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Conceptual functional-by-design optimisation of the antioxidant capacity of *trans*-resveratrol, quercetin, and chlorogenic acid: Application in a functional tea

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A B S T R A C T

Designing functional foods as delivery systems may become a tailored strategy to decrease the risk of non-communicable diseases. Therefore, this work aims to optimise a combination of *t*-resveratrol (RES), chlorogenic acid (CHA), and quercetin (QUE) based on antioxidant assays and develop a functional tea formulation enriched with the optimal polyphenol combination (OPM). Experimental results showed that the antioxidant capacity of these compounds is assay- and compound-dependent. A mixture containing 73% RES and 27% QUE maximised the hydroxyl radical scavenging activity and FRAP. OPM upregulated the gene expressions of heme oxygenase-1, superoxide dismutase, and catalase and decreased the reactive oxygen species generation in L929 fibroblasts. Adding OPM (100 mg/L) to a chamomile tea increased FRAP:39%, DPPH:59%; total phenolic content: 57%, iron reducing capacity: 41%, human plasma protection against oxidation: 67%. However, pasteurisation (63 °C/30 min) decreased only the DPPH. Combining technology, engineering, and cell biology was effective for functional tea design.

1. Introduction

Phenolic compounds are secondary metabolites produced in plants and distributed in fruit seeds, leaves, pulps, skins, cereals, edible roots, and other vegetables. They have at least one aromatic ring bearing one or more hydroxyl groups and can be classified as flavonoids, stilbenes, coumarins, hydroxycinnamic and hydroxybenzoic acids, lignans, and tannins. More than 8,000 compounds are known and frequently consumed in a varied diet. Phenolic compounds are known for their various bioactivities, as demonstrated by multiple *in vitro*, cell-based, *in vivo*, and human interventions (do Carmo et al., 2021).

Phenolic compounds' bioactivities are closely associated with their abilities to scavenge free radicals, stabilise reactive oxygen/nitrogen species (ROS/RNS), and decrease the secretion of pro-inflammatory cytokines and chemokines in multiple organs/tissues (Turner et al., 2014). The resonance brought about the aromatic ring, the number of hydroxyl groups, and the nature of substitutions on the aromatic rings play a crucial role in the bioactivity of phenolic compounds (Zamora et al., 2018). Therefore, studying phenolic compounds with distinct structures and bioactivities is pivotal to understanding how to maximise their biological relevance and incentivise their consumption regularly (Romain et al., 2021).

Among the phenolic compounds consumed in a regular diet, *t*-resveratrol (**Fig. 1A – Supplementary material**), quercetin (**Fig. 1B – Supplementary material**), and chlorogenic acid (**Fig. 1C – Supplementary material**) deserve attention because they are commonly found in various fruits and vegetables. 3,5,4′-Trihydroxy-*trans*-stilbene, or *t*-resveratrol (RES), contains three OH groups and can be found in peanuts, grapes and their products, cocoa, and berries. RES has antioxidant and anti-inflammatory activities (Santos et al., 2022), improves vascular function in hypertensive individuals (DiNatale & Crowe-White, 2022) and regulates glucose homeostasis in type-2 diabetes (Mahjabeen et al., 2022).

1,4,5-Trihydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate), 3-(3,4-dihydroxycinnamoyl)quinic acid, also known as chlorogenic acid (CHA), contains two OH groups and can be found in

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many vegetables, especially in herbal teas, fruits, and coffee (Santos et al., 2020). Because of its antioxidant capacity, CHA is effective against hyperglycemia-induced pathological alteration both *in vitro* and *in vivo* (Preetha Rani et al., 2022), has antihypertensive and anti-inflammatory effects *in vivo* (Wang et al., 2022), and improves cardiovascular, liver, and metabolic responses (Bhandarkar et al., 2019). 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one, also known as quercetin (QUE), is one of the most abundant flavonoids in vegetables, fruits, herbs, and wine (Santos et al., 2020). QUE contains four OH groups that have been linked to multiple bioactivities, such as antioxidant and anti-inflammatory (Chen et al., 2022), anti-atherosclerotic (Luo et al., 2022), and immunomodulatory and gut microbiota modulation effects (Zhou et al., 2022). Considering the alleged health benefits of phenolics, food technologists have incorporated these compounds into different foods and beverages to boost the functional effects and to extend the food's shelf life (Santos et al., 2018).

To date, the is no research on the combined use of RES, QUE, and CHA in potentially functional food, which instigates the research on mechanisms of actions and interaction between these compounds (i.e., antagonism, synergism, and additive effects). Nonetheless, in a previous study (Xu, Gao, & Granato, 2021), our group optimised the combined use of three tea catechins (i.e., epigallocatechin gallate - EGCG, epigallocatechin - EGC, and epicatechin gallate - ECG) to maximise the chemical antioxidant activity. Results showed that 67.4% ECG and 32.6% EGCG was the optimal combination of phenolics. This mixture was added to chrysanthemum tea at 800 mg/L and decreased the ROS generation in OVCAR-3, HEK293 and HFL1 cells. After pasteurisation, the enriched tea did not lose its chemical antioxidant activity.

Developing food models and prototypes that deliver bioactive compounds, such as QUE, CHA, and RES, is paramount for functional food design. For phenolic compounds, targeting antioxidant and anti-inflammatory effects is a way to contribute to decreased odds of cardiovascular and inflammation-lead diseases and oxidative stress. In the long term, reduced incidence of cardiovascular disease will ultimately contribute to public health and comply with the United Nations Sustainable Development Goals (i.e., goal 3 – good health and well-being). Therefore, the aims of this work are: i) analyse the synergistic effects and optimise a combination of RES, CHA, and QUE based on *in vitro* antioxidant assays, ii) analyse the translation of test-tube results into cellular antioxidant activity (CAA), and iii) compare the total phenolic content (TPC) and antioxidant activity of chamomile tea added with the optimal phenolic mixture (OPM) before and after slow pasteurisation.

2. Materials and methods

2.1. Chemical reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri-(2-pyridyl)-s-triazine (TPTZ), ascorbic acid, gallic acid, trichloroacetic acid, chlorogenic acid (5-*O*-caffeoylquinic acid), quercetin, ferric chloride ($FeCl_3$), hydrogen peroxide (H_2O_2), neocuproin, copper (II) chloride dihydrate, 1,10-phenanthroline, and iron (II) sulfate heptahydrate were obtained from Sigma-Aldrich (Darmstadt, Germany). Glacial acetic acid was provided by J. T. Baker (Mallinckrodt Baker Inc., Utrecht, the Netherlands). Anhydrous sodium carbonate, sodium acetate trihydrate, ammonium acetate, and sodium hydroxide were obtained from Panreac Quimica S.A (Barcelona, Spain). Penicillin, streptomycin, and heat-inactivated fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). *t*-Resveratrol (98% purity) was obtained from Extrasynthese (Lyon, France). Aqueous solutions were prepared using ultrapure water.

2.2. Experimental design

A simplex-centroid design with augmented points (n = 10 combinations) was used to study the effects of quercetin, chlorogenic acid, and *t*-resveratrol on the *in vitro* antioxidant capacity (Table 1). For this purpose, single (n = 3), binary (3), and ternary (n = 4) mixtures were tested. This experimental setup allows for the identification of possible synergism, antagonism, and additional effects. Each bioactive compound was diluted in methanol at 1 mmol/L for proper comparison on a molar basis. After combining the samples according to the proportions in Table 1, the solutions were tested for antioxidant capacity using different assays. After each solution preparation, the antioxidant capacity measurement was done using a randomised order to avoid carry-over effects.

