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Effects of tea catechin extracts from BB35 and purple (TRFK 306) tea clones on the gene expression of *Egfr*, *App*, *Bcl2*, *Dnmt*, *Casp3*, *Hif1a*, *Gadd45b* and *Psmb5* genes involved in triple negative breast cancer diseases: *In silico* and *in vitro* study☆

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ABSTRACT

Tea has been shown to contain metabolites that exhibit antioxidant, anti-inflammatory, anti-diabetic, anti-cancer and *anti*-cardio vascular diseases properties. This study aimed to carry out an *in silico* assessment of catechins on the genes previously shown to be involved in Triple Negative Breast Cancer (TNBC) disease and also to evaluate the effects of catechin extracts from purple (TRFK306) and BB35 tea clones on the expression patterns of genes in the 4T1 TNBC cell line as mentioned above. Identification and quantification of different catechin contents in these two tea clones were performed by using High-Performance Liquid Chromatography (HPLC). *In silico* assessment including Absorption, Distribution, Metabolism and Excretion (ADME) study for drug-likeness evaluation, Drug target prediction, Protein-protein interaction (PPI) network analysis and construction, Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment and analysis for targets and Molecular docking study was conducted. Simplified Molecular Input Line Entry System (SMILES) of 4 different tea catechins showed a target on 111 genes in TNBC and have high binding affinity to the receptor-binding pocket of chain A of Epidermal Growth Factor Receptor (EGFR) Extracellular Domains. Catechin extracts promoted the downregulation of expression of 6 genes (*Egfr, App*, *Bcl2*, *Dnmt, Hif1a* and *Psmb5*) and the upregulation of 2 genes (*Casp3* and *Gadd45b*) in the 4T1 TNBC cell line. Catechin extracts from purple tea clones showed higher activity on gene expression levels of *Egfr, Bcl2* and *Casp3* than catechin extracts from BB35 tea clone.

1. Introduction

Breast cancer is the second leading cause of mortality and morbidity among cancer diseases worldwide [1]. TNBC is the most aggressive breast cancer and most difficult to treat mainly due to its poor prognosis; it is considered as breast cancer lacking the expression of Progesterone Receptor (PR), Oestrogen Receptor (ER), and with downregulation of gene expression of Human Epidermal Growth Factor Receptor-2 gene (HER-2) [2,3,4]. With no prior history of breast cancer, Triple Negative Breast cancer (TNBC) is very difficult to diagnose based on the morphological and immunohistochemistry assessment alone [5]. Treatment of TNBC is based mainly on chemotherapy, radiotherapy and surgery. TNBC is one of the sub-types of breast that respond well to the

☆ *Egfr:* Epidermal growth factor receptor. *App*: Amyloid-beta precursor protein. *Bcl2:* B-cell lymphoma 2. Dnmt*:* DNA (cytosine-5)-methyltransferase1. *Casp:* Caspase-3. *Hif1a:* Hypoxia-inducible factor 1-alpha. *Gadd45b*: DNA-damage-inducible beta. *Psmb5:* Proteasome subunit beta type-5. TNBC: Triple Negative Breast Cancer; ADME: Absorption, Distribution, Metabolism and Excretion PPI: Protein-protein interaction; GO: Gene Ontology; KEGG: Kyoto Encyclopaedia of Genes and Genomes, SMILES: Simplified Molecular Input Line Entry System.

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Abbreviation

Egfr	Epidermal growth factor receptor
App	Amyloid-beta precursor protein
Bcl2	B-cell lymphoma 2
Dnmt	DNA (cytosine-5)-methyltransferase1
Casp	Caspase-3
Hif1a	Hypoxia-inducible factor 1-alpha
Gadd45b	DNA-damage-inducible beta
Psmb5	Proteasome subunit beta type-5
TNBC	Triple Negative Breast Cancer
ADME	Absorption, Distribution, Metabolism and Excretion
PPI	Protein-protein interaction
GO	Gene Ontology
KEGG	Kyoto Encyclopaedia of Genes and Genomes
SMILES	Simplified Molecular Input Line Entry System

standard chemotherapy drugs. However, more than 70% of the patient do not achieve a complete response thus causing higher mortality rates compared to the non-TNBC subtypes [5].

Catechins (flavan-3-ols) are the polyphenols found in numerous plant sources including many herbs, vegetables, fruits, and beverages (grapes, red wines, strawberries, green tea, kiwi, gooseberries, etc) [6]. Fresh tea leaves, Green algae (Acetabularia ryukyuensis), Red algae (Chondrococcus hornemannii), black grapes, strawberries and red wines are reported to have a high content of catechins [7]. Tea catechin content is varietal (clonal) dependent and depends on the different modes of tea processing as reported in our previous findings; BB35 tea clone and purple (TRFK 306) tea clone were reported to contain high concentrations of catechins and Epigallocatechin gallate (EGCG), respectively [3]. Tea, mainly green tea is known as one of the best sources of catechins among the most consumed beverages; many *in vitro* and *in vivo* studies reported the association between its consumption and the decrease in the risks of breast cancer diseases [8]. Catechins were reported to have antioxidant activity *in vitro* and *in vivo* [6]. They exhibit anticancer effects on various cancers and related disorders by affecting molecular mechanisms involving angiogenesis, extracellular matrix degradation, the regulation of cell death and multidrug resistance [6,9,10]. Green tea catechins are bioactive compounds showed to have the capacity to prevent cancer progression by modifying functional processes such as cellular multiplication, metastasis, and cell differentiation [11]. Epigallocatechin gallate (EGCG) which is one of the eight catechins [3] showed the capacity to initiate apoptosis in mammary carcinoma cells *in vitro* [12]. Catechins extracts showed antiproliferative activity against different cancer cell lines like HeLa human cervical cancer cells [13], TNBC 4T1cells and HS578T cells [3,14], Breast cancer MCF-7 cells [15]. Tea catechins were reported to have synergism with some chemotherapy drugs and with anti-oxidant natural substances to reduce the dose or to enhance the anti-cancer effects [3,16]. The main challenge of using tea catechins in the treatment of cancer is their limited efficient systemic studies [14]. The evaluation of catechin-gene targets and gene expression of TNBC-related genes can contribute to the use of tea catechins in the treatment of breast cancer diseases.

This study aimed to carry out an *in silico* assessment of the effect of catechins in TNBC, and to determine the gene expression of eight genes; *Egfr, App*, *Bcl2*, *Dnmt, Casp3*, *Hif1a*, *Gadd45b* and *Psmb5* genes after treatment of TNBC cell (4T1) line with tea catechin extracted from BB35 and purple (TRFK 306) tea clone.

2. Materials and methods

2.1. Sample collection and catechins isolation

Tea shoot samples were collected from 14 distinct tea clones grown in Rwanda and Kenya; two leaves and buds were taken for each tea shoot sample and 4 samples were collected for each tea clone. The samples were directly brought to the laboratory by using cooler box. Plant identification and authentication were completed in the Botanical Garden of the INES Ruhengeri-Institute of Applied Sciences, Rwanda and the accession number (INSH2346) was given by the botanist. Isolating the catechins was accomplished by using the techniques described by [17]. Steaming the fresh tea leaves for 40 s at 100 °C was followed by drying them for 40 min at 100 °C, 35 °C for 40 min, and 80 °C for 90 min, and finally, ground. After combining 10 g of ground tea with 200 mL of 40% ethanol, the mixture was placed in a sonicator at 40 °C for 2 h. The filtration of the mixture was done, followed by the evaporation of ethanol by using the vacuum rotary evaporator at 45 °C and then the remaining volume after evaporation, was adjusted by using distilled water to a volume of 200 mL and mixed with an equal volume of ethyl acetate and waited for 30 min for partition. The layer of ethyl acetate was collected and 200 mL of fresh ethyl acetate was added two times to the remaining aqueous layer. All layers of ethyl acetate collected were evaporated by using a vacuum rotary evaporator at 40 °C. The remaining mixture containing catechins and caffeine, was adjusted to a final volume of 200 mL by using distilled water followed by three times of the decaffeination process using dichloromethane (200 mLx3). The decaffeinated top layers were retained and, the bottom layers of dichloromethane were eliminated. The aqueous solution containing catechins was freeze-dried by using mrc freeze dryer FDL-10N-50-8 M.

2.2. HPLC analyses

Four different main catechins: (−)-Epigallocatechin (EGC), (−)-Epicatechin (EC), (−)-Epigallocatechin gallate (EGCG), and (−)-Epicatechin gallate (ECG) were identified and quantified by using HPLC method and respective HPLC standards. All HPLC standards were purchased from Solarbio Life Sciences Company, Beijing, China: EC (cat #: SE8100), ECG (cat #: SE 8110), EGCG (cat #: SE 8120) and EGC (cat #: SE 8130); the purity was >98%. The HPLC method used was described in our previous study[3] and by following the method developed by [18]. Shimadzu HPLC system equipped with SIL-20 A HT auto-sampler and Shimadzu SPD-M20A Prominence Diode Array Detector set at wavelength 254 nm was used. CTO-10AS VP oven was set at 40 °C and HPLC column: OCG-4252-E0 Luna® 5 μm C18 (2) (250 × 4.6 mm) was used. The mobile phase was prepared as follow: water: acetonitrile (87:13) containing 0.05% Trifluoroacetic acid (TFA) (V/V), the isocratic mode was used and the flow rate was set at 1 mL/minute with the injection volume of 20 μL. Shimadzu LabSolution CS software was used for HPLC analysis and for making calibration curves with 5 different levels of concentrations of HPLC standards.

