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# Functional Robustness of Flavonoidic Mixtures Across Different Subclasses: Identification of Redundant Individual Members Through Compositional Perturbation

**Alex Xiong Gao [1,2], Man-Man Sun [3], Christina Lok-Pan Yik [1,2], Iris Wai-Ting Ma [1,2], Doria Jia-Yu Zhu [1,2], Xin-Meng Li [3], Tina Ting-Xia Dong [1,2] and Karl Wah-Keung Tsim [1,2,*]**

[1] Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China; gaoxiong@ust.hk (A.X.G.)
[2] Shenzhen Key Laboratory of Edible and Medicinal Bioresources, HKUST Shenzhen Research Institute, Hi-Tech Park, Nanshan, Shenzhen 518000, China
[3] Key Laboratory of High Magnetic Field and Ion Beam Physical Biology, Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei 230031, China; mmsun@iim.ac.cn (M.-M.S.)
* Correspondence: botsim@ust.hk; Tel.: +852-2358-7332

**Abstract**

Flavonoids commonly occur not in isolation but as compositionally variable mixtures whose structurally diverse members often share antioxidant and other biological properties. This functional overlap may render flavonoid mixtures that are intrinsically robust to compositional variation, a possibility we investigated here using our previously established equimolar mixture of 20 flavonoids (MIX20). First, each flavonoid was individually depleted, and equivalence to MIX20 was assessed using two one-sided tests (TOSTs) with prespecified 80–125% bounds. Most leave-one-out mixtures were statistically equivalent to MIX20 across cell-free antioxidant assays, cellular oxidative damage, and antioxidant response element reporter activation, indicating substantial functional redundancy. Second, we generated 20 biased mixtures, each containing one flavonoid at 50% of the total flavonoid concentration. Their retained bioactivities across assays supported resilience to substantial compositional changes. The generality and boundaries of these findings were further examined using flavonoid subsets derived from MIX20 and an independent eight-flavonoid panel, with functional verification extended to α-glucosidase inhibition, mitochondrial membrane potential, cytotoxicity, and paraquat-induced oxidative stress in *Caenorhabditis elegans*. These results establish a systematic framework for assessing compositional robustness in the tested flavonoid mixtures and may provide a basis for future evaluation of other phytochemical systems.

**Keywords:** holism; coarse-graining; TOST; leave-one-out; polyphenols; diversity; phytochemicals

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## 1. Introduction

Flavonoids are a diverse class of phytochemicals abundant in dietary sources, including tea, fruits, vegetables, and medicinal plants, and have been associated with multiple biological effects [1]. Within these plant-derived matrices, flavonoids exist as complex mixtures of structurally related congeners [2]. These molecules exhibit substantial variation in their heterocyclic backbone (C-ring) oxidation state, glycosylation patterns, and peripheral modifications, such as methylation, acylation, and sulfation. Despite considerable structural diversity, studies of both extracts and purified flavonoids from natural products often

converge on similar functional readouts—such as antioxidant capacity, cytoprotection, and enzyme modulation—across population, biochemical, and molecular levels [3]. A plausible explanation is that the shared pharmacophoric features confer tolerance to compositional heterogeneity. These molecules share core reactive sites; therefore, compositional changes within a complex mixture may not substantially alter the overall functional output. Under this hypothesis, robustness—understood as the preservation of outputs under compositional or contextual perturbations—would help explain the observed reproducibility of these readouts across diverse flavonoidic profiles [4,5].

Despite the intuitive appeal of robustness as an explanation for convergent functional readouts, rigorous tests within a controlled, compositional framework have not been performed. Real-world flavonoidic systems are effectively "black boxes": complex matrices and crude extracts contain many incompletely characterized constituents. As a result, systematic single-component perturbations—required to quantify robustness—are often not feasible [6]. Meanwhile, prevailing flavonoid research typically emphasizes individual flavonoidic structures and their mechanisms. In contrast, principles governing complex mixtures—linking whole-system behavior to the contributions of individual flavonoids—remain underexplored.

This study aimed to evaluate the functional robustness of a defined flavonoid mixture under controlled compositional perturbations across various cell-free antioxidant assays and in vitro cell models [7,8]. Inspired by approaches used in microbial community research [9,10], we employed MIX20—a fully defined, equimolar synthetic mixture of 20 flavonoids spanning five major subclasses—as a tractable "white box" framework [11]. The components of MIX20 were selected for their dietary prevalence and prominence in the literature. Two sequential perturbation strategies were used. First, we employed a leave-one-out (LOO) experimental design tailored to MIX20 to assess its functional redundancy, as established by statistical equivalence [12,13]. Second, we utilized heavily biased formulations—in which a single flavonoid was increased to constitute 50% of the total flavonoid concentration—to probe robustness in a broader context. Overall, this work provides a generalizable methodology for evaluating mixture-level robustness and offers an explanatory framework for the functional stability observed in complex dietary matrices.

## 2. Materials and Methods

### *2.1. Chemicals*

All 28 flavonoids (>98% purity, confirmed by HPLC) were purchased from Chengdu DeSiTe Biological Technology (Chengdu, China) and dissolved in DMSO to yield 50 mM stock solutions. MIX20 was prepared by pooling equimolar volumes of the 20 individual stocks to achieve a total flavonoid concentration of 50 mM (i.e., 2.5 mM each). Additional mixture stocks were prepared using the same procedure to maintain a total stock concentration of 50 mM. DMSO, DPPH, ABTS, FRAP reagents, JC-1, α-glucosidase, and *p*-nitrophenyl-α-D-glucopyranoside (pNPG) were sourced from Yuanye Biotechnology (Shanghai, China); tert-butyl hydroperoxide (tBHP), MTT, FUDR, and paraquat were purchased from Sigma-Aldrich (St. Louis, MO, USA); and all culture media and supplements were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The formulations of all flavonoid mixtures and the corresponding concentrations of each component in the 50 mM stocks are listed in Supplementary Table S1.

### *2.2. Antioxidant Capacities*

The antioxidant capacities of individual flavonoids and their mixtures were evaluated using DPPH, ABTS, and FRAP assays [14–16]. For DPPH assay, 100 μL of sample was mixed with 100 μL of 0.25 mM DPPH in ethanol, incubated in the dark at room temperature

for 30 min, and absorbance was measured at 517 nm. For ABTS assay, the ABTS radical cation (ABTS•+) was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate in the dark at room temperature for 12 h, followed by dilution to an absorbance of 0.70 ± 0.05 at 734 nm; 20 µL of sample was then combined with 180 µL of this working reagent, and absorbance was recorded at 734 nm after 6 min. For both DPPH and ABTS assays, scavenging activity (%) was calculated as 100 × [1 − (A_sample − A_sample blank)/(A_control − A_reagent blank)]. For FRAP assay, the working reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM FeCl3·6H2O in a 10:1:1 (*v*/*v*/*v*) ratio; 20 µL of sample was mixed with 180 µL of FRAP reagent in a 96-well plate, incubated for 10 min at 37 °C, and absorbance was measured at 593 nm to determine reducing activity after blank subtraction. Unless otherwise specified, all absorbance measurements were performed using a Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific).

### *2.3. α-Glucosidase Inhibition Assay*

Inhibitory activity against α-glucosidase was assessed using a microplate colorimetric method [17]. Briefly, 50 µL of sample was preincubated with 100 µL of enzyme solution (1 U/mL in 0.2 M PBS, pH 7.0) at 37 °C for 10 min. The reaction was initiated by adding 50 µL of 5 mM pNPG, and the absorbance of the released *p*-nitrophenol was measured at 405 nm after 30 min. The enzyme inhibition (%) was calculated relative to the control after subtracting reagent blanks.

