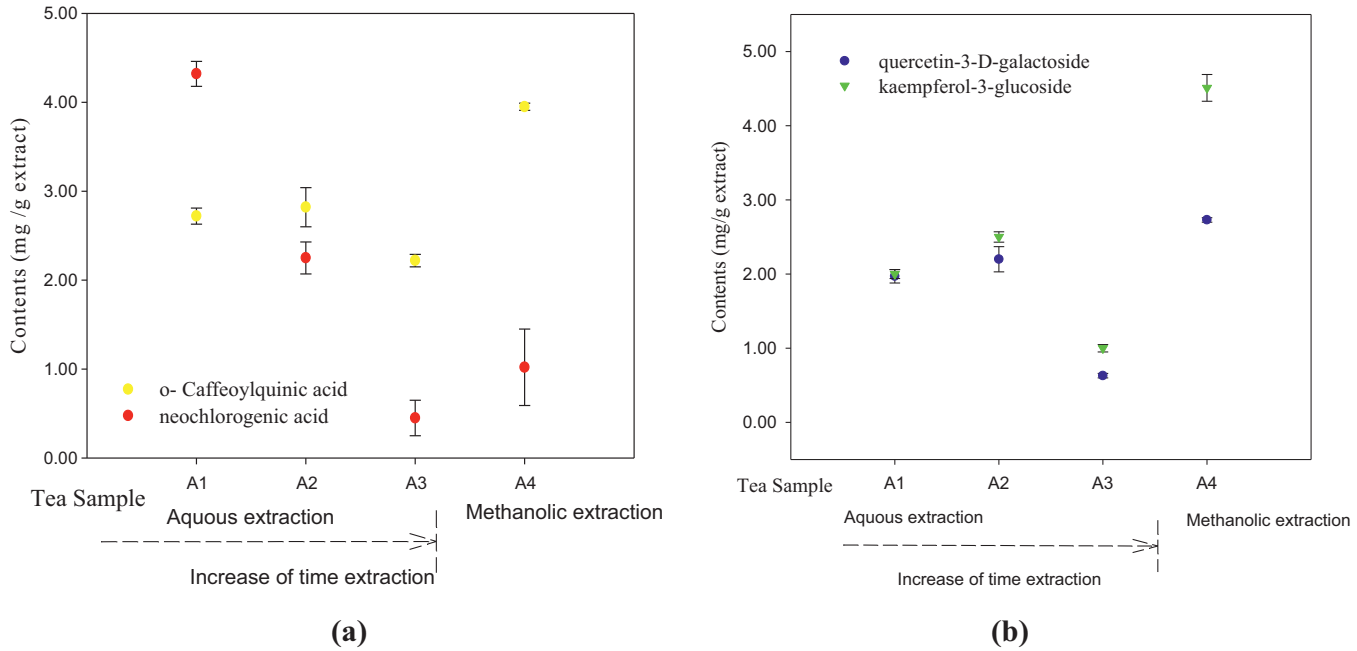


Fig. 4. (a) Graphical representation of the content o-caffeoylquinic acid (o-CQA) and neochlorogenic acid (NCA); (b) Graphical representation of quercetin-3-D-galactoside (Q-3Gal) and kaempferol-3-glucoside (K-3G), in green tea extracts from the Azores as determined by HPLC and expressed in mg/g. **A1**, infusion (5–7 min); **A2**, infusion (30 min); **A3**, maceration (48 h); **A4**, methanolic extract.

