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Purple tea catechins exhibit high antiproliferative activity and synergism with cisplatin against the triple-negative breast cancer cell line 4T1

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Abstract: The objectives of this study were the selection of the best tea clones with high catechin content among the known tea clones in Rwanda and Kenya, the examination of their antiproliferative effects on the triple-negative breast cancer (TNBC) cell line (4T1), and an evaluation of their combination index with cisplatin. The quantification of catechin contents in 14 different tea clones and 5 different processed teas was performed by high-performance liquid chromatography (HPLC). A comparative study of antiproliferative activities of catechin extracts from purple, TRFK306, and BB35 tea clones on the TNBC cell line (4T1) was undertaken, and their combination index (CI) with cisplatin and the dose reduction index (DRI) were determined. The catechin extract from BB35 had the highest concentration of total catechins (817.81±24.2 mg/g DW). After 72 h, the catechin extracts from TRFK306 showed a high IC_{50} of 68.68±3.30 μg/mL. The catechin extracts from TRFK306 showed the best synergism with cisplatin (CI=0.59), and they reduced the doses of cisplatin with the highest DRI=3.74493. Catechin extracts from purple tea showed higher antiproliferative activity and synergism with cisplatin against the TNBC cell line.

Keywords: tea catechins; tea clone; 4T1 cell line; combination index (CI); Dose Reduction Index (DRI)

INTRODUCTION

The tea plant (*Camellia sinensis*) from which the beverage tea is processed is an evergreen plant in the family of Theaceae, a genus of *Camellia* with many overlapping morphological, biochemical, and physiological characteristics [1]. *C. sinensis* consists of two main varieties, var. *sinensis* and var. *assamica,* generally known as China and Assam varieties [2]. A third variety, considered a subspecies of *C. assamica*, is *C. sinensis* var. *assamica* spp. *lasiocalyx* and is known as the Cambod variety [3,4]. Purple tea (referred to as TRFK 306) is one of the tea clones developed by the Tea Research Foundation of Kenya (TRFK) and is a variety of assamica [5].

Tea in the dried form, obtained from processing of apical shoots of tea plants, is one of the most consumed beverages globally [6,7]. Three main types of tea are obtained after its processing: fermented black tea, partially fermented red and oolong tea, and non-fermented green and white tea [2]. The major phenolics found in tea leaves include tea catechins. There are eight catechins (-)-catechin (C), ((-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), (-)-gallocatechin (GC), (-)-catechin gallate (CG), and (-)-gallocatechin gallate (GCG). The main types are (-)-epigallocatechin (EGC), (-)-epicatechin (EC), (-)-epigallocatechin gallate (EGCG), and (-)-epicatechin

gallate (ECG) [7]. Tea catechin contents depend on the tea variety, tea clones, and environmental stresses [3].

Tea has aroused interest among scientists due to the health benefits of its components, including phenolics, alkaloids and amino acids [6]. The health benefits include antioxidant, anticancer, antidiabetic, antiinflammatory, and anti-cardiovascular disease activities. The catechins are responsible for the anticancer activities of tea [2,7].

Breast cancer is a leading healthcare issue among women globally [8]. Triple-negative breast cancer (TNBC) is the most aggressive type of breast cancer. It is characterized by the lack of expression of the estrogen receptor (ER) and progesterone receptor (PR) and the absence of overexpression of the human epidermal growth factor receptor-2 gene (HER-2) [9]. Due to the lack of these specific therapeutic molecular targets, the chemotherapy of TNBC is difficult [10]. As an alternative treatment for TNBC, neoadjuvant chemotherapy regimens have been developed, resulting in significant improvements in the prognosis of TNBC patients [11]. Platinum-based chemotherapy based on the cis-structured platinum compound, cisplatin, is based on the induction of DNA cross-linking that subsequently leads to cell death [11]. However, treatment with cisplatin is toxic and produces inflammation [10]. Dietary herbs have immunomodulatory effects and enhanced therapeutic effects [12]. Combining herbal drugs with chemotherapy has shown promising results in treating and managing cancer [13].

This research aimed to select the best tea clones with high catechin contents among known tea clones in Rwanda and Kenya, to examine their antiproliferative effects on the TNBC cell line 4T1, and to evaluate their combination index with cisplatin.

MATERIALS AND METHODS

Sample collection sites

Tea samples (tea clones, different grades of green tea) were collected from Rutsiro tea factory, Western Province, Rwanda, and in the Ngere tea factory, Murang'a County, Kenya. Black and green tea were collected in supermarkets in Juja, Kiambu County in Kenya (Supplementary material).

Description of samples

Three different types of samples were collected: samples of fresh leaves of tea (14 different tea clones; 4 samples of each tea clone), different processed tea grades from tea factories (3 different tea grades; 3 samples on each grade), and samples of processed tea found on the market (green tea: 3 samples, black tea: 3 samples) (Supplementary Table S1).

Plant material collection and authentication

One hundred g per sample of fresh tea shoots comprised of two leaves and buds were used; four samples were collected from each tea clone. The fresh tea leaves were transported directly to the laboratory for analysis. Plant identification and authentication were made in the herbarium of the botanical garden of INES Ruhengeri-Institute of Applied Sciences, Rwanda, with the accession number INSH2346.

Chemicals and reagents

Analytical grade chemicals were used in the analyses; those used in HPLC analysis were all for HPLC grade and were purchased from certified supply companies. The HPLC standards (-)-epigallocatechin (>98%), (-)-epicatechin (>98%), (-)-epigallocatechin gallate (>98%), and (-)-epicatechin gallate (>98%), resazurin, and cisplatin standard were purchased from Solarbio Life Sciences company, Beijing, China. RPMI 1640 was purchased from BioConcept Ltd, Allschwil, Switzerland. Eagle's Minimum Essential Medium (EMEM) and fetal bovine serum were purchased from Sigma-Aldrich, USA.

Extraction of tea catechins

Preparation of tea samples and the extraction of tea catechins followed the methods used by [14] with some modifications. The fresh tea leaves were steamed in the oven at 100°C for 40 s followed by drying in three steps: first at 100°C for 40 min, then at 35°Cfor 40 min and finally dried at 80°C for 90 min. The dried tea leaves were ground using a blender, the powder was packed in black zip-lock aluminum pouches and stored in the fridge at 4°C. An ultra-sound-assisted method was used for extraction. Ground tea (10 g) was mixed with 200 mL of ethanol 40% and then placed in a sonicator at

40°C for 2 h. The mixture was filtered, and the ethanol was evaporated by using a vacuum rotary evaporator at 45°C. After rotary evaporation, the final volume was adjusted to a final volume of 200 mL with distilled water.