### *2.4. Cell Cultures*

PC12 cells (ATCC CRL-1721, Manassas, VA, USA) were cultured in DMEM with 10% FBS, 5% HS, and 1% penicillin–streptomycin (100 U/mL penicillin; 100 µg/mL streptomycin); HepG2 cells (ATCC HB-8065) and IEC-6 cells (ATCC CRL-1592) were maintained in DMEM with 10% FBS and 1% penicillin–streptomycin. All cell lines were incubated at 37 °C in a humidified 5% $CO_2$ atmosphere.

### *2.5. Transfection of DNA Constructs and Luciferase Assay*

Detached cells were suspended at a density of 300,000 cells/mL and transfected with either pARE-luc or pNF-κB-luc reporter plasmids using jetPRIME reagent (Polyplus, New York, NY, USA) according to the manufacturer's protocol. The transfected cells were seeded into 96-well plates and incubated for 24 h. The culture medium was then replaced with low-serum DMEM containing the designated flavonoid treatments. After a further 24 h of incubation, luciferase activity was measured using a commercial luciferase assay kit on a luminometer (Promega, Madison, WI, USA).

### *2.6. Cell Viability*

Cell viability was evaluated using the MTT assay. Cells were seeded in 96-well plates at 10,000 cells per well for toxicity screening and 25,000 cells per well for tBHP cytoprotection assays. Treatments were administered in basal DMEM. Following incubation, the medium was replaced with MTT solution (0.5 mg/mL in basal DMEM) for 3 h. Formazan crystals were solubilized in 100 µL DMSO with agitation for 15 min. Absorbance was measured at 570 nm (with reference correction at 650 nm) and expressed as a percentage relative to the untreated control.

### *2.7. Mitochondrial Membrane Potential*

Mitochondrial membrane potential was evaluated using the JC-1 fluorescent probe. Cells were incubated with 1 µg/mL JC-1 in HBSS for 20 min at 37 °C. After washing,

cells were treated with the designated formulations in HBSS for another 20 min at 37 °C. Fluorescence was measured on a FlexStation 3 microplate reader (Molecular Devices, San Jose, CA, USA). The membrane potential was quantified as the blank-corrected fluorescence ratio of red aggregates (excitation/emission: 525/595 nm) to green monomers (excitation/emission: 490/530 nm).

### *2.8. C. elegans Assays*

Synchronized wild-type *Caenorhabditis elegans* (N2) embryos were obtained by bleaching gravid adults, allowed to hatch to the L1 stage at 20 °C, and cultured in 24-well plates containing 500 µL of medium per well, with heat- and UV-killed OP50 provided as the food source. The worms were exposed to the indicated flavonoid formulations from the L1 stage until 24 h after reaching the L4 stage. All treatment groups were matched for vehicle concentration, and the final DMSO concentration did not exceed 0.5%. At the L4 stage, FUDR was added to a final concentration of 100 µM to prevent progeny production. Before paraquat exposure, the adult worms were allowed to settle by gravity and briefly transferred to unseeded NGM plates to remove the test formulations. For the paraquat challenge, one adult worm was transferred to each well of a 96-well plate containing 100 µL of medium supplemented with 35 mM paraquat and 100 µM FUDR. Each experimental group included 30 worms. The plates were maintained at 20 °C with shaking at 120 rpm. Survival was assessed every 8 h based on responsiveness to gentle tapping. A worm was scored as dead if it failed to respond to gentle tapping. Each treatment group included 30 worms per independent experiment, and three independent experiments were performed.

### *2.9. Statistical Analysis*

For LOO comparisons, the response ratio relative to the MIX20 baseline was natural-log-transformed and evaluated using the TOST procedure. The equivalence bounds were set at $[\log(0.80), -\log(0.80)]$ ($\alpha = 0.05$), corresponding to 0.80 and 1.25 on the original scale. The data are reported as back-transformed geometric mean ratios (GMRs) with 90% confidence intervals (CIs), and equivalence was declared if the 90% CI fell entirely within the predefined bounds. For 50%-biased robustness tests, statistical significance for each treatment group relative to the control was evaluated using one-sample Wilcoxon signed-rank tests, with *p*-values adjusted for multiplicity using the Benjamini–Hochberg false discovery rate (FDR) procedure. Results were presented as geometric means (GMs) with 95% CIs, with the *n* values reported in the figure legends denoting independent experiments, and statistical significance was defined as adjusted $p < 0.05$ (*). For the *C. elegans* paraquat model, survival was analyzed using the log-rank (Mantel–Cox) test. Each treatment group was compared with the vehicle control, with the Benjamini–Hochberg FDR correction applied for multiple comparisons. Survival data from three independent experiments were pooled for the analysis. Results are presented as median survival times with 95% CIs, and statistical significance was defined as adjusted $p < 0.05$ (*). All statistical analyses were performed using R software (version 4.4.1).

## 3. Results

### *3.1. Antioxidant Properties of MIX20 Under LOO Perturbation*

System-level robustness can arise from redundancy. Accordingly, we first evaluated whether the artificial mixture of 20 flavonoids, named MIX20, could exhibit compositional redundancy using a total-concentration-matched LOO design. MIX20 was an equimolar, fully defined mixture of widely studied flavonoids spanning five equally weighted subclasses, i.e., flavone, flavonol, flavanone, flavanol and isoflavone (shown in Figure 1). In the master stock, the total flavonoid concentration was fixed at 50 mM (2.5 mM each in

the 20-component formulation). For each LOO formulation, we omitted one pre-defined component and re-normalized the remaining components to maintain equimolarity at the same total concentration of 50 mM. Consequently, the concentration of each component in each MIX20-derived LOO formulation was 50 mM divided by the number of retained components (e.g., 50 mM ÷ 19 components). This total-concentration-matched design was used to isolate the consequences of compositional perturbation from those caused by changes in overall mixture concentration.

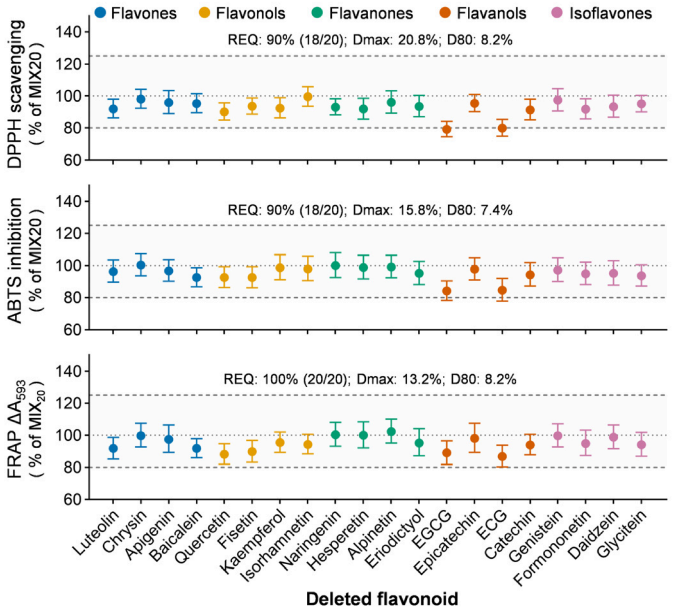



**Figure 1. TOST on antioxidant capacity relative to the MIX20 baseline.** Individual 50 mM flavonoid stocks were mixed in equal volume to create the MIX20 and 20 LOO variant stocks, maintaining the total flavonoid concentration of all mixtures at 50 mM (see Supplementary Table S1). MIX20 and LOO variants were tested for DPPH and ABTS inhibition, as well as reducing power via FRAP, at a final flavonoid concentration of 25 μM (with individual components at 25/20 μM for MIX20 or 25/19 μM for each LOO variant). Values are reported as GMR with 90% CI ($n = 8$) and expressed as percentages relative to MIX20 (MIX20 = 100%, indicated by a dotted line). Equivalence to the MIX20 baseline was assessed using TOST with ± 20% bounds (represented by dashed lines, corresponding to a linear range of 80% to 125%). REQ—the equivalence rate, defined as the proportion of contrasts whose 90% CIs lie entirely within the prespecified equivalence bounds; Dmax—the worst-case absolute deviation, defined as the maximum observed deviation from baseline among all contrasts; D80—the 80th-percentile absolute deviation, computed across the LOO contrasts to represent typical upper-quantile drift; EGCG—epigallocatechin gallate; ECG—epicatechin gallate.