2.3. In vitro antioxidant capacity

Cupric-ion reducing antioxidant capacity (CUPRAC) was estimated utilising the copper (II)-neocuproine (Cu(II)–Nc) reagent as the chromogenic agent, and the results were expressed as mg of ascorbic acid equivalents per litre (mg AAE/L). The DPPH free-radical scavenging activity was measured in methanol using a DPPH concentration of 0.10 mmol/L, and the results were expressed as mg AAE/L. The ferric-reducing antioxidant power (FRAP) was quantified using a standard spectrophotometric method, and the results were expressed as mg AAE/L. The hydroxyl radical scavenging activity was estimated using the 1,10-phenanthroline-H_2O_2 spectrophotometric method, and the results were expressed as mg of gallic acid equivalents per litre (mg GAE/L). All analyses were conducted in quadruplicate and followed the procedures and experimental conditions outlined elsewhere (do Carmo et al., 2021; Pap et al., 2021).

Table 1
Antioxidant capacity of *t*-resveratrol, quercetin, and chlorogenic acid and their binary and ternary mixture in relation to simplex centroid design of experiments).

t-Resveratrol	Quercetin	Chlorogenic acid	Hydroxyl radical scavenging activity (mg GAE/L)	FRAP (mg AAE/L)	CUPRAC (mg AAE/L)
1	0	0	38.73 ± 0.43^{a}	9.01 ± 1.21^{f}	38.37 ± 4.78^{de}
0	1	0	23.54 ± 1.57^{c}	24.66 ± 0.87^{b}	136.02 ± 24.00^{a}
0	0	1	2.54 ± 0.60^{h}	35.48 ± 0.08^{a}	42.00 ± 7.50^{de}
0.5	0.5	0	30.56 ± 0.36^{b}	8.70 ± 1.61^{f}	67.89 ± 28.07^{cd}
0.5	0	0.5	12.09 ± 0.95^{ef}	22.80 ± 1.26^{bc}	17.02 ± 8.93^{e}
0	0.5	0.5	5.83 ± 1.01^{g}	31.48 ± 2.56^{a}	89.24 ± 8.18^{bc}
0.66	0.17	0.17	18.99 ± 0.10^{d}	16.12 ± 2.40^{de}	76.85 ± 18.24^{cd}
0.16	0.67	0.17	13.24 ± 0.60^{e}	13.45 ± 2.09^{ef}	134.20 ± 18.33^{ab}
0.16	0.17	0.67	9.73 ± 1.27^{f}	19.89 ± 1.16^{cd}	39.28 ± 13.72^{de}
0.34	0.33	0.33	14.28 ± 0.62^{e}	18.05 ± 1.65^{cde}	32.53 ± 5.67^{de}
P value (homoscedasticity)			0.869	0.966	0.765
P value (one-way ANOVA)			<0.001	<0.001	<0.001

Note: AAE = ascorbic acid equivalent. GAE = gallic acid equivalent. Different letters in the same column represent significantly different ($p < 0.05$) treatments. For the phenolic compounds, coded values represent the relative percentage in relation to 100% (1 = 100%, 0.5 = 50%, 0.33 = 33%, and 0.17 = 17%).

2.4. Response surface modelling and optimisation

Response Surface Methodology (RSM) was used to model the experimental data (i.e., antioxidant capacity results) into a cubic regression equation (Eq. (1), where linear (b_i), quadratic (b_{ij}), and cubic effects (b_{ijk}) on the response, ($\hat{y}$), (i.e., antioxidant activity) were estimated using the triplicate values (n = 40 data points per antioxidant assay):

$$\hat{y} = \sum_{i=1}^{3} b_i x_i + \sum_{i<j}^{3} \sum_{j}^{3} b_{ij} x_i x_j + \sum_{i<j}^{3} \sum_{j<k}^{3} \sum_{k}^{3} b_{ijk} x_i x_j x_k \quad (1)$$

Only significant ($p < 0.05$) coefficients were used to obtain the polynomial regression equations, and triangular plots were obtained to represent the RSM models. To assess the statistical significance of each model, the determination coefficient (R^2) and the adjusted R^2 were estimated using the TIBCO Statistica 13.3 software. The desirability function for multi-targeted optimisation maximised the antioxidant capacity based on the FRAP and hydroxyl radical scavenging activity. For this purpose, 100 iterations were used to obtain the optimal phenolic mixture, and the D-value was calculated. The optimal phenolic mixture (OPM) was tested for its antioxidant capacity (FRAP and hydroxyl radical scavenging activity). The relative error, which considers the predicted and the observed values, was calculated and compared with the ±95% confidence interval.

2.5. Application of the optimal phenolic mixture in a tea beverage model

To assess the effects of OPM on the antioxidant capacity in a tea beverage model, chamomile tea (Thompson's family tea, Dublin, Ireland) was used. The infusion was prepared by adding 2 g of chamomile powder into 100 mL of ultrapure water at 95 °C, manually stirred for 10 min, and then cooled to 25 °C using a water bath. OPM in solid form was added to two treatments at 100 mg of OPM per litre of chamomile tea. The mixture was magnetically stirred for 5 min, whereby one was subjected to slow pasteurisation at 63 °C for 30 min to unveil the effects of thermal treatment on the TPC and antioxidant activity of chamomile tea formulations. The chamomile tea was filtered using a qualitative paper and divided into three treatments: i) unpasteurised chamomile tea (C, negative control), ii) chamomile tea added with OPM at 100 mg/L (CT), and iii) chamomile tea added with OPM at 100 mg/L and pasteurised (PCT).

The TPC was assessed using the Folin-Ciocalteu phenol reagent, and the results were expressed as mg GAE/L. The antioxidant capacity (FRAP, section 2.3) of each tea sample was measured in triplicate and expressed as mg AAE/L. The DPPH free radical scavenging activity of teas was also measured in triplicate. For this purpose, a methanolic DPPH solution at 0.10 mmol/L was prepared, and readings were recorded at 517 nm after 30 min reaction in the dark. Results were expressed as mg AAE/L. The hydroxyl radical scavenging activity and the iron-reducing capacity were assessed in triplicate using the H_2O_2-1,10-phenanthroline and Prussian Blue assays, respectively, and the results were expressed as mg GAE/g (J. S. Santos et al., 2020, Pap et al., 2021). All analyses were run in quadruplicate.

The inhibition of copper-induced human plasma oxidation was evaluated six times using a spectrophotometric procedure (Liu & Huang, 2015). Human blood was obtained from a healthy 39-year-old male in heparinised tubes. After centrifugation (1200 *g* for 10 min), human plasma was diluted 40-fold with phosphate saline buffer (5 mmol/L, NaCl at 150 mmol/L, pH 7.35). Plasma (50 μL) and each tea sample (50 μL) were mixed in a 96-well plate. After 15 min incubation at 37 °C, lipid peroxidation was initiated by $CuCl_2$ (150 μmol/L, 100 μL). The production of conjugated dienes was monitored at 245 nm for 120 min at 5 min intervals. The difference in absorbance between 120 min and 0 min was calculated using ascorbic acid (25–150 mg/L) as the standard. The results were expressed as mg AAE/L. The biological protocol was approved by the University of Limerick Research Ethics Committee (protocol 2023_02_01_S&E).

2.6. Cellular antioxidant activity

To assess the effects of RES, QUE, and the OPM on cell viability, mouse fibroblast L929 cells (kindly donated by Prof. Fuliang Hu, Zhejiang University, China) were cultured in DMEM (high glucose) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated fetal bovine serum in a humidified incubator at 37 °C and 5% CO_2 and dissolved RES with anhydrous ethanol at 5 mg/mL. QUE was dissolved in DMSO at 5 mg/mL. Stock solutions were stored at 4 °C in the dark and diluted with a complete medium at the time of use, with the final concentration of ethanol/DMSO in the cell culture medium not exceeding 0.1% (v/v).

Cells (3.5×10^4 cells/mL) were inoculated uniformly in 96-well plates on two different days, whereby three plates were used to ensure reproducibility and statistical significance. After the cell growth reached 70%, they were treated with varying concentrations of RES, QUE, and the OPM for 2 h, and stimulated with 600 μM H_2O_2 for 24 h. After treatment for 24 h, 10 μL of CCK-8 (Dojindo, Kumamoto, Japan) were added to each well and incubated for 1 h. Absorbance values were measured at $\lambda = 450$ nm and the cell viability was expressed as a percentage to the untreated cells.