2.3. In silico study

2.3.1. ADME study for drug-likeness evaluation

To evaluate the drug-likeness of different main catechins from both BB35 and purple tea clone, pharmacokinetic (PK)) parameters (Absorption, Distribution, Metabolism and Excretion: ADME) were evaluated. The evaluation of those pharmacokinetic parameters is based on Lipinski's rule of five (RO5) which describes the relationship between pharmacokinetic and physicochemical parameters. Molecules or candidate drugs should not be orally taken as medicine if two or more of those rules of 5 are not met [19]. The PubChem online database (https://pubchem.ncbi.nlm.nih.gov/) was used to collect the Canonical Simplified Molecular Input Line Entry System (SMILES) of 4 different tea catechins analysed by HPLC (ECG, EC, EGCG and EGC) [20], then after,

those SMILES were submitted to SwissADME (http://www.swissadme.ch/) for drug-likeness evaluation [21].

2.3.2. Drug target prediction

Drug target prediction was done in three major steps: firstly, general targets of SMILES of 4 different tea catechins was done, secondly the prediction of disease-related genes, and lastly the screening out of only potential targets of TBNC-related genes. Prediction of general targets of 4 different tea catechins was done by using 3 different databases: BindingDB, Suiss TargetPrediction and PharmMapper databases. Each catechin SMILES was uploaded in BindingDB database https://www.bindingdb.org/rwd/bind/chemsearch/marvin/FMCT.jsp), similarity was set at ≥0.7 [22] and the target gene names were converted into gene IDs from UniProtKB (https://www.uniprot.org/) [23]. Similarly, each SMILES was uploaded into Suiss TargetPrediction database (www.swissstargetprediction.ch/), only gene targets with probability of at least 0.10 similarity were selected [24]. Again each SMILES was uploaded into PharmMapper databases (https://www.lilab-ecust.cn/pharmmapper/), *Mus musculus* was selected as the target species, only gene with normalized ≥0.7 were selected as the target gene [25] and their names were converted into gene IDs from UniProtKB (https://www.uniprot.org/) [23]. The genes from all these databases were combined to remove duplicates.

The prediction of TNBC-related genes was done by using GenCards database (https://www.genecards.org/) [26], the Comparative Toxicogenomics Database (CTD) (https://ctdbase.org/) [27] and DisGeNET platform (https://www.disgenet.org/search) [28]. Triple Negative Breast Cancer (TNBC) was input to search disease-related genes. All genes from those 3 databases/platforms were combined to remove the duplicates. Finally, the potential genes related to TNBC and catechins target genes were intersected in the Venn diagram by using the Bioinformatics and Evolutionary Genomics platform (https://bioinformatics.psb.ugent.be/webtools/Venn/).

2.3.3. Protein-protein interaction (PPI) network analysis and construction

Common genes (intersection) between catechins' target genes and genes related to Triple Negative Breast Cancer were uploaded to the STRING V12.0 database (https://string-db.org/); the organism was set as *Mus musculus*, the confidence score and the false discovery rate (FDR) stringency were set at 0.400 and 5 respectively [29]. Topology analysis of the PPI network was done by Cytoscape v3.10.1 software. The 10 key gene targets were filtered out by using the Maximal Clique Centrality (MCC) algorithm of Cytoscape v3.10.0 software.

2.3.4. The analysis of GO and KEGG enrichment of target genes

The Gene Ontology (GO) and Kyoto Encyclopaedia for Genes and Genomes (KEGG) enrichment analysis [30] was conducted to investigate the biological process of the catechins' gene targets involved in TNBC. The targets were mapped to the ShinyGO 0.77 enrichment tool (http://bioinformatics.sdstate.edu/go/) and the terms with rate (FDR) cut-off = 0.05, species = mouse, false discovery. For GO enrichment analysis, the biological process (BP), the cellular component (CC), and the molecular function (MF) categories were considered [31].

2.3.5. Molecular docking study

The molecular docking study was performed to get a deeper understanding of the binding modes and to predict potential interactions between ligands (SMILES of 4 different tea catechins (ECG, EC, EGCG and EGC)) and the receptor-binding pockets of Human Epidermal Growth Factor and Receptor Extracellular Domains (PDB ID:1IVO) [32]. This protein (receptor) was chosen based on the fact that Epidermal growth factor (EGF) is involved in the regulation of cell proliferation and differentiation process through binding to the EGF receptor (EGFR) extracellular region [33]; and its abnormal activation plays substantial role in initiating the oncogenic transformation of cells and their subsequent invasive capabilities and eventual escape from the primary tumour in various cancers including breast cancer [34]. Those aforementioned SMILES of 4 different tea catechins were retrieved from the PubChem online database (https://pubchem.ncbi.nlm.nih.gov/) in SDF format [20] which was then transformed into PDB format by using PyMol [35].

The 3D crystal structure of Human Epidermal Growth Factor and Receptor Extracellular Domains (1IVO) was retrieved from the online RCSB Protein Data Bank (https://www.rcsb.org/) [36]. Ligands and receptor preparation and molecular visualization were done by using UCSF Chimera 1.16 software. Autodock Vina 1.1.2 software was used for molecular docking. BIOVIA Discovery Studio Visualizer 4.5 software was used to visualize the docking results and receptor-ligand interaction on the 2D diagram.

2.4. Cell culture and treatment

The 4T1 breast cancer cell line was bought from ATCC (Manassas, VA, USA). Cisplatin standard was bought from Solarbio Life Sciences, Beijing, China (cat #: IC0440). The 4T1 cells were cultured in the cell culture laboratory of the Centre of Traditional Medicine and Drug Research of Kenya Medical Research Institute (CTMDR-KEMRI). RPMI 1640 (with 25 mM HEPES and L-glutamine) medium supplemented with 10% Fetal Bovine Serum (FBS), and 1% Penicillin-streptomycin was used to grow the cells and incubation was done by using a CO_2 incubator set at 37 °C and 5% CO_2. The half-maximal inhibitory concentration (IC50) of catechin extracts and cisplatin was determined and reported in the previous publication [3]. The 4T1 cells for gene expression assessment, were grown and treated with IC50 concentration in T75 cell culture flasks and for an exposure time of 24, 48 h and 72 h.

2.5. Gene expression pattern analyses

The 4T1 breast cancer cells were cultured in T75 cell culture flasks to reach 70–80% confluency and treated with IC_{50} of catechin extracts and cisplatin standard for 24 h, 48 h and 72 h. Total RNA was extracted from treated cells utilizing a Total RNA extraction kit (Solarbio Life Sciences company, Beijing, China) and by following the manufacturer's instructions. The purity and concentration of RNA were assessed by using NanoDrop OneC UV–Vis Spectrophotometer (Thermo Scientific) followed by assessment of 28 S and 18 S bands on 1% agarose gel electrophoresis. cDNA synthesis was done by using Accuris qMax First Strand cDNA synthesis Flex Kit (ACCURIS Life Science Reagents, Edison, NJ 08818, USA). Total RNA extracted from different samples were diluted and adjusted at 150 ng/μL before cDNA synthesis, and the procedures were performed according to the manufacturer's instructions. Quantitative Real-Time PCR (qPCR) was performed by using qTOWER3 - Real-Time PCR Thermal Cycler (analytic Jena). cDNA from different samples were diluted and adjusted at 50 ng/μL and HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX), 5x kit (SOLIS BIODYNE, Estonia) was used for qPCR. A final volume of 20 μL containing 4 μL of HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX), 0.5 μL of each of the primers, 1 μL of template cDNA and 14 μL of nuclease-free water was prepared and qPCR condition was set as follows: Initial activation at 95 °C for 10 min to activate the polymerase, denaturation at 95 °C for 15 s, annealing at 58.5 °C for 30 s and extension at 72 °C for 30 s. The primer sequences were as follow: *Gapdh*: forward primer: 5′-GCCTCCTCCAATTCAACCCT-3′, reverse primer: 5′-CTCGTGGTTCACACCCATCA-3'; *App:* forward primer: 5′TCCGAGAGGTGTGCTCTGAA-3′, reverse primer: 5′-TGGCTTCCAGCCTCTCTTTG-3'; *Bcl2*: forward primer: 5′-CCAACGGGGAAACACCAGAA-3′, reverse primer: 5′-AGTTCCACAAAGGCATCCCAG-3'; *Casp3*: forward primer: 5′- AGCTTGGAACGGTACGCTA-3′, reverse primer: 5′-GGCCCATGAATGTCTCTCTG-3'; *Dnmt*1: forward primer: 5′-AGCTGTTCTGTCGTCTGCAA-3′, reverse primer: 5′-GCCATTTCTGCTCTCCAGGT-3'; *Egfr*: forward primer: 5′-TGCCAGAATGTGAGCAGAGG-3′, reverse primer: 5′-AGGTGATGTTCATGGCCTGG-3'; *Gadd45b*: forward primer: 5′-CAGCGTGGTCTTGTGCCT-3′, reverse primer: 5′-CGGTTGTG

CCCAATGTCT-3'; *Hif1a*: Forward primer: 5′-CCTGTAAGCAAGGAGC-CAGAA-3′, reverse primer: 5′-GTGGCAACTGATGAGCAAGC-3'; *Psmb5:* forward primer: 5′-GCTACAGCGGGTGCTTACAT-3'; reverse primer: 5′-TTCCCAGAAGCTGCAATCCG-3′

2.6. Statistical analysis

An excel data sheet was used to calculate each catechin content percentage. One-way ANOVA in R software, version R 4.2.1 with a P value set at 0.05 was used to determine the significance variations of each type of catechin content between different groups, and Least Significant Difference Test (LCD) of R software was used to compare different means of catechins in different groups. Tukey multiple comparison tests (TMCT) in GraphPad Prism 8.0.2 software was used for comparison of relative gene expression of different genes.

