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Special Issue - Potential of Food By-Products

Potential of Food By-Products

Valorization and Optimization of Agro-Food By-Products from *Punica granatum* Bark and *Juglans regia* Husk Combined with *Syzygium aromaticum* for Enhanced Antioxidant and Anti-Inflammatory Activities: A D-Optimal Mixture Design Approach

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ABSTRACT

Background: The sustainable valorization of agri-food processing by-products has emerged as a scientifically promising strategy for the development of natural functional ingredients rich in bioactive compounds, contributing simultaneously to the circular bioeconomy and the formulation of health-promoting food and nutraceutical products.

Aims: The present study aimed to optimize a multi-component plant-derived formulation comprising *Syzygium aromaticum* (clove), *Punica granatum* bark (pomegranate), and *Juglans regia* husk (walnut) employing a D-optimal ABCD mixture design.

Materials and Methods: A ten-run D-optimal ABCD mixture design was employed to optimize the proportions of three plant-derived powders — *Syzygium aromaticum* (clove), *Punica granatum* bark, and *Juglans regia* husk — with TPC, TFC, and DPPH radical scavenging activity as response variables. The optimized formulation was characterized by LC-MS/MS for phenolic compound profiling. Antioxidant capacity was assessed using DPPH, ABTS, FRAP, and ferrous ion chelating assays. Anti-inflammatory activity was evaluated by BSA denaturation inhibition, and cytotoxicity by hemolytic activity assay on human erythrocytes.

Results: The optimal formulation comprised 58% pomegranate bark, 30% clove and 12% walnut husk. LC-MS/MS revealed that cloves was the richest source of acid (7,947.87 ± 255.68 µg/g) and quinic acid (5,235.29 ± 79.53 µg/g), pomegranate bark was characterized by catechin, epicatechin, and citric acid, and walnut husk by catechin, quercetin, and citric acid. The optimized mixture revealed a more enriched phenolic profile than the individual extracts, with substantial levels of rutin, kaempferol, naringenin, catechin, and epicatechin concentrations, suggesting synergistic phytochemical interactions. IC_{50} values for DPPH, ABTS, ferrous iron chelating and BSA denaturation inhibition were 50.97 ± 0.31, 94.84 ± 1.34, 71.15 ± 0.72, and 4.33 ± 0.15 µg/mL, respectively; EC_{50} for ferric reducing power was 170.83 ± 0.58 µg/ml. Hemolytic activity was negligible (13.38%) at the highest concentration tested (5 mg/mL), confirming erythrocyte biocompatibility.

Conclusions: The present study demonstrates that D-optimal mixture design constitutes an effective methodological strategy for the optimization of multi-component plant formulations, enabling the identification of synergistic combinations with enhanced phytochemical composition and biological activities.

Keywords: *Syzygium aromaticum*; *Punica granatum* Bark; *Juglans regia* Husk; Phytochemical Interactions; D-Optimal Mixture Design; Antioxidant Activity; Anti-Inflammatory Activity.

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1 INTRODUCTION

In recent years, the valorization of plant resources and agri-food by-products has emerged as a major research focus, particularly in the context of the circular and sustainable economy. The agri-food industries generate substantial quantities of plant residues—bark, husks, skins, and pomace, that have traditionally been considered as low-value plant-based waste, despite constituting particularly rich sources of bioactive compounds. These biomasses are increasingly recognized as strategic raw materials for the formulation of

natural ingredients for the food, pharmaceutical, and cosmetic industries (Fernandez *et al.*, 2025).

Plant by-products contain a considerable diversity of secondary metabolites, including phenolic compounds such as flavonoids, tannins, phenolic acids, and various aromatic derivatives. These molecules, recognized for their antioxidant, antimicrobial, and anti-inflammatory properties, exhibit strong technological and functional potential (Zhu *et al.*, 2024) and have been extensively investigated across a range of food and pharmaceutical applications (Djemaa-Landri *et al.*, 2021; El-Maati*et al.*, 2016).

Among the most promising residues, pomegranate peel, walnut husks, and specific spices such as cloves are distinguished by their exceptionally high polyphenol content. Pomegranate peel has been the subject of numerous recent investigations demonstrating pronounced antioxidant activity together with efficacy against several post-harvest fungal pathogens, highlighting its potential as a natural preservative (Nawaz *et al.*, 2025). Walnut husk, for its part, is recognized to contain compounds such as juglone, which exhibit antimicrobial, antifungal, and anti-inflammatory properties of considerable industrial interest (Zhu *et al.*, 2024). Spices rich in essential oils, such as cloves, are similarly employed in food formulations owing to their antioxidant properties and their capacity to enhance the oxidative stability of lipid-rich products. Collectively, these plant materials offer complementary phenolic profiles for the development of multifunctional natural formulations, thereby fostering growing interest in advanced extraction techniques capable of improving the efficiency, selectivity, and sustainability of phenolic compound recovery (Khan *et al.*, 2024).

Research conducted in 2025 has emphasized the necessity to integrate these by-products into a sustainable valorization framework, particularly through their utilization as sources of natural polyphenols in food or nutraceutical formulations (Shabhazi *et al.*, 2025). Within this context, experimental design methodology constitutes an essential tool for optimizing the combined use of such biomasses. Mixture designs, in particular, enable the systematic investigation of the effect of the relative proportions of different plant extracts on the experimental responses under study, while facilitating the identification of potential synergistic interactions among the matrices studied. Such approaches are especially more appropriate to the development of optimized, high-value-added natural ingredient formulations.

More recently, mixture design methodology has been successfully applied to combinations of plant extracts and essential oils to optimize synergistic antioxidant and/or antimicrobial activities, notably in formulations based on pomegranate peel (Skenderidis *et al.*, 2021) and clove (Nacer-Eddine *et al.*, 2026). These studies demonstrate the effectiveness of mixture designs in identifying optimal formulations while substantially reducing the number of experimental trials required. However, to the best of our knowledge, no previous study has reported the simultaneous combination of pomegranate peel, walnut husk, and clove employing an ABCD-type mixture design integrating multi-response optimization (TPC, TFC, and DPPH), LC-MS/MS characterization, and a comprehensive evaluation of the antioxidant, anti-inflammatory, and cytotoxic activities of the resulting optimized formulation.

Accordingly, the present study aimed to employ an ABCD-type mixture design to optimize a combined formulation of pomegranate peel, walnut husk, and clove. The optimized formulation was evaluated through multiple experimental responses, including total phenolic content (TPC), total flavonoid content (TFC), DPPH radical-scavenging activity, in addition to anti-inflammatory activity and cytotoxicity, and was further characterized by LC-MS/MS, with the overarching objective of developing a high-value natural formulation for the sustainable valorization of plant resources.

2 Materials and Methods

2.1 Plant Material Preparation

Dried flower buds of *Syzygium aromaticum* (clove) were purchased from a local retail market in *Oued Semar*, Algiers, Algeria (semi-arid Mediterranean climate) in September 2020. *Punica granatum* bark was collected from mature fruits harvested in the Akbou region, Bejaia, Algeria (sub-humid Mediterranean climate) in October 2020. Following collection, the bark was thoroughly rinsed with distilled water and subsequently air-dried in the absence of light at room temperature (22 ± 2 °C) for approximately one week until constant mass was attained. Green husks of *Juglans Regia L.* were collected at the immature green developmental stage in the Tizi N'Berber region, Bejaia, Algeria (sub-humid Mediterranean climate) in August 2020. The husks were rinsed with distilled water and oven-dried at 40°C for one week until constant mass was attained. Following drying, each plant material was reduced to a fine powder employing a laboratory electric grinder, sieved through a 250 µm mesh sieve, and stored at ambient temperature (22 ± 2°C) in hermetically sealed opaque containers, protected from light and moisture until extraction.