To assess the intracellular antioxidant capacity of RES, QUE, and OPM, L929 cells (3.5×10^4 cells/mL) were inoculated uniformly in 12-well plates. After the cells grew to 70%, they were treated with different concentrations of the compounds for 2 h, then stimulated with 600 μmol/L H_2O_2 for 6 h. The gene expression of some oxidative stress- and inflammation-related genes, including superoxide dismutase (SOD), selenoprotein thioredoxin reductase 1 (Txnrd), nuclear factor-erythroid factor 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and catalase (CAT) were analysed by quantitative real-time polymerase chain reaction (qRT-PCR). Sequences of the primers for qRT-PCR can be found in **Table 1 – Supplementary material**.

Removal of the culture medium, total cellular RNA was extracted using the RNA extraction kit (Beijing CarryHelix Biotechnology Co. Ltd., Beijing, China) according to the instructions provided by the reagent vendor. The concentration and purity of RNA samples were determined by a NanoDrop spectrophotometer (ND2000, NanoDrop Technologies, Wilmington, DE, USA). The cDNA template was synthesised by reverse transcription with 1 μg of total RNA using the PrimeScriptTM RT Master Mix Reverse Transcription Kit (TaKaRa, Dalian, China). The reverse transcription products were stored at −20 °C, and primers were synthesised by Sangon Biotech (Shanghai, China). Real-time fluorescent qPCR was performed using the TB Green Premix Ex Taq II kit (TaKaRa, Dalian, China) in the QuantStudio 1 real-time fluorescent quantitative PCR system. Data were analysed using the 2-ΔΔCt method, and β-actin was used to normalise the expression of the target gene.

A 2′,7′-dichlorofluorescin diacetate (DCFH-DA) probe (Solarbio Life Sciences, Beijing, China) was used to determine ROS production, which can be oxidised to fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. Treated L929 cells were incubated with 10 μmol/L DCFH-DA for 30 min at 37 °C and washed thrice with the basal medium. Fluorescence was then examined with a confocal laser scanning microscope (Olympus FV1200, Yokohama, Japan) using excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence intensity was quantified by Image J software, and a statistical histogram was drawn by GraphPad Prism 8 software.

2.7. Statistical analyses

Experimental data were collected using Microsoft Excel 2010, and basic statistics (average, standard deviation) were calculated. One-way analysis of variances (ANOVA) was used to highlight differences in the mean values, and Tukey's HSD test was used to separate the means.

Probability values below 0.05 were used to reject the null hypothesis. The basic and inferential statistical analyses were performed using TIBCO Statistica 13.3 (TIBCO Statistica Inc, Tulsa, OK, USA).

3. Results and discussion

3.1. RSM modelling of in vitro antioxidant capacity and optimisation

Table 1 shows the antioxidant capacity of RES, QUE, and CHA and their binary and ternary combinations using CUPRAC, FRAP, and hydroxyl radical scavenging capacity. RES (38.73 mg AAE/L) presented the highest mean value for the hydroxyl radical scavenging activity, whereas CHA (2.54 mg AAE/L) presented the opposite behaviour. QUE (23.54 mg AAE/L) showed an intermediate value, and its combination with RES (0.5:0.5) provided the second-highest antioxidant capacity (30.56 mg AAE/L). These results corroborate the observations made by (Liang & Kitts, 2015), who found that CHA is a weak free radical scavenger compared to flavonoids but still shows other mechanisms of antioxidant action *in vivo*.

However, the other binary and ternary combinations did not present any additive or synergistic antioxidant effects, as formally tested by RSM (Table 2). In fact, the binary combinations RES/QUE, QUE/CHA, and the ternary combinations of phenolics enhanced the antagonistic antioxidant potential (i.e., decreasing the antioxidant potential), as the regression coefficients were significant ($p < 0.05$), negative, and had similar or even higher values compared to that of RES. These observations can be visualised in Fig. 1A, where the 2-dimensional response plot depicts the different antioxidant capacity values for all the experimental results. The RSM model explained 99% ($R^2 = 0.990$) of data variability, confirming its potential to be used for prediction purposes.

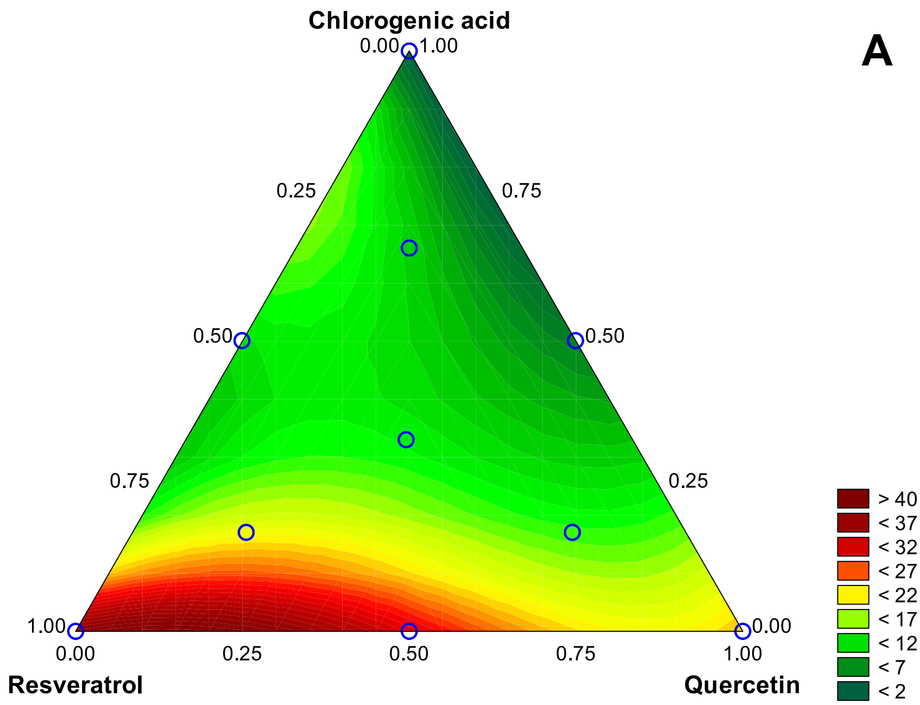
CHA (35.48 mg AAE/L) presented the highest FRAP value, followed by QUE (24.66 mg AAE/L), whereas RES (9.01 mg AAE/L) showed the lowest FRAP value. The binary combination of CHA/QUE (0.5:0.5) also showed a high FRAP value (31.48 mg AAE/L,) followed by the CHA/RES (0.5:0.5) combination (22.80 mg AAE/L). To better understand the experimental data, RSM was applied (Table 2), and the regression model explained 95% ($R^2 = 0.950$) of data variability. It shows that the regression coefficient of the binary combination between RES and QUE (0.5:0.5), which also presented a low FRAP value (8.70 mg AAE/mL), and the regression coefficient of the ternary combination of phenolics showed a significant ($p < 0.05$) antagonistic effect on FRAP. This effect may be because the tested ternary combinations showed approximately 50% less antioxidant capacity than that of CHA and clearly shows that in a model solution, phenolics interact and may bring about changes in the antioxidant capacity if blended with other compounds. The response plot (Fig. 1B) shows the experimental results and can be used to observe the effects of each phenolic compound and their combinations on the FRAP values.

For CUPRAC, QUE and the combination of QUE/RES/CHA (0.67:0.16:0.17) presented the highest antioxidant potential (136.02 mg AAE/L). For this specific assay, we hypothesise that the QUE's reducing potential of the Cu^{2+} ion may be linked to the *o*-catechol group in the B-ring that seems central to the CUPRAC (Silva et al., 2002). On the other hand, the CUPRAC mean values of RES (38.37 mg AAE/L) and CHA (42.00 mg AAE/L) were approximately 3.5-fold less than that of QUE. These results are in line with the original report of the CUPRAC method, where (Apak et al., 2008) compared the antioxidant capacity of different phenolic compounds and observed that QUE (4.38 Trolox equivalent) had a higher CUPRAC value than CHA (2.47 Trolox equivalent). The binary (17 to 89 mg AAE/L) and ternary (32 to 134 mg AAE/L) combinations between the three phenolic compounds showed no additive or synergistic antioxidant effects. These observations were further confirmed using multiple regression (Table 2), as the regression coefficients for the binary and ternary combinations were deemed nonsignificant ($p > 0.05$). Nevertheless, the RSM regression equation was able to explain 73% of data variability (e.g., $R^2 = 0.73$), implying that the model can be used for the prediction of CUPRAC values in future experiments. CUPRAC results displayed in Table 1 can be better visualised in the 2-dimensional fitted response plot of Fig. 1C.