3. Results

3.1. Catechin content in different catechin extracts from BB35 and purple tea clones

Among 14 tea clones assessed, BB35 tea clone was found to have high concentration of catechins. Purple (TRFK306) tea clones was found to have very high concentration of EGCG. EGCG is known to have more anti-cancer and anti-oxidant activities than other catechins. For that reason, catechins extracts from these 2 tea clones were retained and used in *vitro* studies. Comparison of catechin contents of all tea clones analysed were reported in our previous publication [3] and are presented in supplementary materials (Supplementary Table S1). Catechin extracts from BB35 tea clones had 162.44 ± 26.30 mg g^{-1}, 122.53 ± 17.15 mg g^{-1}, 442.76 ± 20.52 mg g^{-1} and 90.07 ± 20.40 mg g^{-1} for EGC, EC, EGCG and ECG, respectively. Catechin extracts from purple (TRFK 306) tea clone had 52.15 ± 10.61 mg g^{-1}, 88.98 ± 9.68 mg g^{-1}, 552.2 ± 10.61 mg g^{-1} and 104.88 ± 16.86 mg g^{-1} for EGC, EC, EGCG and ECG, respectively.

3.2. ADME study for drug-likeness evaluation of tea catechins

The SMILES of 4 catechins (EGC, EC, EGCG and ECG) used in this study are presented in Fig. 1. ADME study of catechins was done to evaluate its drug likeness which can be used in the development of pharmaceuticals. The results of prediction of drug likeness, physiochemical properties, ADME parameters, pharmacokinetic properties, druglike nature and medicinal chemistry of 4 different tea catechins are presented in Table 1.

In terms of physicochemical properties, EC had the lowest molecular weight among all 4 catechins analysed (MW of 290.27 g/mol), followed by EGC with the MW of 306.27 g/mol, then after ECG with the MW of 442.37 g/mol; EGCG had the highest MW among other catechins analysed (458.37 g/mol). The good compound must have low MW (below 350 g/mol).

In terms of lipophilicity, all SMILES of catechins were found to be predicted as good candidates as they had log P values below Lipinksi's rule-of-five criteria (<5). EGC had the highest lipophilicity among all catechins (Consensus Log $P_{o/w}$ of 0.42), followed by EC with Consensus Log $P_{o/w}$ of 0.85, then EGCG with Consensus Log $P_{o/w}$ of 0.95 and finally ECG with Consensus Log $P_{o/w}$ of 1.3.

All catechins were water-soluble. EC and EGC had high GI absorption while ECG and EGCG had low GI absorption. All catechins had no blood–brain barrier (BBB) permeant and were found not to inhibit CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4.

In terms of drug-likeness, all catechins were found to respect Lipinski's rules with some minor violations: 0 violation for EC, 1 violation (NH or OH > 5) for ECG and EGC and 2 violations (Nor O > 10 NH or OH > 5) for EGCG. EC, ECG and EGC had favourable Bioavailability Score (0.55) while EGCG had low bioavailability score (0.17).

In terms of lead likeness, EC and EGC were found to have lead likeness as they have MW below 350 g/mol, while ECG and EGCG were

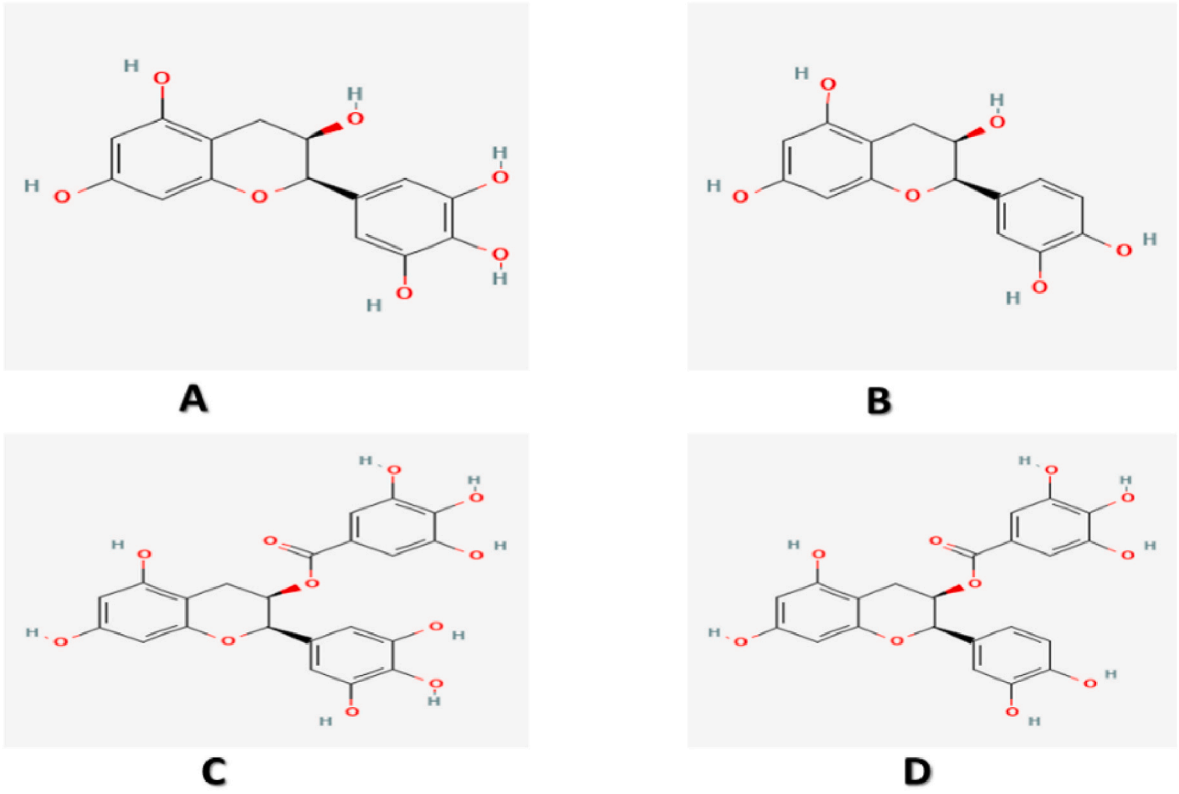


Fig. 1. SMILES of catechins. A: EGC, B: EC, C: EGCG, D: ECG. Source: PubChem (https://pubchem.ncbi.nlm.nih.gov/)[20].

Table 1
Drug likeness parameters of 4 tea catechins Different parameters like physicochemical properties (Lipophilicity and water solubility), pharmacokinetics parameters, drug likeness and medical chemistry of SMILES of 4 catechins were evaluated.

Properties	EC	ECG	EGCG	EGC
Physicochemical Properties				
MW (g/mol)	290.27	442.37	458.37	306.27
Lipophilicity				
Log $P_{o/w}$ (iLOGP)	1.47	1.7	1.53	0.98
Log $P_{o/w}$ (XLOGP3)	0.36	1.53	1.17	0
Log $P_{o/w}$ (WLOGP)	1.22	2.2	1.91	0.93
Log $P_{o/w}$ (MLOGP)	0.24	0.05	−0.44	−0.29
Log $P_{o/w}$ (SILICOS-IT)	0.98	1.04	0.57	0.49
Consensus Log $P_{o/w}$	0.85	1.3	0.95	0.42
Water Solubility				
Class	Soluble	Soluble	Soluble	Soluble
Pharmacokinetics				
GI absorption	High	Low	Low	High
BBB permeant	No	No	No	No
P-gp substrate	Yes	No	No	No
CYP1A2 inhibitor	No	No	No	No
CYP2C19 inhibitor	No	No	No	No
CYP2C9 inhibitor	No	No	No	No
CYP2D6 inhibitor	No	No	No	No
CYP3A4 inhibitor	No	No	No	No
Log K_p (skin permeation)	−7.82 cm/s	−7.91 cm/s	−8.27 cm/s	−8.17 cm/s
Drug likeness				
Lipinski	Yes, 0 violation	Yes, 1 violation NH or OH > 5	Yes, 2 violations Nor O > 10 NH or OH > 5	Yes, 1 violation NH or OH > 5
Ghose	Yes	Yes	Yes	Yes
Veber	Yes	No, 1 violation TPSA> 140	No, 1 violation TPSA> 140	Yes
Egan	Yes	No, 1 violation TPSA> 131.6	No, 1 violation TPSA> 131.6	Yes
Muegge	Yes	No, 2 violations TPSA> 150, H-don>5	No, 3 violations TPSA> 150, H-acc >10, H-don>5	No, 1 violation H-don>5
Bioavailability Score	0.55	0.55	0.17	0.55
Medicinal Chemistry				
PAINS	1 alert: catechol_A	1 alert: catechol_A	1 alert: catechol_A	1 alert: catechol_A
Brenk	1 alert: catechol	1 alert: catechol	1 alert: catechol	1 alert: catechol
Lead likeness	Yes	No, 1 violation MW > 350	No, 1 violation MW > 350	Yes
Synthetic accessibility	3.50	4.16	4.20	5.53