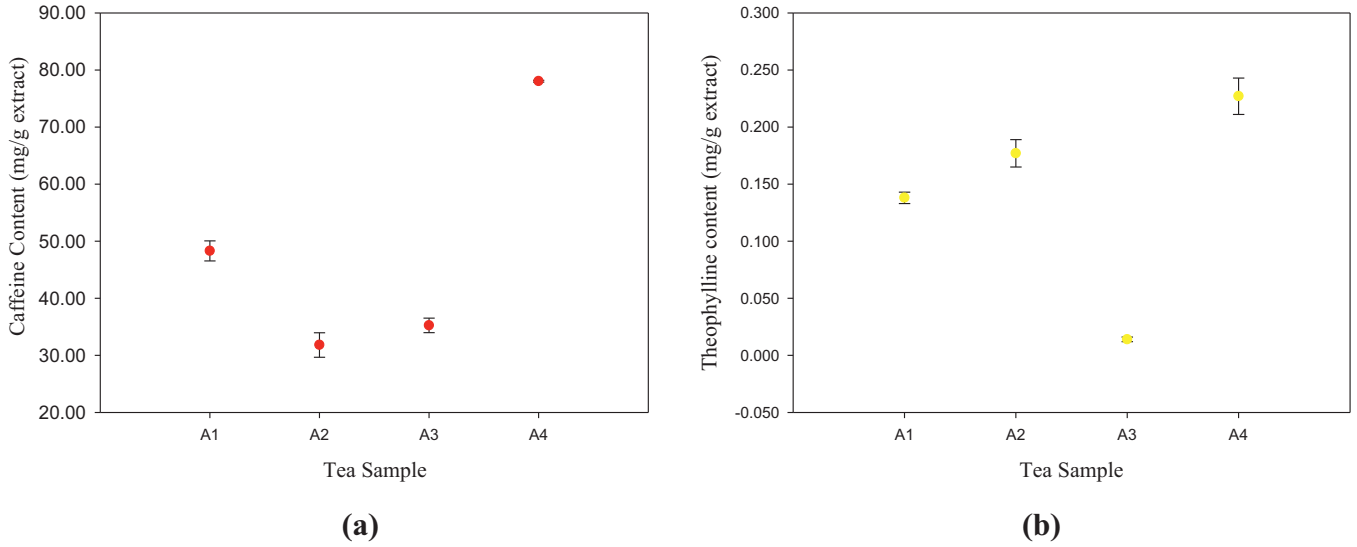


Fig. 5. (a) Graphical representation of the content caffeine and (b) theophylline in green tea extracts from the Azores as determined by HPLC and expressed in mg/g. **A1**, infusion (5–7 min); **A2**, infusion (30 min); **A3**, maceration (48 h); **A4**, methanolic extract.

Antioxidant properties

Free-radical scavenging activity has been determined by using (DPPH•). Table 5 shows the results obtained for Azorian green tea extracts **A1** and **A4** in comparison to previously published data. Studies performed with *C. sinensis* var. *sinensis* revealed an IC_{50} antioxidant value of 23.26 μg/ml (Uchenna et al., 2010), which was significantly different from those reported by Saito and colleagues who worked with *C. sinensis* var. *assamica* extracts (Saito et al., 2007). Their IC_{50} values ranged from 8.33 to 16.10 μg/ml, which are within the range obtained for the infusion extracts studied in the present work. Other authors found higher values than ours, but the standard they used was ascorbic acid instead of GA (Manian et al., 2008; Jigisha et al., 2015). Limitations of the DPPH• assay have been previously discussed by several authors. Deng et al. (2011) found that different standards and a lack of standardization of the ratio of DPPH• to antioxidants complicate comparisons between published results. Moreover, the pigments that may co-exist in the extracts may also interfere with the measurements, if they absorb at similar wavelengths as the DPPH• radical.

Several studies claimed that the DPPH• scavenging capacity of tea is principally due to the presence of catechins, especially EGCG (Nanjo et al., 1999). Many attempts were undertaken to correlate the DPPH• scavenging activities with the presence of catechins, yet, in most of the cases, poor correlations were found (Lin et al., 1998; Manian et al., 2008; Nanjo et al., 1999) Other factors may also influence the measured results, *e.g.* (1) other antioxidant substances beyond catechins, (2) the dependence of the antioxidant power by the catechins' structure, (3) different flavonoid concentrations extracted by