Isolation of tea catechins and decaffeination

Isolation of tea catechins was performed by ethyl acetate/dichloromethane solvation [14]. Equal volumes of tea extracts (200 mL) and ethyl acetate were mixed for 30 min, followed by a partition of the mixture between an aqueous layer and ethyl acetate. The ethyl acetate layer was collected, and the remaining aqueous layer was mixed with 200 mL of ethyl acetate two times, followed by extraction of each layer of ethyl acetate on the top of the aqueous layer. The collected layers of ethyl acetate-containing tea catechins and caffeine were evaporated by the vacuum evaporation method at 40°C. After evaporation, the remaining extract was adjusted to a final volume of 200 mL by distilled water. The decaffeination process was performed three times by using 200 mL of dichloromethane. The bottom layers of dichloromethane were eliminated, and the decaffeinated aqueous layers were retained. The collected aqueous solution was dried by freeze drying.

HPLC analysis

Catechin standards were prepared following the manufacturer's protocols. The freeze-dried extract of tea containing the catechins was diluted in HPLC-grade water (20 mg/mL). Before HPLC analysis, the catechin standard solution and the tea extract solutions were filtered using a syringe filter (pore size: 0.22μm). HPLC analysis was carried out by following the method developed by [15] with some modifications. The HPLC system used in this study was Shimadzu-equipped with SIL-20A HT auto-sampler and a Shimadzu SPD-M20A Prominence Diode Array Detector, and the wavelength was set at 254 nm. The HPLC column was OCG-4252-E0 Luna® 5 μm C18 (2) (250 x 4.6 mm) kept in a CTO-10AS VP oven at 40°C. The isocratic mode was used; the mobile phase was at a ratio of water:acetonitrile of 87:13. It contained 0.05% trifluoroacetic acid (TFA) (vol/vol). The flow rate was 1 mL min^{-1}. The injection volume was 20 μL. The specific peaks of the compounds were identified by comparing their retention time and absorbance with the standards. The calibration curve was constructed using Shimadzu LabSolutions CS software with five levels of different concentrations of standards.

Cell lines and culture conditions

Both the 4T1 mammary carcinoma cell line and the Vero CCL-81 cell line were obtained from ATCC (Manassas, VA, USA). The 4T1 cells were grown in RPMI 1640 supplemented with 25 mM HEPES and L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. Vero CCL-81 cells were grown in EMEM media supplemented with 10% FBS, 1% penicillin-streptomycin, 1% L-glutamine and 1% HEPES. All cells were grown in T75 cell culture flasks and incubated at 37°C and 5% CO_2.

Resazurin metabolic assay

The resazurin metabolic assay is based on the reduction by living cells of oxidized blue dye (resazurin reagent) into a pink product, resorufin [16]. The half-maximal inhibitory concentration (IC_{50}) and the half-maximal cytotoxic concentration (CC_{50}) of two different tea catechin extracts from two different tea clones (purple tea, TRFK 306) and the BB35 and cisplatin standard on 4T1 mammary carcinoma cells and Vero CCL-81 cells, respectively, were determined. The cells at 80-90% confluency were washed with 8 mL phosphate-buffered saline (PBS) twice, detached by 1 mL of 0.25% trypsin-EDTA, incubated for 3-4 min, then counted by a hemocytometer after staining with 0.4% trypan blue. The cells (4T1 mammary carcinoma cells and Vero CCL-81 (normal) in suspension were seeded in 96-well plates at a density of 1×10^4 cells in100 μL of growth media per well and incubated at 37°C and 5% CO_2 for 24 h for cell attachment. The seeding media was then aspirated and replaced by 100 μL of fresh media with different working concentrations of drugs (500 μg/mL, 375 μg/mL, 250 μg/mL, 125 μg/mL and 25 μg/mL) for catechin extracts and 150 μg/mL, 75 μg/mL, 18.75 μg/mL, 3.75μg/mL and 1.875 μg/mL for cisplatin standards prepared from stock solutions. Dimethyl sulfoxide (DMSO 0.5%) was used as a solvent control. The treated cells in plates were incubated as described above for 24 h, 48 h, and 72 h. For determination of cell viability, 20 μL of resazurin (0.15 mg/mL) were added to each well and incubated at 37°C for 3 or 4 h, then

the absorbance was read at a wavelength of 570 nm and a reference wavelength of 600 nm using a plate reader Multiskan Go (Thermo Scientific, USA).

The percentage of cell viability was calculated using the following formula:

$$\%\ \text{cell viability} = \frac{(Abs.treated\ cell - abs.blank)}{(Abs.untreated\ cells - abs.blank)} x100 \quad [17]$$

with the blank containing media and resazurin.

Each experiment was conducted in triplicate. The graph of percentage cell viability against the concentration of drugs was constructed. IC_{50}, IC_{40}, IC_{30}, IC_{20}, IC_{10} and CC_{50} were calculated using nonlinear regression (curve fit), GraphPad Prism 8.0.2 software.

Determination of the selectivity index (SI)

The selectivity index indicates the ability of drugs or extracts to selectively kill the cancer cells while sparing normal cells [18]. It was calculated as the ratio of the CC_{50} of normal cells (Vero CCL-81) over the IC_{50} of cancer cells (4T1 mammary carcinoma cells).

Combination of tea catechin extracts and cisplatin

The CI and the DRI determined whether tea catechins exhibit synergism with cisplatin when applied to 4T1 mammary carcinoma cells. This was obtained by slightly modifying the protocol used by [17]. Different combinations of the inhibitory concentrations of catechin extracts and cisplatin were used as follows: IC_{40}+IC_{10}, IC_{30}+IC_{20}, IC_{20}+IC_{30}, and IC_{10}+IC_{40} (catechin extracts+cisplatin). Catechin extracts from BB35 tea and those from purple tea were each combined with cisplatin. On the same plate, the treatment of cells with IC_{10}, IC_{20}, IC_{30}, IC_{40}, and IC_{50} of each drug was performed. Other procedures were the same as those used for the determination of the IC_{50} and CC_{50} by the resazurin metabolic assay. The following formulae were used to calculate the CI and the DRI:

$$\text{Combination Index (CI)} = \frac{(D)_1}{(Dx)_2} + \frac{(D)_2}{(Dx)_2} \quad [19]$$

$$\text{Dose Reduction Index (DRI); } (DRI)_1 = \frac{(Dx)_1}{(D)_1};$$

$$(DRI)2 = \frac{(Dx)_2}{(D)_2} \quad [19]$$

where D is the dose (or concentration of the drug), $(Dx)_1$ and $(Dx)_2$ are D_1 and D_2 alone, respectively, that can inhibit a system by x%. The results were analyzed using CompuSyn software developed by Ting-Chao Chou and Nick Martin [20].