Mw: Molecular weight; log $P_{o/w}$: The *n*-octanol/water partition coefficient; GI: Gastrointestinal; BBB: *The* blood–brain barrier; *P*-gp: *P*-glycoprotein; CYP1A2: Cytochrome P4501A2; CYP2C19: Cytochrome P450 2C19; CYP2C9: Cytochrome P450 2C9; CYP2D6: Cytochrome P450 2D6; CYP3A4: Cytochrome P450 3A4; TPSA: Topological Polar Surface Area.

found to not have lead likeness as their MW are above 350 g/mol. All catechins analysed showed good permeation or absorption as they had MW which is below 500 g/mol) as predicted by the rule of 5', according to SwissADME tools, ECG and EGCG cannot be considered as lead compound as such due to MV > 350.

3.3. Drug target prediction

The prediction of genes targeted by the aforementioned SMILES was done by using 3 different databases (BindingDB, Suiss Target Prediction and PharmMapper) and the results of genes targeted by each SMILES and in each database, are presented in supplementary materials (Supplementary Table S2). After combining genes targeted by all SMILES in each database and after removing the duplicate target genes, 50 targets were identified by BindingDB, 15 targets were identified by Suiss TargetPrediction and 71 targets were identified by PharmMapper. The duplicate targets from those 3 databases were removed and 124 targets were retained.

GeneCard database, CTD database and DisGeNET platform were used to predict TNBC-related genes. 5766 targets were identified by the GeneCard database, 14,309 target genes were identified by the CTD database and 1663 target genes were identified by using the DisGeNET platform. All those genes from 3 databases, were pooled together and the duplicate genes were removed, 15,473 genes were retained as unique. 124 catechin-targeted genes and 15,330 TNBC-related genes were uploaded into Venn diagram to search the intersection between genes targeted by catechins and TNBC-related genes. The results from the intersection are presented in Venn diagram (Fig. 2).

The analysis of the relationship between genes targeted by tea catechins and TNBC-related genes revealed that 111 genes were intersected in the Venn diagram, means that 4 catechins analysed can target 111 genes in TNBC. Among 124 genes targeted by catechins, only 13 genes were found to not belong to TNBC-related genes.

3.4. Protein-protein interaction (PPI) network analysis

A total number of 111 intersected targets between catechin-target proteins and TNBC-related targets were used to construct the PPI network (Fig. 3). The PPI network had 107 nodes, 920 edges, average node degree was 17.2, average local clustering coefficient was 0.634, the expected number of edges was 334 and PPI enrichment p-value was <1.0e-16. Ten key gene targets filtered out by using the Maximal Clique

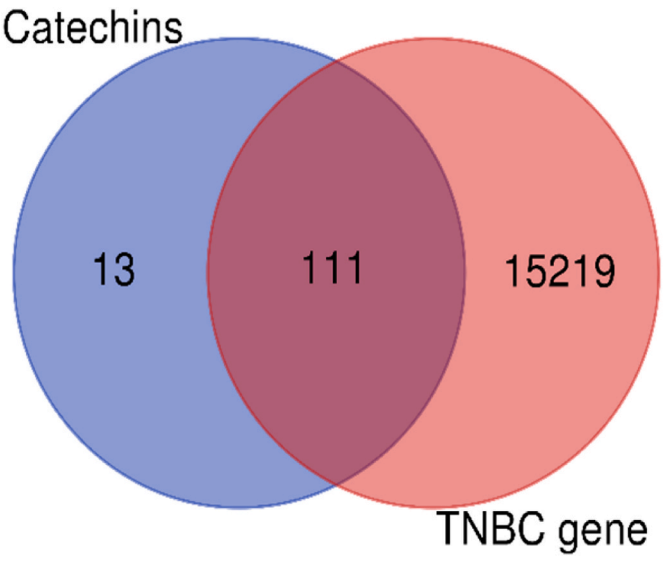


Fig. 2. Venn diagram of the intersection between genes targeted by catechins and TNBC-related genes.

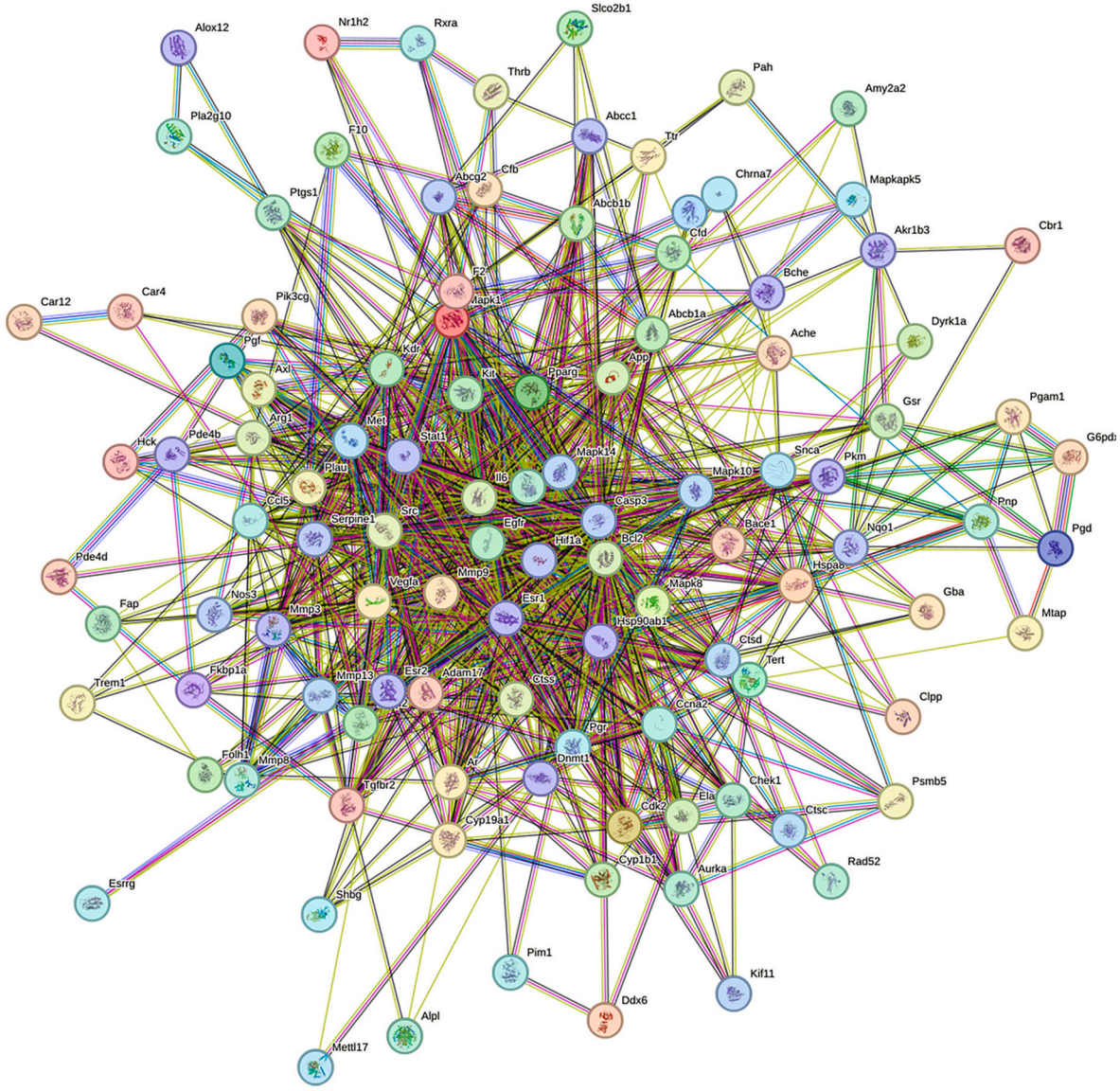


Fig. 3. Protein-protein interaction (PPI) network of catechin target and TNBC-related protein.

Centrality (MCC) algorithm of Cytoscape v3.10.1 software was namely *Casp3, Mapk14, Pparg, Mmp3, Serpine1, Src, Bcl2, Egfr, Mmp9* and *Kdr* (Fig. 4).

