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EVALUATION OF THE ANTIOXIDANT CAPACITY AND CHARACTERIZATION OF PHENOLIC COMPOUNDS OBTAINED FROM TEA (*CAMELLIA SINENSIS*) FOR PRODUCTS OF DIFFERENT BRANDS SOLD IN COLOMBIA.

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Abstract

The active compounds from natural sources, have become an interest within the scientific community, especially phenolic compounds due to their effects on health and nutraceutical use as food,[1]. The consumption of green tea in Colombia is a recent trend and the market is continuously growing, then the most common commercially available types of green tea were tested in this study; Oriental, Lipton, Hindu and Jaibel . The objective of this work was to determine the total polyphenol content and in-vitro antioxidant capacity of green tea commercialized in Colombia. The antioxidant capacity was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging assay and the oxygen radical absorbance capacity (ORAC) assay. A similar profile was observed for the antioxidant capacity determined by both methods; 748.09 ± 39.76 to 1138.45 ± 78.03 µmol Trolox equivalent / g dry sample (µmol TE/g sample) for DPPH assay, and 740.83± 37.81 to 1588.05± 21.72 03 µmol Trolox equivalent / g dry sample for ORAC assay. The total polyphenol concentration in green tea was found to vary 93.76 ± 1.38 to 127.74 ± 3.32 mg gallic acid equivalents (GAE) / g sample, the total flavonoid content (TFC) vary 10.42 ± 0.52 to 29.15 ± 2.04 mg catechin equivalent/g sample. The antioxidant activities were well correlated with the total polyphenol content (r^2 =0.9911) for the ORAC method and total flavonoids (r^2= 0.997) with DPPH assay. In general the behavior of all samples was Oriental≥ Lipton> Hindu> Jaibel both TPC and TFC as the biological activity. A method for identifying the catechins present, caffein, organic and phenolic acids in green tea was developed by high performance liquid chromatography (HPLC); the chromatographic profiles showed the presence of ten compounds including the most abundant were, gallic acid, caffeine and epigallocatechin gallate (EGCG) to which they areattributes his antioxidant capacity.
This is the first systematic screening for the identification of polyphenols and antioxidant activity in tea commercialized in Colomiba.

Keywords: Antioxidant activity, green tea, high-throughput, phenolic content.

Introduction

Tea is an infusion of *Camellia sinensis*, is one of the most consumed beverage in the word, due to sensory properties, stimulating effects and potential health benefits. The sinesis plant is originally from Southeast China, gradually expanded to India, Sri Lanka, and many tropicals and subtropical countries, [1]. Tea can be divided into three categories on the basis of fermentation process, green tea (non-fermented), black tea (post-fermented) and oolong tea (semi-fermented) [2]. The American Medicinal Association shows that green tea may reduce cholesterol levels, high blood pressure and reduce the risk of strokes. The National Cancer Institute reports that the antioxidants in green tea can prevent various types of cancer [3]. Chemical composition of tea is complex: polyphenols(catechins and flavonoids), alkaloids (caffein, theobromine, etc), amino acids, glucosides, proteins, valatile compounds, minerals and trace elements [4]. The tea components are affect by diferents variables like a cultivar type, growth conditions, horticultural practices (mechanical-or- hand plucking age of leaves) and the technologies used for manufacturing [5]. The major compounds in green tea is catechins, which are flavonols; these in turn are class of flavonols which are polyphenols [6]. Studies sugest that the health benefits of green tea are significant due to the presence of catechin, because are capable of reducing the amount of free radical, and prevent the formation of pro-inflammatory compounds [7]. Green tea contains six primary catechins compounds namely catechin (C), gallocatechin (GC), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) [3]. The chemical structure of catechins have been related to their antioxidant capacity, this depend on the number and position of hidroxyl groups [8].

Therefore the present study was carried out to determinate total phenolic compounds and flavonoids, set HPLC method to identify some cathechins, caffeine, galic acid, ferrulic and clorogenic acid and compared antioxidant activity in different brands of tea sold in Colombia, in order to establish a relationship between structure and biological activity.

Methods

Tea samples

Were selected the most representative green tea brands accordingly to information of the specially shops. These tea samples were purchased from super- market. Four brands were used; Oriental, Hindu, Lipton and Jaibel. Each tea brands was analysed in triplicate.

Extraction of green tea compounds

A mass of 0.2 g of each tea was weighed and mixed with 10 mL of acetic acid. 0.1%, and reflux extraction method was used for 30 minutes. Then allowed to cool to room temperature and centrifugated at 7500 rpm for 15 minutes. The supernatant was decanted and brought to 15 mL, aliquoted and stored at 4°C for subsequent analysis.

Total phenol content (TPC)

Total phenol content in tea extracts was determinated by high-throughput assay according to the modified Folin-Ciocalteu method described by E. a Ainsworth and K. M. Gillespie, [9]. The extracts were diluted (1:50) in destiled water, 50 µL of each sample, standar or methanol blank were added to 96-well-microplate. Then 75 µL Folin reagent (1:10) and 75 µL of $Na_2 CO_3$ 7.5% were added and homogenized, the absorbance was read at 760 nm after 60 minutes in a microplate reader (Thermo Multiscan Go, serie number 1510-01100, SkanIt Software 3.2.1.4 RE for Multiskan GO (es)) The phenolic compounds were quantified using calibration curves of gallic acid and expressed as mg Galic acid equivalent / g sample (mg GAE/g sample).