The plant materials selected for this study correspond to minimally processed natural plant-derived ingredients. With the exception of washing, drying, grinding, and sieving, no chemical treatment, additive, or purification process was applied prior to extraction, in accordance with the principle of minimal processing of plant-derived ingredients and the

sustainable valorization of agri-food by-products (Difonzo *et al.*, 2022).

2.2 Preparation of Extracts

The extraction of polyphenolic compounds from the powder mixtures of pomegranate bark, walnut husk, and clove was performed in accordance with the method described by Romani *et al.* (2006), with minor modifications. Each powder mixture was prepared according to the proportions defined by the ABCD mixture design (Table 1), and a total mass of 1 g of each mixture was accurately weighed for each experimental run. Extraction was carried out by maceration in 100 mL of ethanol/water (70:30, v/v) for 2 hours 30 minutes at ambient temperature (22 ± 2°C). The resulting extract was subsequently centrifuged at 4,000 rpm for 20 minutes at 20°C, and the supernatant was filtered through Whatman No. 1 filter paper prior storage at 4°C pending analysis.

2.3 Experimental Design

Mixture Design

An experimental design methodology was employed to minimize the number of experimental runs required, whilst enabling the identification of the optimal formulation and the characterization of interaction effects among the three component variables — clove, pomegranate bark, and walnut husk — with respect to the measured experimental responses, namely TPC, TFC, and DPPH antiradical activity. Following the construction and validation of the mixture design across the different response variables, the component proportions were estimated in order to optimize the mixture compositions and to identify the most favorable formulation variants (Khokhar *et al.*, 2010).

Mixture Design Constraint

In a mixture design comprising n components, each component constitutes a defined proportion of the complete mixture. When the proportions of the first component $(n - 1)$ components are specified, the proportion of the remaining component n is determined automatically as the sum of all component proportions must equal unity. The component fractions X_i are expressed as values ranging from 0 to 1 — equivalent to percentages ranging from 0 to 100% — subject to the fundamental compositional constraint presented in Equation 1 (Fadil *et al.*, 2018).

$$0 \le X_i \le 1 \quad \sum X_i = 1 \qquad \text{(Eq. 1)}$$

Where X_i denotes the proportional content of component i. Equation 1 is referred to as the fundamental constraint of mixture designs (Fadil *et al.*, 2018).

Experimental Matrix and Mathematical Model

A ten-run D-optimal (ABCD) mixture design was selected for the optimization study. This design was specifically designated for its appropriateness in formulation studies in which the experimental factors are constrained by a constant sum, as defined by Equation 1. In contrast to response surface designs such as the Central Composite Design (CCD) and the Box–Behnken design — which are intended for independent variables varying freely within an unconstrained experimental domain — the ABCD mixture design explicitly incorporates the compositional constraint whilst efficiently estimating linear, binary, and ternary interaction effects with a limited number of experimental runs, rendering it particularly appropriate to the optimization of formulations based on natural plant-derived ingredients (Galvan *et al.*, 2021). The ten experimental runs were distributed as follows: pure (single-component) mixtures, located at the vertices of the simplex triangle (Experiments 1, 2, and 3); binary mixtures at equal proportions (0.5/0.5) (Experiments 4, 5, and 6); a ternary mixture with equal proportions of all three components (Experiment 7); and ternary mixtures with unequal proportions (Experiments 8, 9, and 10). The precise proportions of each component — clove, pomegranate bark, and walnut husk — for each of the ten experimental runs are presented in Table 1.

To express the measured responses as a function of the combination-based autonomous variables, an incomplete third-degree (special cubic) regression model was employed (Fadil *et al.*, 2018). This model constitutes a specific form of the complete third-degree polynomial model for mixture experiments, incorporating linear terms, binary interaction terms, and a single ternary interaction term, whilst excluding the pure cubic terms, which are generally not estimable with a limited number of experimental runs (Cornell, 2002). The model is expressed as follows:

$$Y = \alpha_1X_1 + \alpha_2X_2 + \alpha_3X_3 + \alpha_{12}X_1X_2 + \alpha_{13}X_1X_3 + \alpha_{23}X_2X_3 + \alpha_{123}X_1X_2X_3 + \varepsilon \quad \text{(Eq. 2)}$$

where:

- Y = response variable (TPC, TFC, or DPPH radical scavenging inhibition)
- X_1, X_2, X_3 = fractional proportions of the three mixture components
- α_1, α_2, α_3 = coefficients of the linear terms
- α_{12}, α_{13}, α_{23} = coefficients of the binary interaction terms
- α_{123} = coefficient of the ternary interaction term
- ε = residual error term

2.4 Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) was determined according to the Folin–Ciocalteu colorimetric method described by

Taga *et al.* (1984). An aliquot of 100 μL of extract was combined with 2 mL of 2% (w/v) aqueous sodium carbonate (Na_2CO_3) solution. After 2 minutes, 100 µL of Folin–Ciocalteu reagent diluted to 50% was incorporated, and the resulting mixture was thoroughly agitated and incubated in the dark at ambient temperature (22 ± 2°C) for 30 minutes. Absorbance was subsequently measured at 750 nm utilizing a UV-Vis spectrophotometer (UV-1800, Shimadzu Europa GmbH, Germany). Results are expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE)/g DW), calculated from a gallic acid calibration curve.

2.5 Determination of Total Flavonoids Content (TFC)

Total flavonoid content (TFC) was determined according to the aluminum chloride colorimetric method described by Djeridane *et al.* (2006). A volume of 1 mL of extract was combined with 1 mL of aluminum chloride ($AlCl_3$) solution at 2% (w/v), agitated thoroughly, and incubated in the dark at ambient temperature (22 ± 2°C) for 15 minutes. Absorbance was measured at 430 nm employing a UV-Vis spectrophotometer. Results are expressed as milligrams of quercetin equivalent per gram of dry plant matter (mg QE/g DW), calculated from a quercetin standard calibration curve.

2.6 Determination of Antioxidant Activity of the Optimal Plant Mixture

DPPH Radical Scavenging Activity

The 2,2' diphenyl-1-picrylhydrazil (DPPH) radical scavenging activity was determined according to the protocol described by Alam *et al.* (2013). Two hundred microliters of the extract of the optimal three-plant mixture (clove/pomegranate bark/walnut husk, 30%/58%/12%) were combined with 2 mL of DPPH solution (0.5 mM in methanol) and incubated in the dark at ambient temperature (22 ± 2°C) for 30 minutes. Absorbance was subsequently measured at 517 nm. Antioxidant capacity was expressed as the percentage inhibition of DPPH radical scavenging activity and calculated according to the following equation:

$$\text{DPPH radical inhibition (\%)} = [(A_{contol} - A_{Sample}) / A_{control}] \times 100 \quad \text{(Eq.3)}$$

Where $A_{control}$ represents the absorbance of the control and A_{sample} represents the absorbance of the sample tested.

ABTS Radical Scavenging Activity

The ABTS radical cation decolorization assay was performed according to the method described by Alonso-Carrillo *et al.* (2017). Solutions of ABTS (7 mM) and potassium persulfate (2.45 mM) were prepared, combined, and incubated in the dark at ambient temperature (22 ± 2°C) for 12–16 hours prior to use. The resulting $ABTS^{+\bullet}$ solution was diluted with ethanol to achieve an initial absorbance of 0.70 ± 0.02 at 734 nm. One thousand microliters of the diluted $ABTS^{+\bullet}$ solution was then combined with 100 µL of extract at varying concentrations (3.13, 12.50, 25.00, 50.00, 100.00, and 150.00 µg/mL). The mixture was allowed to react for 6 minutes at ambient temperature, and absorbance was measured at 734 nm.