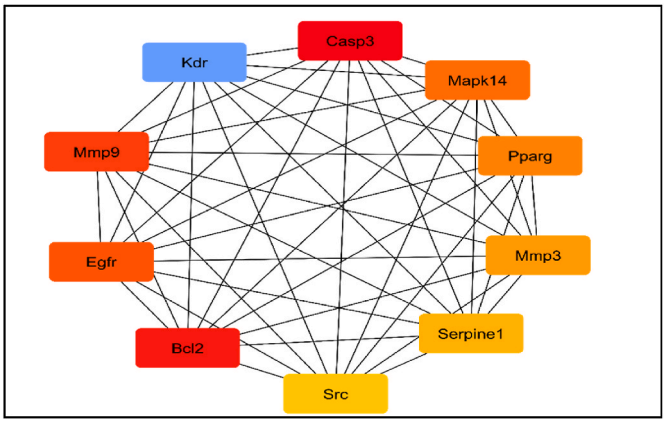


Fig. 4. Ten key gene targets in the interaction network.

3.5. The analysis of GO and KEGG enrichment of target genes

To investigate the function of catechin target genes in the biological process (BP), cellular component (CC), Molecular function (MF) and in the pathway of TNBC, 111 catechin targeted genes involved in TNBC, were enriched in a total of 1536 GO elements (FDR<0.05). There were 1000 BP, 146 CC and 390 MF protein targets in Gene Ontology. The top 10 pathways for each GO enrichment are presented on the dot plot chart (Fig. 5). Top 10 catechin-targeted BP are: Response to UV-A, Mast cell chemotaxis, Vagina development, Prostate gland growth, Cellular response to UV-A, Mast cell migration, Mitotic centrosome separation, Thrombin-activated receptor signalling pathway, Centrosome separation, Epithelial cell differentiation involved in prostate gland development. (Fig. 5).

The top 10 catechin targeted CC are the Endosome lumen, Nuclear envelope lumen, Glycinergic synapse, Voltage-gated calcium channel complex, Caveola, Terminal bouton, Rough endoplasmic reticulum, Plasma membrane raft, Perikaryon, Extracellular organelle (Fig. 5). For MF are ABC-type xenobiotic transporter activity, Steroid hormone receptor activity MAP kinase activity, Efflux transmembrane transporter activity, Histone kinase activity, Nuclear receptor activity, Ligand-

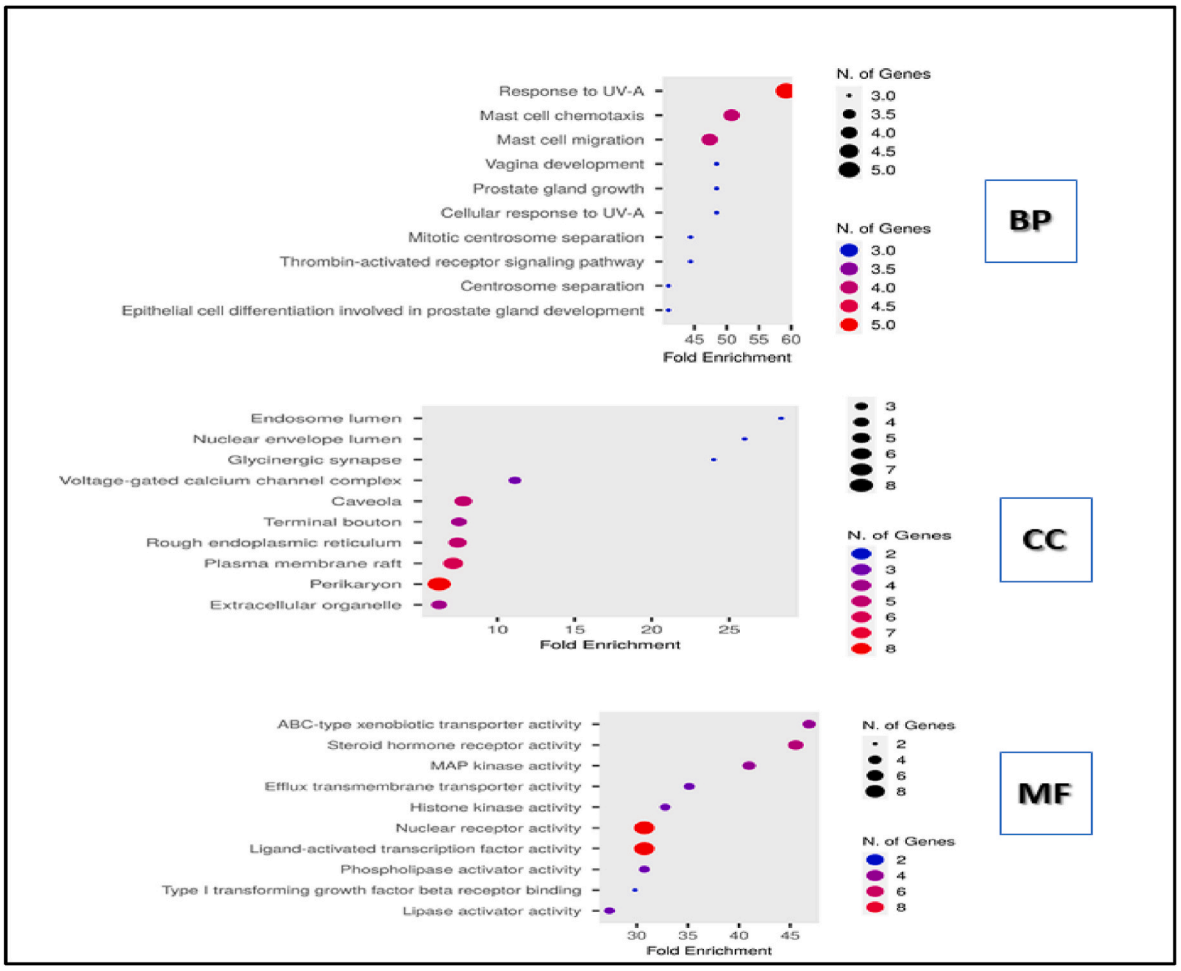


Fig. 5. Catechin GO enrichment analysis. At the Y-axis are the names of GO pathways, and at the X-axis are the fold enrichment. The size and colour of bubbles represent the number of genes; large bubbles indicate many genes involved in the pathway.

activated transcription factor activity, Phospholipase activator activity, Type I transforming growth factor beta receptor binding, Lipase activator activity (Fig. 5). GO enrichment and analysis showed that among the top 10 BP, response to UV-An involved more catechin gene targets in TNBC (5) than in other BP and among the top 10 MF, nuclear receptor activity and ligand-activated transcription factor activity involved many catechin gene targets in TNBC (8 genes for each) than in other MF. response to UV-A biological process (BP) has been shown from decades to relation with carcinogenesis.

The following catechin target genes are found in the TNBC signalling pathway: Egfr, Kit, Akt Erk1/2 (Mapk 1) (Fig. 6. A.). The 10 examples of catechin targeted pathways in KEGG are AGE-RAGE signalling pathway in diabetic complications, Endocrine resistance, IL-17 signalling pathway, Oestrogen signalling pathway, Relaxin signalling pathway, Fluid shear stress and atherosclerosis, MicroRNAs in cancer, Hepatitis B, Lipid and atherosclerosis, Pathways in cancer.

3.6. Molecular docking study

The 4 SMILES of catechins were docked into a receptor-binding pocket of chain A of EGFR Extracellular Domains (PDB ID: 1IVO) that has 4 chains and 8 receptor-binding pockets for *N*-acetyl-beta-D-glucosamine (NAG) ligand (Fig. 7. A). Chain A was used, it has 5 receptor-binding pockets for NAG (Fig. 7. B). The characteristics of binding site amino acids are presented in Table 2.