Total flavonoid content. (TFC)

Total flavonoid content in tea extracts was determinated by high-throughput. Briefly 15 µL of $NaNO_2$ 5% was added in each well, except the blank (15 µL of water) and mixed with 100 µL of tea extract (1:25) and/or Catechin standard in 96 well-microplate and left durnig 5 minutes followed by 15 µL of $AlCl_3$, mixed and left for 6 minutes at room temperature in the dark; finally 70 µL of NaOH 1M was added. The absorbance was read in 5 minutes at 500 nm over a microplate reader (Thermo Multiskan Go, serie number 1510-01100, SkanIt Software 3.2.1.4 RE for Multiskan GO (es)). The flavonoids contents were quantified using calibration curves of Catechin and expressed as mg catechin equivalent/g sample. (mg CE/g sample.

Antioxidant Activity

DPPH assay

The DPPH assay was carried out by high-throughput according to the modified method of R. Fernandez-Orozco, [10] . A volume of 25 µL of tea extrac (150 ppm in Methanol) and/or Trolox standar with the

respective controls; Positive control was Hidroquinone 1000 ppm, negative control was Methanol 96%, in a 96-well plate was added, followed by 100 µL of 50 ppm of DPPH metanolic solution to each well, except the blank sample. The mixture was incubated in the dark at room temperature for 30 minutes, and the absorbance at 517 nm was measured by a microplate reader (Thermo Multiskan Go, serie number 1510-01100, SkanIt Software 3.2.1.4 RE for Multiskan GO (es)). The scavenging capacity was calculated as: % A.A= [A $c_{(-)}$ – A_{tea} /$Ac_{(-)}$]*100, where $Ac_{(-)}$ is the absorbance of the control and A_{tea} is the absorbance of the tested sample. Trolox was used as standar. Free radical scavenging capacities of tea were expressed as µmol Trolox Equivalent/ g sample. (µmol TE/g sample)

ORAC assay

Antioxidant activity of the diferent teas was also assessed with the ORAC (Oxigen Radical Absorbance Capacity) assay acording to the method of K. M. Gillespie [11]. Briefly in each well of a solid white 96 well-microplate, 187 µL of 80 nM florescein disolved in 75 mM PBS (phosphate buffered saline) was added followed by 31 µL of tea previously diluted 1000 times in PBS. After 15 minutes incubation in the dark at 37 ˚C, 31 µL of 140 mM AAPH [2,2'-azobis 2-methylpropionamidine) dihydrochloride] were rapidly added to each well and fluorescense recorded from the top every 120 second until fluorescence decayed, using excitation wavelength of 493 nm and an emmision filter of 515 nm using a fluorescence Spectrophotometer (Varian, Cary Eclipse, version 1.1(135)) The net AUC (area under the fluorescence decay curve) for each sample/satandard was obteined by subtracting the area of the blank sample (PBS). Antioxidant activity was expressed as µmol Trolox equivalent/ g sample (µm TE/g sample) using the linear regression value obtained from the trolox calibration curve.

Determination of tea compounds by High Pressure Liquid Chromatogrphy (HPLC).

Tea infusions were analysed on a reverse phase high performance liquid chromatographic system (Hitachi LaChrom with ultraviolet detector (Hitachi L-2420 UV-Vis) an injection volume of 20 µL, Column Oven L-2300, Pum L-2130. The separations were performed using a C18 reverse phase column (Ultra AQ C18 150 x 3.2 mm i.d, 3 µm Particle, Ser# 12031031M, Cat# 9178313, Lot#110836P) and column temperature was maintained at 35 ˚C. Software used was EZChrom Elite® data system, version 3.3.1 SP1) the phase mobile consisted of acetic acid (0.5% in water, solvent A) and mix of acetonitrile: Etile acetate: acetic acid 0.1% in water (10:2:88 (v/v) solvent B). Gradient elution procedure is presented in table 1. Peaks were identified by comparing sample retention times to those of autentic standard. UV- detection was perfromed at 270 nm. Before the HPLC analysis, the extracts were filtered through a polytetrafuoroethylene (PTFE) membrane cartridge.

Experimental design

All measurements were carried out in triplicate and the results are statistically analyzed using the Grhapad prims program to determine the average value and standard error and ANOVA, tukey´s Multiple Comparision Test, (significant $p< 0.05$), were performed to determine significant differeces.

Results

Total phenol content (TPC)

The total phenolic content of the 4 brands green tea are shown in figure 1 and table 2, were quantified using calibration curves of gallic acid (5-100 mg/L) performed every day of the assay. The total phenolic compounds were found between 93.76- 127.74 mg galic acid Equivalent /g sample. The highest levels was measured in Oriental brand (127.74 ±3.32 mg equivalent Galic acid / g sample), similar amounts were also obtained in Lipton (114.70 ±4.61 mg Galic acid equivalent / g sample) and Hindu (99.06 ± 2.96 mg Galic acid equivalent / g sample) , while Jaibel contained the lowest amount (93.76 ± 1.38 mg Galic acid equivalent / g sample).

Total flavonoid content. (TFC)

The total flavonoid content of the tea extract were relatively low compared with TPC, and ranged from 10.42 ± 0.52- 29.6 ± 1.43mg catechin equivalent / g sample, (see figure 2 and table 2) and quantified using calibration curve of catechin (5-70 mg/L) performe every day of the assay. The highest levels was measured in Oriental brand (29.61 ±1.43 mg catechin equivalent / g sample), similar amounts were also obtained in Lipton (29.15 ± 2.04 catechin equivalent / g sample) and Hindu (21.02± 0.77 mg catechin equivalent / g sample), while Jaibel contained the lowest amount (10.42± 0.52 mg catechin equivalent / g sample).