Ferric Reducing Antioxidant Power (FRAP) Assay

One milliliter of extract at varying concentrations (6.25, 25, 50, 100, 150 µg/mL) was combined with 2.5 mL of phosphate buffer (0.2 M, pH: 6.6) and 2.5 mL of 1% (w/v) aqueous potassium hexacyanoferrate [$K_3Fe(CN)_6$] solution. Following incubation at 50°C for 30 minutes, 2.5 mL of 10% (w/v) trichloroacetic acid solution was combined and the mixture was centrifuged for 10 minutes. An aliquot of 2.5 mL of the resulting supernatant was combined with 2.5 mL of distilled water and 0.5 mL of 0.1% (w/v) aqueous ferric chloride ($FeCl_3$) solution, and absorbance was measured at 700 nm. A higher absorbance value is indicative greater ferric reducing antioxidant activity. Results are expressed as the effective concentration required to produce 50% of the maximum absorbance (EC_{50}), as described by Allane and Benamara, (2010).

Metal Chelating Activity Assay

The ferrous ion chelating activity of the phenolic extract of the optimal mixture was determined by the method described by Gulcin *et al.* (2003). Two hundred and fifty microliters of extract at varying concentrations (3.13, 6.25, 12.50, 100.00, 150.00 μg/mL) were combined with 25 μL of ferrous chloride ($FeCl_2$) solution (2 mM) and 50 μL of ferrozine solution (5 mM). The mixture was thoroughly agitated and subsequently incubated in the dark at ambient temperature (22 ± 2°C) for 10 minutes. Absorbance was measured spectrophotometrically at 562 nm employing a spectrophotometer (8500 II; Bio-CromGmb, Zurich, Switzerland). Results are expressed as IC_{50} and the percentage inhibition of ferrozine-Fe2+ complex formation was calculated utilizing the following equation:

$$\text{\% Inhibition} = 100 \times [(A_0 - (A_1 - A_2)) / A_0]$$

where A_0 denotes the absorbance of the control reaction (without extract); A_1 denotes the absorbance in the presence of extract and ferrozine; and A_2 denotes the absorbance in the presence of extract, ferrozine, and $FeCl_2$. The control contained neither $FeCl_2$ nor ferrozine.

2.7 Assessment of In Vitro Anti-Inflammatory Activity

BSA Denaturation Inhibition Assay

The capacity of the phenolic extracts to inhibit heat-induced bovine serum albumin (BSA) denaturation — as a proxy for anti-inflammatory activity — was evaluated

according to the method described by Abbou *et al.* (2019). A test solution of 0.5 mL, comprising 0.45 mL of 2% (w/v) aqueous BSA solution and 0.05 mL of extract at varying concentrations (1.25, 2.50, 3.75, 6.25, 7.50 µg/mL), was prepared and incubated at 37°C for 20 minutes, followed by heat treatment at 70°C for a further 20 minutes. Following cooling to ambient temperature, absorbance was measured at 660 nm. The percentage inhibition of protein denaturation was calculated according to the following equation:

$$\text{Inhibition (\%)} = [(A_0 - A_1) / A_0] \times 100$$

where A_0 denotes the absorbance of the control solution (heat-treated BSA in the absence of extract) and A_1 denotes the absorbance of the test solution containing plant extract.

Cytotoxicity Assay (Hemolytic Activity)

Venous blood samples were obtained from healthy volunteers by qualified medical personnel at the polyclinic laboratory. Residual blood samples of sufficient remaining volume, exhibiting no visible signs of hemolysis, lipemia, or icterus, were selected for analysis. Following completion of routine laboratory analyses, all samples were stored at 4°C and processed within 2 hours of collection. All samples were irreversibly anonymized prior utilization, in strict accordance with the principles of the Declaration of Helsinki and applicable local regulations governing the secondary use of anonymized biological samples. Blood samples were centrifuged at 3,000 rpm for 10 minutes and the supernatant was discarded. The erythrocyte pellet was washed three times with physiological saline (0.9% NaCl). Following the final wash, red blood cells (RBCs) were resuspended to yield a 10% (v/v) suspension in 0.9% NaCl for cytotoxicity assay (Abbou *et al.*, 2019; Oyedapo and Famurewa, 1995).

For the hemolytic activity assay, 1 mL of the RBC suspension was combined with 1 mL of the extract at varying concentrations (1, 2, 3, 4, and 5 mg/mL) and incubation for 10 minutes at ambient temperature (22 ± 2°C). Samples were subsequently centrifuged at 3,000 rpm for 10 minutes, and the absorbance of the supernatant was measured at 540 nm to quantify hemoglobin release as an index of hemolytic activity. Complete hemolysis induced by distilled water was assigned a value of 100% hemolysis (Pagano and Faggio, 2015). Hemolysis (%) was calculated as follows:

$$\text{Hemolysis \%} = [(A_0 - A_1) / A_0] \times 100$$

where A_0 denotes the absorbance of the control (distilled water without extract) and A_1 denotes the absorbance in the presence of the extract.

2.8 Phenolic Compound Profiling by LC-MS/MS

Approximately 25 mg of lyophilized sample was accurately weighed and solubilized in 70% (v/v) aqueous ethanol. Each sample solution was subsequently prepared at dilution ratios of 1:2 and 1:10. Quantification was performed utilizing the most extensively diluted extract (1:10 dilution) wherever analytically feasible. Chromatographic analysis was carried out employing an HPLC-MS/MS system comprising a Waters Alliance 2695 instrument (Waters®, Ireland) equipped with a quaternary pump, solvent degasser, autosampler, and column oven, integrated with a Waters 996 photodiode array detector (PDA/DAD) (Waters®, Ireland). Separation was achieved on a LiChrospher® 100 RP-18, 5 µm column (250 × 4 mm) maintained at 35 ◦C, with an injection volume of 20 µL. The mobile phase consisted of a binary gradient system comprising 0.1% (v/v) formic acid in water (Eluent A) and acetonitrile (Eluent B), delivered at a flow rate of 0.3 mL/min. Spectral absorption was monitored across the wavelengths range of 210–700 nm utilizing a photodiode array detector. Tandem mass spectrometry (MS/MS) detection was performed through a Micromass® Quattro Micro triple quadrupole instrument (Waters®, Ireland), equipped with an electrospray ionization (ESI) source operating at 120°C and applying a capillary voltage of 2.5 kV. Analytical conditions were individually optimized for each reference standard, and all analyses were conducted in multiple reaction monitoring (MRM) mode to maximize selectivity and sensitivity. System control, data acquisition, and data processing were performed via MassLynx software (version 4.1). High-purity nitrogen (N_2) served both as a drying gas and a nebulizing gas, whilst ultra-high purity argon (Ar) was employed as the collision gas. Whenever analytically feasible, two MRM transitions were monitored for the identification and quantification of each compound, with a maximum permissible deviation of 15% between the MRM1/MRM2 transition ratios.

2.9 Statistical Analysis

The reliability and adequacy of the model were evaluated through the coefficient of determination (R^2) and the F-test at a 95% confidence level, performed with JMP software (version 10.0; SAS Institute Inc., Cary, NC, USA). The F-ratio, derived from the comparison of regression and residual mean squares, was employed to assess the statistical significance of each model and its capacity to account for the observed experimental variability. A model was considered statistically significant when the calculated F-value exceeded the corresponding critical F-value at the specified confidence level, indicating an adequate fit to the experimental data. The Student's *t*-test was applied to evaluate the statistical significance of individual regression coefficients, with higher absolute *t*-values and lower corresponding *p*-values indicating a greater contribution of the corresponding factors. Experimental design construction, mathematical modeling, and optimization were carried out through JMP 10.0. Isoresponse curves were initially examined to identify potential compromise regions across the response variables,

followed by the application of the desirability function approach to determine the optimal combination of mixture component proportions simultaneously satisfying all response criteria. Confirmatory experimental validation runs were subsequently performed to verify the predictive reliability of the proposed optimized model

3 RESULTS

3.1 Total Phenolic Content (TPC)

The results of the ten experimental runs, along with their respective measured responses — total phenolic content (TPC), total flavonoid content (TFC), and DPPH radical scavenging inhibition — are presented in Table 1. Among the pure single-component samples, the clove extract recorded the highest TPC content (290.41 ± 2.67 mg GAE/g of extract), followed by the binary mixture of Experiment 5 (263.35 ± 0.40 mg GAE /g of extract) and ternary mixtures of Experiments 8 and 10 (223.95 ± 1.2 mg GAE /g of extract, 255.79 ± 2.41 mg GAE /g of extract, respectively). The walnut husk extract exhibited the lowest TPC value (47.04 ± 4.82 mg GAE /g of extract), which accounts for the comparatively reduced TPC contents recorded in Experiments 4, 6, and 9 (155.11 ± 4.28 mg GAE/g of extract, 187.50 ± 0.53 mg GAE /g of extract and 167.56 ± 2.81 mg GAE /g of extract, respectively) — experimental runs in which walnut husk constituted an equal (binary mixture) or greater (ternary mixture) proportion of the formulation.