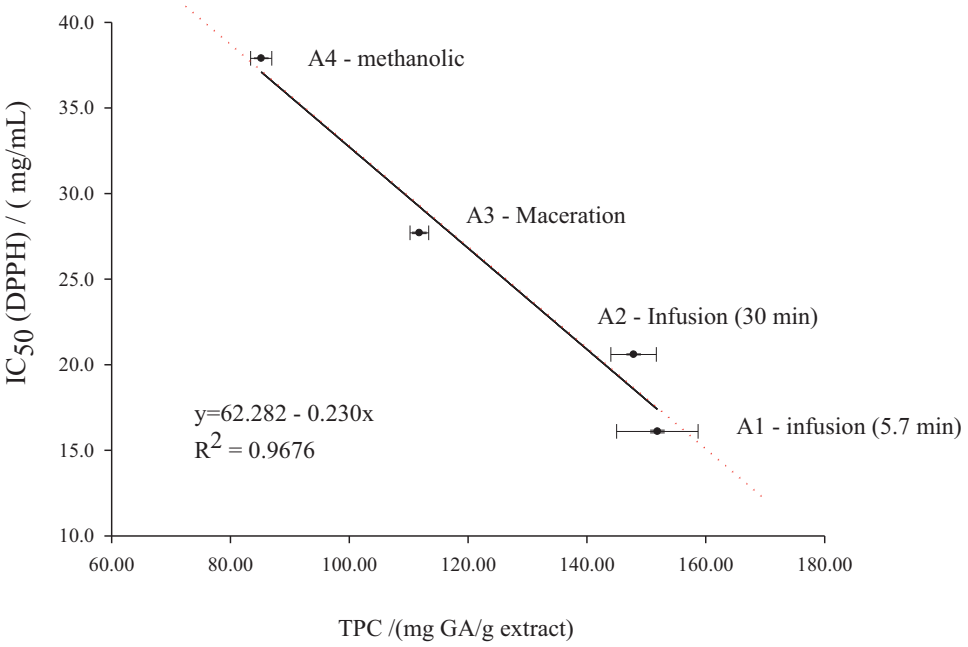


Fig. 6. Graphical representation of DPPH• IC_{50} *vs.* total phenolic content (TPC) for green tea samples from the Azores. Infusions gives higher contented of extracted of phenolics, followed by maceration and methanol extraction. y=IC_{50} (DPPH•) and x =TPC.

Table 4
Content of L-theanine, caffeine and theophylline in green tea extracts from the Azores as determined by HPLC and expressed as mg/g. **A1**, infusion (5–7 min); **A2**, infusion (30 min); **A3**, maceration (48 h); **A4**, methanolic extract.

Tea sample	L-Theanine	Caffeine	Theophylline
A1	± 2.45[a]	48.31 ± 1.76	0.138 ± 0.005
A2	182.45 ± 7.35	31.82 ± 2.14	0.177 ± 0.012
A3	341.76 ± 12.09[ca]	35.25 ± 1.28	0.014 ± 0.002
A4	–	78.04 ± 0.16	0.227 ± 0.016

*Values expressed as mean ± standard deviation obtained from three measurements per replicate. For each parameter, different lowercase superscripts indicate statistically significant differences ($p < 0.05$).

Table 6
Total phenolic contents and antioxidant activity obtained for green tea extracts from the Azores.

Sample	TPC* (mg GAE/g extract)	IC_{50} (DPPH•)* (μg/ml)
A1	151.843 ± 6.857[a]	16.1 ± 0.08[a]
A2	147.850 ± 3.828[a]	20.6 ± 0.03[a]
A3	111.793 ± 1.575[b]	27.7 ± 0.04[b]
A4	85.179 ± 1.776[c]	37.9 ± 0.02[c]

* Values expressed as mean ± standard deviation obtained from 3 measurements per replicate. For each parameter different lowercase superscripts indicate statistically significant differences ($p < 0.05$).

different extraction methods, and (4) antagonistic or synergistic effects in complex plant compound mixtures (Pereira et al., 2013). Furthermore, the total DPPH• scavenging activity dependent on the presence of additional antioxidant compounds such as glycosylated flavonols, oxyaromatic acids and phenolic acids and their derivatives has already been suggested by other studies (Lin et al., 2008; Horzic et al., 2009). Hence, the antioxidant capacity of green tea is not determined by single phytochemical but by a large number of different phenolics.

The values Table 6 show the TPC values and the IC_{50} values for the DPPH• assay measured in the **A1-A4** samples. At first glance, TPC values appeared to be dispersive and not correlated with those obtained from the DPPH• assay, but a closer look revealed an interesting trend as illustrated in Fig. 6. Depending on the extraction method, a linear relationship between the DPPH• IC_{50} and TPC values was observed (see the dashed linear regression lines in Fig. 6). Since the R^2 regression value was close to 1, a line for linear regression was plotted in the graphic representation. As expected, this line showed a negative slope, clearly indicating that the antioxidant capacity varied among the extraction methods: infusion-based extraction showed lower IC_{50} values followed by maceration and finally by methanolic extraction. The antioxidant activities of the infusions and methanolic extracts were probably due to the higher catechin contents, while the high concentration of GA in the maceration extract suggests that this substance may be the main contributor to the antioxidant activity, as previously described by other authors (Battestin et al., 2008). Methanolic extract contained higher amount of EGCG, ECG and higher TCC. Nevertheless, it revealed lower antioxidant DPPH• activity, clearly suggesting that the antioxidant capability of the phenolic compounds depends on its nature, as pointed out by several other studies (Amic et al., 2003; Grzesik et al., 2018; Meo et al., 2013).