Antioxidant Activity

The antioxidant activity of the tea infusions was

evaluated using two independent assays, DPPH and ORAC. A calibration curve of trolox (6.5 - 100 uM) allowed to compare antioxidant activity in different brands of tea expressed as µmol Trolox equivalent / g dry sample. The results obtained from DPPH assay reported in the figure 3 and table 2, shown values ranged from 748.09 ± 39.76 to 1138.45 ± 78.03 µmol Trolox equivalent / g dry sample, similar to the values obtained by ORAC assay reported in the figure 4, which have a range from 740.83± 37.81 to 1588.05± 21.72 03 µmol Trolox equivalent / g dry sample. The Oriental brand tea had the highest DPPH and ORAC values while the Jaibel brand showed the lowest.

Determination of tea compounds by High Pressure Liquid Chromatogrphy (HPLC)

The aromatic structural similiarity of the green tea catechins made the separation difficult, but HPLC method has become a technique to separate properly [12]. Comparing the retention times established by mixing standards (see figure 7), allowed to identify and separate at least ten of the eleven compounds in the tea extracts. (See figure 8 to 11). The peaks corresponding to gallic acid, caffeine and EGCG are the most abundant in all brands of tea. Jaibel presented differences in composition and antioxidant activity as it did not lodged catechin and was the only one that showed even in small proportions ferulic acid.

Discussion

Currently, nutraceuticals are becoming a part of the daily diet, because the current lifestyle could generate many diseases which lead to the scientific community to the search for natural sources of compounds that help to maintain a balance in the consumer health. At present, the consumption of green tea increased due to studies that reported a number of health benefits associated with consumption reducing cardiovascular diseases, action against some cancers, inflammatory diseases, diabetes and weight loss [5]. The present study was carried out to determinate how antioxidant activity varies from the diferents brands of green tea in order to associate the antioxidant activity with the content of flavonoids and phenolic compounds to establish a relationship between the structure and the ability to remove free radicals. Tea extraction is one of the most factors affecting the analytical results; the authors recommend water as the solvent of choice due to toxicological reasons. Is said to a temperature of 80 °C and a time of 30 minutes are the optimal conditions, while the water to tea ratio and the particle size (around 1 mm) are of importance[7], [13]. The differents samples were different particle size and the extracts showed diferents colours especially Jaibel that was pink and the other ones were yellow. The Folin-Ciocalteu assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phophotunsting acid complexes form blue which allows a masure of total phenols and other oxidation substrates. Total phenolic content (TPC) of green tea are presented en table 2, the highest content was Oriental (127.74 ± 3.32 mg acid galic equivalent/g sample) and the lowest was Jaibel (93.76 ± 1.38 mg acid galic equivalent/g sample). In general the four brands showed a decreasing behavior Oriental≥Lipton>Hindu>Jaibel. An analysis of variance ANOVA (tukey´s Multiple Comparision Test, significant $p< 0.05$), identified significant differeces between Oriental, Hindu and Jaibel. (ab, cd,cd) and no significant differences were found between Oriental and Lipton(ab,ab) (Figure 1).

D. Horžić et al compared TPC between herbals infusions and tea, the results showed that green tea have much higher content of phenols (1380-1830 mg/L gallic acid equivalents) as flavonoids (1070 – 1280 mg/L gallic acid equivalents) and these results are comparable with those obtained in this study; if expressed in mg/L gallic acid equivalents (table 2) a range between 1250.12 to 1703.22 mg/L acid gallic equivalent (Jaibel and Oriental) is obtained [14]. Another study evaluated the TPC in differents brands marketed in Chile, showed that the ranged of total phenols was 947.6-1678 mg/L gallic acid equivalents in green tea and 880.7 to 1822.5 mg/L gallic acid equivalents in black tea[15] . In a study of several brands of the green, of commercially available tea in Argentina, also determined that generally green tea leaves had higher total phenol content (14 to 21 g/100 g) [16], these latest are relatively close to our results. The results of TPC also were in accordance with A. Luximon-Ramma [12] showing content from 62 to 107 mg/g for total phenols content in nine commercially black tea, and 184 ± 36 mg/g in infusions of fresh tea leaves, however, It is important to note the difference in the extraction process. As one possible reason why in our study a higher content was obtained due to temperature conditions and extraction time. In general all samples tested showed high levels of flavonoids and phenolic compounds. The differences found between brands may be due to manufacturing process used by each industry, particle size and other compounds as if Jaibel who contains hibiscus and lemon peel.

Total Flavonoid content (TFC) of green tea are presented en table 2, the highest content was Lipton (29.15 ± 2.04 mg catechin equivalent/g sample) and Oriental (29.61 ± 1.43 mg catechin equivalent/g sample), while the lowest was Jaibel (10.42 ± 0.52 mg catechin equivalent/g sample). In general the four brands showed a decreasing behavior Liptonl≥Oriental >Hindu >Jaibel. An analysis of variance ANOVA (tukey´s Multiple Comparision Test, significant $p< 0.05$), identified significant differeces between Oriental, Hindu and Jaibel. (a, b, c) and no significant differences were found between Oriental and Lipton(a). (Figure 2).