Table 1 shows that each phenolic compound (i.e., RES, QUE, and CHA) showed distinct antioxidant capacity, highlighting that the results are assay- and chemical structure-dependent. For example, RES showed the highest hydroxyl radical scavenging effect but the lowest FRAP. Similarly, CHA showed the lowest hydroxyl extreme scavenging effect,

Table 2
Assessing the effects of *t*-resveratrol (A), quercetin (B), and chlorogenic acid (C) and on the chemical antioxidant activity using response surface modelling.

	Regression Coefficient	Standard Error	*t*-Value	*p*-Value	−95% Confidence limit	+95% Confidence limit
Hydroxyl radical scavenging activity						
A (RES)	38.312	0.659	58.163	<0.001	36.945	39.678
B (QUE)	23.127	0.659	35.102	<0.001	21.760	24.493
C (CHA)	2.371	0.724	3.278	0.003	0.871	3.872
AB	−34.368	3.499	−9.823	<0.001	−41.623	−27.112
BC	−29.093	3.498	−8.317	<0.001	−36.347	−21.839
ABC	−45.634	21.111	−2.162	0.042	−89.416	−1.852
AB(A-B)	50.434	11.857	4.254	<0.001	25.844	75.023
AC(A-C)	−122.839	11.809	−10.402	<0.001	−147.330	−98.349
R^2	0.990					
R^2_{adj}	0.987					
Ferric-reducing antioxidant power (FRAP)						
A (RES)	8.763	1.137	7.709	<0.001	6.411	11.114
B (QUE)	24.755	1.137	21.779	<0.001	22.404	27.106
C (CHA)	35.614	1.052	33.860	<0.001	33.439	37.790
AB	−34.443	5.992	−5.748	<0.001	−46.839	−22.046
ABC	−102.033	33.843	−3.015	0.006	−172.042	−32.024
AB(A-B)	53.432	20.378	2.622	0.015	11.275	95.588
AC(A-C)	85.248	20.492	4.160	<0.001	42.858	127.639
R^2	0.950					
R^2_{adj}	0.938					
Cupric ion reducing antioxidant capacity (CUPRAC)						
A (RES)	24.911	10.420	2.391	0.024	3.492	46.330
B (QUE)	140.752	9.862	14.272	<0.001	120.480	161.024
C (CHA)	36.356	10.420	3.489	0.002	14.937	57.776
AC(A-C)	386.218	187.090	2.064	0.049	1.649	770.788
R^2	0.735					
R^2_{adj}	0.705					



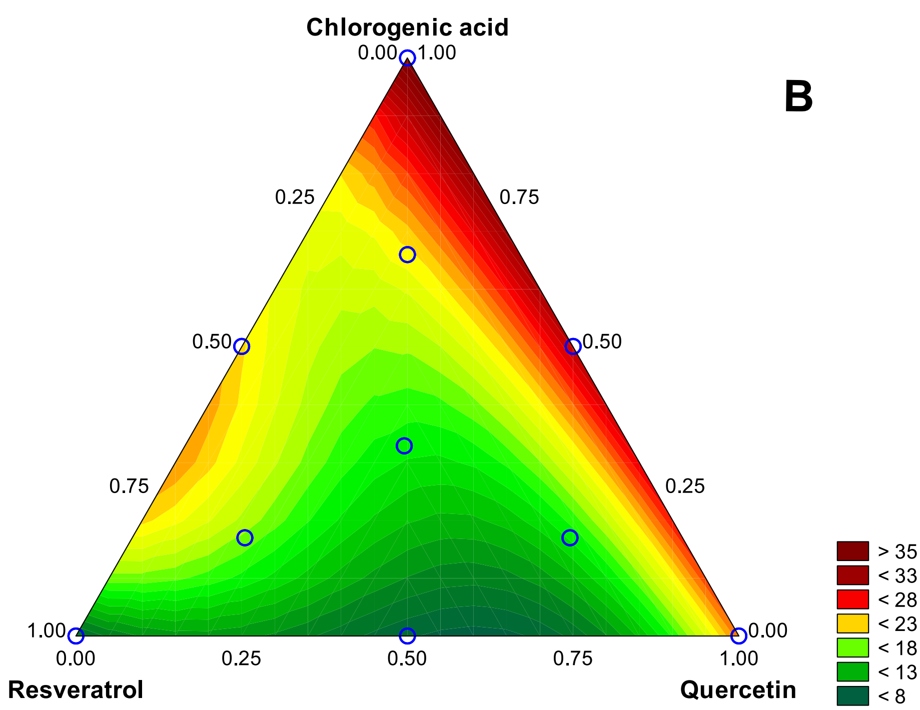


Fig. 1. Triangular response plots for the effects of *t*-resveratrol, quercetin, and chlorogenic acid on the hydroxyl radical scavenging activity (A), ferric-reducing antioxidant power (B), and cupric-ion reducing antioxidant capacity (C).

but the highest FRAP and QUE showed the highest CUPRAC value and intermediate values for the other assays. Our results reiterate the need to assess the antioxidant capacity of phenolic compounds and food-derived extracts using more than one *in vitro* assay.

The hydroxyl radical scavenging activity, CUPRAC, and FRAP employ the single electron transfer (SET) mechanism of action, but the former also encompasses the hydrogen atom transfer (HAT) mechanism. Still, the two later assays rely on the reducing capacity of metals (e.g., iron for FRAP, and copper for CUPRAC), while the former depends on using a free radical (e.g.,·OH). SET-based antioxidant assays measure the phenolic compound's (R-OH) ability to transfer one electron to reduce ROS, primarily free radicals, transforming these reactive species into less reactive substances. Although these assays employ the exact mechanism of action, each has its features, especially regarding the pH of the reaction medium, temperature, reaction time, and solvents. For example, the FRAP assay is conducted at pH 3.6 for 30 min, whereas the reaction medium of CUPRAC is buffered at pH 7 and the reaction time is also 30 min. Additionally, the temperature of the FRAP assay is monitored at 37 °C to simulate the human body, while for the other methods, the temperature is not monitored. Therefore, the antioxidant capacity of

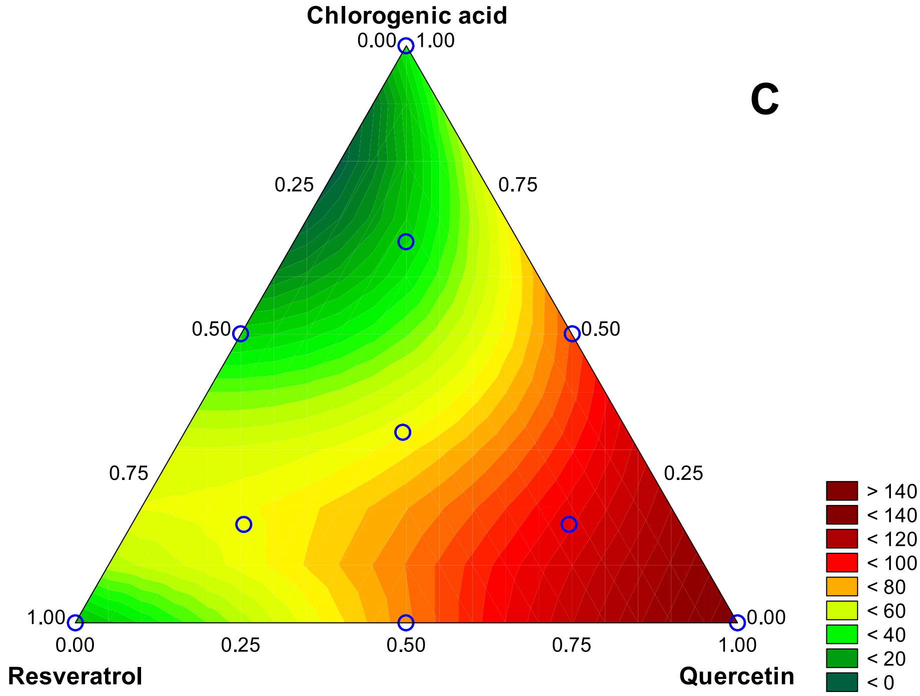


Fig. 1. (*continued*).

bioactive substances highly depends on the pH, temperature, and solvents because they determine the antioxidant's ionisation and deprotonation potential and, consequently, its reducing capacity. All in one, no single assay translates *in vivo* effectiveness, and multiple test tube-based assays should be used to characterise pure compounds and, more importantly, food extracts.