The TOST comparisons were conducted at an equal molar concentration of flavonoids. Equivalence was visualized using forest plots of GMRs with 90% CIs relative to the 100% baseline. The equivalence bounds were set to [0.80, 1.25], based on the widely used 80–125% bioequivalence criterion commonly adopted in regulatory settings (e.g., for generic drugs). For statistical summarization, we consolidated figure-level metrics in each figure to provide a system-wide overview of robustness across the LOO comparisons. These metrics include: (i) the equivalence rate (REQ), defined as the proportion of contrasts whose 90% CIs lie entirely within the prespecified equivalence bounds; (ii) the 80th-percentile absolute deviation (D80), computed across the LOO contrasts to represent typical upper-quantile drift; and (iii) the worst-case absolute deviation (Dmax), defined as the maximum observed deviation from baseline among all contrasts.

Assessments of MIX20's functional redundancy were first performed using three cell-free antioxidant assays (i.e., DPPH, ABTS, and FRAP) at three concentrations

(Figures 1 and S1). At 25 μM, the representative forest plots showed that the 90% CIs for the GMRs of most LOO formulations lay entirely within the prespecified equivalence bounds of 0.80–1.25 relative to the MIX20 baseline (Figure 1). Only one to two LOO contrasts exhibited larger deviations, e.g., epigallocatechin gallate (EGCG) and epicatechin gallate (ECG), where 90% CIs partially crossed an equivalence bound. The other two tested concentrations, i.e., 10 μM and 40 μM, showed similar consistency. The omission of any single flavonoid member did not lead to functional collapse, indicating redundancy within the mixture (Figure S1A,B).

To determine whether the observed functional redundancy extended to cellular systems, we tested the LOO mixtures in cell cultures. Cultured cells (PC12, HepG2, and IEC-6), pre-treated with most LOO formulations at 10 μM, showed statistically equivalent protection against the tBHP-induced oxidative damage (with REQ > 75%) (Figure 2). Notably, the omission of baicalein modestly reduced the protective effect relative to MIX20, with a Dmax of ~24–29% indicating a contributory role for this member within the mixture. We also evaluated cytoprotective signaling using pARE-luc (a reporter plasmid driven by the antioxidant response element), and pNFκB-luc (a reporter plasmid driven by nuclear factor kappa-light-chain-enhancer of activated B cells) in the transfected cultures (Figure 3). Both reporter assays are commonly used to evaluate adaptive cellular stress responses. Again, only a few perturbations had 90% CIs that slightly crossed an equivalence bound; the REQ was higher than 90%, and the Dmax and D80 values were lower than 14% across cells and reporters. In addition, similar response profiles were observed for pARE-luc activation in both HepG2 and PC12 cells (Figure 3). Collectively, the viability assay and reporter assay indicate that functional redundancy may be largely preserved in cellular contexts.

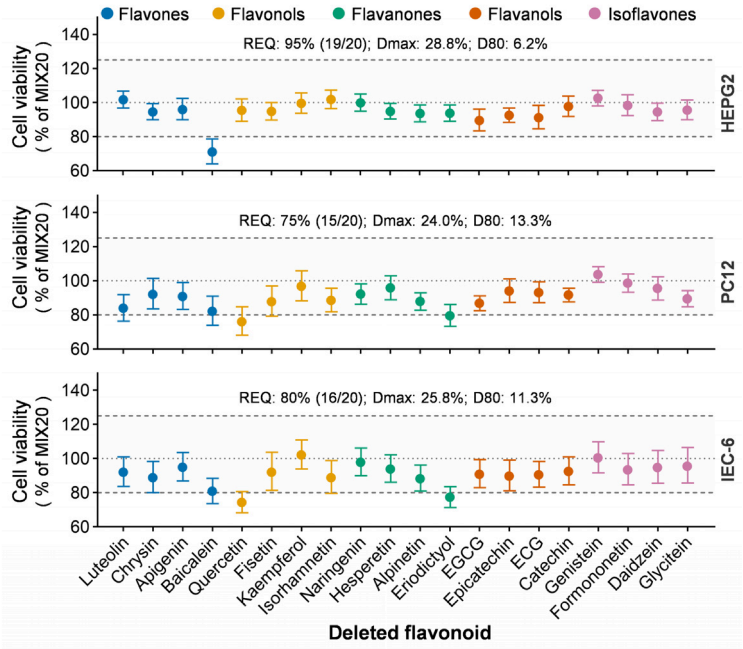



**Figure 2. TOST on the protection against tBHP-induced toxicity in cell cultures.** HepG2, PC12, and IEC-6 cells were exposed to MIX20 or individual LOO variants at a final flavonoid concentration of 10 μM (with individual components at 10/19 μM for each LOO variant) for 1 h, followed by 8 h of tBHP treatment at 100 μM. Cell viability was assessed by MTT. Values are reported as GMR with 90% CI ($n = 8$) and expressed as percentages relative to MIX20 (MIX20 = 100%, indicated by a dotted line). Equivalence to the MIX20 baseline was assessed using TOST with ± 20% bounds (represented by dashed lines, corresponding to a linear range of 80% to 125%). REQ—the equivalence rate; Dmax—the worst-case absolute deviation; D80—the 80th-percentile absolute deviation.