Table 5
Free radical scavenging activity by DPPH• (μg/ml) of green tea.

Tea Grade	DPPH• / IC_{50} (μg/ml)			
	Methanolic	Infusion	Standard	Ref.
Assam green tea	75.30 ± 0.11	80.10 ± 0.03	Ascorbic acid	Jigisha et al., 2015
Kashmir green tea	81.30 ± 0.02	79.10 ± 0.03	Ascorbic acid	Jigisha et al., 2015
Uttarakhand green tea	79.30 ± 0.02	81.50 ± 0.05	Ascorbic acid	Jigisha et al., 2015
Green tea mean		23.26	BHT	Uchenna et al., 2010
Green tea		8.33 to 16.10	BHT	Saito et al., 2007
Green tea		19.50	BHT	Manian et al., 2008
Azores	37.90 ± 0.20	16.1 ± 0.8	BHT	This work

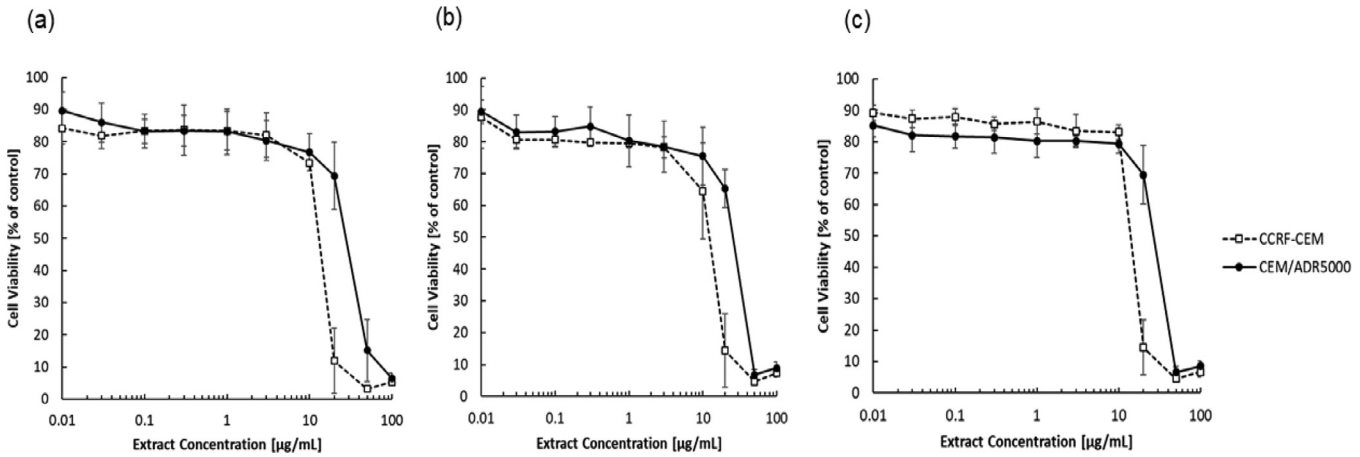


Fig. 7. Cytotoxic effect of green tea extracts from the Azores with (a) H_2O, (b) 70% MeOH:30% H_2O or (c) 50% CH_2CL_2: 50% MeOH. Resazurin reduction assays were carried out to determine dose response curve. Cell viability of CCRF-CEM and CEM/ADR5000 cells is represented by mean values ± SD of three independent experiments with each six parallel measurements values are expressed as percentage survival of untreated control.

Table 7
Cytotoxicity (IC_{50} values) of green tea extracts from the Azores with different solvent towards sensitive CCRF-CEM and multi-drug resistant CEM/ADR5000 leukemia cell lines.

Solvent	CCRF-CEM IC_{50} [µM]	CEM/ADR5000 IC_{50} [µM]	Degree of resistance[a]
H_2O	14.09 ± 1.88	27.91 ± 4.26	1.98
70% MeOH:30% H_2O	12.13 ± 2.62	25.31 ± 1.75	2.09
50% CH_2CL_2: 50% MeOH	12.40 ± 3.34	26.35 ± 2.60	2.13

[a] The degree of resistance was determined as the ratio of IC_{50} value of multi-drug resistant CEM/ADR5000 cells divided by the IC_{50} value of sensitive CCRF-CEM.