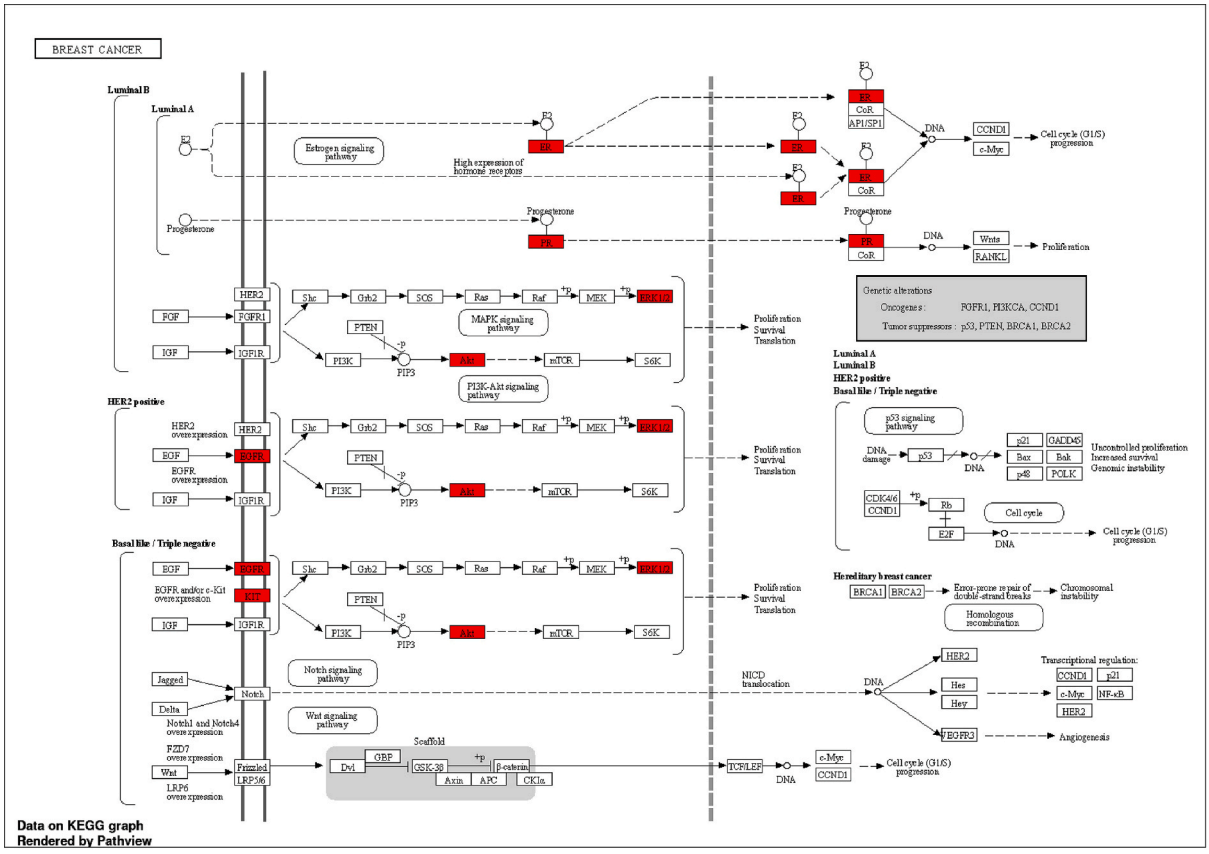
All catechins analysed showing the binding affinity to the receptor 1IVO in the range of −8.1to −8.4 kcal/mol. EC, ECG, EGCG and EGC binding to 1IVO with a binding energy of −8.2 kcal/mol, −8.1 kcal/mol, −8.3 kcal/mol and −8.4 kcal/mol, respectively (Table 3).

EC binds onto the receptor-binding pocket of chain A of 1IVO specifically on the following residues: Thr339, Tyr292, Arg310, Lys311, Ser291, Gly288, Cys287, Ser340, Lys375, Glu376, Val312, Glu293, Ala286, Thr378 with the following bonds: van der Waals, conventional hydrogen bond and Pi alkyl (Fig. 8. A).

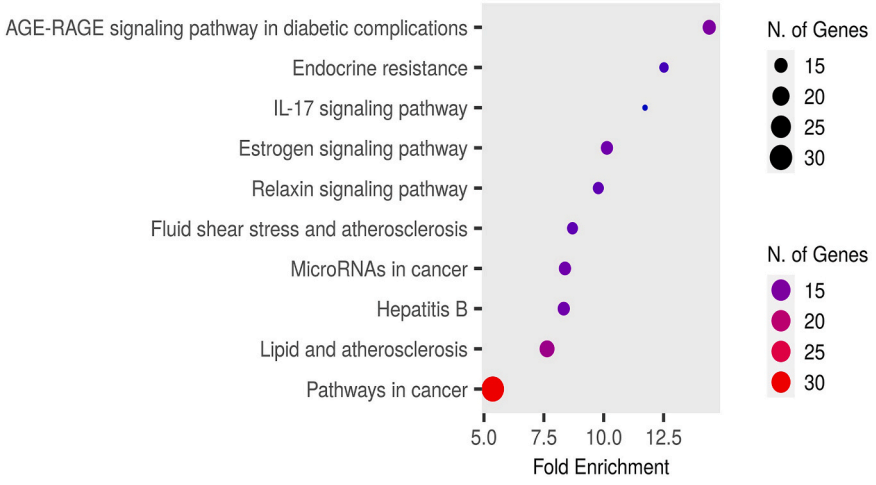
EGC binds onto the receptor-binding pocket of chain A of 1IVO specifically on the following residues: Thr339, Tyr292, Arg310, Lys311, Thr378, Cys287, Ser340, Lys375, Glu376, Val312, Glu293, Ser291, Gly288, Ala286 with the following bonds: van der Waals, conventional hydrogen bond, unfavourable donor-donor bond and Pi alkyl (Fig. 8. B).

EGCG binds onto the receptor-binding pocket of chain A of 1IVO specifically on the following residues: Val36, Ala62, Asn86, Ala265, Phe230, Leu38, Arg84, Lys229, Cys227, Val226, Glu60, Lys4, Val6, Arg231Thr266, Glu3 with the following bonds: van der Waals, conventional hydrogen bond, carbon hydrogen bond, unfavourable donor-donor bond, Pi-alkyl, pi-cation, pi-anion (Fig. 8. C.).

ECG binds onto the receptor-binding pocket of chain A of 1IVO specifically on the following residues: Met294, Arg300, Glu295, Lys375, Glu376, Glu293, Thr378, Tyr292, Cys287, Gly288, Ser340, Val312, Arg310, Lys311, Thr339 with the following bonds: van der Waals, conventional hydrogen bond, unfavourable donor-donor bond, Pi-pi T-shaped, pi-alkyl (Fig. 8. D.).



(A)



(B)

Fig. 6. KEGG signalling pathway analysis for targets. **A.** Breast cancer pathway and target genes of catechins. The catechin target genes are highlighted in red in the pathway. **B.** Catechin KEGG enrichment analysis. At the Y-axis are the names of KEGG pathways, and at the X-axis are the fold enrichment. The size and colour of bubbles represent the number of genes; large bubbles indicate many genes involved in the pathway.

3.7. Gene expression pattern analyses

To assess the gene expression levels of 8 different genes, the Livak method ($\Delta\Delta C_T$ method) was used and the *Gapdh* gene was used as a housekeeping gene. Cisplatin was used as a standard chemotherapy drug and as a positive control. Among 8 genes assessed, 6 genes were downregulated while 2 genes were upregulated in treated samples. Downregulated genes are Epidermal growth factor receptor (*Egfr*), Amyloid-beta precursor protein (*App*), B-cell lymphoma 2 (*Bcl2*), DNA (cytosine-5)-methyltransferase1 (*Dnmt1*), Hypoxia-inducible factor 1-alpha (*Hif1a*) and Proteasome subunit beta type-5 (*Psmb5*). Upregulated genes are Caspase-3 (*Casp3*) and Growth arrest and DNA-damage-inducible, beta (*Gadd45b* Downregulated genes as well as upregulated genes showed different levels of expression in different cell treatments (Fig. 9).

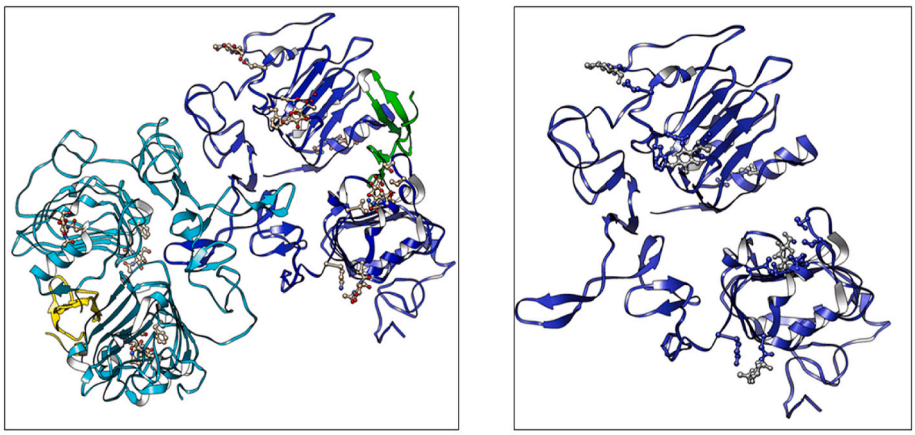


Fig. 7. The 3D crystal structures of Human Epidermal Growth Factor and Receptor Extracellular Domains (1IVO). **A.** whole structure. Each colour represents a specific chain or ligands (blue: chain A, cyan: chain B, green: chain C, yellow: chain d, grey with red dots: NAG ligands).; **B.** structure of chain A (blue colour).

Table 2
Characteristics of binding site amino acids.

Amino acids	Hydrophobicity	Pka	Avg. Isotropic displacement	Interactions	PDB secondary	Secondary
A: THR358	−0.7		77.231	Van der Waals	Undefined	Turn
A: THR360	−0.7		72.414	Van der Waals	Undefined	Turn
A: SER326	−0.8		72.262	Van der Waals	Undefined	Coil
A: MET 294	1.9		132.189	Van der Waals	Undefined	Coil
A: LYS 229	−3.9	10.4	87.454	Van der Waals	Undefined	Coil
A: ILE316	4.5		40.078	Van der Waals	Undefined	Coil
A: ILE 327	4.5		37.385	Van der Waals	Undefined	Coil
A: ILE332	4.5		57.016	Van der Waals	Helix	Turn
A: ASP323	−3.5	3.9	76.306	Van der Waals	Undefined	Turn
A: ASN328	−3.5		71.224	Covalent hydrogen bond	Helix	Coil
A: PHE321	2.8		58.355	Van der Waals	Helix	Helix
A: VAL312	4.2		142.941	Van der Waals	Undefined	Coil
A: ASN 331	−3.5		126.241	Covalent hydrogen bond	Helix	Helix
A: ASN328	−3.5		71.224	Covalent bond	Helix	Coil
A: GLU376	−3.5	4.3	61.009	Van der Waals	Sheet	sheet

Fifteen amino acids are found in the binding sites used for docking study (see Table 3). Among those amino acids, six of them are hydrophobic. Ligand is interacted with the receptor with 1 covalent bond, 2 covalent hydrogen bonds and 13 Van der Waals bonds.