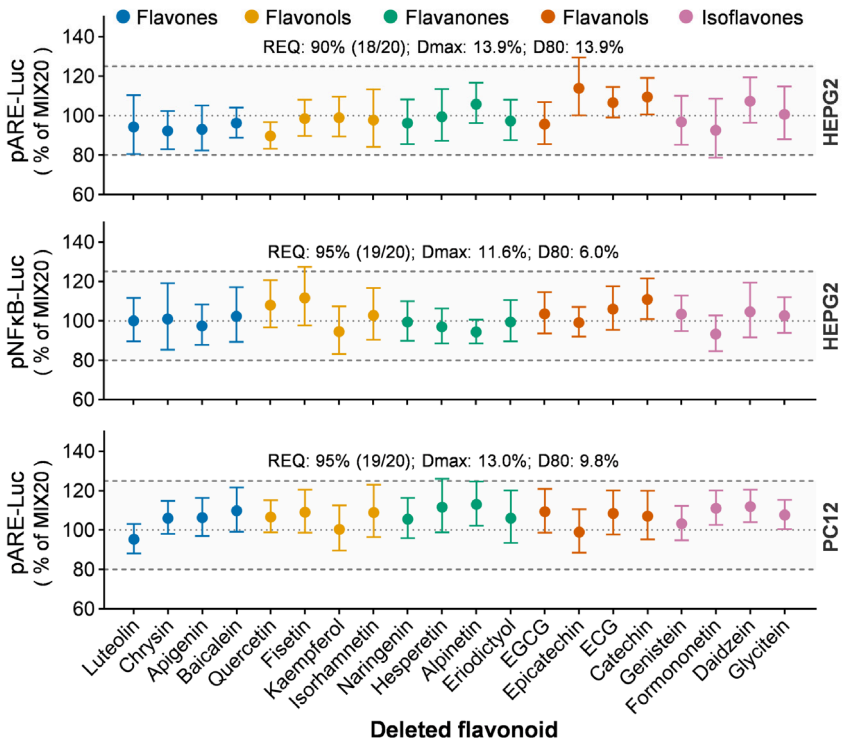



**Figure 3. TOST on the activation of reporter constructs in transfected cells.** HepG2 cells were transfected with pARE-Luc and pNF-κB–Luc, and PC12 cells were transfected with pARE-Luc. Following transfection, cells were treated with 10 μM MIX20 or individual LOO variants, as shown in Figure 1, for 24 h in DMEM containing 1% FBS and 0.5% horse serum. Luciferase activity was measured by luminometry. Values are reported as GMR with 90% CI ($n$ = 8) and expressed as percentages relative to MIX20 (MIX20 = 100%, indicated by a dotted line). Equivalence to the MIX20 baseline was assessed using TOST with ± 20% bounds (represented by dashed lines, corresponding to a linear range of 80% to 125%). REQ—the equivalence rate; Dmax—the worst-case absolute deviation; D80—the 80th-percentile absolute deviation.

### *3.2. Functional Redundancy Analysis Based on the LOO Method*

In addition, the LOO panel of MIX20 was evaluated for α-glucosidase activity, cell toxicity and mitochondrial membrane potential [18]. Most contrasts for α-glucosidase inhibition and mitochondrial depolarization fell within the prespecified equivalence region, whereas the cytotoxicity assay showed a higher proportion of nonequivalent contrasts, with a REQ of 65% and a D80 of 18.9% (Figure 4A–C). The greater variability may have resulted from the high flavonoid concentration used in the cytotoxicity assay. Notably, omission of EGCG produced a pronounced deviation (a Dmax of 61%) from the baseline in α-glucosidase inhibition assay (Figure 4A), suggesting that EGCG might act as a key node in the mixture's functional network. This indicates that the functional robustness of the flavonoid mixture generalizes across multiple, mechanistically distinct readouts.

In addition to the MIX20 formulation, we applied the LOO perturbation to internal subsets of MIX20. By partitioning the MIX20 matrix, we generated two independent MIX10 formulations (MIX10a and MIX10b). Each was an equimolar mixture of 10 flavonoids, consisting of two components from each of the five subclasses (Figure 5A,B). In both MIX10 sets, the majority of single-component omissions remained within the prespecified equivalence bounds in the antioxidant capacity assays, i.e., ABTS and FRAP assays. Interestingly, the distribution patterns of both MIX10a and MIX10b series among the two assays were very similar, including the TOST parameters, i.e., ~90% REQ. However, in the distinct mixture of flavonoids "MIX8," which contained eight dietary flavonoids (wogonin, galangin, pinocembrin, GCG, biochanin A, cardamonin, taxifolin, cyanidin) not included in the MIX20

configuration, the results were different. Under the LOO perturbation, none of the MIX8 LOO contrasts had 90% confidence intervals entirely within the prespecified equivalence region (Figure S2A,B). This suggests that redundancy is not unlimited. Rather, redundancy appears to be context-dependent and may be constrained by the number of constituents and the functional diversity of the individual flavonoids.

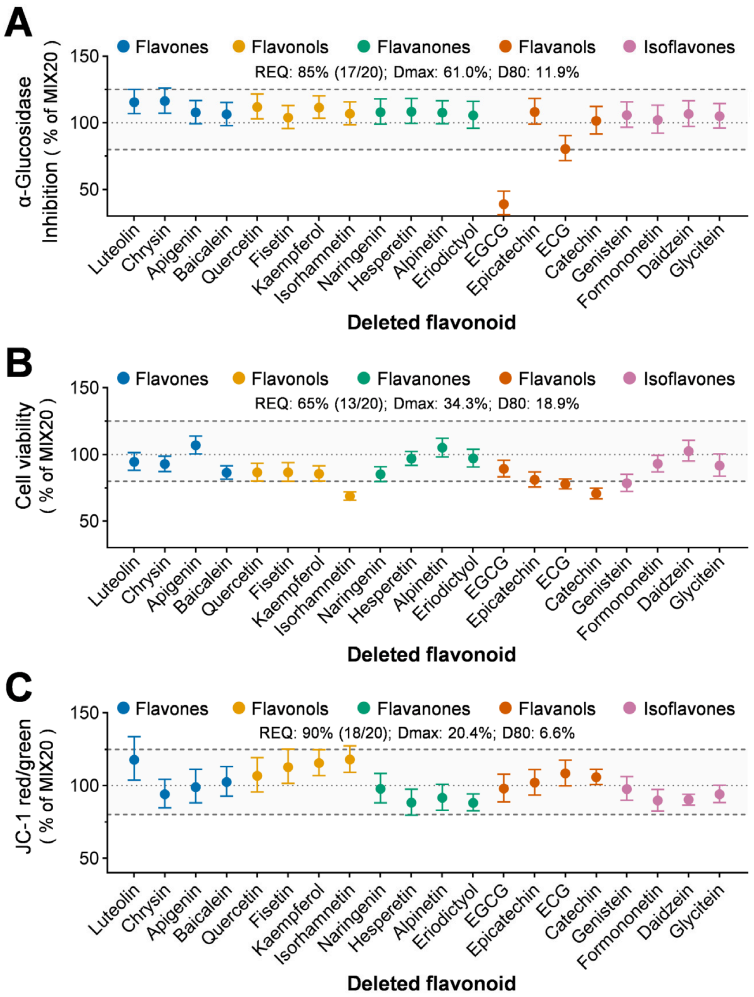



**Figure 4. TOST in the non-antioxidant assays.** MIX20 and LOO variants, with the same doses as those listed in Figure 1, were tested for (**A**) α-glucosidase inhibition at 25 μM, (**B**) cytotoxicity in HepG2 cells at 50 μM (with individual components at 50/19 μM for each LOO variant), and (**C**) rapid perturbation of mitochondrial membrane potential in PC12 cells at 2 μM (with individual components at 2/19 μM for each LOO variant). Values are reported as GMR with 90% CI ($n = 8$) and expressed as percentages relative to MIX20 (MIX20 = 100%, indicated by a dotted line). Equivalence to the MIX20 baseline was assessed using TOST with ± 20% bounds (represented by dashed lines, corresponding to a linear range of 80% to 125%). REQ—the equivalence rate; Dmax—the worst-case absolute deviation; D80—the 80th-percentile absolute deviation.

To further interrogate this notion, we increased the magnitude of compositional change to challenge redundancy in MIX20. Instead of single LOO perturbations, we generated 10 MIX12 variants by removing two entire flavonoid subclasses (four flavonoids each) from the MIX20 baseline (Figure 6A,B). As expected, larger removal produced greater functional fluctuation in the antioxidant assays. In both antioxidant assays, seven of the ten variants had 90% CIs that did not lie entirely within the prespecified equivalence bounds, corresponding to an REQ of 30% (Figure 6A). Surprisingly, the cellular pARE-luc and pNF-κB-luc responses in the transfected cells remained comparatively stable in 9 out of

the 10 variants, suggesting that redundancy also depends on assay modality, particularly for readouts engaging biological homeostatic mechanisms (Figure 6B). The concentration used for MIX20 was not saturating. These findings indicate that equivalence and functional retention capture distinct aspects of mixture robustness.