3.2 Total Flavonoids Content (TFC)

As presented in Table 1, the ternary mixture in which pomegranate bark constituted the predominant component (Experiment 8), yielded the highest total flavonoid content (14.41 ± 0.18 mg EQ/g of extract), followed by the binary mixture of Experiment 5 and the pure pomegranate bark sample of Experiment 3, which exhibited closely comparable TFC values of 13.01 ± 0.89 and 13.9 ± 0.04 mg/g EQ of extract, respectively. The binary mixture of Experiment 6 and the pure clove sample of Experiment 1 recorded the lowest TFC values, at 8.78 ± 0.32 and 7.91 ± 0.18 mg/g EQ of extract, respectively.

3.3 DPPH Radical Scavenging Activity

Flavonoids are well documented in the literature to exhibit a broad spectrum of biochemical and biological activities, including antioxidant properties that are positively correlated with radical scavenging capacity (El-Maati *et al.*, 2016). The DPPH radical scavenging method was employed in the present study as one of the most widely adopted and validated approaches for the evaluation of antioxidant capacity in plant-derived extracts (Brahmi *et al.*, 2017).

The results summarized in Table 1 demonstrate that the various mixture combinations exhibited significant variability in their scavenging activity against the DPPH radical. Experiments 3 (pomegranate bark), 5 (binary mixture) and 8 (ternary mixture) displayed the highest antiradical activity, with DPPH inhibition percentages of 80.23 % ± 0.09%, 78.79 % ± 0.09% and 79.31 % ± 0.72%, respectively. In contrast, the lowest radical scavenging activity was recorded in Experiments 1 (pure walnut husk) and Experiment 6 (binary mixture), with inhibition percentages of 29.21 % ± 3.96% and 39.40 % ± 6.28%, respectively. The observed pattern of DPPH radical scavenging activity was broadly consistent with the TFC results, suggesting a positive and proportional relationship between flavonoid content and radical scavenging capacity across the experimental combinations.

Table 1. Different Combinations Generated by the Design of the Selected Mixture and the Responses Recorded for Each Experiment

Experience	Clove	Pomegranate bark	Walnut husk	TPC (mg GAE/g DW)	TFC (mg QE/ g)	DPPH (%)
1	0	0	1	47.05±5.24	7.91±0.10	29.21± 3.96
2	1	0	0	290.41±15.69	10.54±0.09	71.82± 1.93
3	0	1	0	194.32±1.06	13.94±0.04	80.23± 0.09
4	0	0.5	0.5	155.11±1.87	11.62±0.10	76.19 ±0.77
5	0.5	0.5	0	263.35±0.40	13.00±0.89	78.79 ± 0.096
6	0.5	0	0.5	187.50±0.53	8.79± 0.32	39.39± 6.28
7	0.33	0.33	0.33	265.34±2.41	11.84± 0.01	74.21± 0.96
8	0.16	0.66	0.16	223.96±1.20	14.42±0.18	79.31± 0.72
9	0.16	0.16	0.66	167.56± 11.32	10.25± 0.26	59.23± 1.98
10	0.66	0.16	0.16	255.79±2.41	10.68± 0.33	72.70± 0.48

3.4 Experimental Design Results

3.4.1 Statistical Validation of the Model

Analysis of variance (ANOVA) results, presented in Table 2, confirmed the statistical significance and adequacy of the regression models for all three response variables. The

Table 2. Analysis of Variance for the Adjusted Model

Response	Source	DF	SS	MS	F	*p*-value
DPPH	R	6	2679.4	446.57	32.01	0.036
	r	2	27.9	13.95		
	total	8	2707.29			
	R^2	0,989				
TPC	R	6	45609.3	7601.55	28.47	0.0343
	r	2	533.91	266.96		
	total	8	46143.22			
	R^2	0,988				
TFC	R	6	28.70	4.78	28.26	0.0346
	r	2	0.34	0.17		
	total	8	29.04			
	R^2	0,988				

DF: *Degrees of Freedom;* ***SS:*** *Sum of Squares;* ***MS:*** *Mean Square;* ***F:*** *F-ratio;* ***R:*** *Regression;* ***r:*** *Residual;* ***R2:*** *Coefficient of determination.*

calculated F-ratios for TPC, TFC, and DPPH were 28.47, 28.26, and 32.01, respectively, are approximately 1.5 times higher than the theoretical value ($F_{(0.05,\ 6,\ 2)}$ = 19.33) at the 95% confidence level by a factor of approximately 1.5, thereby confirming the statistical significance of the regression. Furthermore, the associated *p*-value of 0.003 ($p < 0.05$) provides additional confirmation that the main regression effect is statistically significant. The coefficients of determination ($R^2 \approx 0.99$) for all three response variables — TPC, TFC, and DPPH — indicated an excellent concordance between the experimentally observed values and the values estimated by the fitted models, confirming that the selected special cubic regression model provides an adequate and highly reliable representation of the experimental data.

3.4.2 Regression Coefficients and Fitted Models

The regression coefficients of the fitted models for each response variable (TPC, TFC, and DPPH) were assessed for statistical significance at the 5% significance level ($p < 0.05$) (Table 3). All statistically significant terms — encompassing both individual component effects and binary or ternary interaction terms — were retained in the final model, whilst coefficients with *p*-values exceeding 0.05 were excluded. For TPC (Equation 1), the most significant terms corresponded to the effects of the pure components α_1 (clove) and α_2 (pomegranate bark). For TFC (Equation 2), the linear effects of all three pure components (α_1, α_2, and α_3) were found to be statistically significant. For DPPH radical scavenging activity (Equation 3), both the pure component effects and the binary interaction term between walnut husk and pomegranate bark (α_{23}) were identified as statistically significant contributors to the response variability.

The final fitted mathematical models expressing each response variable as a function of the three mixture component fractions are as follows:

TPC (Eq. 1):

$$\hat{Y}_{TPC} = 284.788\ x_1 + 195.36\ x_2$$

TFC (Eq. 2):

$$\hat{Y}_{TFC} = 10.40\ x_1 + 13.93\ x_2 + 8.02\ x_3$$

DPPH (Eq. 3):

$$\hat{Y}_{DPPH} = 72.40\ x_1 + 79.84\ x_2 + 30.05\ x_3 + 86.96\ x_2x_3$$

where x_1, x_2, and x_3 represent the fractional proportions of clove, pomegranate bark, and walnut husk, respectively.