Another crucial aspect of antioxidants' effectiveness is associated with their chemical structure: the number and position of phenolic hydroxyls (Rice-Evans et al., 1996). For example, for flavonoids, such as QUE, the *ortho*-hydroxylation in the B ring enhance the antioxidant potential by maximising the stability of the QUE's phenoxyl radical form when electrons or hydrogen atoms are transferred to a ROS. For stilbenes and phenolic acids, the number and position of phenolic OH play a crucial role in the antioxidant capacity. According to (J. Chen et al., 2020), with less than four phenolic hydroxyl groups on the benzene ring (e.g., in the CHA structure), the antioxidant activity of phenolic acids is proportional to the number of phenolic hydroxyl groups.

Regarding the optimisation procedure (**Fig. 2 – Supplementary material**), a mixture containing 73% RES and 27% QUE was regarded as the optimum combination of phenolics to increase the *in vitro* antioxidant capacity based on the hydroxyl radical scavenging activity and FRAP. This combination was further analysed for its antioxidant capacity, and the results are shown in **Table 2 - Supplementary material**. For both assays, the experimental results are within the ±95% confidence interval and are very close to the predicted mean values. Therefore, it is possible to conclude that RSM could effectively optimise the combination of different polyphenols regarding their antioxidant activity. Considering the experimental conditions, the RSM models generated for these antioxidant assays can be used to predict the antioxidant activity of different combinations of RES, QUE, and CHA in future studies.

3.2. Cellular antioxidant activity in mouse fibroblast L929 cells

The effects of RES (0.25 to 2 μg/mL), QUE (0.5 to 4 μg/mL), and OPM (0.5 to 2 μg/mL) on L929 cell viability were assessed (Fig. 2). Results show that QUE (up to 4 μg/mL), cell viability was over 90 %, while RES (up to 1 μg/mL) did not impact cell viability. Still, at 2 μg/mL, cell viability was approximately 80%. On the other hand, OPM (up to 0.5 μg/mL) did not change cell viability, but at 2 μg/mL, cell viability was roughly 65%. The maximum concentration of RES used in the present study was 2 μg/mL, and the maximum concentration of QUE was 4 μg/mL, respectively. tRES and QUE at 1 μg/mL did not show cytotoxicity to L929 cells, thus 1 μg/mL of RES and QUE were mixed in the ratio of 73:27 (w/w) to form the optimal polyphenol combination (OPM), as also indicated by the RSM optimisation procedure. The final concentration of OPM was 0.5 μg/mL. Next, the *in vitro* antioxidant capacity of RES, QUE, and OPM using an H_2O_2-induced oxidative stress model was assessed in L929 fibroblasts. As shown in Fig. 3A, the cell viability of fibroblasts treated with OPM (78–83%) was higher ($p < 0.05$) than that of RES (~61%) but similar ($p > 0.05$) to that of QUE (~75%). OPM at 0.125 μg/mL showed better ($p = 0.004$) antioxidative activity in rescuing H_2O_2-induced cell viability losses (11.48%) than RES (-8.98%) or QUE treated alone (5.14%, $p = 0.072$).

Both RES and OPM at 0.5 μg/mL significantly induced gene expression of *HO1* (Fig. 3B) in L929 fibroblasts pre-treated with H_2O_2. In contrast, QUE and OPM at 0.125 μg/mL did not affect the expression of *HO1*. *HO1* is a well-known detoxification gene in redox regulation that plays an essential role in the cellular response to oxidative stress. In fact, *HO1* is an Nrf2-regulated gene that plays a critical role in preventing vascular inflammation and macrophage differentiation and polarisation to specific subtypes (Loboda et al., 2016). Regarding the Txnrd expression (Fig. 3C), both RES and QUE increased its gene expression, and similar results were obtained with OPM at 0.125 and 0.5 μg/mL. *Txnrd* is a crucial enzyme for protecting cells against intracellular reduction–oxidation (e.g., ROS generation) reactions by scavenging free radicals and suppressing the oxidative action of ROS (Lee et al., 2019). Results from Fig. 3D show that compared to the cell solely treated with H_2O_2, the effects of RES, QUE, and OPM at the concentrations tested on Nrf2 were marginal. Considering the results obtained herein, we hypothesise that the antioxidant effect of OPM might not be related to the regulation of the Nrf2 pathway. Nrf2 is a regulator of resistance to oxidants (e.g., ROS), thus, increasing its expression by the treatment with bioactive compounds would add protection from oxidative damage in the long term.