Statistical analysis

The percentages of each catechin were analyzed using an Excel data sheet. The significance determination of variations of each type of catechin content between different groups was conducted by one-way ANOVA in R software, version R 4.2.1, with a P value set at 0.05. Means comparison was performed by the least significant difference (LCD) test. GraphPad Prism 8.0.2 software was used for IC and CC_{50} calculation, and Tukey's multiple comparison test (TMCT) was used to compare different treatments.

RESULTS

HPLC profiles of catechin elution

Catechin compounds were eluted in the following order: (-)-epigallocatechin (EGC), (-)-epicatechin (EC), (-)-epigallocatechin gallate (EGCG), and (-)-epicatechin gallate (ECG) (Fig. 1A and B). The tea catechin extracts

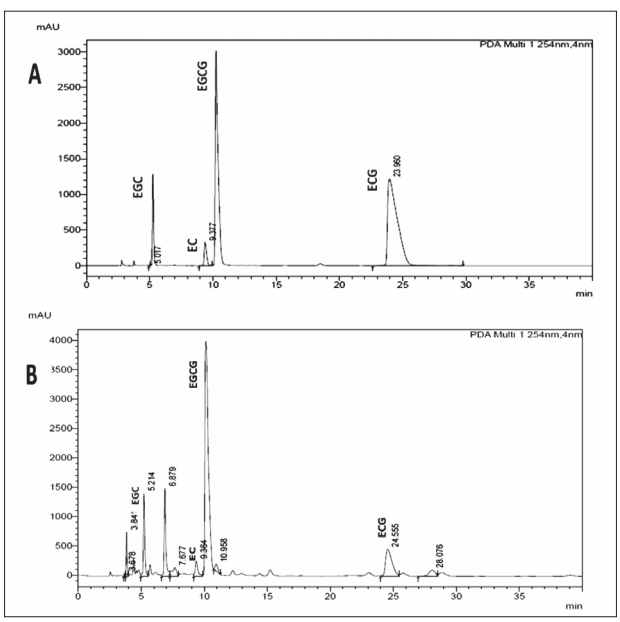

Fig. 1. HPLC profiles of catechin elution. **A** – Peaks of mixed catechin standards. **B** – Peaks of sample of tea catechin extracts. The HPLC catechin standards used were pure as shown by the presence of only four peaks corresponding to the four catechins mixed.

contained four main catechin polyphenols (EGC, EC, EGCG and ECG), as well as other compounds (Fig. 1).

Comparison of the catechin content in different tea clones

Four different catechins, EGC, EC, EGCG, and ECG, in different tea clones, were evaluated and compared among 14 tea clones. For EGC content, there was a significant difference in some of the 14 tea clones analyzed. Tea clone SFS and TRFK 371/3 had the highest EGC contents with mean values of 232.19±9.01 mg/g and 214.78±15.75 mg/g, respectively, and there was no significant difference between these two clones. There were no significant differences between TRFK 301/4, TRFK 31/8, TRFK 6/8, and BB35 clones, and no significant differences between TRFK 100/5, TRFK 18/52, TRFK 303/577, TRFK 7/3, IB475, and KAG 501 clones. Purple (TRFK306) tea clones had the lowest EGC content, with 52.15±10.61 mg/g (Table 1).

For the EC content, significant differences were observed between some of the 14 analyzed tea clones. The clone with the highest EC content was TRFK 301/4, with a mean of 169.16±34.89 mg/g. There were no significant differences in EC contents between TRFK 100/5, TRFK 18/52, TRFK 6/8, TRFK 7/3, IB475, and KAG 501. The TRFK 371/3 and SFS10 clones had the lowest EC content, with 67.344±15.07 mg/g and 58.6±15.11 mg/g, respectively, and there was no significant difference between these two clones (Table 1).

For the EGCG content, significant differences were detected in some of the 14 tea clones that were analyzed. The tea clone with the highest EGCG content was the purple (TRFK306) clone, with a mean value of 552.2±10.61 mg/g. There were no significant differences between the following tea clones: TRFK 100/5, TRFK 301/4, TRFK 31/8, and TRFK 6/8. The tea clones that had the lowest EGCG content were TRFK 371/3 and IB475, and there was no significant difference between them, with mean values of 256.62±10.61 mg/g and 313.9±53.74 mg/g, respectively (Table 1).

For the ECG content, significant differences were noted in some clones. The clone that had the

Table 1. Comparison between catechin contents among different tea clones. The comparison was performed by one-way ANOVA, each catechin content as presented in each column was compared with different tea clones.

S/N	Name	EGC (mg g^{-1} DW)*±SD	EGC(%age)	EC (mg g^{-1} DW)*±SD	EC(%age)	EGCG (mg g^{-1} DW)*±SD	EGCG(%age)	ECG (mg g^{-1} DW)*±SD	ECG (%age)	TOTAL! (mg g^{-1} DW)±SD
1	TRFK 100/5	100.55±12.51 ^{c}	16.16%	124.44±26.57 ^{b}	20.00%	326.27±34.26^{def}	52.45%	70.84±14.16^{ef}	11.39%	622.11±11.45^{k}
2	TRFK 18/52	96.73±15.03 ^{c}	14.13%	127.65±7.99 ^{b}	18.65%	364.4±20.18 ^{cd}	53.24%	95.73±3.18^{cde}	13.99%	684.51±27.65^{g}
3	TRFK 301/4	155.289±6.45 ^{b}	21.32%	169.16±34.89 ^{a}	23.23%	331.93±18.635^{def}	45.57%	71.952±8.36^{ef}	9.88%	728.33±13.91^{e}
4	TRFK 303/577	93.62±4.35 ^{c}	14.01%	120.01±11.01^{bcd}	17.96%	363.98±11.57^{cde}	54.47%	90.62±4.21^{cdef}	13.56%	668.24±8.01^{h}
5	TRFK 31/8	159.27±28.48 ^{b}	23.86%	84.46±14.93 ^{de}	12.65%	342.28±27.60^{def}	51.27%	81.56±17.63^{def}	12.22%	667.57±7.48^{i}
6	TRFK 371/3	214.78±15.75 ^{a}	34.99%	67.344±15.07 ^{e}	10.97%	256.62±10.61^{g}	41.81%	75.10±3.182^{def}	12.23%	613.84±7.08^{j}
7	TRFK 6/8	162.89±23.32 b	23.04%	139.98±9.29 ^{b}	19.80%	338.69±12.97^{def}	47.90%	65.45±11.83^{f}	9.26%	707.01±12.38^{f}
8	TRFK 7/3	82.14±14.73 cd	13.81%	136.8±16.40 ^{b}	23.01%	307.34±4.74^{efg}	51.69%	68.35±3.1^{f}	11.49%	594.63±12.64^{n}
9	BB10	52.43±5.69 ^{d}	8.76%	117.03±6.45^{bcd}	19.56%	317.68±26.41^{fg}	53.10%	111.11±14.11^{bc}	18.57%	598.24±18.28^{m}
10	BB35	162.44±26.30 ^{b}	19.86%	122.53±17.15 ^{bc}	14.98%	442.76±20.52 ^{bc}	54.14%	90.07±20.40^{cdef}	11.01%	817.81±24.24^{a}
11	IB475	93.75±7.14 ^{c}	15.63%	127.90±14.85 ^{b}	21.33%	313.9±53.74^{g}	52.34%	109.12±18.29^{bc}	18.20%	599.72±11.24^{l}
12	KAG 501	103.23±3.01 ^{c}	13.01%	130.05±17.17 ^{b}	16.39%	431.15±50 ^{b}	54.33%	129.1±4.85^{ab}	16.27%	793.53±9.93^{c}
13	PURPLE (TRFK306)	52.15±10.61 ^{d}	6.53%	88.98±9.68^{cde}	11.15%	552.2±10.61 ^{a}	69.18%	104.88±16.86^{bcd}	13.14%	798.2±10.15^{b}
14	SFS10	232.19±9.01 ^{a}	31.71%	58.6±15.11 ^{e}	8.00%	302.34±5.38^{fg}	41.29%	139.08±8.18^{a}	18.99%	732.21±16.83^{d}
F value		35.93	NA	7.667	NA	15.08	NA	6.178	NA	17.91
P value		0.0001	BA	0.0001	NA	0.0001	NA	0.0001	NA	0.001