The results of TFC also were in accordance with A. Luximon-Ramma [12] showing content from 15 to 26 mg/g for total flavonoids in nine commercially black tea, and 34± 5 mg/g in infusions of fresh tea leaves, while in another study the extraction was performed with methanol:HCl (50: 1) for 1 hour, and showed a lower content of flavonoids (0,671 ±0.041 g/kg) [17] than those reported in this study.

The antioxidant activity was determinted by aplications of the DPPH and ORAC method. An estimate of the antioxidant properties of pure compounds or extracts are their ability to trap free radicals, one of the most popular is the method employing stable, 2,20-diphenyl-1-picrylhydrazylradical ($DPPH^{\cdot}$) [18]. During this assay, the purple chromogen radical is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine.

The reduction of the purple chromogen radical by hydrogen-donating antioxidants is monitored by the decrease of optical density at long wavelengths (515–520 nm)[19]. The oxygen radical absorbance capacity (ORAC) assay has been widely used to measure the antioxidant activity of nutraceuticals, pharmaceuticals and foods, measure the classical ability of an antioxidant to quench free radicals by hydrogen donation[11]. The results are considered by some to be of biological relevance as a reference for antioxidant effectiveness. The antioxidant activity of green tea are presented in table 2, the highest content was Lipton (1123.39± 147.87 µmol equivalent Trolox/g sample) and Oriental (1138.45 ± 78.03 µmol equivalent Trolox/g sample) determined by DPPH assay, while the lowest was Jaibel (748.09 ± 39.76). In general the four brands showed a decreasing behavior Liptonl≥Oriental >Hindu >Jaibel. An analysis of variance ANOVA (tukey´s Multiple Comparision Test, significant $p< 0.05$), identified significant differeces between Oriental and Jaibel. (a, b) and no significant differences were found between the rest of samples. See figure 3. The results obtanied by ORAC allowed to see that the highest antioxidant activity was to Oriental tea (1588.05 ± 21.72) and the lowest was Jaibel (740.83 ± 37.81), and their behavior of all samples was Oriental>lipton>Hindu>Jaibel. Overall antioxidant activity of all samples showed the same behavior assessed by two methods; however ORAC data obtained are slightly higher. The ORAC assay provides a measure of both the general and specific antioxidant action of plant tissue extracts and can be used in combination with DPPH assay to compare and get better results, both methods are rapid, simple, low cost and used for food, beverages and plants. Jaibel showed the lowest levels of phenolic compounds, flavonoids and antioxidant activity, possibly due to the addition of hibiscus and lemon peel that could mask other compounds.

M. Jeszka-Skowron and A. Zgoła-Grześkowiak showed that the antioxidant activity of two out of four pure green tea infusions was 2- or even 3-fold higher than green tea with fruits or quince, green tea witht leemon had good results, these findings could provide information that additives such as jasmine petals and lemon skin are excellent antioxidants, but it is also probable that cheaper teas of worse quality were used for the production of the aromatized teas [20]. The antioxidant activity of Lipton was compared by other author (1000 µmol equivalent Trolox/g sample) who determined the antioxidant capacity of twenty-four commercial green tea varieties give values close to those obtained in our study[21].

The different antioxidant capacity exhibited by polypehnols is consistent with their chemical structure in regard to number and position of phenolic hidroxyl groups [22]. The Oriental and Lipton tea extract showed better antioxidant activity compared to Jaibel and simultaneously these two samples showed the highest and lowest content of phenolic compounds and flavonoids respectively, showing a corelacion between the activity-structure because the antioxidant activity determined by the ORAC method showed a correlation with the total phenolic content (r^2 =0.9911) see figure 5 and DPPH with total flavonoids (r^2= 0.997) see figure 6 . Green tea is an unfermented tea, for this reason is rich in polyphenols. A tipical chromatogram of a tea extracts is shown in figure 7, while table 2 shown lists the retention time of the standars. Comparing the retention times established by mixing standards, allowed to identify at least 10 of the 11 compounds in the extracts. The peaks corresponding to gallic acid, caffeine and EGCG are the most

abundant in all brands of tea (Figure 8-11). Though also it evidenced the presence of Trigonelline, GC, EGC, chlorogenic acid, catechin, EC, Ferulic acid and ECG. The antioxidant activity may be related to the high content of these compounds especially EGCG who has more hydroxyl groups within the structure, also compared to the literature reported where higher contents in tea. These results can be compared with those reported by C. Wu, H. Xu, J. Héritier, and W. Andlauer[23] where EGCG was the major catechin in all tea varieties, ranging from 44.6% to 53.7% of the total catechins, similar to most tea varieties in China. On the other hand the extraction process also influenced the results because the reflux extraction method has been used most often in the extraction of green tea caffeine and catechins[24]. In our study Jaibel presented differences in composition and antioxidant activity as it did not lodged catechin and was the only one that showed even in small proportions ferulic acid, possibly by the addition of hibiscus and lemon peel that could mask other compounds; while other brands that do not contain additives showed good results that correlate both polyphenol content and antioxidant activity.