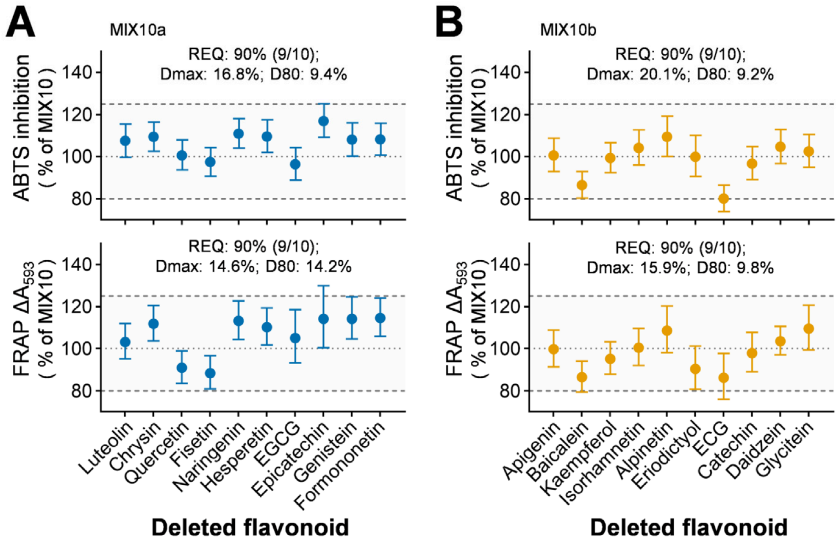



**Figure 5. TOST on antioxidant capacity of MIX10.** The flavonoids included in MIX20 were divided into two 10-flavonoid subsets, MIX10a (**A**) and MIX10b (**B**), ensuring an even subclass distribution (see Supplementary Table S1). The two MIX10 mixtures and all corresponding LOO variant stocks were prepared by mixing equal volumes of the 50 mM individual flavonoid stocks, keeping the total concentration at 50 mM. The LOO variants for MIX10a and MIX10b were evaluated for ABTS and FRAP inhibition at a final flavonoid dose of 25 μM (with individual components at 25/10 μM for MIX10 or 25/9 μM for each LOO variant). Values are reported as GMR with 90% CI ($n = 8$) and expressed as percentages relative to MIX10a or MIX10b (MIX10 = 100%, indicated by a dotted line). Equivalence to the MIX10 baseline was assessed using TOST with ± 20% bounds (represented by dashed lines, corresponding to a linear range of 80% to 125%). REQ—the equivalence rate; Dmax—the worst-case absolute deviation; D80—the 80th-percentile absolute deviation.

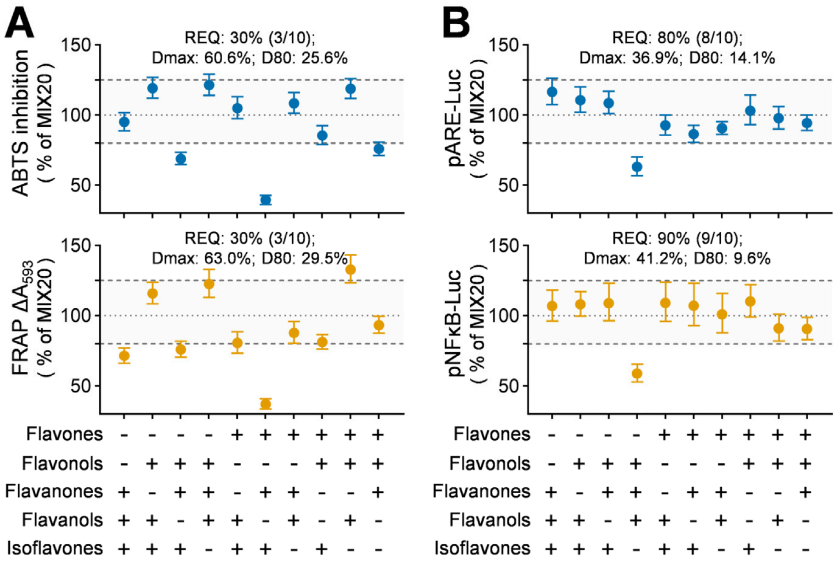



**Figure 6. TOST of two subclasses-depleted MIX20.** MIX20 comprised five subclasses, with four individual flavonoids in each. By systematically excluding pairwise subclass combinations from MIX20, ten distinct MIX12 variants (each containing three subclasses) were generated. Each variant stock was prepared by mixing equal volumes of the 12 corresponding 50 mM individual flavonoid

stocks. All mixture variants were tested for (**A**) the inhibition of ABTS and FRAP at a total flavonoid dose of 25 μM (with individual components at 25/12 μM for each MIX12), and (**B**) in luciferase assays at 10 μM (with individual components at 10/12 μM for each MIX12). Values are reported as GMR with 90% CI ($n$ = 8) and expressed as percentages relative to MIX20 (MIX20 = 100%, indicated by a dotted line). Equivalence to the MIX20 baseline was assessed using TOST with ± 20% bounds (represented by dashed lines, corresponding to a linear range of 80% to 125%). REQ—the equivalence rate; Dmax—the worst-case absolute deviation; D80—the 80th-percentile absolute deviation.

### *3.3. The Biased Perturbation Formulations Reveal the Robustness of the Mixture*

The biased perturbations were introduced to test whether the functions of MIX20 could be retained under large, single-component weighting. Specifically, we combined the flavonoid member at 1:1 with its corresponding LOO mixture, yielding 20 formulations in which a single component accounted for 50% of the total flavonoid content (i.e., 50%-weighted, single-compound-biased mixtures). Because these comparisons were not framed as equivalence tests around MIX20 fixed at 100% baseline, we analyzed the outcomes using conventional hypothesis testing versus the control rather than TOST. A perturbed mixture was considered to retain function when it remained significantly active relative to the corresponding control in the expected direction. The recurrence of function retention across compositionally biased formulations provided a complementary measure of mixture robustness under substantial compositional perturbation.

We first evaluated the antioxidant capacity using DPPH, ABTS, and FRAP assays. All biased mixtures consistently retained antioxidant activities, and several of them outperformed MIX20, i.e., baicalein, EGCG, and ECG, suggesting stronger contributions from the heavily weighted components and/or non-linear interactions among flavonoids (Figure 7). We then applied the same cellular assays used in the LOO evaluation. In cultured HepG2 cells, 14 of the 20 variants significantly protected against tBHP-induced oxidative stress, whereas in PC12 cells, 18 of 20 did so (Figure 8A). The luteolin-biased formulation showed prominent protection in both cell lines. Consistently, the pARE-luciferase reporter assay in HepG2 cells revealed significant activation across 18 of the 20 biased mixtures, whereas the apigenin- and chrysin-biased formulations were less effective (Figure 8B). Collectively, the retention of functional activity across most biased formulations and experimental readouts supports the functional robustness of MIX20 to broad shifts in flavonoid composition.

We then extended this analysis to additional mixture architectures. Internally, MIX10a-based biased subsets, in which one flavonoid contributed 50% of the total concentration, showed a similar pattern: all variants exhibited significant antioxidant activity (Figure 9A), particularly the 50% EGCG-biased formulation. Externally, the eight-flavonoid panel outside the scope of MIX20 also retained function in most of the biased configurations (Figure S2). For an additional stability test, these eight flavonoids were further partitioned into eight equally distributed MIX4 subsets, as described in Supplementary Table S1. Then, each MIX4 stock was mixed with MIX20 stocks at a 1:1 ratio to generate eight MIX24 formulations, with each 50 mM stock containing 25 mM MIX4 and 25 mM MIX20 (Figure 9B). All MIX24 variant formulations retained antioxidant activity, demonstrating over 50% inhibition in the ABTS assay and an approximately 0.5-unit increase in absorbance at 593 nm in the FRAP assay. These results indicated that the MIX20-based functional profile was robust and not abrogated by introducing external components into the matrix.