3.4.3 Isoresponse Contour Plots and Mixture Optimization

The isoresponse contour plots for TPC, TFC, and DPPH radical scavenging activity (Figure 1), constructed as a function of the proportional contributions of the three mixture components, provide a comprehensive visualization of all possible combinations of operating conditions capable of achieving a target response value. These plots enabled the identification of the two most influential components — pomegranate bark and clove — as the principal drivers of all three responses, whilst confirming that the contribution of walnut husk, despite its lower individual potency, was not negligible at its optimal inclusion level of 12%. In Figure 1,

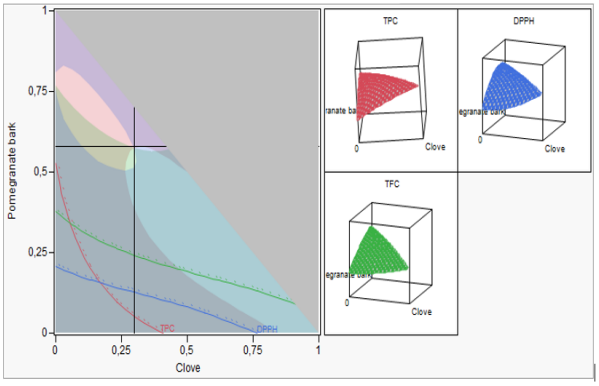

Figure 1. Isoresponses Graph Demonstrating the Areas of Optimal Compromises for TPC (red), TFC (green) and DPPH Inhibition (blue) Obtained by Fixing the Proportion of Walnut Husk and Varying the Proportions of Pomegranate Bark and Clove

Table 3. Estimated regression coefficients for the incomplete cube regression model

	Term	Coefficient	Estimation	Standard error	t-student	*p*-value
	Clove	X_1	284.77857	15.83888	17.98	0.0031*
	Pomegranate bark	X_2	195.15875	16.30861	11.97	0.0069*
	Walnut husk	X_3	49.31369	15.83888	3.11	0.0895
	Clove*Pomegranate bark	X_{12}	74.37464	79.71534	0.93	0.4493
TPC	Clove*Walnut husk	X_{13}	68.36633	79.64766	0.86	0.4811
	Pomegranate bark*Walnut husk	X_{23}	143.94489	79.71534	1.81	0.2127
	Clove*Pomegranate bark*Walnut husk	X_{123}	1336.6288	533.9595	2.50	0.1293
	Clove	X_1	10.409527	0.398805	26.10	0.0015*
	Pomegranate bark	X_2	13.938952	0.410633	33.95	0.0009*
	Walnut husk	X_3	8.026611	0.398805	20.13	0.0025*
	Clove*Pomegranate bark	X_{12}	2.8222782	2.007143	1.41	0.2949
TFC	Clove*Walnut husk	X_{13}	-1.772464	2.005439	-0.88	0.4700
	Pomegranate bark*Walnut husk	X_{23}	3.0465223	2.007143	1.52	0.2684
	Clove*Pomegranate bark*Walnut husk	X_{123}	15.258482	13.4445	1.13	0.3741
	Clove	X_1	72.232586	3.620697	19.95	0.0025*
	Pomegranate bark	X_2	79.917161	3.728075	21.44	0.0022*
	Walnut husk	X_3	30.054996	3.620697	8.30	0.0142
	Clove*Pomegranate bark	X_{12}	11.276237	18.22257	0.62	0.5991
DPPH	Clove*Walnut husk	X_{13}	-41.93646	18.2071	-2.30	0.1478
	Pomegranate bark*Walnut husk	X_{23}	86.975777	18.22257	4.77	0.0412*
	Clove*Pomegranate bark*Walnut husk	X_{123}	271.61423	122.0608	2.23	0.1560

the red, blue, and green regions correspond to the TPC, DPPH, and TFC response surfaces, respectively.

The optimal mixture proportions, as determined by the desirability function approach and clearly illustrated on the mixture simplex graph (Figure 2), were identified as: 30% clove, 12% walnut husk, and 58% pomegranate peel, yielding a composite desirability value of 82.50%. The predicted optimal response values for this formulation were: TPC = 242.17 ± 10.50 mg GAE/g of extract; TFC = 13.12 ± 1.80 mg QE/g of extract; and DPPH radical scavenging inhibition = 88.50 ± 4.50%.

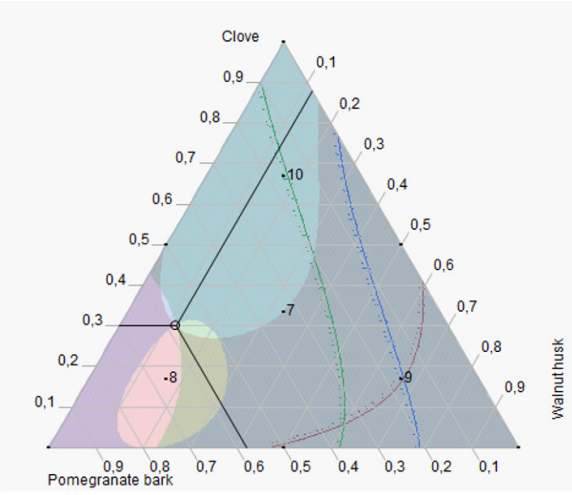

Figure 2. Optimal Compromise Zones for TPC, TFC, and Percentage DPPH Inhibition

Experimental validation of the proposed optimal formulation was performed by preparing and analyzing the mixture at the identified optimal proportions. As presented in Table 4, no statistically significant difference was observed between the predicted and experimentally determined values for any of the three response variables, thereby confirming the predictive adequacy and reliability of the fitted model for this optimization approach.

3.5 Antioxidant Activities of the Optimal Plant Mixture Extract

Four complementary antioxidant assays were performed to evaluate the antioxidant activity of the phenolic extract of the optimal plant mixture (Table 5). The results obtained from the metal chelating activity, DPPH radical scavenging, and ABTS cation decolorization assays are expressed as IC_{50}. The ferric reducing antioxidant power (FRAP) is expressed as the effective concentration required to achieve an absorbance of 0.5 at 700 nm (EC_{50}).

Table 4. Predicted and Observed Values for the Test Point Performed for the Optimal Mixture (Clove / Pomegranate Bark / Walnut Husk (30% / 58% / 12%)

Components of the mixture	Proportion (%)	Response	Predicted value	Observed value
Clove	30	TPC	257.95 ± 47.9a	242.17 ±10.5a
Pomegranate bark	58	TFC	13.13 ± 1.2[a]	13.12 ±1.8[a]
Walnut husk	12	DPPH	83.81 ± 10.9[a]	88.5 ±4.5[a]

Data are given as mean values ± standard deviations. Mean within the same line sharing the same letter is not different (P>0.05)

3.5.1 DPPH and ABTS free radical scavenging assay

The phenolic extract of the optimal three plant mixture was evaluated for its anti-free radical scavenging activity employing both the DPPH and ABTS radical cation decolorization assays, with results expressed as IC_{50} values (Table 5). In accordance with established convention, a lower IC_{50} value is indicative of superior radical scavenging potency.

With respect to DPPH radical scavenging activity, the IC_{50} value recorded for the optimal mixture extract is presented in Table 5. Regarding $ABTS^{+}$• radical scavenging activity, the optimal plant mixture extract demonstrated an IC_{50} of 94.84 ± 1.34 µg/mL, a value marginally higher than that recorded for the synthetic reference antioxidant BHA (IC_{50} = 56.25 ± 0.19 µg/mL), indicating that the extract possesses significant radical scavenging capacity, albeit slightly lower than that of the reference compound under the experimental conditions applied.

3.5.2 Ferric Reducing Antioxidant Power (FRAP)

The reducing power of bioactive compounds is widely regarded as a relevant and reliable indicator of their antioxidant potential, as it reflects the capacity of electron-donating species to reduce ferric ions (Fe^{3+}) to their ferrous form (Fe^{2+}) (Karray *et al.*, 2021). The optimal plant mixture extract demonstrated significant ferric reducing capacity, with an EC_{50} of 170.83 ± 0.57 ug/mL (Table 5). This value was marginally higher than the EC_{50} recorded for BHA (124.33 ± 0.58 µg/mL), indicating that whilst the reducing power of the optimal mixture extract was slightly inferior to that of the synthetic reference standard, it nonetheless reflects a substantive antioxidant reducing capacity attributable to the richness of the extract in electron-donating phenolic constituents.