Acknowledgments

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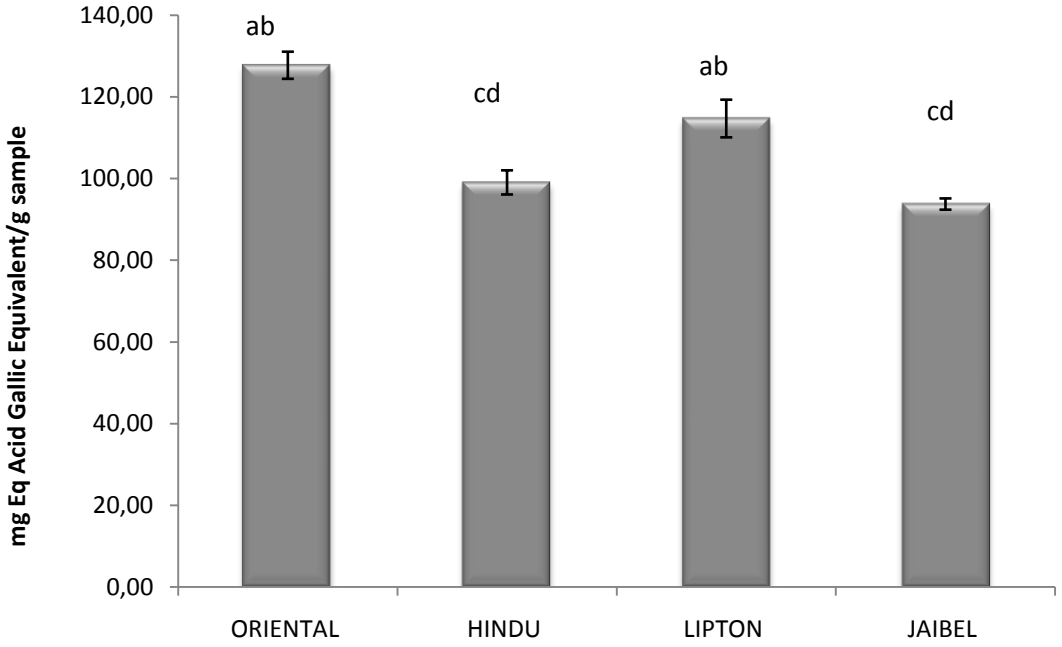

Figure 1. Total phenol content (TPC).

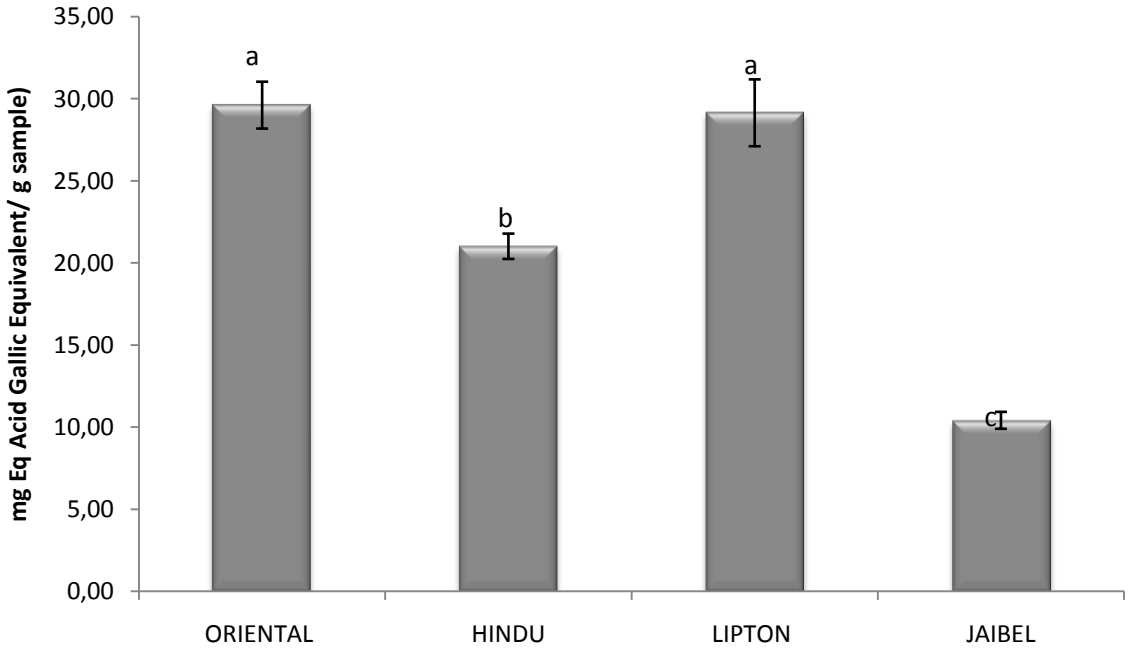

Figure 2. Total flavonoid content. (TFC)