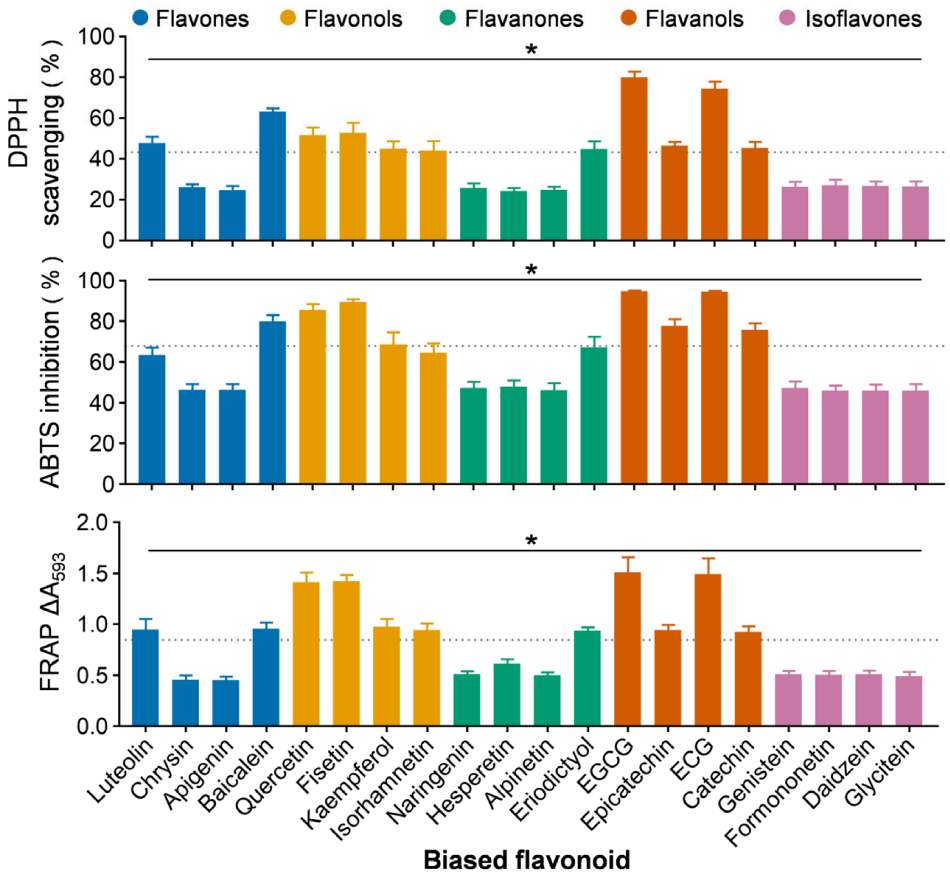



**Figure 7. Functional retention of biased variants in antioxidant assays.** Each 50 mM individual flavonoid stock was mixed 1:1 ($v/v$) with the 50 mM LOO stock containing the other 19 flavonoids, thereby generating 20 biased mixtures in which the indicated flavonoid comprised 50% of the total concentration (i.e., 25 mM, with each of the remaining flavonoids at 25/19 mM). MIX20 and all of its biased variants were evaluated for DPPH and ABTS inhibition, as well as for their reducing power via FRAP assay, at a final flavonoid concentration of 25 µM (with the biased component at 12.5 µM and each remaining individual flavonoid at 12.5/19 µM). The MIX20 level is indicated by a dotted line. Values are expressed as percentages of inhibition or as increased absorbance at 593 nm, reported as GM with 95% CI ($n = 8$). Significance was assessed by one-sample Wilcoxon signed-rank tests with Benjamini–Hochberg FDR correction. * Adjusted $p < 0.05$ vs. control.

Next, we evaluated assays beyond redox readouts, including α-glucosidase inhibition, high-concentration cytotoxicity, and mitochondrial membrane potential depolarization (Figure 10A–C). Across these modalities, the results consistently support the conclusion that systematically constructed biased formulations tend to retain the functions observed within MIX20, thereby supporting functional robustness across these assay modalities. Corresponding to the reduced α-glucosidase inhibition observed in the LOO perturbation in Figure 4, the biased EGCG variant here showed prominent inhibition, almost attaining 100% inhibition. The luteolin-biased formulation deviated markedly from the other variants, consistent with our previous observation that luteolin is highly toxic at high concentrations and is very efficient in inducing depolarization.

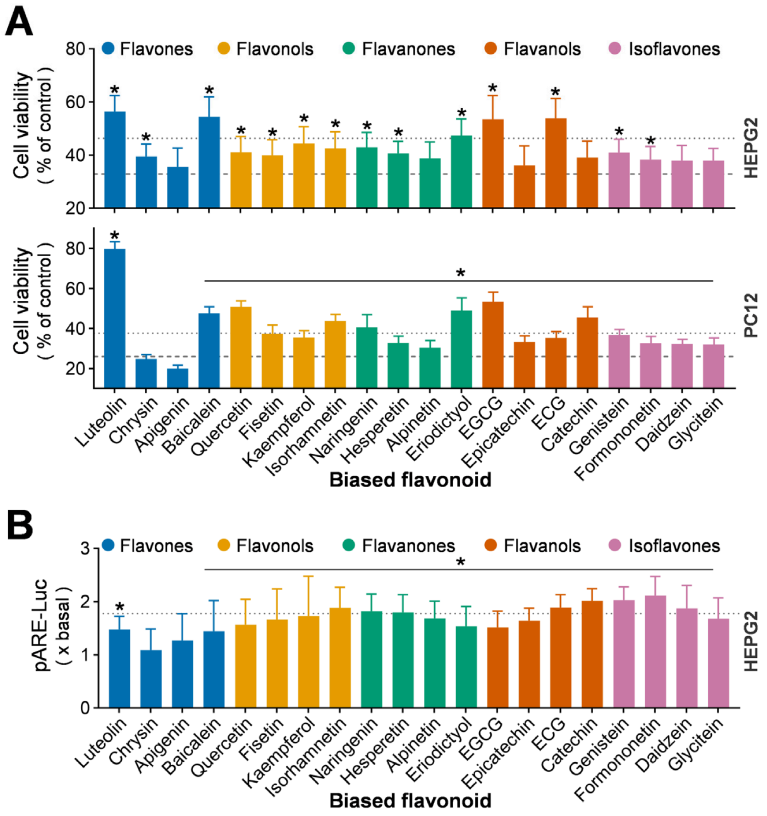



**Figure 8. The biased variants in protection against tBHP-induced toxicity and activation of protective reporter elements**. (**A**) HepG2 and PC12 cells were exposed to all individual biased variants at a final flavonoid dose of 10 μM (with the biased component at 5 μM and each remaining individual flavonoid at 5/19 μM) in DMEM for 1 h, followed by 8 h of tBHP treatment at 100 μM. Cell viability was assessed by MTT assay. (**B**) HepG2 cells were transfected with pARE-Luc. Following transfection, cells were treated with 10 μM individual biased variants in DMEM containing 1% FBS and 0.5% horse serum. Then, 24 h later, luciferase activity was measured by luminometry. The vehicle control group is indicated by a dashed line, and the MIX20 level is indicated by a dotted line. Values are expressed as percentages of the control or as fold changes, reported as GM with 95% CI ($n = 8$). Significance was assessed by one-sample Wilcoxon signed-rank tests with Benjamini–Hochberg FDR correction. * Adjusted $p < 0.05$ vs. the vehicle control in (**A**) or the control in (**B**).