Groups with the same letters in the same column are not significantly different at P=0.05.

* mg g^{-1} of dried weight of tea extracts (DW). EGC – epigallocatechin; EC – epicatechin; EGCG – epigallocatechin gallate; ECG – epicatechin gallate; ! – the total amount of catechins, which are combined with all four individual catechins.

highest ECG content was SFS10, with a mean value of 139.08±8.18 mg/g. The TRFK 3/7, and TRFK 6/8 clones had the lowest ECG contents, with values of 68.35±3.1 mg/g and 65.45±11.83 mg/g, respectively; there was no significant difference between these two clones (Table 1).

For the catechin content, significant differences were observed. The tea clone with the highest catechin content was BB35, with a value of 817.81±24.24 mg/g. The second tea clone with a high catechin content was clone purple (TRFK306), with a value of 798.2±10.15 mg/g. The TRFK7/3 clone had the lowest catechin content of 594.63±12.64 mg/g (Table 1).

Comparison of the catechin contents in different processed teas

As in tea clones, 4 different catechins in processed tea were evaluated and compared. For the EGC content, there was a significant difference among the different groups. The processed tea with the highest EGC content was the green tea FBOP, with a value of 160.9±12.30 mg/g. No significant difference was found between the green tea BOP1 and the green tea PEAKOE. The group with the lowest EGC content was green tea bought from the market, with a mean value of 109.94±2.74 mg/g. No EGC was detected in the black tea group (Table 2).

For the EC content, there was a significant difference between the five different groups of processed tea. The group of processed tea with the highest EC content was green tea PEAKOE, with a mean value of 136.30±5.14 mg/g. The green tea FBOP and the green tea obtained from the market exhibited no significant differences in EC content. The black tea group had the lowest EC content of 36.80±6.12 mg/g (Table 2).

EGCG content did not exhibit any significant difference in the different groups of processed tea. The groups of green tea PEAKOE and green tea BOP1 had the highest EGCG contents with mean values of 425.7±11.12 mg/g and 414.7±16.12 mg/g, respectively, and there was no significant difference between these two groups of processed tea. Apart from the black tea group, which had the lowest EGCG content of 11.44±12.45 mg/g, the green tea obtained on the market had the lowest EGCG content with a mean value of 282.9±25.83 mg/g (Table 2).

There was a significant difference in ECG content among the five groups. The processed tea with the highest ECG content was the green tea obtained from the market, with a mean value of 144.655±3.57 mg/g. Green teas BOP1 and PEAKOE did not display a significant difference in ECG content, with values of 90.85±7.14 mg/g and 100.55±6.70 mg/g, respectively. ECG was not detected in the black tea group (Table 2).

For the total catechin content, a significant difference was observed for the green tea BOP1 and green tea PEAKOE, which had the highest total catechin

Table 2. Comparison between catechin contents among different processed teas. The comparison was done by using one-way ANOVA, each catechin content as presented in each column was compared among processed teas.

S/N	Name	EGC (mg g^{-1} DW)*±SD	EGC (%age)	EC (mg g^{-1} DW)*±SD	EC (%age)	EGCG (mg g^{-1} DW) *±SD	EGCG (%age)	ECG (mg g^{-1} DW)*±SD	ECG (%age)	TOTAL! (mg g^{-1} DW) ±SD
1	BLACK TEA	ND	NA	36.8±6.12^{c}	NA	11.44±12.45^{d}	NA	ND	NA	NA
2	Green tea BOP1	158.5±9.85^{ab}	19.97%	129.65±6.14^{ab}	16.33%	414.7±16.12^{a}	52.25%	90.85±7.14^{b}	11.45%	793.7±13.56^{a}
3	Green tea MARKET	109.94±2.74^{b}	16.87%	115.82±15.34^{b}	17.77%	282.9±25.83^{c}	43.40%	144.655±3.57^{a}	21.96%	653.315±21.89^{b}
4	Green tea PEAKOE	153.95±8.25^{ab}	18.85%	136.30±5.14^{a}	16.69%	425.7±11.12^{a}	52.14%	100.55±6.70^{b}	12.31%	816.5±10.12^{a}
5	Green tea FBOP	160.9±12.30^{a}	23.63%	115.84±2.24^{b}	16.88%	320.49±24.14^{b}	47.07%	83.65±17.9^{c}	12.28%	680.88±14.45^{b}
F value		13.35	NA	3.799	NA	304.6	NA	2.311	NA	17.91
P value		0.001	NA	0.05	NA	0.0001	NA	0.05	NA	0.01

Groups with the same letters in the same column are not significantly different at P value = 0.05.
* mg g^{-1} of dried weight of tea extracts (DW).
EGC – epigallocatechin; EC – epicatechin; EGCG – epigallocatechin gallate; ECG – epicatechin gallate; ! – the total amount of catechins, which are combined with all four individual catechins.
ND – non-detectable; NA – not applicable, BOP – broken orange pekoe; FBOP – flowery broken orange pekoe
Values are expressed as means ± standard deviation of the means..