Human cells contain various antioxidant agents, such as compounds

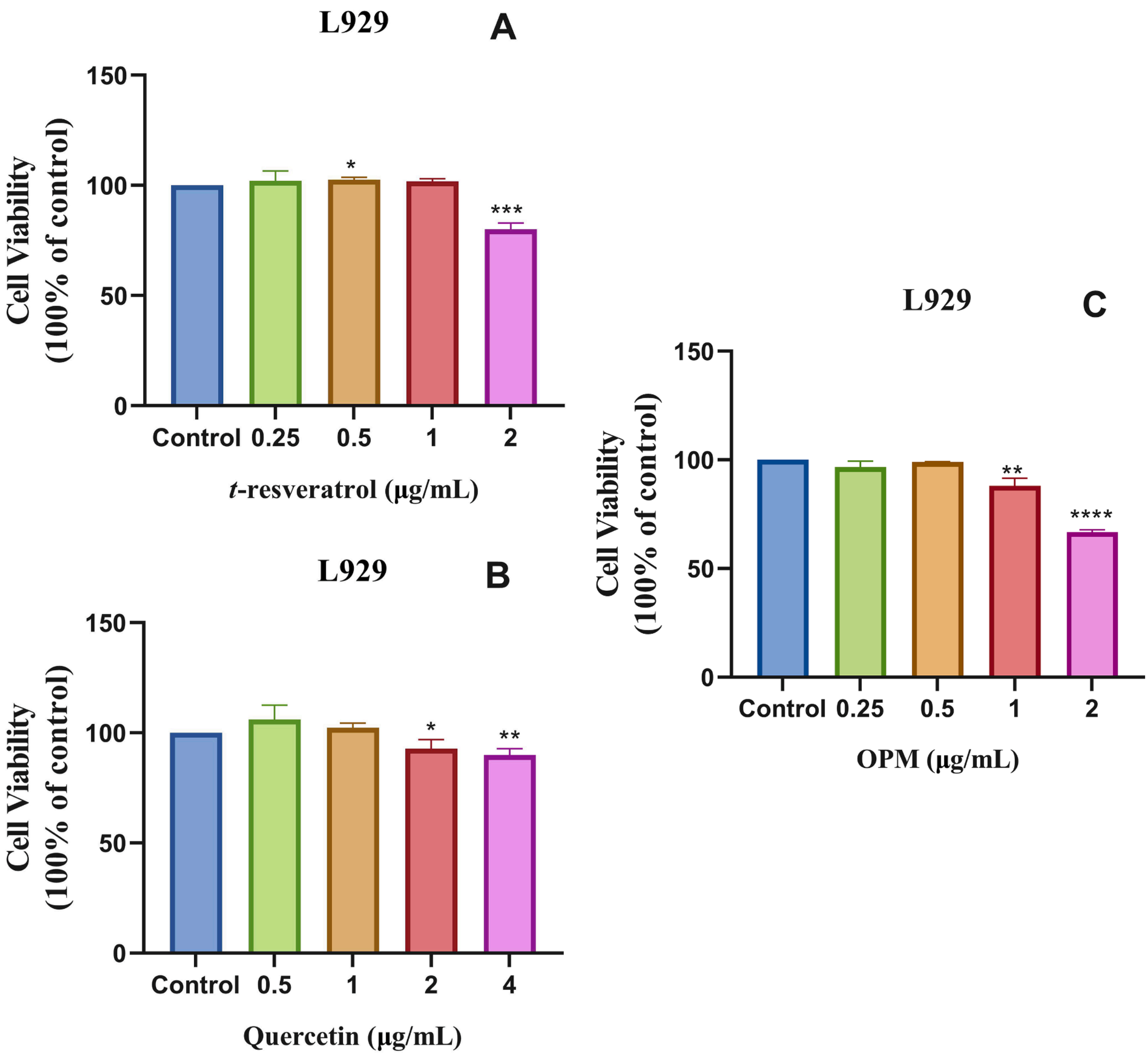


Fig. 2. Safe concentrations of *t*-resveratrol (A), quercetin (B) and optimised phenolic mixture - OPM (73 % *t*-resveratrol + 27% quercetin) on L919 fibroblasts. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the control group.

and enzymes, to prevent or repair the damage caused by ROS and to regulate redox-sensitive signalling pathways. Endogenous enzymes, such as CAT and SOD, are responsible for the antioxidant defence by converting superoxide radicals and H_2O_2 into water, thus not damaging cell homeostasis and function. More specifically, SOD converts the superoxide radical into H_2O_2 and molecular oxygen, while CAT converts H_2O_2 into water and oxygen. The *SOD* results (Fig. 3E) showed that RES (1 μg/mL) and OPM at 0.5 μg/mL could overexpress *SOD*, but QUE (1 μg/mL) and OPM at 0.125 μg/mL were ineffective in increasing *SOD* mRNA expression. A dose-dependency was observed in cells treated with OPM at 0.125 and 0.5 μg/mL, while OPM at 0.5 μg/mL had higher $p < 0.05$) *SOD* expression than RES, showing a synergism between QUE and RES. Finally, REV, QUE, and OPM increased the mRNA expression of *CAT* compared to the control (cells treated with H_2O_2) (Fig. 3F). However, the increase in *CAT* expression provided by the treatment of fibroblasts with QUE or RES was higher ($p < 0.05$) than that of OPM at 0.5 μg/mL. It is well known that the dietary consumption of polyphenol-rich foods increases the gene expression and activity of *CAT* and *SOD*. The overexpression of antioxidant enzymes is not always dependent on the polyphenol dose (He et al., 2023; Xie et al., 2023).

Treating cells with H_2O_2 induces the formation of intracellular ROS, such as hydroxyl and peroxyl radicals and superoxide anions, inducing loss of mitochondrial membrane potential, decreasing cell viability, and enhancing cell apoptosis. Therefore, controlling ROS generation is central to maintaining homeostasis and cell viability (Park, 2013). Regarding intracellular ROS generation (Fig. 3G), the pre-treatment of L929 cells with 0.125, 0.5 μg/mL OPM significantly inhibited the overproduction of ROS compared to the cells solely treated with H_2O_2 (maximum ROS production). There was a dose-dependency effect on the cellular antioxidant activity as the OPM at 0.5 μg/mL inhibited ROS generation more remarkably than at 0.125 μg/mL. These results align with those reported elsewhere, where increasing concentrations of durian shell extracts (10, 50, and 100 μg/mL) decreased the malondialdehyde formation in HepG2 cells (He et al., 2023). Overall, these data suggested the protective effects of OPM on H_2O_2-induced oxidative stress in fibroblasts by counteracting intracellular ROS generation and increasing enzymatic gene expression (e.g., *SOD*, *HO-1*, and *CAT*), as illustrated in Fig. 3H. Further studies should focus on measuring antioxidant enzymatic activity (e.g., *CAT* and *SOD*) and the level of oxidation end products (e.g., malondialdehyde) to corroborate the observations obtained herein and ascertain the exact mechanism of antioxidant action of OPM.