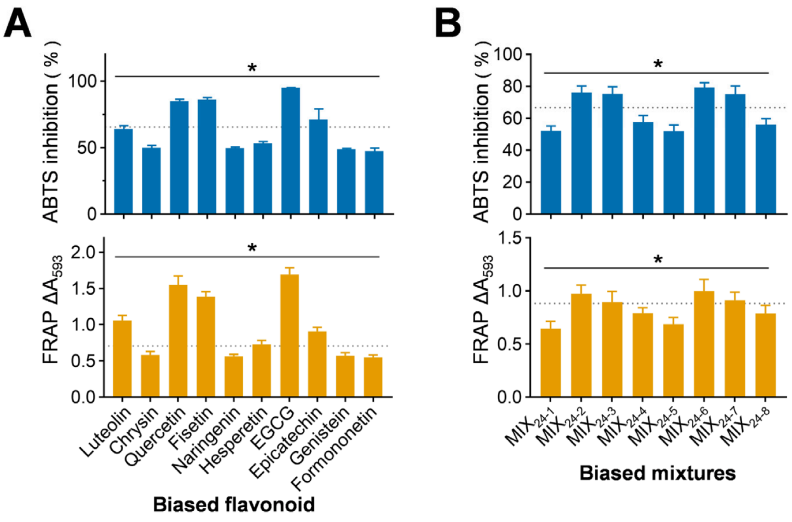



**Figure 9. Generalization of formulation robustness in antioxidant assays:** (**A**) 50 mM stocks of biased variants corresponding to MIX10a (identical to those shown in Figure 5) were created by mixing

a 50 mM individual stock of the biased flavonoid with the corresponding 50 mM LOO stock containing the remaining 9 flavonoids. The resulting biased variant mixtures were tested for the inhibition of ABTS and FRAP at a total flavonoid dose of 25 µM (with the biased component at 12.5 µM and each remaining individual flavonoid at 12.5/9 µM). (**B**) The eight flavonoids not included in MIX20, as indicated, were used as an external panel. Eight groups of 4 flavonoids were sampled from these 8 flavonoids, and 8 different 50 mM MIX4 stocks were created by equally mixing the 4 flavonoid stocks at 50 mM. Then, each MIX4 stock was mixed with MIX20 stocks at a 1:1 ratio to form 8 different 50 mM MIX24 variants, each containing 25 mM MIX4 and 25 mM MIX20 (Supplementary Table S1). These variants were tested for ABTS and FRAP inhibition at a final flavonoid concentration of 25 µM (with the MIX4 and MIX20 components each at 12.5 µM). The levels of MIX10a and MIX20 are indicated by dotted lines. Values are expressed as percent inhibition, reported as GM with 95% CI ($n = 8$). Significance was assessed by one-sample Wilcoxon signed-rank tests with Benjamini–Hochberg FDR correction. * Adjusted $p < 0.05$ vs. control.

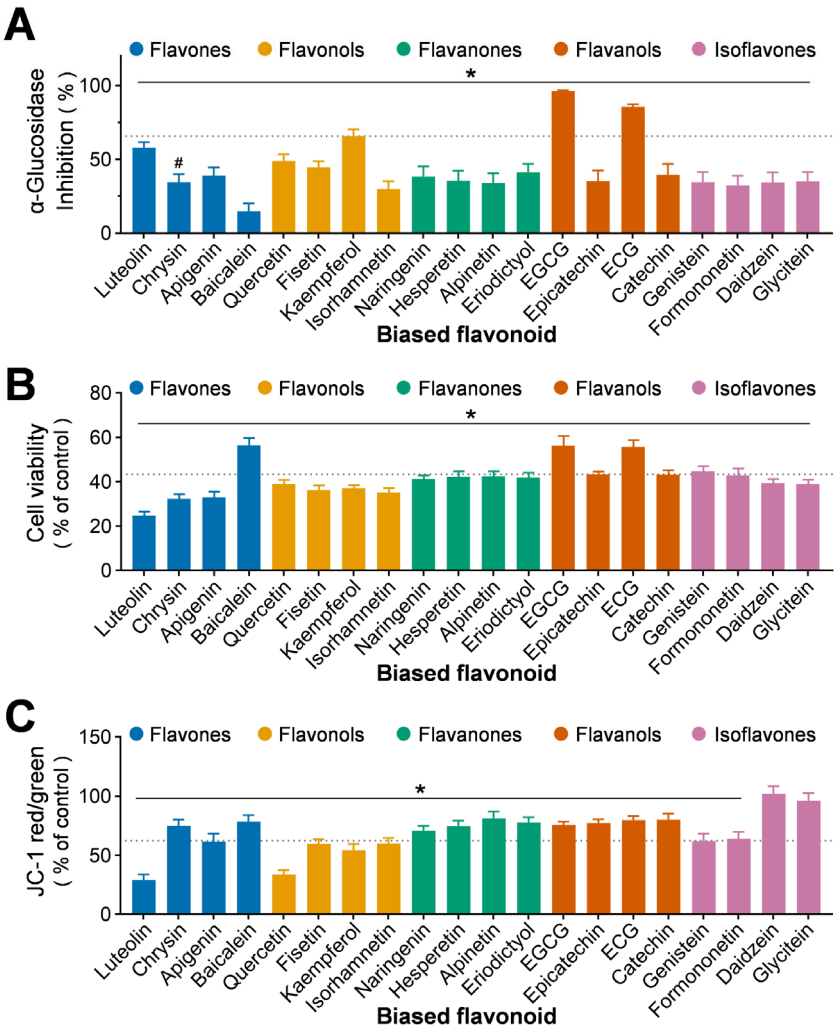



**Figure 10. Functional retention of biased variants in non-antioxidant assays.** MIX20 and its biased variants were tested for (**A**) alpha-glucosidase inhibition at 25 µM (with the biased component at 12.5 µM and each remaining individual flavonoid at 12.5/19 µM), (**B**) cytotoxicity in HepG2 cells at 50 µM (with the biased component at 25 µM and each remaining individual flavonoid at 25/19 µM), and (**C**) rapid perturbation of mitochondrial membrane potential in PC12 cells at 2 µM (with the biased component at 1 µM and each remaining individual flavonoid at 1/19 µM). The MIX20 level is indicated by a dotted line. Values are expressed as percentages of inhibition or as percentages of the control, reported as GM with 95% CI ($n = 8$). Significance was assessed by one-sample Wilcoxon signed-rank tests with Benjamini–Hochberg FDR correction. * Adjusted $p < 0.05$ vs. control.

As an in vivo extension of the analysis, we employed a *C. elegans* paraquat toxicity assay. All tested concentrations (10 to 100 μM) of MIX20 increased survival rate under paraquat stress. Specifically, the 50 μM concentration, which had the highest median survival time at approximately 60 h, was selected for subsequent comparisons (Figure 11A). Although the larger compositional shifts can more easily fail TOST equivalence bounds, such variants may still retain function. Therefore, we tested ten "leave-two-subclasses-out" variants as comparators to MIX20 at 50 μM (Figure 11B). Consequently, all ten MIX12 variants retained significant protection against paraquat-induced stress, with median survival times ranging from approximately 53 to 65 h.