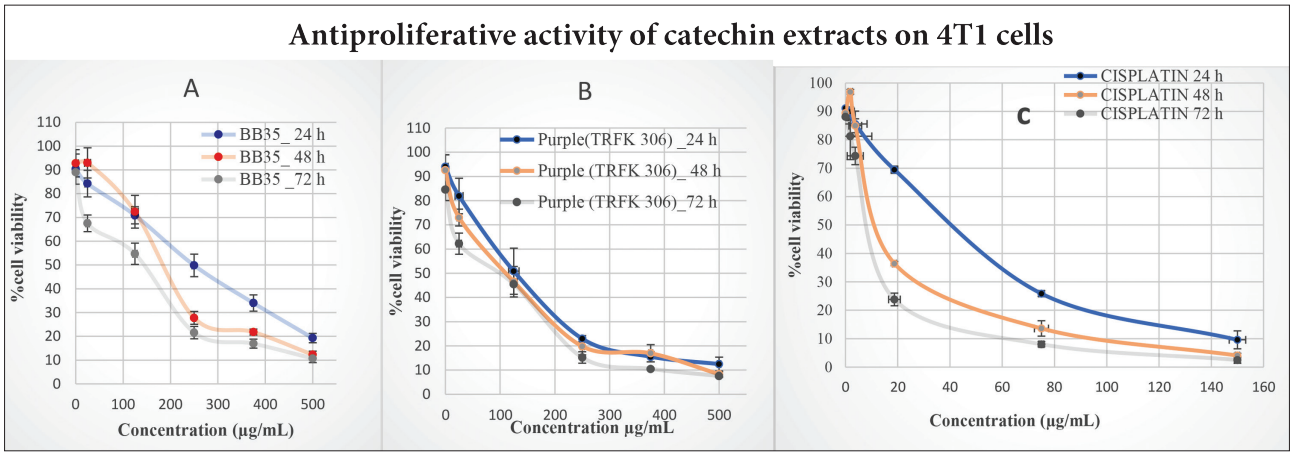

Fig. 2. Antiproliferative activity of catechin extracts on 4T1 mammary cancer cells; BB35 tea clones (**A**), catechin extracts from purple TRFK 306 tea clones (**B**), and cisplatin (**C**) on 4T1 mammary carcinoma cells. The values were calculated from three different experiments (n=3). The coefficient of determination (r^2) was above 0.95 for all curves. IC_{50} of all drugs was decreased as the time of drug exposure of the cells increased.

contents of 793.7±13.56 mg/g and 816.5±10.12 mg/g, respectively. The green tea FBOP and green tea obtained on the market did not significantly differ in their catechin contents, which were 680.88±14.45 mg/g and 653.315±21.89 mg/g, respectively (Table 2). Two tea clones with the highest catechin content, BB35, and purple (TRFK306), were used for further analysis.

Antiproliferative activity of catechin extracts on 4T1 cells

The half maximal inhibition concentration (IC50) of catechin extracts and cisplatin on 4T1 mammary carcinoma cells were determined after 24 h, 48 h, and 72 h. The IC_{10}, IC_{20}, IC_{30}, and IC_{40} were calculated and used for combination index determination. Catechin extracts from BB35, and purple (TRFK 306) clones demonstrated antiproliferative activities against 4T1 cells. Their antiproliferative activities increased with increasing drug exposure time. The IC_{50} values for BB35 were 270.9±9.23 µg/mL, 199.7±5.75 µg/mL, and 79.71±2.96 µg/mL for exposure times of 24 h, 48 h, and 72 h, respectively. The IC_{50} values for purple TRFK 306 tea were 118.0±3.09 µg/mL, 99.70±5.69 µg/mL, and 68.68±3.30 µg/mL for exposure times of 24 h, 48 h, and 72 h, respectively. The IC_{50} values of cisplatin were 40.15±1.02 µg/mL, 16.87±0.99 µg/mL, and 10.69±0.37 µg/mL for exposure times of 24 h, 48 h, and 72 h, respectively (Fig.2).

Comparison of antiproliferative activities of tea catechin extracts from BB35, the purple TRFK 306 tea clone, and cisplatin

The catechin extracts from the purple tea had higher antiproliferative activities on 4T1 mammary carcinoma cells than those from BB35 tea clones at 24 h and 48 h at P<0.001, but there was no significant difference (P>0.05) after 72 h of exposure. Cisplatin displayed higher antiproliferative activity than both catechin extracts (P <0.0001 at all exposure times (24h, 48h, and 72h)) (Fig.3).

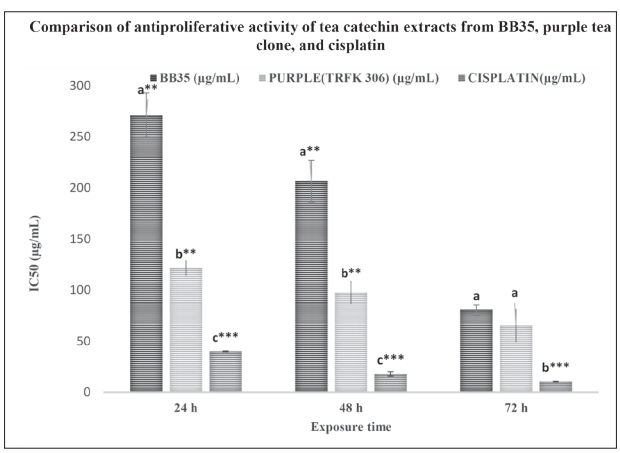

Fig. 3. Comparison of the antiproliferative activity of tea catechin extracts from BB35, the purple tea clone, and cisplatin. Different IC_{50} of each tested drug were plotted against the exposure time for the drug. The same letter in the same exposure time means that there was no significant difference. *** – P value at 0.0001, ** – P value at 0.001.

Table 3. Selectivity index. The half-maximal cytotoxic concentration of catechin extracts from BB35, purple TRFK 306, and cisplatin on normal cells (Vero CCL-81) was divided by the half-maximal inhibitory concentration of the catechin extracts from BB35, purple TRFK 306 tea clone, and cisplatin on the cancer cells line (4T1).

Cell lines	Exposure time		
	Catechin extract from BB35		
	24 h	48 h	72 h
Vero CCL-81 cell line CC_{50} (ug/mL)	381.7±20.49	320.04±23.96	268.4±15.4
4T1 cell line IC_{50} (ug/mL))	270.9±9.23	199.0±5.75	79.71±2.96
Selectivity index	1.4	1.6	3.36
	catechin extract from purple tea		
Vero CCL-81cell line CC_{50} (ug/mL)	320.0±32.42	259.5±22.4	210.1±19.96
4T1 cell line IC_{50} (ug/mL))	118.0 ± 3.09	99.7±5.11	68.44±3.30
Selectivity index	2.71	2.6	3.07
	cisplatin		
Vero CCL-81 cell line CC_{50} (ug/mL)	47.7± 3.74	43.02±6.68	15.48±1.29
4T1 cell line IC_{50} (ug/mL))	40.15±0.24	16.87±0.99	10.69±0.37
Selectivity index	1.188	2.55	1.44

$CC_{50:}$ – half maximal cytotoxic concentration; IC_{50} – half maximal inhibitory concentration. Values are expressed as means±standard deviation of the means.