3.5.3 Ferrous Iron Chelation Capacity

Iron, whilst an essential micronutrient for biological systems, can promote oxidative damage to proteins, lipids, and other macromolecules when present in its free ferrous form (Fe^{2+}), primarily through its participation in the Fenton reaction and its capacity to form the ferrozine–Fe^{2+} colored complex (Jiang *et al.*, 2014). Iron-chelating agents confer protection against such oxidative damage by sequestering free Fe^{2+} ions, thereby inhibiting lipid peroxidation and other iron-catalyzed oxidative reactions. Ferrozine is capable of forming stable, quantifiable colored complexes with Fe^{2+}; in the presence of effective chelating agents, this complex formation is disrupted, resulting in a measurable reduction in absorbance (Gulcin *et al.*, 2003).

Table 5. Antioxidant and anti-inflammatory activities of the optimal mixture extract

Activity	Calculated parameter	Extract concentration(µg/mL)	BHA (µg/mL)
RSA-DPPH	IC50	50.97±0.31[b]	87.81±3.71[a]
RSA-ABTS	IC50	94.84± 1.34[a]	56.25 ±0.19[b]
Ferric reducing power	EC50	170.83±0.57[a]	124.33 ±0.58[b]
Iron chelation capacity	IC50	71.15 ± 0.72[a]	56.87±0.21[b]
Anti-inflammatory activity	IC50	4.33± 0.15	/

Data are given as mean values ± standard deviations. Mean within the same line sharing the same letter is not different (P>0.05)

The results presented in Table 5 indicate that the phenolic extract of the optimal plant mixture exhibited significant ferrous ion chelating activity, with an IC_{50} of 71.15± 0.72 µg/mL. this value was comparable to that of the synthetic chelating agent ethylenediaminetetraacetic acid (EDTA), the reference standard, which exhibited an IC_{50} of 56.87± 0.21µg/mL.

3.6 *In vitro* Anti-Inflammatory Activity

3.6.1 Inhibition of BSA Denaturation

Protein denaturation recognized as a primary molecular mechanism underlying the development and perpetuation of

inflammation, involving the irreversible alteration of protein conformation and the consequent loss of biological function, which may trigger or exacerbate the inflammatory response (Brown and Mackey, 1968). The optimized phenolic extract of the three-plant mixture exhibited pronounced inhibition of heat-induced BSA denaturation, with an IC_{50} of 4.33 ± 0.15 ug/mL (Table 5), indicative of a marked *in vitro* anti-inflammatory potential at very low extract concentrations.

The antioxidant and anti-inflammatory activities observed for the optimized mixture may be rationalized through a set of complementary molecular mechanisms. The DPPH and ABTS radical-scavenging activities are principally attributable to hydrogen atom transfer (HAT) and single-electron transfer (SET) mechanisms, facilitated by hydroxyl-rich phenolic compounds such as gallic acid and catechins, which stabilize reactive radical species through electron delocalization across their aromatic ring systems (Platzer *et al.*, 2021). The ferrous ion chelating activity, in turn, may be attributed to the capacity of catechol and galloyl groups— characteristic of gallic acid and quinic acid — to form thermodynamically stable complexes with Fe^{2+} ions, thereby inhibiting Fenton reaction-mediated hydroxyl radical generation and attenuating iron-catalyzed oxidative damage (Andjelkovic *et al.*, 2006). The *in vitro* anti-inflammatory activity, as assessed by inhibition of BSA denaturation, may be attributed to non-covalent interactions between polyphenolic constituents and albumin through hydrogen bonding and hydrophobic interactions, thereby stabilizing its native conformation of the protein against heat-induced denaturation (Feng *et al.*, 2023).

Furthermore, the significant enrichment of the optimized mixture in rutin, catechin, and epicatechin compared with the individual plant extracts suggests the occurrence of complementary phytochemical interactions within the mixture. Such synergy has been demonstrated in the literature to enhance antioxidant capacity through co-solubilization and mutual protection of phenolic compounds during extraction, resulting in greater bioactive compound stability and biological activity than would be predicted from the individual extracts in isolation (Bernal-Mercado *et al.*, 2018). Collectively, these complementary mechanisms provide a mechanistic rationale for the superior antioxidant and anti-inflammatory activities of the optimized formulation compared with the individual plant extracts.

3.6.2 Cytotoxicity Assessment

The capacity of certain bioactive substances to induce hemolysis of human erythrocytes constitute one of the most critical safety parameters in the evaluation of compounds intended for pharmaceutical or nutraceutical applications (Pietkiewicz *et al.*, 2010). The hemolytic activity of the phenolic extract of the optimal plant mixture was accordingly assessed across a range of concentrations.

The results demonstrated no statistically significant differences in the percentage of hemolysis across the extract concentrations of 1, 2, 3, 4 mg/mL, with only a low percentage of hemolysis (13.38%) recorded at the highest concentration tested (5 mg/mL). These findings confirm the absence of significant cytotoxic effects on human erythrocytes at all concentrations evaluated, including at the highest tested concentration of 5 mg/mL, and are consistent with the biosafety profile expected of polyphenol-rich plant extracts intended for use in food or health applications.

Table 6. HPLC-MS/MS Analysis of Clove, Pomegranate, Walnut, and Optimized Mixture Extracts

Compounds	Clove (µg/g)	Pomegranate (µg/g)	Walnut (µg/g)	Mixture (µg/g)
Gallic acid	7947 ± 256^{a}	748 ± 31^{c}	2076 ± 108^{b}	1952 ± 62^{b}
Quinic acid	5235 ± 80^{a}	39 ± 2.7^{c}	1035 ± 68^{b}	1100 ± 18^{b}
Rutin	<0.8	11 ± 0.4^{b}	8.7 ± 0.5^{b}	23 ± 1.4^{a}
Kaempferol	202 ± 7.1^{a}	2.4 ± 0.01^{c}	29 ± 0.5^{b}	33 ± 1.3^{b}
Naringenin	73 ± 1.2^{a}	0.9 ± 0.1^{c}	14.8 ± 0.6^{b}	15 ± 0.1^{b}
Catechin	<0.4	194 ± 42^{a}	90 ± 2.6^{b}	232 ± 3.9^{a}
Epicatechin	<0.4	31 ± 6.5^{ab}	21 ± 0.6^{c}	45 ± 1.2^{a}
Apigenin	1.1 ± 0.1^{a}	0.5 ± 0.4^{a}	0.8 ± 0.04^{a}	0.9 ± 0.2^{a}
3.4.5-Trimethoxycinnamic acid	2.2 ± 0.3^{a}	0.2 ± 0.0^{b}	0.25 ± 0.07^{b}	0.5 ± 0.1^{b}
Vanillin	353.44 ± 8.5^{a}	2.5 ± 0.1^{d}	64 ± 2^{c}	78 ± 3.6^{b}
Quercetin	261 ± 11^{a}	18 ± 0.8^{c}	127 ± 1.8^{b}	117 ± 0.4^{b}
Phloretin	<0.1	<0.1	< 0.1	< 0.1
Ferulic acid	41 ± 0.6^{a}	0.9 ± 0.2^{c}	10.4 ± 1.8^{b}	10 ± 0.1^{b}
Citric acid	8372 ± 226^{c}	12376 ± 102^{a}	11087 ± 42^{b}	10962 ± 63^{b}
Cinnamic acid	22 ± 0.6^{a}	2 ± 1.69^{b}	5.5 ± 0.7^{b}	5 ± 0.6^{b}

Data are expressed as mean values ± standard deviations. Mean within the same line sharing the same letter is not different ($p > 0.05$)

3.7 LC-MS/MS Phenolic Profiling

LC-MS/MS analysis of the individual extracts of clove, pomegranate bark, and walnut husk, as well as of the optimal plant mixture, revealed distinct phenolic and organic acid profiles. The results are summarized in Table 6.