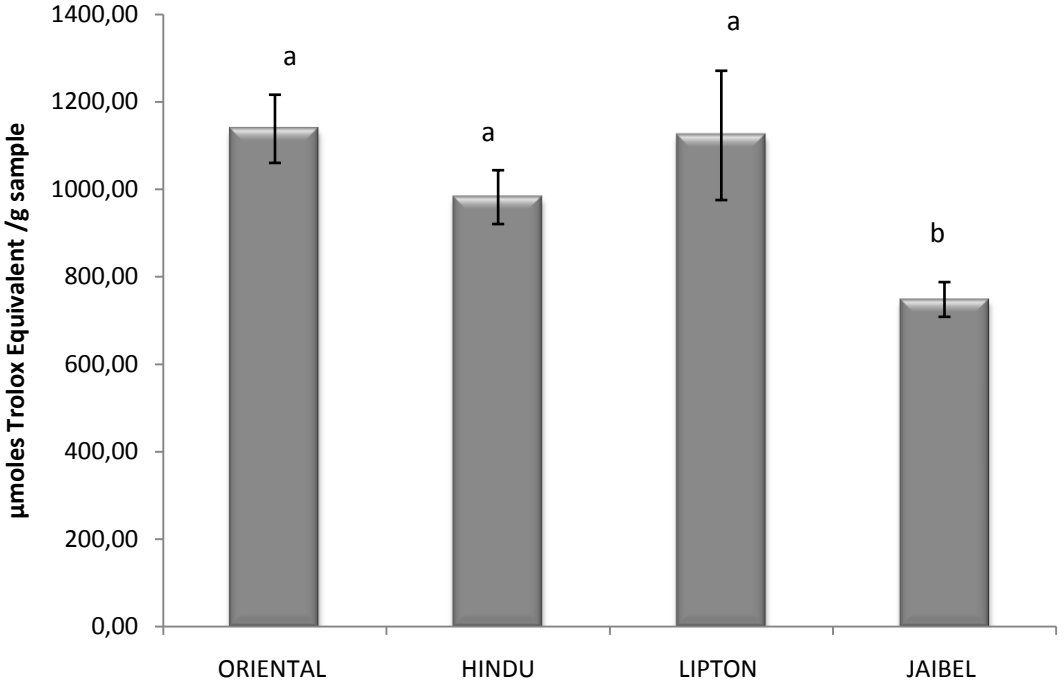

Figure 3. Antioxidant activity by DPPH

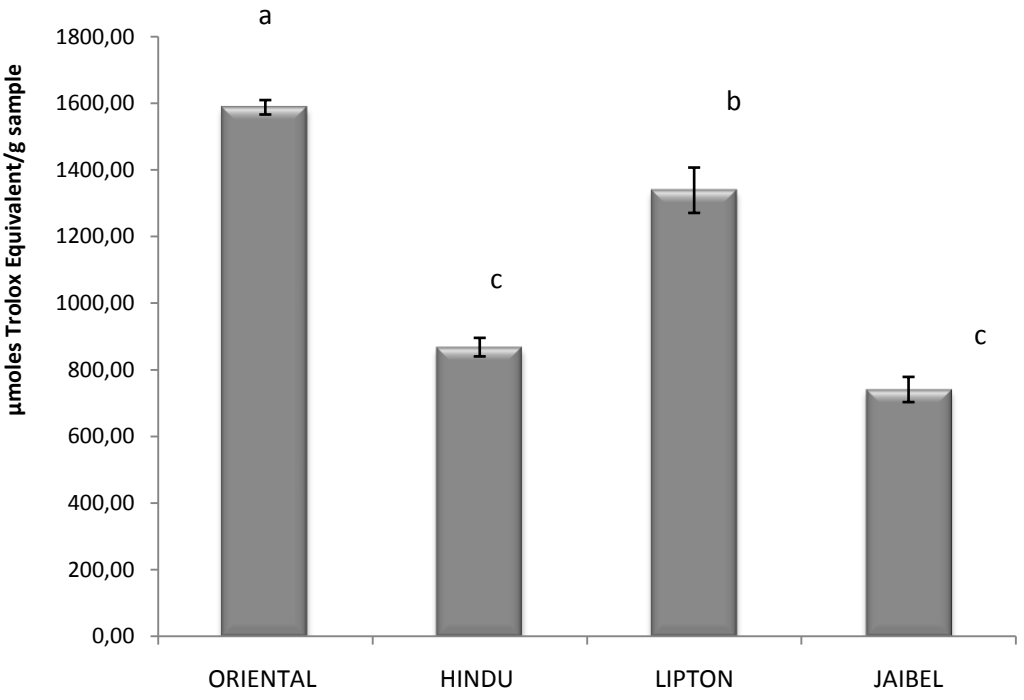

Figure 4. Antioxidant activity by ORAC

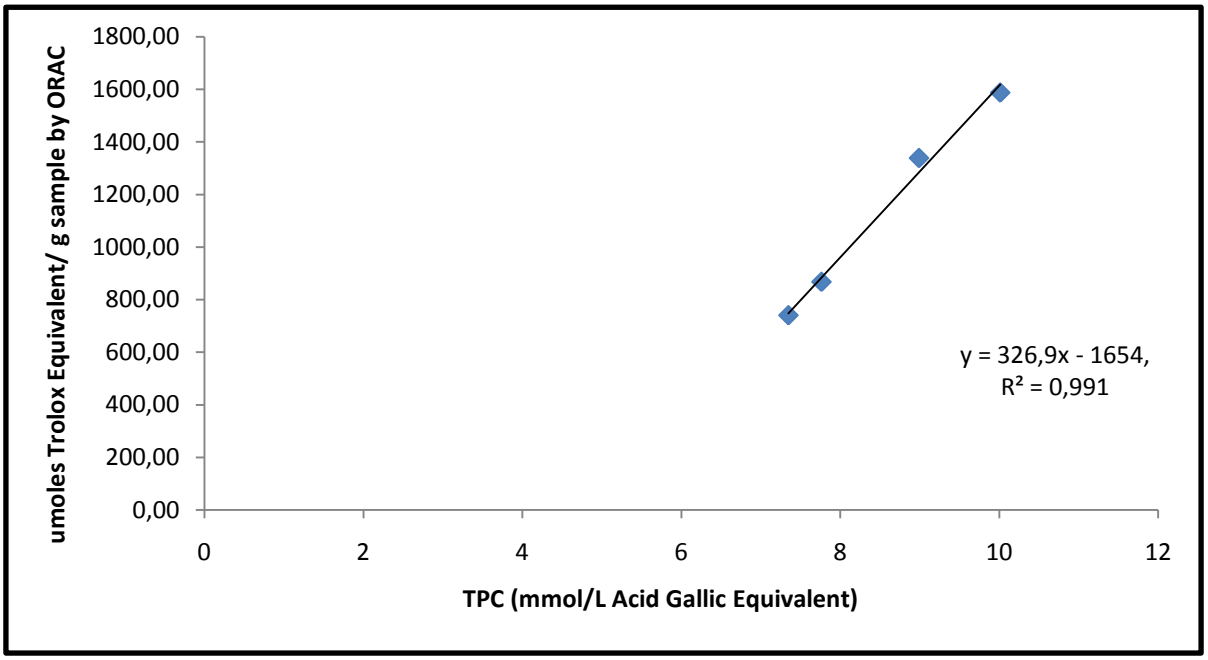

Figure 5. Correlation ORAC Vs TPC

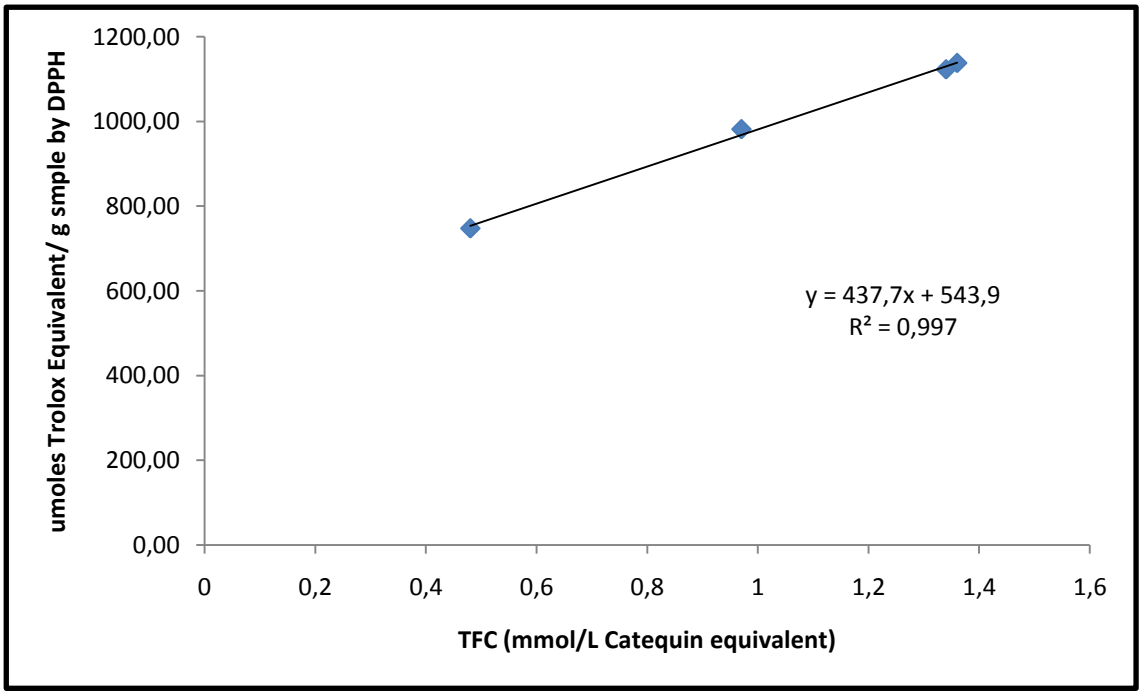

Figure 6.Correlation DPPH Vs TFC

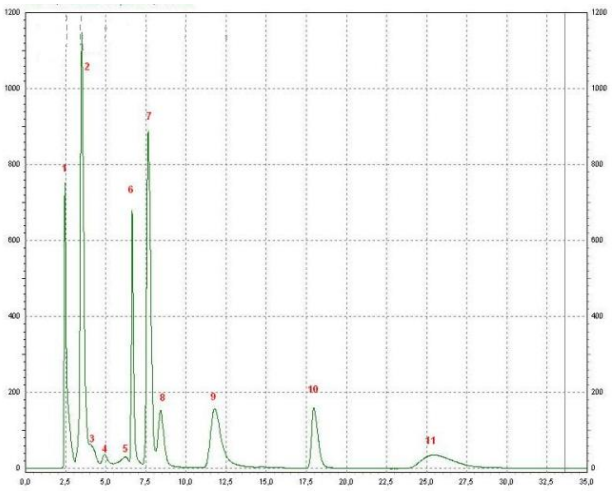

Figure 7. HPLC Chromatogram of the standars

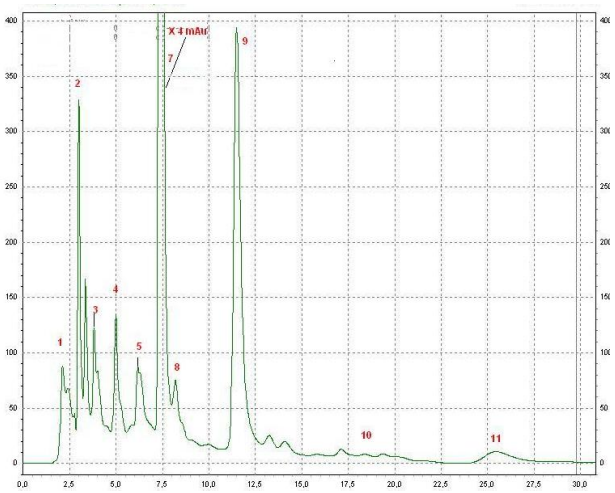

Figure 8. HPLC Chromatogram of the Jaibel green tea extract

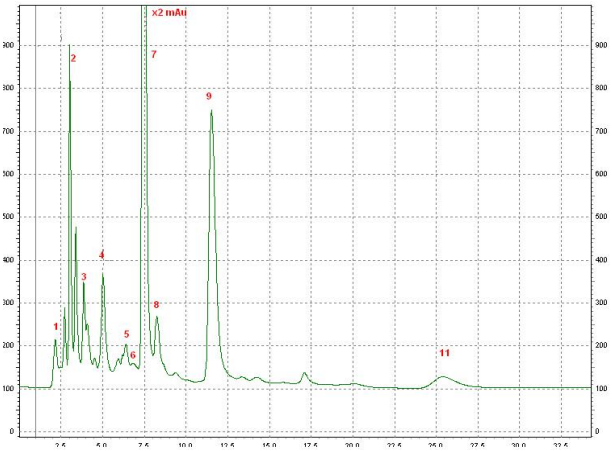

Figure 9. HPLC Chromatogram of the Lipton green tea extract

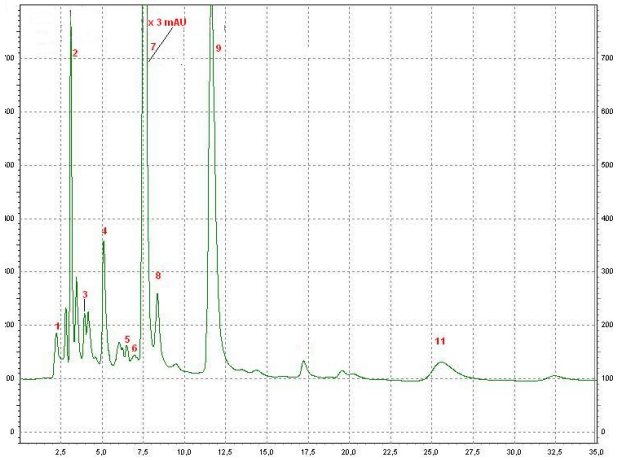

Figure 10. HPLC Chromatogram of the Lipton green tea extract