Selectivity index (SI)

The half-maximal cytotoxic concentration (CC_{50}) in Vero CCL-81 cells was determined for selective index calculation. The CC_{50} for the BB35 catechin extract was 361.8±20.49 µg/mL (mean±SD), 320±23.96 µg/mL, and 268.4±15.4 µg/mL after exposure times of 24 h, 48 h, and 72 h, respectively. The CC_{50} for the purple (TRFK 306) tea was 320±32.42 µg/mL (mean±SD), 259.5±22.4 µg/mL, and 210±19.96 µg/mL after 24 h, 48 h, and 72 h, respectively, while the CC_{50} for cisplatin was 47.7±3.74 µg/ml (mean±SD), 43.02±6.68 µg/mL, and 15.48±1.29 µg/mL (mean±SD) after 24 h, 48 h and 72 h, respectively. The selectivity indices of different agents found in the tea catechin extracts from the BB35 tea clone, the purple (TRFK 306) tea clone, and cisplatin were calculated based on the IC_{50} obtained on normal (CCL-81) and cancer cells (4T1 mammary carcinoma cells).

The catechin extracts from tea exhibited a higher selectivity index than cisplatin. The selectivity indices were 3.36 and 3.07 for the catechin extracts from BB35 tea and the purple (TRFK306) clone, respectively, and 1.44 for cisplatin. The SI of catechins increased with the drug exposure time (Table 3).

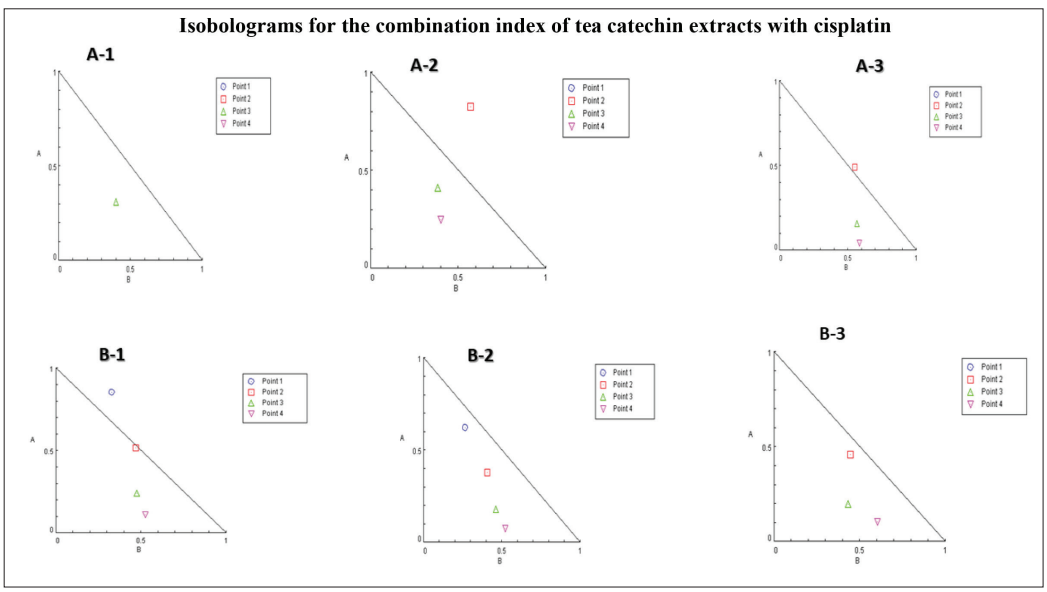

Fig. 4. Isobolograms for the combination index of tea catechin extracts with cisplatin: combinations of catechin extracts from the BB35 tea clone with cisplatin (A-1, A-2, and A-3 for 24 h, 48 h, and 72 h, respectively); the combination of catechin extracts from the purple tea clone with cisplatin (B-1, B-2, and B-3 for 24 h, 48 h, and 72 h, respectively). Point 1: IC_{40}+IC_{10} (catechin extracts+cisplatin); Point 2: IC_{30}+IC_{20} (catechin extracts+cisplatin); Point 3: IC_{20}+IC_{30} (catechin extracts+cisplatin); Point 4: IC_{10}+IC_{30} (catechin extracts+cisplatin). The points that are below the diagonal (CI<1) indicate synergism, ones that are on the diagonal (CI=1) indicate additive activity, and those above the diagonal (CI>1) indicate indifference or antagonism.

Isobolograms of drug combination of tea catechin extracts with cisplatin

The catechins extracted from the BB35 and purple TRFK 306 teas were each combined with cisplatin to assay for synergism, and the CI and DRI were determined. Catechin extracts from both tea clones showed synergism with cisplatin in different combinations. Catechin extracts from the purple TRFK306 tea exhibited higher synergism with cisplatin than the catechin extract from BB35.

The catechins from purple TRFK 306 showed synergism with cisplatin in some combinations as follows: at 24 h, two different combinations of the catechin extracts from purple tea clones and cisplatin had synergism with a CI of 0.63 and 0.71. One combination (IC_{30} of catechins from purple tea clones+IC_{20} of cisplatin) showed an additive activity of 0.98 (≈1). At 48 h, all combinations of catechin extracts from the purple tea and cisplatin exhibited synergism, with CIs ranging from 0.59-0.88. At 72 h, three different combinations of catechin extracts from purple tea and cisplatin showed synergism with CIs ranging from 0.63-0.90 (Fig. 4). The catechins from BB35 showed synergism with cisplatin in some combinations: at 24 h, one combination of the catechin extracts from BB35 and cisplatin possessed synergism with a CI of 0.71. At 48 h, two combinations of catechin extracts from BB35 and cisplatin exhibited synergism with CI values of 0.65 and 0.79. At 72 h, two combinations of catechin extracts from BB35 and cisplatin showed synergism with CI values of 0.62 and 0.72. One combination showed an additive activity of 1.04 (≈1) (Fig.4).

The dose reduction index (DRI) of different drug combinations

The DRI of catechin extracts from BB35 and purple TRFK306 combined with cisplatin was determined. The DRI from different drug combinations (the same as used for determination of the combination indices) are presented in Table 4. Of the different combinations of catechin extracts from BB35 with cisplatin and the combination of catechin extracts of purple TRFK306 with cisplatin, the best DRI was obtained for the combinations of IC_{20} of catechin extracts with IC_{30} of cisplatin at 24 h, 48 h, and 72 h.