3.7.1 Phenolic Characterization of Individual Plant Extracts

The phenolic profiles of the individual plant extracts suggest distinct contributions to the overall biological activity of the system. Clove exhibited the highest concentrations of gallic acid (7,947.87 ± 255.68 µg/g) and quinic acid (5,235.29 ± 79.53 µg/g) among the three individual extracts (Table 6), indicative of a particularly strong antioxidant potential, further corroborated by the presence of kaempferol, quercetin, and citric acid. Pomegranate bark was found to contain 1.47-fold more citric acid than clove, along with catechin and epicatechin (Table 6), suggesting a dual contribution to both antioxidant and anti-inflammatory activities, despite its comparatively lower gallic acid content (747.52 ± 30.79 µg/g). Walnut husk, characterized by the presence of citric acid, quinic acid, catechin, and quercetin, similarly exhibited a phytochemical profile consistent with antioxidant and anti-inflammatory biological activities.

3.7.2 Phenolic Characterization of the Optimal Plant Mixture

The optimal three-plant mixture demonstrated an enriched and more diverse phytochemical profile relative to the individual single-plant extracts, which is anticipated to confer enhanced biological activities through synergistic phytochemical interactions. The mixture retained substantial concentrations of gallic acid (1,952.79 ± 61.98 µg/g), quinic acid (1,100.25 ± 18.49 µg/g), and citric acid (10,962.00 ± 63.00 µg/g) (Table 6), supporting its antioxidant activity. Notably, the mixture exhibited significantly elevated concentrations of rutin (22.80 ± 1.39 µg/g), catechin (232.00 ± 3.90 µg/g), epicatechin (45.00 ± 1.20 µg/g), and further flavonoids as displayed in Table 6 suggesting the occurrence of complementary phytochemical interactions, that may enhance the antioxidant and anti-inflammatory properties of the formulation.

The optimal mixture presented a more balanced and enriched profile than any of the individual extracts. Gallic acid (1,952.79 ± 61.98 µg/g) and quinic acid (1,100.25 ± 18.49 µg/g) concentrations in the mixture were lower than those recorded for pure clove but higher than those of pomegranate bark and walnut husk (Table 6), reflecting a substantial antioxidant potential. Most notably, the mixture demonstrated a marked increase in rutin (22.80 ± 1.39 µg/g) relative to clove (< 0.8 µg/g), pomegranate bark (10.65 ± 0.37 µg/g), and walnut husk (8.66 ± 0.53 µg/g), alongside elevated concentrations of kaempferol (33.41 ± 1.26 µg/g) and naringenin (15.57 ± 0.07 µg/g) levels (Table 6).

4 Discussion

The findings of the present study shed light on the complex interactions that occur between plant-derived antioxidant compounds and their capacity to collectively determine the overall biological activity of a mixed formulation. The systematic analysis conducted herein revealed several critical principles governing the preparation of optimized antioxidant mixture from plant extracts. The formulation was optimized to maximize total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity across a three-component plant matrix system comprising clove, pomegranate bark, and walnut husk and the resulting optimal formulation was subsequently characterized for its anti-inflammatory and cytotoxic properties. It is well established in the literature that bioactive compounds within complex plant matrices can interact in ways that either attenuate or augment the overall antioxidant activity within a sample (Bassolé and Juliani, 2012). phytochemicals interactions have been reported to produce additive, antagonistic, indifferent, or synergistic effects on biological activity, depending upon the chemical nature and relative proportions of the constituent compounds (Ouedrhiri *et al.*, 2016).

Total phenolic content (TPC), total flavonoid content (TFC), and DPPH radical-scavenging activity were selected as the primary optimization response variables to identify the optimal formulation. A D-optimal ABCD mixture design was subsequently employed to model the combined effects of the three plant powders and determine the optimal proportional contribution of each component.

Total Phenolic Content

The TPC values varied considerably across the plant matrices. The clove extract exhibited the highest TPC (290.41 ± 2.67 mg GAE/g of extract), a value closely consistent with that reported by El-Maati *et al.* (2016) (293 mg GAE/g of extract). Pomegranate bark ranked second, with a TPC of 194.32 ±3.74 mg GAE/g of extract, marginally lower than the value of 205.07 mg GAE/g of extract reported by Marsoul *et al.* (2020). In contrast, walnut husk displayed the lowest TPC (47.05 ± 4.82 mg GAE /g of extract), a value broadly comparable to that reported by Taheri *et al.* (2020) (61.34 mg GAE/g of extract). These findings indicate that the phenolic richness of the individual plant matrices differs markedly, and confirm that clove and pomegranate bark constitute the principal contributors to the TPC of the optimized formulation.

Total Flavonoid Content

The TFC of the clove extract was consistent with the value reported by El-Maati *et al.* (2016) (12 mg EQ g^{-1} of extract). The TFC of pomegranate bark (13.94 mg/EQ g^{-1} of extract) was slightly higher than that reported by Jaisinghani *et al.* (2018), (10 mg/g EQ extract). In contrast, the TFC of walnut husk (7.91 ± 0.10 mg QE/g of extract) was approximately two-fold higher than the value of 4.15 mg QE/g of extract reported by Kulačanin *et al.* (2020). These observations further support the major contribution of pomegranate bark to the flavonoid richness of the optimized formulation.

The botanical composition of the mixture formulation plays a critical and determinant role in shaping its phytochemical profile, and the systematic adjustment of component proportions through mixture design methodology constitutes an effective strategy for the targeted modulation of the phytochemical composition and bioactivity of the formulation.

The discrepancies in TPC and TFC observed between the present study and previous published data may be attributable to multiple factors, including cultivar type, geographical origin, climatic conditions, maturity stage at harvest, agricultural practices, post-harvest storage conditions, and extraction procedures — in particular solvent composition and extraction methodology (Poyrazoğlu *et al.*, 2002). Two additional factors specific to the plant matrices investigated in the present study warrant particular attention. With respect to pomegranate bark, the available evidence indicates that the phenolic content of the peel progressively decreases during fruit ripening, with earlier harvest stages associated with higher phenolic content and antioxidant activity; early harvest therefore represents the optimal stage for valorizing pomegranate bark as a phenolic-rich co-product. Similarly, immature walnut husks exhibited the highest phenolic and condensed tannin contents, with both declining progressively as the fruit develops and matures. Collectively, these considerations indicate that the maturity stage at harvest constitutes a major determinant of the phytochemical composition of both pomegranate bark and walnut husk, and may therefore partially account for the quantitative differences in TPC and TFC observed between the present study and previously published data.

DPPH Radical Scavenging Activity

The DPPH radical-scavenging activities recorded for the individual plant extracts were broadly consistent with values reported in the published literature. The DPPH inhibition percentage obtained for pomegranate bark was comparable to the 80.12% reported by Fourati *et al.* (2020) , whilst the value recorded for clove was in close agreement with the 75.37% reported by Saraswathi *et al.* (2020). The inhibition percentage recorded for walnut husk in the present study was marginally higher than the 15.92% reported by Latos-Brozio and Masek (2019). Interestingly, mixture formulations enriched in pomegranate bark — in particular Experiments 8 and 10 — consistently exhibited higher DPPH radical scavenging activity than other formulations, suggesting that the antioxidant capacity of the mixtures is governed not only by the total phenolic concentration but also by the qualitative composition and the structural diversity of the constituent phenolic compounds. This observation is further supported by the findings of Kainama *et al.* (2020), who demonstrated that TPC and TFC contributed 18.8% and 24.0%, respectively, to the total radical scavenging capacity of plant extracts, highlighting the differential and complementary contributions of these compound classes. The LC-MS/MS profiling data further supports this interpretation, as the optimized formulation was reported to be enriched in catechin, epicatechin, and rutin, flavonoids well recognized for their high hydrogen-donating capacity and efficient free radical-scavenging properties.