4. Discussion

EGCG content in catechin extracts from these two tea clones used *in vitro* study was high compared to other catechins analysed. This higher concentration of EGCG than other catechins corroborates the previous findings [37,38].

High MW of EGCG and ECG might be the cause of their low GI absorption which the latter in turn causes their low bioavailability (M. J [39]). reported that the absorption depends on the physical-chemical properties of molecules (pka, hydrophilicity, size, molecular configuration and solubility) and their conjugated derivatives. The bioavailability of EGCG is lower than other catechins, this finding corroborates the findings of [40] who found that EGCG has an absolute bioavailability of 0.14 after oral administration in male Sprague Dawley rats. This cannot abolish their chemopreventive properties as they can synergistically conjugate with other catechins and increase their bioavailability as [41] found that methylated EGCG showed a higher inhibitory effect than EGCG alone. EGCG and ECG cannot be considered as lead compounds as such due to MW > 350; for that, lead optimization must be done to improve the MW and to reduce any other deficiencies that can be found in their structure [42]. A drug candidate having log P values beyond Lipinksi's rule-of-five criteria (>5) is likely to be associated with undesired drug properties, such as rapid metabolic turnover, poor aqueous solubility, high plasma protein binding, and tissue accumulation; in addition, a drug candidate with too high lipophilicity can likely be associated with *in vivo* toxicity [43,44].

The PPI network constructed by using the catechin target genes in TNBC showed significantly more interactions than expected (an enrichment P value below 1.0e-16), according to STRING V12.0 database, proteins had higher interaction among themselves than what would be expected for a random set of proteins of the same size and degree of distribution found in the genome. As reported by [29], such an enrichment indicates that the proteins are at least partially connected as a group. The network constructed showed ten key catechin target genes. Those genes are reported to be involved in cancer development, progression and treatment: *Casp3* gene [45], *Mapk14* gene [46], *Pparg* gene [47] ([48], *Mmp3* and *Mmp9* genes [49,50], *Serpine1*gene [51–53], *Bcl2* gene [54,55], *Egfr* gene [56,57,58], *Kdr* gene [59]. These results could confirm the anti-proliferative effects of catechins as reported in previous reports on the anticancer effects of tea catechins [3,8,12,13,60].

GO enrichment and analysis revealed that catechins target key BP, CC and MF; this could confirm their role in the prevention and treatment of different cancer diseases: The damages caused by UV-A to biomolecules such as DNA damage, and lipid peroxidation are strongly implicated in both cell death and cell transformation to malignancies [61]. Nuclear receptors play a key role in the regulation of physiological processes and are known to have pro-oncogenic and ant-oncogenic activities [62]. Many Transcription Factors (TFs) have been reported to be

Table 3
The binding affinity of catechins onto the receptor-binding pockets of chain A of 1IVO. The binding affinity energy of SMILES of 4 catechins are expressed in Kcal/mol.

Compound	Binding energy (Kcal/mol)	RMSD l.b.	RMSD u.b.	Residue in contact	Interaction type
EC	−8.2 −7.8	0.00 0.821	0.00 1.535	Thr339, Tyr292, Arg310, Lys311, Ser291, Gly288, Cys287, Ser340, Lys375, Glu376, Val312, Glu293, Ala286, Thr378	10 Van der Waals, 4 conventional hydrogen bonds and 1 Pi alkyl
EGC	−8.4 −8.4	0.00 0.005	0.00 1.774	Thr339, Tyr292, Arg310, Lys311, Thr378, Cys287, Ser340, Lys375, Glu376, Val312, Glu293, Ser291, Gly288, Ala286	9 Van der Waals, 3 conventional hydrogen bond, 1 unfavourable donor-donor bond and 1 Pi alkyl
ECG	−8.1 −8.1	0.00 1.523	0.00 1.774	Met294, Arg300, Glu295, Lys375, Glu376, Glu293, Thr378, Tyr292, Cys287, Gly288, Ser340, Val312, Arg310, Lys311, Thr339	9 Van der Waals, 5 conventional hydrogen bond, 2 unfavourable donor-donor bonds, 1 Pi-pi T-shaped,2 pi-alkyl
EGCG	−8.3 −8.3	0.00 0.005	0.00 1.448	Val36, Ala62, Asn86, Ala265, Phe230, Leu38, Arg84, Lys229, Cys227, Val226, Glu60, Lys4, Val6, Arg231Thr266, Glu3	7 Van der Waals, 7 conventional hydrogen bonds, 1 carbon hydrogen bond, 1 unfavourable donor-donor bond, 3 Pi-alkyl, 1 pi-cation, 1 pi-anion

RMSD: root-mean-square deviation, is calculated based on the best mode of binding and uses only the heavy atoms; two types of RMSD are presented: l.b, lower bound; u.b. Upper bound.

critical for cancer development and progression [63]. KEGG enrichment and analysis showed that 30 catechin target genes were involved in cancer pathways and 16 genes were involved in breast cancer pathways, this is confirmed the role of catechins in the prevention and treatment of different cancer diseases as reported in the previous findings [3,8,12,13,60].

Molecular docking revealed that SMILES of all 4 catechins bound onto the receptor binding pocket of chain A of EGFR Extracellular Domains (PDB ID: 1IVO). The binding affinity of ligands to the receptors is determined by the binding energy (Kcal/mol); the high binding affinity is shown by the low binding energy. All SMILES of 4 catechins showed slightly similar to the binding affinity of the Erlotinib compound used to treat some cancers. Saini et al. [64] reported that the Erlotinib-EGFR complex had −7.5 kcal/mol and [65] found the binding affinity of −9.34 kcal/mol. This suggests that catechins can work as EGFR inhibitors. Our findings are different from those of [66] who did molecular docking of EGCG and its derivatives at the ATP-binding pocket of EGFR and found that EGCG binding affinity was −5.18 kcal/mol and its derivatives had a binding affinity of 0.47 kcal/mol, and 1.81 kcal/mol for [4″-*O*-(2‴,3‴,4‴,6‴-tetra-*O*-butyryl-β-D-glucopyranosyl)]-(−)-epigallocatechin-3-gallate and [4′-*O*-(2‴,3‴,4‴,6‴-tetra-*O*-butyryl-β-D-glucopyranosyl)-4″–(2‴,3‴,4‴,6‴-tetra-*O*-butyryl-β-D-glucopyranosyl)]-(−)-epigallocatechin-3-gallate, respectively.

Catechin extracts from purple (TRFK306) tea clone showed higher effects on gene expression than ones from BB35 tea clones, these may be due to its high concentration in EGCG content as the latter is reported in previous findings that it has higher anti-proliferative activity than other catechins [3,67,68]. Purple tea, in addition to catechin content, contains anthocyanins which are reported to have anti-cancer effects [69,70]. A high effect on gene expression was found after 48 h, this may be due to that most *in vitro* experiments on the cell line are conducted when the cells are in the log phase of growth, consequently, the cells progress into the station phase and then death phase as the exposure time is increasing e [71,72]so at 48 h, the cells might have been already in the stationary phase. At this phase, cellular metabolism is still active and the expression of the genes essential for cell survival is high at this phase even if the cells cease to grow [73].

Downregulation of gene expression level of the following genes, *Egfr, App, Bcl2, Dnmt1, Hif1a*, and *Psmb5* was due to the antiproliferative activity of catechin extracts and cisplatin on the 4T1 TNBC cell line. These genes are upregulated in cancer development: *Egfr* gene expression is involved in the regulation of breast carcinoma development [74]; *App* gene is upregulated in much various cancer including breast cancer and it promotes the metastasis of breast cancer cells by acting on the MAPK signalling pathway [75]; *Bcl2* upregulation is involved in development of breast cancer by inhibiting apoptosis mainly in Luminal breast cancer [55]; *Dnmt1*is involved in development of breast cancer by inducing MEG3 hyper-methylation [76] its overexpression is associated with development of TNBC subtype [77]. *Hif1a* plays a significant role in breast cancer migration and invasion by mediating hypoxia-induced translation of mRNA-encoding genes (Zhao ji [78]) its overexpression is found in brain metastasis from breast cancer [79]. The *Psmb5* gene is overexpressed in TNBC [80] [81]). Catechins extracts provoked the upregulation of *Casp3* and *Gadd45b* genes. *Casp3* is considered a key enzyme that triggers a series of events that lead to apoptosis [82]. The overexpression of *Gadd45b* has been found to inhibit the growth of various tumour cell lines [83]. This overexpression of *Casp3* and *Gadd45b* gene in the 4T1 TNBC cells treated with catechin extracts explains the antiproliferative activity of the latter on 4T1 cells. Catechin extracts from purple and its combination with cisplatin caused higher expression of *Casp3* than catechin extract from BB35 tea clones and its combination with cisplatin, these was due to the high content of EGCG in purple tea clones [67]. found that EGCG promotes overexpression of *Casp3* and apoptosis in prostate cancer cell lines. EGCG which is present in high concentration in purple tea was reported in our previous publication to have synergism with cisplatin [3] and this might be the reason for the high *Casp* gene expression of the combination of cisplatin with catechin extracts from purple tea clone. EGCG due to having 3 rings: A, B and D might have been the reason for its synergism with cisplatin, as rings B and D were reported to be involved in the inhibition of proteasome activity; the inhibitors of the latter were approved to cure different cancers and cisplatin is one of the proteasome inhibitors [84,85].