Table 4. Dose reduction index (DRI) of different drug combinations. The following table presents the DRIs of different combinations of catechin extracts from BB35 tea clones with cisplatin and catechin extracts from the purple TRFK 306 tea clone with cisplatin. Different combinations of inhibitory concentrations of catechin extracts and cisplatin were used as follows: IC_{40}+IC_{10}, IC_{30}+IC_{20}, IC_{20}+IC_{30}, IC_{10}+IC_{40} (catechin extracts+cisplatin).

BB35 tea extract and cisplatin					
	Fa	**Dose of BB35 (μg/mL)**	**Dose of cisplatin (μg/mL)**	**DRI BB35**	**DRI cisplatin**
24 h	0.309	173.006	23.2912	0.80095	2.42213
	0.18	112.93	14.7558	0.66902	0.90582
	0.647	403.02	57.5691	3.22932	2.48786
	0.166	106.494	13.8576	1.34089	0.44934
48 h	0.378	158.645	11.369	0.9494	2.67379
	0.414	167.599	12.3897	1.21448	1.75218
	0.717	266.983	25.6868	2.44043	2.59096
	0.793	310.422	32.5259	4.02884	2.48669
72 h	0.113	9.57198	2.99114	0.18003	1.08335
	0.492	69.8795	8.23468	2.04266	1.80942
	0.64	126.723	11.1516	6.34568	1.75809
	0.744	205.139	14.2529	23.0804	1.71165
purple tea extract and cisplatin					
	Fa	**Dose purple (μg/mL)**	**Dose of cisplatin (μg/mL)**	**DRI purple**	**DRI cisplatin**
24 h	0.39	103.255	29.2769	1.17069	3.0446
	0.456	124.088	34.625	1.93254	2.12554
	0.592	180.065	48.6405	4.12993	2.10201
	0.662	220.715	58.573	9.06427	1.89926
48 h	0.523	113.736	15.9235	1.61122	3.74493
	0.561	127.977	17.3785	2.64089	2.45771
	0.648	169.53	21.4054	5.53659	2.1591
	0.708	209.597	25.0504	13.6545	1.91517
72 h	0.23335	29.4906	4.25865	0.6112	1.54243
	0.57987	72.1799	10.1515	2.18992	2.23062
	0.72216	104.997	14.6046	5.06989	2.30248
	0.7011	98.8053	13.768	9.60207	1.65342

DRI – dose reduction index, Fa – fraction affected (percentage inhibition/100), DRI BB35 – dose reduction index of catechin extracts from BB35 tea clones, DRI purple – dose reduction index of catechins extracts from purple (TRFK 306) tea clones. The doses presented in the table are the ones that can produce the respective Fa if used alone.

DISCUSSION

The catechin content in different tea clones and processed tea was analyzed by HPLC, after which the catechin extracts from BB35 and purple TRFK306 were selected for further analyses. The results of HPLC analysis showed that catechin contents are tea-clone

dependent, showing that each tea clone is unique. Previous studies have revealed that the level of polyphenol is associated with not only varieties and clones but also with the soil and environmental conditions of the site where the tea was grown [21]. Some tea clones used in this study were genetically improved for commercialization purposes, which could be the reason for their higher content of catechins than other tea clones.

The concentration of EGCG was high in all groups analyzed. These results validate previous results from different studies [6,22]. While EGCG is the most potent catechin that inhibits the growth of cancer cells, other catechins in green tea act synergistically to enhance the inhibitory effect of EGCG [23]. The concentrations of catechins in tea clones were in the following order: EGCG>EGC≥EC≥ECG; there was no significant difference between EGC and EC concentration and between EC and ECG concentration, but EGC concentration was higher than that of ECG. These results corroborated previous results [24], where the concentration of catechins in the tea leaves of *Camellia sinensis var. sinensis* followed the order EGCG>EGC>ECG. On the other hand, in the leaves of *C. sinensis* var. *assamica*, the order was EGCG>ECG>EC; however, EGCG was the highest and catechins (C) the lowest in the following order: EGCG>GCG>EGC>ECG>EC>C [25].

The purple tea cloneTRFK306, which, in addition to certain tea constituents is found in green tea, also contains anthocyanins [26] and higher concentrations of EGCG than other types of catechins. This result agrees with the results of [27], who found that the main catechins in purple tea were EGCG, ECG, and EGC, with EGCG being the most abundant in leaves and flakes. The low levels of EGC and EC compared to the levels of EGCG and ECG may be due to the glycosylation of some leucoanthocyanidins to anthocyanins [21,29]. Catechin and gallocatechin are synthesized from the leucocyanidin by the action of leucoanthocyanidin 4-reductase (LAR), but in contrast to the biosynthesis of EC and EGC, leucoanthocyanidins are not the direct precursors. Anthocyanidin synthase has to convert leucoanthocyanidins to anthocyanidins, which may be glycosylated to anthocyanins [29]. The total catechin content in black tea was much lower than in all groups of green tea analyzed. This finding is in agreement with the previous study [28], where green tea had a significantly higher catechin content than black tea. This low catechin content in black tea is due to its fermentation that causes the oxidation of catechins by polyphenol oxidases (PPO) (EC 1.10.3.1), where catechins are converted into complex products, theaflavins and thearubigins [2].

Cisplatin was used as a positive control and as the reference chemotherapy drug. Based on the results of its antiproliferative effect on 4T1 mammary cells, extracts from BB35 and purple tea clones showed antiproliferative activity against 4T1 cell lines. The antiproliferative activity of these extracts is attributed to the high content of catechins. Tea catechins have been shown to exhibit anticancer activities on different cancer cell lines such as prostate cancer, colon cancer, hepatocellular carcinoma, etc. [12,29-31]. The catechin extracts from purple tea clones showed higher antiproliferative activity than those from the BB35 clones. This may be attributed to the high content of EGCG, which is known to have higher anticancer activity than other types of catechins [34,35]. The antiproliferative activities of both tested catechin extracts increased with the increasing exposure time to the different treatments, implying that the IC50 values of each active component decreased as the exposure time was extended. This phenomenon may be because most *in vitro* antiproliferative experiments are conducted when cells are in the exponential growth phase; consequently, the duration of exposure increases as the cells progress into the stationary and death phases [36,37]

The catechin extracts from both tea clones were non-toxic to normal cells as the selectivity indices were >1. Safe drugs should have high cytotoxicity and a low inhibitory concentration, meaning they must kill the cancer cells and spare normal cells [38]. Different acceptable values of SI have been reported. For example, in [39], it was assumed that the sample with SI≥10 was a potential candidate for further investigation; in [40], it was proposed that samples with SI≥3 were a prospective anticancer drug candidate; in [39-41], it was suggested that a drug with SI≥2 could be an appropriate anticancer drug candidate. In [44], research on different *Eugenia* and *Syzygium* sp. extracts against Gram-negative and Gram-positive bacteria suggested that a non-toxic bioactive compound should have SI>1. Due to the many variations of acceptance of SI value, further work and validations are important in determining and validating the minimum value of the

SI. Nevertheless, drugs with an SI value below 1 are considered toxic as they kill normal cells.