The optimal plant mixture extract demonstrated a notably low DPPH IC_{50} value (50.97 ± 0.31 µg/mL), indicative of strong radical-scavenging capacity, which was superior to that of the synthetic reference antioxidant BHA (IC_{50} = 87.81 ± 3.7 µg/mL). This result is consistent with the IC_{50} of 53.54 µg/mL reported by Saraswathi *et al.* (2020) for clove extract under comparable experimental conditions. These findings demonstrate that the optimized formulation successfully preserved the high antioxidant potential of clove while simultaneously benefiting from the complementary phytochemical contributions of pomegranate bark and walnut husk.

Furthermore, the optimized formulation exhibited substantially lower IC_{50} values for both DPPH and ABTS scavenging activity compared with the values reported by Zeghad *et al.* (2019) for pomegranate bark extract (DPPH IC_{50} = 600 ± 0.003 µg/mL; ABTS IC_{50} = 580 ± 0.003 µg/mL) and markedly outperformed the modest DPPH inhibition (15.92 ± 0.08%) and ABTS inhibition (19.84 ± 0.99%) reported for walnut husk by Latos-Brozio and Masek (2019). The improved antioxidant performance of the optimized formulation relative to the individual plant extracts may be attributable to three-component mixture, which integrates the phenolic acid-rich profile of clove with the flavonoid-rich profile of pomegranate bark whilst retaining the bioactive compounds from walnut husk. Collectively, the optimized mixture represents a promising natural source of antioxidants with potential applications in functional foods and nutraceutical formulations.

Ferrous Ion Chelating Activity and Anti-Inflammatory Activity

Yuan *et al.* (2005) reported that compounds with iron chelating activity generally contain two or more of the following functional groups: –OH, –SH, –COOH, $–PO_3H_2$, –CO, $–NR_2$, –S, and –O. Several of these functional groups are present in the phenolic compounds identified in the optimized formulation by LC-MS/MS analysis — including gallic acid, catechin, epicatechin, rutin, and quercetin — which may account for the pronounced Fe^{2+}chelating activity of the formulation and its capacity to limit metal-catalyzed oxidative reactions through sequestration of free ferrous ions.

Beyond its antioxidant properties, the optimized formulation additionally exhibited significant *in vitro* anti-inflammatory activity, with an IC_{50} value of 4.33 ± 0.15 µg/mL in the BSA denaturation assay. This activity is likely attributed to the concerted action of the identified phenolic constituents, particularly flavonoids and phenolic acids, which have been extensively reported to inhibit heat-induced protein denaturation, scavenge reactive oxygen species, and modulate inflammatory pathways through the regulation of pro-inflammatory mediators and signaling cascades (Cory *et al.*, 2018; Prince *et al.*, 2019).

Cytotoxicity and Hemolytic Activity

The optimal formulation induced a low percentage of erythrocyte hemolysis (13.38%) at the highest concentration tested (5 mg mL^{-1}), confirming its compatibility with human erythrocytes under the experimental conditions applied and indicating a favorable safety profile for potential biomedical, nutraceutical, or food applications.

LC-MS/MS Profiling and Phytochemical Synergy

LC-MS/MS analysis provided further mechanistic support for the observed biological activities by demonstrating that the optimized formulation contained significantly higher concentrations of rutin, catechin, and epicatechin than any of the individual plant matrices. Specifically, rutin increased from < 0.80 µg/g in clove, 10.65 ± 0.37 µg/g in pomegranate bark, and 8.66 ± 0.53 µg/g in walnut husk to 22.80 ± 1.39 µg/g in the optimised formulation. Catechin reached 232.00 ± 3.90 µg/g in the mixture compared with < 0.4, 194.00 ± 42.00, and 90.00 ± 2.60 µg/g in clove, pomegranate bark, and walnut husk, respectively. Epicatechin increased to 45.00 ± 1.20 µg/g compared with < 0.4, 31.00 ± 6.50, and 21.00 ± 0.60 µg/g. These marked elevations in flavonoid concentrations in the mixture relative to the individual components suggest the occurrence of synergistic interactions that promote flavonoid stability and extraction, which is consistent with previous investigations demonstrating that combining phenolic compounds can promote co-solubilization and mutual protection of the most labile compounds during extraction (Bernal-Mercado *et al.*, 2018).

These findings additionally support the prospective utilization of the optimized formulation as a natural functional ingredient. However, direct incorporation into food matrices should preferably be conducted at concentrations below the IC_{50} values determined *in vitro,* in order to preserve sensory acceptability and consumer palatability. A sensory-by-design approach, exploiting the specific polyphenol–protein and polyphenol–polysaccharide interactions occurring within the food matrix, may reduce undesirable organoleptic attributes such as bitterness and astringency while preserving the antioxidant potential of the formulation (Ferreira *et al.*, 2026). Incorporation trials in real food systems, complemented with descriptive and hedonic sensory evaluations, therefore represent a logical next step prior to considering industrial-scale applications.

Study limitations and perspectives

The present study is subject to several limitations that should be duly acknowledged and considered in the interpretation of its findings. First, the antimicrobial activity of the optimized mixture was not evaluated. Whilst the primary objective of this investigation was to characterize its antioxidant and anti-inflammatory properties, the assessment of antimicrobial potential would constitute a valuable extension, particularly in light of the increasing global challenge of antimicrobial resistance. Recent studies employing analogous mixture design approaches with pomegranate by-products have highlighted the relevance of this strategy by demonstrating synergistic antioxidant and antimicrobial activities, together with *in vivo* validation (Skenderidis *et al.*, 2021). Second, the synergistic interactions among the extracts were inferred from the statistical significance of the interaction terms within the mixture model; however, the Fractional Inhibitory Concentration (FIC) index, a standardized metric commonly employed for the rigorous characterization of interactions in antimicrobial studies, was not determined. The calculation of the FIC indices will therefore be incorporated into future investigations. Third, the potential influence of the optimized mixture on the composition and functionality of the commensal intestinal microbiota was not assessed and warrants dedicated investigations employing validated *in vitro* and *in vivo* models. Finally, as all findings were derived exclusively from *in vitro* assays, they do not fully recapitulate the bioavailability, metabolic transformation, pharmacokinetic profile, and systemic toxicity of the investigated compounds in a living organism. Future research will therefore focus on the *in vivo* validation of the biological activities identified *in vitro*, the evaluation of antimicrobial activity together with FIC determination, the assessment of the mixture's effects on the microbiota, and the exploration of its potential applications in pharmaceutical, nutraceutical, and food products.

5 Conclusions

The present study demonstrated that a D-optimal mixture design constitutes a rigorous and an effective methodological strategy for the simultaneous optimization of phytochemical composition and biological activity in a formulation combining *Syzygium aromaticum* (clove), *Punica granatum* bark (pomegranate) and *Juglans regia* husk (walnut).The optimized formulation exhibited elevated phenolic and flavonoid contents, alongside remarkable antioxidant and anti-inflammatory activities, low hemolytic activity towards human erythrocytes, and a phytochemical profile enriched in biologically significant constituents — including catechin, epicatechin, rutin, gallic acid, quinic acid, and citric acid — as characterized by LC-MS/MS analysis.

The LC-MS/MS profiling data suggest that the enhanced biological activities of the optimized formulation are associated with a more balanced phytochemical composition resulting from the complementary and synergistic contributions of the three plant matrices. These findings highlight the scientific value and practical utility of combining edible plant materials and agro-food by-products through a rigorous statistical mixture design framework as a strategy for the development of natural formulations with enhanced biological properties.

From an application perspective, the optimized formulation represents a promising source of natural bioactive compounds for incorporation into food, nutraceutical, and pharmaceutical applications. Nevertheless, further investigations — encompassing bioavailability assessment, long-term safety evaluation, stability studies in complex food matrices, comprehensive sensory acceptability testing, and in vivo efficacy validation — are required before practical or industrial implementation of this formulation can be responsibly considered.

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