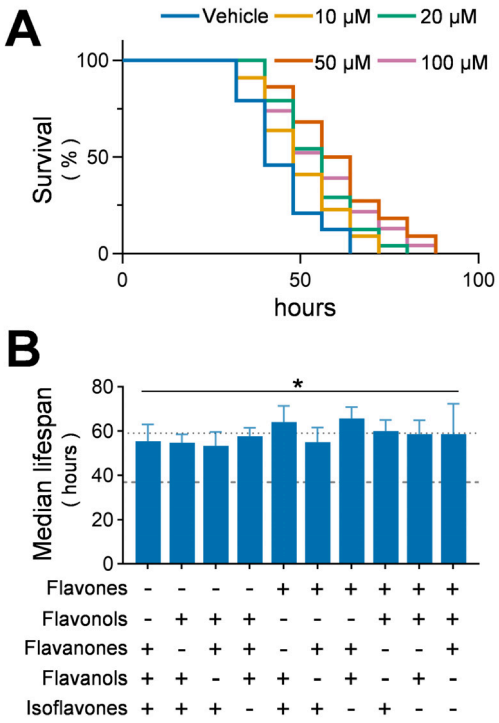



**Figure 11. Functional robustness of MIX20 in paraquat-induced *C. elegans* toxicity.** (**A**) MIX20 at various concentrations (0–100 μM) was applied to C. elegans from the L1 stage until 24 h after reaching the L4 stage. The worms were then transferred to liquid culture containing 35 mM paraquat for stress induction. Each group contained 30 animals per experiment. Three independent experiments were conducted, and one representative set of survival curves is shown. (**B**) MIX20 and all two-subclass-depleted variants described in Figure 6 were applied to *C. elegans* at a final flavonoid concentration of 50 μM, and their protective effects against 35 mM paraquat were evaluated. Survival was analyzed using the log-rank (Mantel–Cox) test, and the median survival times were plotted as a bar graph. The levels of the vehicle control and MIX20 groups are indicated by dashed and dotted lines, respectively. Each treatment group was compared with the vehicle control, with the Benjamini–Hochberg FDR correction applied for multiple comparisons. The results are presented as median survival times with 95% CIs. * Adjusted $p < 0.05$ vs. control.

## 4. Discussion

This study does not focus on specific bioactivities of individual flavonoids or their underlying mechanisms. Instead, we assess whether the system maintains stable functional outputs despite internal perturbations, adopting a holistic perspective of flavonoidic functions [19]. Using a defined mixture of 20 representative flavonoids as a white-box model, we evaluated the system robustness by combining LOO analysis with biased perturbations. The findings from antioxidant and related biochemical assays provide a system-level dissection and help explain the recurrent concordance of flavonoid bioactivities, as reported

across diverse phyto-extracts and single-compound studies. This experimental design offers a generalizable framework for dissecting other phytochemical classes characterized by scaffold-sharing derivatives, although its applicability to such systems requires independent experimental validation. These findings suggest that certain mixture-level bioactivities may be retained despite substantial variation in flavonoid composition.

From an evolutionary perspective, flavonoids diversified extensively during land plant evolution while retaining a shared phenylpropanoid-derived scaffold and broadly conserved physicochemical properties [20,21]. This combination of structural conservation and diversification may have produced two complementary features relevant to mixture-level robustness. Structural diversification allows different flavonoids to cover a broader range of functions, whereas their conserved chemical features can generate overlapping activities for a given function. Consequently, the loss or altered abundance of one constituent may have little effect, either because that constituent contributes minimally under the tested conditions or because its function overlaps with those of other mixture members. Analogous to the buffering effect of biodiversity in ecological systems [22], this combination of functional breadth and partial redundancy may enable flavonoid mixtures to maintain relatively stable outputs despite substantial variation in composition.

Although conceptually plausible, the robustness of functional outputs under compositional perturbation has not, to our knowledge, been formally evaluated in a defined flavonoidic mixture. MIX20 provided a tractable system for concentration-matched LOO analysis, in which the remaining components were proportionally rescaled after each omission to preserve the total concentration. This design evaluated the mixture-level response to constituent loss and compositional redistribution, rather than separating their individual effects or isolating the contribution of the omitted constituent. We further generated biased mixtures in which individual flavonoids accounted for 50% of the total flavonoid concentration. Together, functional equivalence in the LOO mixtures and function retention in the biased mixtures support the system-level robustness of MIX20 under different degrees of compositional perturbation.

Robustness may be regarded as an inherent property of flavonoidic mixtures, although its quantitative expression and interpretation depend on the perturbation design and assay readout. For example, under large compositional perturbations, the degree of functional deviation varied between cell-free antioxidation and cell-based assays, even though overall biological activity was consistently retained. In certain contexts, robustness might even appear trivial. For instance, in antioxidant LOO analyses, the observed responses closely aligned with statistical expectations for the omission of a single component from a 20-constituent mixture. Yet, this predictability is informative, as it captures the collective regularity of the mixture. Notably, the tested responses were not entirely uniform: the omission of specific constituents occasionally produced pronounced, assay-specific outliers, highlighting non-redundant functional nodes within an otherwise robust network.

The robustness framework developed here extends the study of flavonoid mixtures beyond functional characterization toward the characterization of collective regularities. From a complexity-science perspective, this approach describes high-dimensional mixture behavior through condition-dependent parameters and response trends, rather than attempting to resolve the numerous constituent-level interactions, typically examined in studies of synergy and antagonism [23]. Integrating these system-level trends with mechanistic and omics-based findings may provide a broader understanding of flavonoid actions [24,25]. This perspective may also be applicable to other multi-target phytochemical classes characterized by structural diversification around a shared molecular scaffold.

Despite these insights, the immediate generalizability of our findings is constrained by our methodological choices and the specific experimental conditions. First, the MIX20-based

simplification does not fully capture the hierarchical, non-uniform distribution of concentration present in real foods, nor does it account for key physiological processes, such as gut microbial bio-transformation, phase II metabolism, or selective tissue distribution [26,27]. Second, our validation currently relies on a subset of high-throughput bioassays. Future work should therefore include more targeted, molecular-level comparisons and additional in vivo models to determine whether the observed robustness persists under broader conditions. Third, given the proof-of-concept nature of this study and the absence of validated assay-specific equivalence margins, the 80–125% bounds were adopted as a pragmatic benchmark. Appropriate equivalence margins should be established on a case-by-case basis according to the biological relevance and variability of each assay. Finally, beyond formulation perturbations, other classes of perturbations relevant to dietary contexts—such as background matrix components (e.g., polysaccharides and proteins)—should be systematically introduced into the perturbation framework.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antiox15111465/s1, Figure S1: TOST on antioxidant capacity relative to the MIX20 baseline; Figure S2: Generalization of formulation robustness in an independent MIX8; Table S1: Concentration of each flavonoid component in a 50 mM mixture stock solution.

**Author Contributions:** Conceptualization, A.X.G. and K.W.-K.T.; Methodology, A.X.G. and M.-M.S.; Formal Analysis, A.X.G. and M.-M.S.; Investigation, A.X.G., M.-M.S., C.L.-P.Y., I.W.-T.M., D.J.-Y.Z. and X.-M.L.; Data Curation, A.X.G. and M.-M.S.; Writing—Original Draft Preparation, A.X.G. and M.-M.S.; Writing—Review and Editing, C.L.-P.Y., I.W.-T.M., D.J.-Y.Z., T.T.-X.D. and K.W.-K.T.; Visualization, A.X.G. and M.-M.S.; Supervision, T.T.-X.D. and K.W.-K.T.; Project Administration, T.T.-X.D.; Funding Acquisition, K.W.-K.T. All authors have read and agreed to the published version of the manuscript.

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