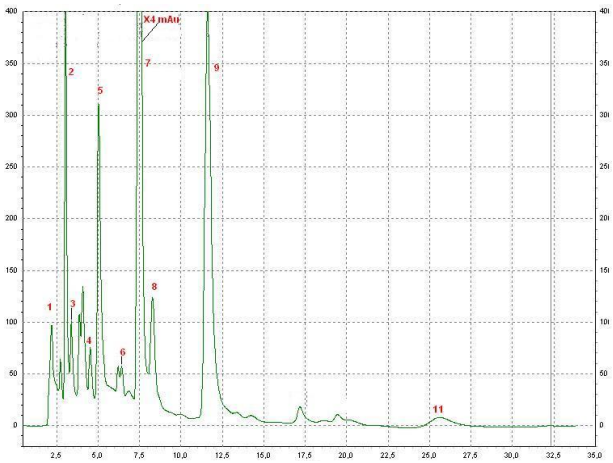

Figure 11. HPLC Chromatogram of the Hindu green tea extract

Table 1. Gradient elution profile for HPLC analysis

Time (min)	Solvent A	Solvent B	Flow (ml/min)
0	10	90	0,5
15	40	60	0,5
17	50	50	07
20	60	40	0,7
25	40	60	0,7
30	20	80	0,7
35	10	90	0,5

Table 2. Resume, TPC, TFC, DPPH and ORAC results of green tea

Sample	*Total phenol content (TPC)*		*Total flavonoid content. (TFC)*		*DPPH assay*	*ORAC assay*	
	mg/g eq AG	*mg/L eq AG*	*mg/g Eq Catechin*	*mg/L eq CAT*	*µmolesTrolox Eq/g sample*	*µmolesTrolox Eq/g sample*	*uM Trolox Eq*
Oriental	127.74 ± 3.32	1703 ±10.01	29.61 ± 1.43	394.85±19.03	1138.45 ± 78.03	1588.05 ± 21.72	21,17±0.29