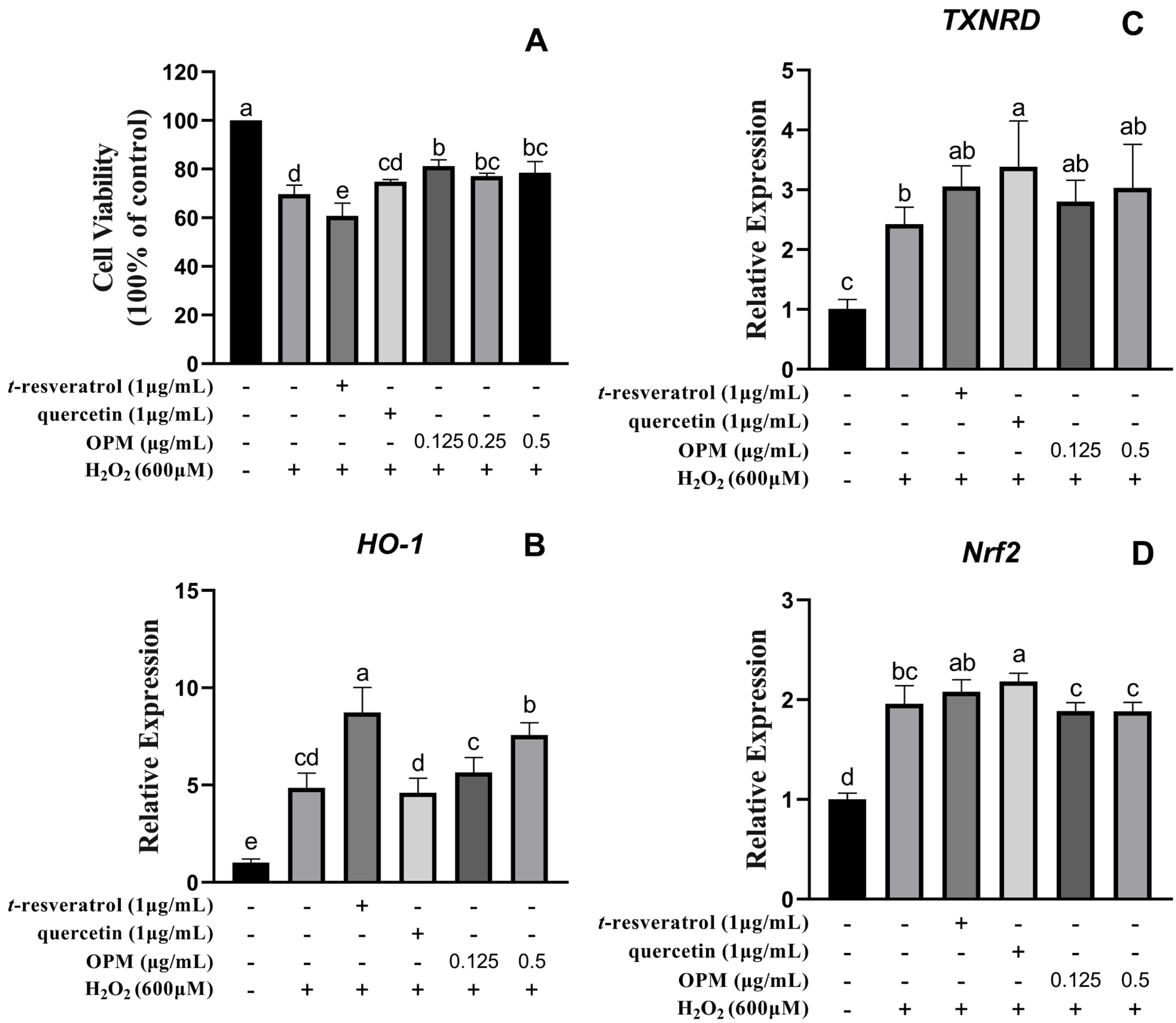


Fig. 3. Effect of *t*-resveratrol, quercetin, and the optimised phenolic mixture (OPM) containing quercetin and *t*-resveratrol (73:27 w/w) on H_2O_2-induced L929 oxidative stress. (A) L929 fibroblasts were pre-treated with/without the indicated concentrations of resveratrol, quercetin, and phenolic mixtures for 2 h, and then stimulated with 600 μM H_2O_2. Cell viability was assessed after 24 h exposure to H_2O_2. (B) Effect of phenolic compounds (e.g., *t*-resveratrol and quercetin) or optimised phenolic mixture (resveratrol and quercetin) on the gene expression of antioxidant-related genes in H_2O_2-challenged L929 cells. The relative mRNA expression of heme oxygenase-1 (*HO1*) (B), selenoprotein thioredoxin reductase 1 (*TXNRD*) (C), nuclear factor-erythroid factor 2-related factor 2 (*Nrf2*) (D), superoxide dismutase (*SOD*) (E), and catalase (*CAT*) (F) was determined using qRT-PCR. Ct values of target genes were normalized to the value of β-actin. The data are shown as mean ± SD from three biological experiments and three technical replicates and were analysed by one-way ANOVA followed by Duncan's test. The means with different superscripts are significantly different. (G) Fluorescence micrograph (×200) of ROS generation obtained from L929 cells within 24 h. (H) ROS generation in L929 fibroblasts treated with H_2O_2. (I) Schematic diagram of the effect of OPM at 0.125 and 0.5 μg/mL on antioxidant enzyme activity and expression. Figures generated with BioRender (https://biorender.com/) Different letters comparing the treatments represent significant differences ($p < 0.05$).

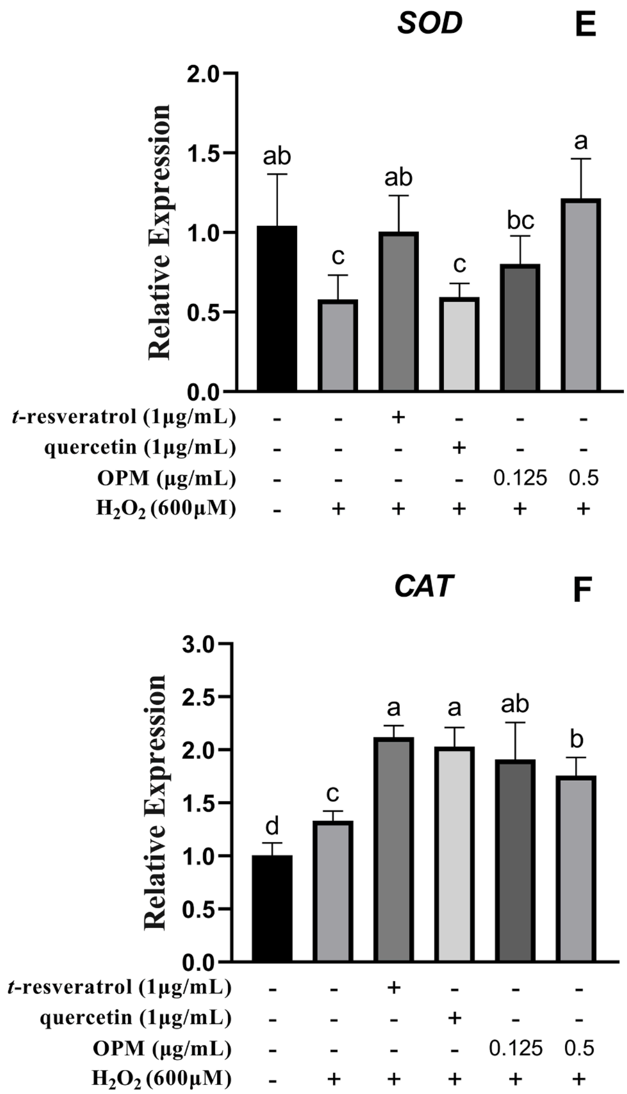


Fig. 3. (*continued*).

3.3. Application of the optimal phenolic mixture in a tea beverage model

In food applications, pasteurisation under different conditions is routinely applied. In the current work, model chamomile tea beverages were prepared as a delivery system for OPM to understand pasteurisation's effect on the final product's overall antioxidant capacity. Results in Fig. 4 show that FRAP (Fig. 4A) increased by 39% when OPM was added, and the pasteurisation did not affect ($p > 0.05$) the FRAP values. Similarly, the DPPH radical scavenging effect (Fig. 4B) of the chamomile tea supplemented with OPM at 100 mg/L had 57% more antioxidant capacity than the regular chamomile tea. Still, the pasteurisation process significantly ($p < 0.05$) decreased the DPPH value, although the pasteurised tea had a 17% higher antioxidant power than the control. CT had 41% more iron-reducing capacity (Fig. 4C) than C (33.31 vs 23.58 mg GAE/L), and the pasteurisation did not change the values ($p > 0.05$). The hydroxyl radical scavenging activity (Fig. 4D) of tea samples was different at 95% confidence, whereby CT and PCT had the highest mean values (84.33 and 86.40 mg GAE/L) than C (76.63 mg GAE/L), implying that the values are similar. Finally, adding OPM to chamomile tea increased the TPC (Fig. 4E) from 148 to 233 mg GAE/L, which is 57% more than the plain tea. Regarding the plasma protection against oxidation (Fig. 4F), plain chamomile tea (227 mg AAE/L) had lower antioxidant activity than CT (379 mg AAE/L) and PCT (348 mg AAE/L). These results show that OPM increased the plasma protection against induced oxidation by 67%, and the result was not unchanged ($p > 0.05$) after slow pasteurisation.

In a similar approach, (J. S. Santos et al., 2018) developed a functional tea composed of white tea, fermented rooibos, and roasted mate. They did not see any significant effects of slow pasteurisation (63 °C/30 min) on the total phenolic content (1240 mg/L unpasteurised vs 1220 mg/L pasteurised) and total reducing capacity (2460 mg/L unpasteurised vs 2225 mg/L pasteurised). On the other hand, Norhayati, Izzreen, and Hooi (2019) developed beverages with added honey as a source of bioactive compounds and observed the opposite behaviour when the pasteurisation at 72 °C/15 s was used: the total phenolic content of the unpasteurised beverage (187 mg/100 g) was higher than the pasteurised counterpart (150 mg/100 g). It is clear that the effects of pasteurisation on the content of bioactive compounds and antioxidant capacity of phenolic-rich beverages are matrix-dependent, and the results obtained here should be confirmed on a larger scale.

Correlation analysis was calculated, and the results showed that the inhibition of copper-induced oxidation was strongly ($p < 0.01$) correlated to FRAP ($r = 0.898$), DPPH ($r = 0.830$), TPC ($r = 0.723$), iron-reducing capacity ($r = 0.639$), and hydroxyl radical scavenging