Anticancer activity of green tea sample from Azores

Green tea and its ingredients such as EGCG and other polyphenols have been reported to exert chemopreventive effects against human cancer stem cells (Wang et al., 2002; Fujiki et al., 2018), to suppress prostate carcinoma (Davalli et al., 2012) and the development of bladder cancer (Miyata et al., 2018). Quercitin and theanine from green tea inhibited human leukemia cells (Calgarotto et al., 2018). Therefore, we investigated the cytotoxicity of Azorean green tea extracts against wild-type and drug-resistant leukemia cells. A major requirement in clinical oncology is not only to have new drugs at hand, but also to have drugs that are able to kill otherwise drug-resistant and refractory tumors.

The aqueous extract, aqueous/methanolic extracts, (70% MeOH:30% H_2O) as well as dichloromethane/methanol, (50% CH_2Cl_2: 50% MeOH) extracts were tested for their potential to inhibit drug-sensitive CCRF-CEM and multidrug-resistant CEM/ADR5000 cancer cells. The latter overexpressed the MDR-conferring drug transporter P-glycoprotein/MDR1 (Kimmig et al., 1990; Gillet et al., 2004). The extracts showed IC_{50} values of 12–14 µM and 25–27 µM against CRF-CEM and CEM/ADR5000 cells, respectively (Fig. 7 and Table 7). The extracts showed low cross-resistances (1.98–2.13), while standard drugs such as doxorubicin were more than 1000-fold less active in multidrug-resistant CEM/ADR5000 cells than in wild-type CEM/CCRF cells (Efferth et al., 2008). This indicates these extracts could be useful to target not only drug-sensitive but also drug-resistant cells.

Conclusion

Green tea grown in the Azores, the oldest plantation of the *C. sinensis* in Europe, was used to determine the chemical composition based on various extraction conditions. The extracts were phytochemically characterized (catechins, oxyaromatic acids, flavonols, alkaloids and theanine) by HPLC-DAD, in order to quantify the amounts of phytochemicals per gram of extract.

The TCC of the maceration extract was significantly lower than the infusion extracts: the catechin amount extracted by maceration was similar to the one obtained from infusions. However, the maceration sample contained significantly lower quantities regarding the remaining catechins. At high temperatures, the amounts of EGCG and ECG diminished, if the extraction time was raised from 5 to 7 to 30 min. By contrast, increasing extraction times led to higher quantities of catechins, epimerized catechins and EGC. Moreover, increasing extraction times also increased the GA amount. The content of this oxyaromatic acid was very high in the maceration sample, indicating that the GA content was raised due to the occurrence of degalloylation of EGCG and ECG in the maceration sample.

Concerning the methanolic extraction, the change of solvent enhanced TCC and methanol facilitated the extraction of high molecular weight catechins (EGCG and ECG). Methanol more effective extracted glycosylated Q-3Gal and K-3G. The *o*-CQA amount extracted by methanol was also significantly higher than the one in the aqueous samples.

The quantity of NCA decreased in a linear manner depending on extraction times. The maceration extract showed the lowest value.

L-theanine was not extracted in methanol, and the amount extracted in aqueous solutions increased with the extraction time. By contrast, higher contents of caffeine and theophylline were extracted by means of methanol. The content of extracted theophylline was significantly lower in the maceration extract.

The anti-oxidant activities (DPPH$^{\bullet}$ assay) and TPC of aqueous (infusions and maceration) and methanolic extracts of Azorean *C. sinensis* were also determined. TPC correlated with DPPH$^{\bullet}$ IC_{50} values in a linear fashion depending on the extraction method. Infusion yielded the highest TPC, while the methanolic extract yielded the lowest one.

Finally, the cytotoxic activity of Azorean green tea samples was screened towards drug-sensitive CCRF-CEM and multidrug-resistant CEM/ADR5000 cancer cells. The highly multidrug-resistant cells revealed only low degrees of cross-resistances, indicating that they might

be useful to target not only drug-sensitive but also drug-resistant and otherwise refractory tumor cells.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

CRediT authorship contribution statement

Sérgio Barreira: Methodology, Investigation. **Carla Moutinho:** Methodology, Investigation. **José Neves:** Methodology, Investigation, Conceptualization. **Ean-Jeong Seo:** Methodology, Investigation, Writing - original draft. **Mohamed-Elamir F. Hegazy:** Investigation, Writing - review & editing. **Thomas Efferth:** Supervision, Writing - review & editing. **Lígia Rebelo Gomes:** Supervision, Conceptualization, Writing - original draft, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phyplu.2020.100001.

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