Hindu	99.06± 2.96	1320.80± 7.76	21.02 ± 0.77	280.25± 10.29	982.20 ± 61.57	1339.03 ± 27.90	11.57±0.37
Lipton	114.70 ± 4.61	1529.38 ± 8.99	29.15 ± 2.04	388.63±27.16	1123.39 ± 147.87	867.95 ± 68.10	17.85±0.91
Jaibel	93.76 ± 1.38	1250.12 ± 7.35	10.42 ± 0.52	138.69±6.89	748.09 ± 39.76	740.83 ± 37.81	9.88±0.50

Table 3. Composition of green tea, determined by HPLC

Standar	Retention Time (tr=min)	# Peak	Presence/Absence			
			Oriental	Lipton	Hindu	Jaibel
Trigonelline	2.5	1	X	X	X	X
Gallic Acid	3.5	2	X	X	X	X
Gallocatechin	4.12	3	X	X	X	X
Epigalocatequina	5.13	4	X	X	X	X
Chlorogenic acid	6.42	5	X	X	X	X
Catechin	6.6	6	X	X	X	
Caffeine	7.63	7	X	X	X	X
Epicatechin	8.41	8	X	X	X	X
EGCG	12.05	9	X	X	X	X
Ferrúlico acid	18.5	10				X
ECG	26	11	X	X	X	X