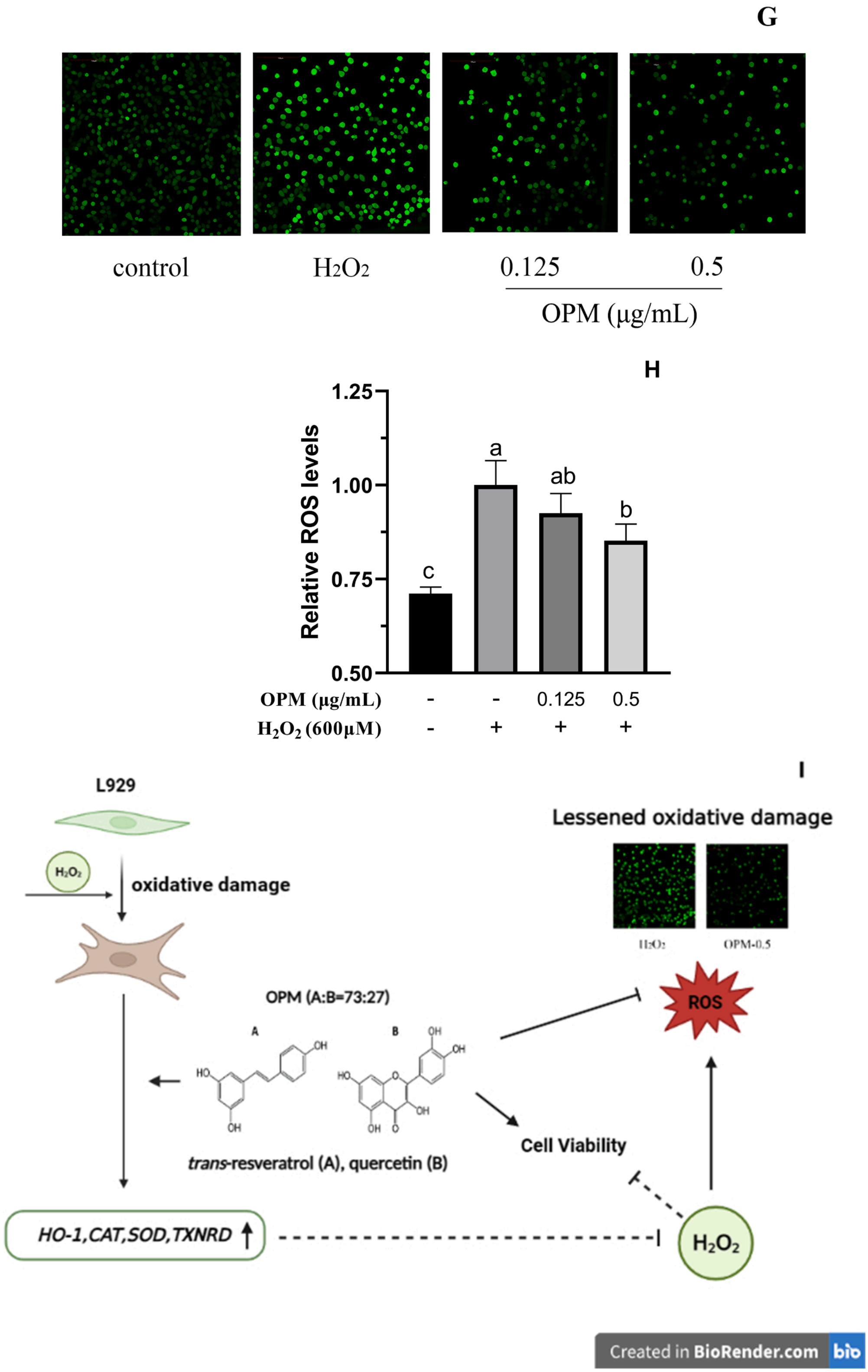


Fig. 3. (*continued*).

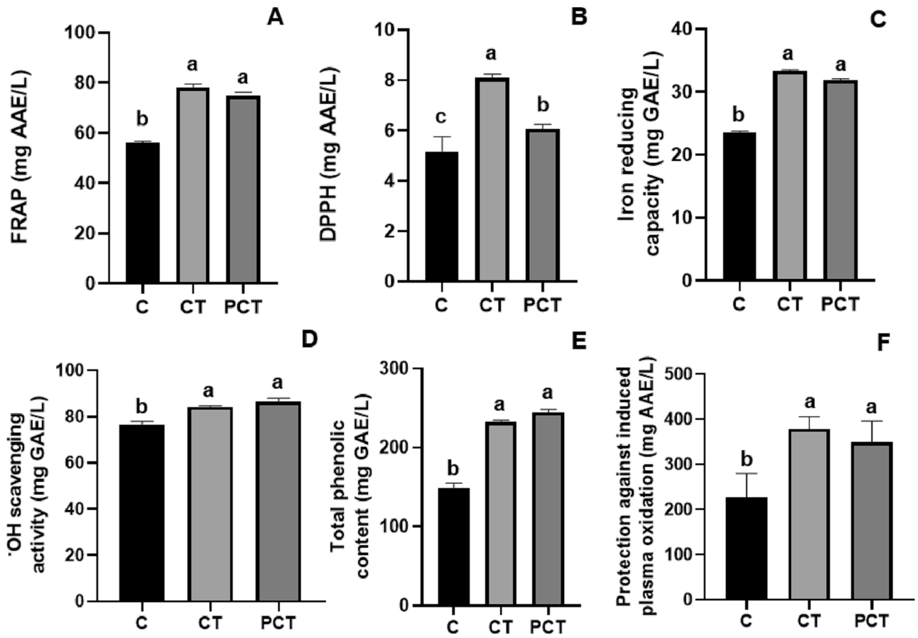


Fig. 4. Effects of slow pasteurisation at 63 °C/30 min on the antioxidant capacity of chamomile tea beverage supplemented with the 100 mg/L of the optimised phenolic mixture - OPM (73 % *t*-resveratrol and 27% quercetin): FRAP (A), DPPH (B), iron reducing capacity (C), hydroxyl radical scavenging activity (D), total phenolic content (E), and protection against induced human plasma oxidation (F). Different letters comparing the tea samples represent statistically different results ($p < 0.05$). GAE = gallic acid equivalent; AAE = ascorbic acid equivalent; C = plain chamomile tea; CT = chamomile tea added with OPM (100 mg/L); PCT = chamomile tea added with OPM (100 mg/L) subjected to slow pasteurisation at 63 °C/30 min.

activity ($r = 0.567$). Although the correlation between chemical antioxidant assays is obvious when a more biologically relevant assay is conducted, such as the plasma oxidation assay, it is central to assess the mechanisms by which bioactive compounds (i.e., phenolics) behave in real-world, complex matrices, such as herbal teas. From these results, it is possible to ascertain that chamomile tea added with OPM can scavenge free radicals (e.g., DPPH and •OH) and reduce prooxidant transition metals (i.e., Fe^{2+} and Cu^{2+}), which might explain the protection of human plasma against induced oxidation.

As a final comment, although this conceptual framework encompassing statistical optimisation, *in vitro* and *ex vivo* chemical antioxidant assays and cellular antioxidant activity seems to work, some limitations are obvious: i) scaling up the process is necessary to know if results are similar to the lab-based tests; ii) the bioavailability of OPM should be tested in a more complex set-up encompassing the digestion and absorption processes (e.g., INFOGEST digestion protocol coupled with Caco-2 monolayer); iii) fate, transport, and bioactivity of OPM and their metabolites should be studied *in vivo* to ascertain the functionality of newly developed food models.

4. Conclusions

A mixture containing 73% resveratrol and 27% quercetin (OPM) was regarded as the optimum combination of phenolics (OPM) to increase the *in vitro* antioxidant capacity. OPM upregulated the expression of HO-1, SOD, and CAT and significantly decreased the ROS generation in L929 fibroblasts, and a dose–response effect was observed. When OPM was added to a chamomile tea, the antioxidant capacity and human plasma protection against copper-induced oxidation were significantly increased by adding OPM at 100 mg/L. However, slow pasteurisation (63 °C/30 min) decreased the free-radical scavenging effect but did not change the total phenolic content, the reducing potential, and human plasma protection against oxidation. Overall, the approach combining food technology, engineering, and cell biology was effective for the functional tea design. However, further studies should focus on the sensory aspects of the beverage, bioavailability after digestion, transport and accumulation, and bioactivity *in vivo*.

CRediT authorship contribution statement

Hazel Corrigan: Methodology, Formal analysis, Investigation. **Aoife Dunne:** Methodology, Formal analysis, Investigation. **Niamh Purcell:** Methodology, Formal analysis, Investigation. **Yuyang Guo:** Methodology, Formal analysis, Investigation. **Kai Wang:** Funding acquisition, Writing – review & editing. **Hongzhuan Xuan:** Funding acquisition, Writing – review & editing. **Daniel Granato:** Conceptualization, Methodology, Validation, Writing – original draft, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136764.

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