Different combinations of different inhibitory concentrations of catechin extracts and cisplatin showed synergistic and additive effects. According to the methods described in [45] and refined later in [46], based on the CompuSyn software developed by Chou and Martin in 2005 [20], the ranges of the CI were defined as CI<I, CI=1, and CI>1 as synergism, additive effects, and antagonism, respectively. Combinations of drugs are classified as very strong synergism (CI<0.1), strong synergism (0.1<CI<0.3), synergism (0.3<CI<0.7), moderate synergism (0.7<CI<0.85), slight synergism (0.85<CI<0.9), nearly additive (0.9<CI<1.1), slight antagonism (1.1<CI<1.2), moderate antagonism (1.2<CI<1.45), antagonism (1.45<CI<3.3), strong antagonism (3.3<CI<10), and very strong antagonism (CI>10) [19]. Based on the above classification of combinations, catechin extracts from purple tea clones and cisplatin showed a higher synergistic effect than the combinations of catechins from BB35 with cisplatin. This high synergistic effect between catechins from the purple tea clone and cisplatin may be due to its higher content of EGCG. EGCG may produce the effect of synergism with cisplatin due to its structure: EGCG has 3 rings, A, B, and D, and rings B, and D are involved in the inhibition of proteasome activity *in vitro*, and proteasome inhibitors have been approved to treat different cancers [47]. Cisplatin is a proteasome inhibitor that induces a dose-dependent inhibition of 3 proteasome protein activities: caspase-like activity, chymotrypsin-like activity, and trypsin-like activity [48]. Good synergism was found in all combinations of catechin extracts from purple tea clones with cisplatin after an exposure time of 48 h. This can be an indicator of the higher synergism of catechin extracts from purple tea with cisplatin. The result showed that catechin extracts could reduce the dose of cisplatin to be used as a synergistic combination, leading to lower doses of constituents and thereby reducing the potential for side effects of the drugs [49].

The DRI of each combination was calculated to establish by how many folds the doses of the drug can be reduced in the combination to produce a given effect as compared to the doses of each drug alone. According to the dose-reduction index introduced by [50] and based on the CompuSyn software developed by [20], DRI>1 is considered to be beneficial. Catechin extracts from purple tea clones showed a greater reduction in cisplatin doses than catechin extracts from BB35 clones. The highest DRIs of cisplatin were obtained in different combinations with catechin extracts from purple tea clones (DRI>3). This higher reduction of cisplatin doses by the catechin extracts from the purple clone than the catechin extracts from BB35 tea clones may be due to the high content of EGCG. Beneficial DRI values obtained in many different combinations of cisplatin with catechin extracts do not necessarily mean that there was synergism in the combinations, as an additive effect or a slightly to moderate antagonism can give DRI>1 based on the formula of calculation of DRI [19]. However, since the synergistic effect of the catechin extracts from purple tea clones and cisplatin has been documented, the high DRI indicates that a combination of cisplatin and the catechin extracts of purple tea clones could be highly beneficial in cancer treatment.

CONCLUSIONS

The BB35 tea clone had the highest total catechins content of all tea clones and processed teas analyzed. The purple TRFK306 tea clone was found to contain the highest EGCG content. This property of the catechin extract of the purple TRFK306 tea clone underlines the observed high antiproliferative effects and synergism with cisplatin against the TNBC cell line 4T1. Its capacity to reduce the doses of cisplatin used in combination against 4T1 cells was evaluated *in vitro*, and the results showed that purple TRFK306 can reduce the doses of cisplatin more than 3-fold while producing the same effects.

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Data availability: Data underlying the reported findings have been provided as a raw dataset, which is available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Ndacyayisenga%20et%20al_Dataset.pdf

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SUPPLEMENTARY MATERIAL

Sample collection sites

- Rutsiro tea factory (western province, Rwanda (1˚56'42"S29˚24'51"E); the following samples were collected: Tea clones (BB10, BB35, IB475, TRFK105/5, TRFK 31/8, TRFK6/8, TRFK301/14, TRFK303/557, TRFK7/3, TRFK18/58) different grades of processed green tea.
- Ngere tea factory, Murang'a county, Kenya (0˚50'16"S36˚48'34"E); the following samples were collected: Tea clones (BB35, TRFK301/4, TRFK31/8, TRFK371/3, TRFK6/8, KAG501, PURPLE TEA (TRFK306), SFS1O)
- Juja, Kiambu county, Kenya (1˚06'27"S37˚00'57"E), Processed and packaged black tea and green tea were collected there.

Supplementary Table S1. Description of samples. Tea clones and processed teas were collected from Rwanda and Kenya.

S/N	Tea clone/Samples type	Varietal types	Description
1.	TRFK 100/5	Assam	Commonly grown in East Africa [5]
2.	TRFK 18/52	Assam	Commonly grown in East Africa [5]
3.	TRFK 301/4	Cambod	Commonly grown in Kenya and Rwanda[21].
4.	TRFK 303/577	Assam	Widely grown in Kenya[5]
5.	TRFK 31/8	Assam	Widely grown in Kenya and East Africa [5]
6.	TRFK 371/3	Assam	Widely grown in Kenya [21].
7.	TRFK 6/8	Assam	Widely grown in Kenya and in Rwanda [21].
8.	TRFK 7/3	Assam	Widely grown in Kenya and in Rwanda [21].
9.	BB10	Assam	Widely grown in Kenya and East Africa [5]
10.	BB35	Assam	Widely grown in East [5]
11.	IB475	Assam	Widely grown in East [5]
12.	KAG 501	Assam	Grown in Kenya
13.	PURPLE (TRFK 306)	Assam	Grown in Kenya
14.	SFS10	Assam	Grown in Kenya
15.	Black tea	NA	Collected from market
16.	Green tea PEKOE	NA	General pekoe grade
17.	Green tea BOP1	NA	Broken Orange Pekoe: Main broken grade
18.	Green tea FBOP	NA	Flowery Broken Orange Pekoe: Coarser and broken with some tips
19.	Green tea (market)	NA	Green tea, already packaged and sent to the market

NA – not applicable