5. Conclusion

The purple (TRFK306) tea clone was found to have the highest concentration of EGCG. This underlines the observed high activity on different gene expressions and high activity when combined with cisplatin in the TNBC cell line 4T1. In *silico* study showed that SMILES of 4 different catechins target different genes in TNBC and have high binding affinity to the receptor-binding pocket of chain A of EGFR Extracellular Domains; this *in silico* study was followed by the laboratory experimental study for gene expression assessment.

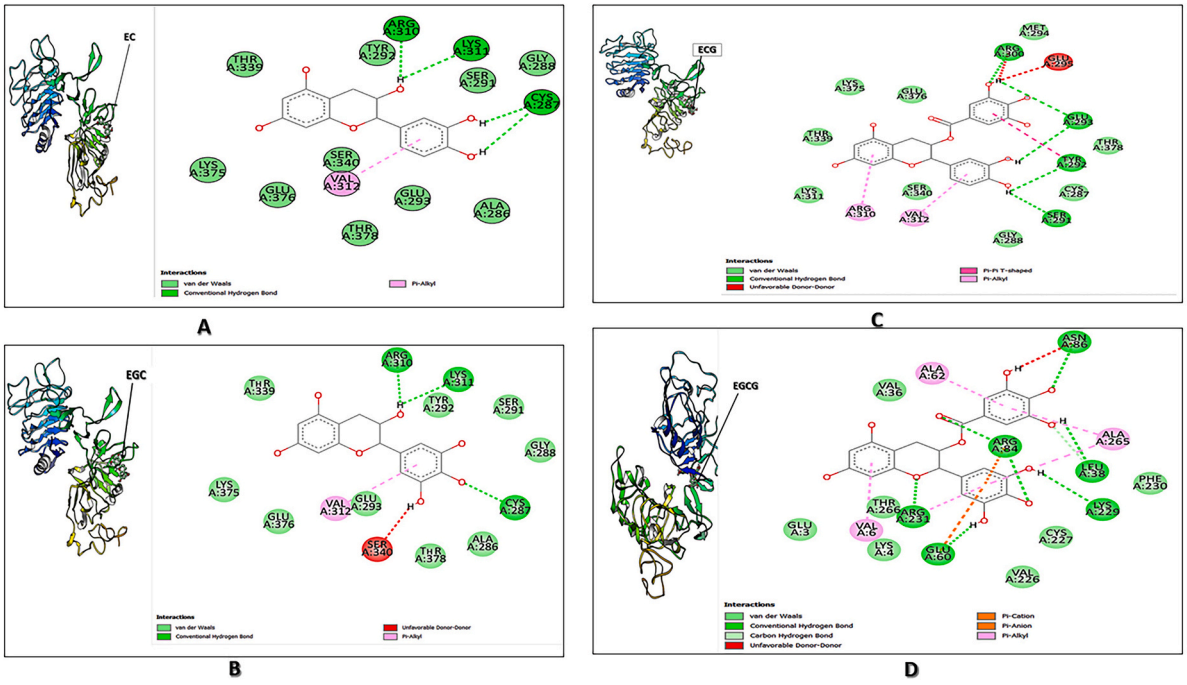


Fig. 8. Binding modes of catechins onto binding-receptor pockets of 1IVO. A. binding mode of EC (left: 3D diagram, right: 2D diagram). **B**. Binding mode of EGC (left: 3D diagram, right: 2D diagram); **C**. Binding mode of ECG (left: 3D diagram, right: 2D diagram). **D**. Binding mode of EGCG (left: 3D diagram, right: 2D diagram). Different interactions: van der Waals (light green), conventional hydrogen bond (green), carbon-hydrogen bond (very light green), unfavourable donor-donor bond (red), Pi-Pi T-shaped (purple), Pi-alkyl (light purple), pi-cation (orange) pi-anion (orange).

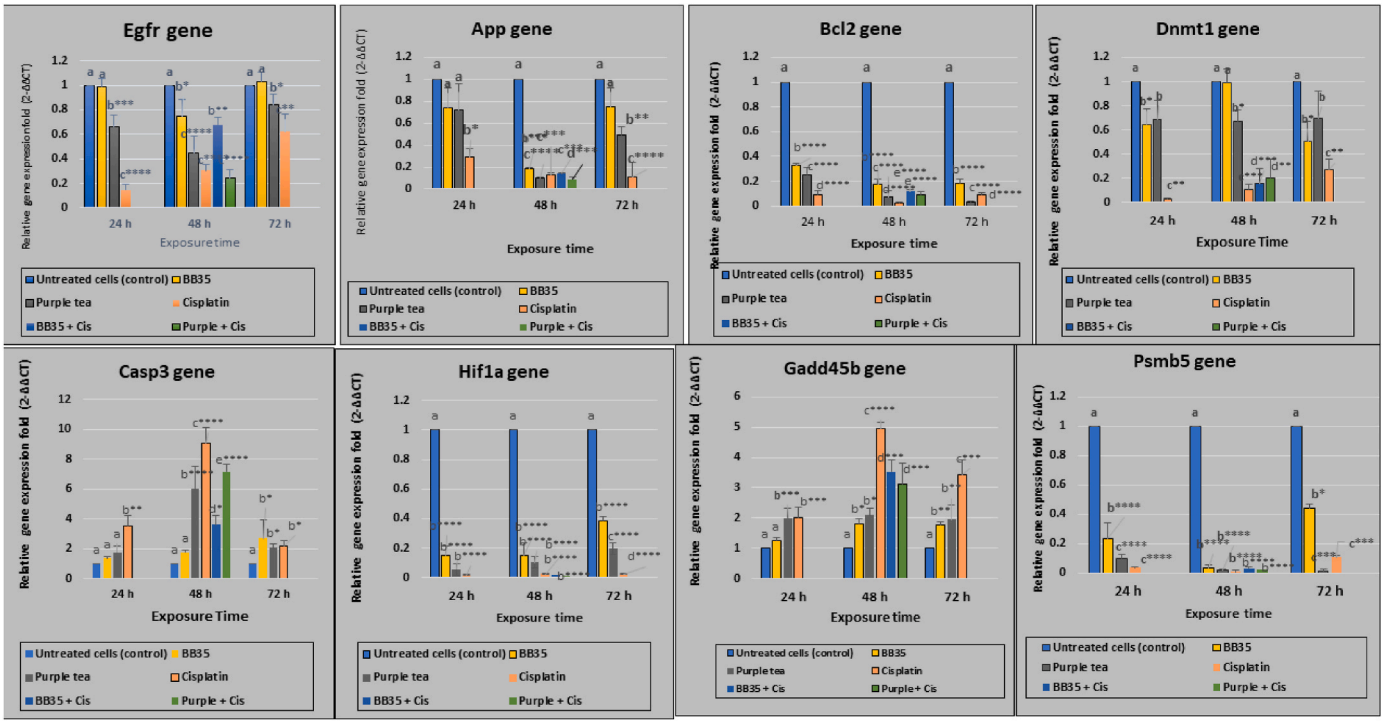


Fig. 9. Expression levels of 8 different genes. Gene expression levels were assessed after exposure times of 24 h, 48 h and 72 h. Effects of a combination of catechin extract from BB35 tea clones and cisplatin and the combination of catechin extracts from purple tea and cisplatin on gene expression levels were assessed at an exposure time of 48 h (we considered the exposure time as those combinations had shown high antiproliferative activities than in other exposure times). The samples with the same letters on the top of bar plots, in the same exposure times mean that there are no significant differences. The data are represented as the means ± SD (N = 3 biological replicates). *p < 0.05, **p < 0.001, ***p < 0.0001 and ****p < 0.0000.

Ethical Statement for Solid State Ionics

Hereby, I/Joseph NDACYAYISENGA/consciously assure that for the manuscript/Effects of tea catechin extracts from BB35 and purple (TRFK 306) tea clones on the gene expression of *Egfr, App*, *Bcl2*, *Dnmt, Casp3*, *Hif1a*, *Gadd45b* and *Psmb5* genes involved in Triple Negative Breast cancer diseases: *in silico* and *in vitro* study/the following is fulfilled.

1) This material is the authors' own original work, which has not been previously published elsewhere.
2) The paper is not currently being considered for publication elsewhere.
3) The paper reflects the authors' own research and analysis in a truthful and complete manner.
4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
5) The results are appropriately placed in the context of prior and existing research.
6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.
7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

The violation of the Ethical Statement rules may result in severe consequences.

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CRediT authorship contribution statement

Joseph Ndacyayisenga: Writing – review & editing, Writing – original draft, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Festus M. Tolo:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Fred Wamunyokoli:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Esther N. Maina:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imu.2024.101469.

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