Research, Society and Development, v. 14, n. 9, e9114949574, 2025
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v14i9.49574

Optimizing the solvent mixture for extracting phenolic compounds with antioxidant and phytotoxicity activity from *Myrcia fallax* leaves using the I-optimal design

Otimização da mistura de solventes para extração de compostos fenólicos com atividade antioxidante e fitotóxica de folhas de Myrcia fallax usando o I-optimal design

Optimización de la mezcla de solventes para la extracción de compuestos fenólicos con actividad antioxidante y fitotoxicidad de hojas de Myrcia fallax utilizando el I-optimal design

Received: 09/15/2025 | Revised: 09/25/2025 | Accepted: 09/26/2025 | Published: 09/27/2025

Murilo Henrique Maciel de Camargo
ORCID: https://orcid.org/0009-0008-7969-627X
University of São Paulo State, Brazil
E-mail: murilo_maciel97@outlook.com
Vittor Paulo Vieira da Costa
ORCID: https://orcid.org/0000-0002-8617-8066
University of São Paulo State, Brazil
E-mail: vittorpcosta@gmail.com
Catarina dos Santos
ORCID: https://orcid.org/0000-0002-9881-527X
University of São Paulo State, Brazil
E-mail: catarina.santos@unesp.br

Abstract
The objective of this study was, through an I-optimal design, to enhance the extraction of total phenolic content (TPC) and total flavonoid compounds (TFC) from *Myrcia fallax* (A. Rich) DC leaves from a mixture of three solvents of Ethanol, Methanol, and water (maximum 50%) to get the best yield, antioxidant and phytotoxic results. Methanol provided higher percentage yields of extraction, while for Total Phenolic Content (TPC) and Total Flavonoid Content (TFC), better values were given to methanol-water. Also, pure ethanol had better results regarding antioxidant activity using DPPH and the ABTS methods. For phytotoxicity activity evaluation, extracts from methanol-water were indicated due to their ability to inhibit radicle length and stimulate hypocotyl growth compared to the control. Then, the I-optimal design allowed us to correlate the *Myrcia fallax* extracts with their biological activities from solvent-different polarity mixtures.
Keywords: *M.fallax*; I-optimal; Antioxidant activity and phytotoxicity.

Resumo
O objetivo deste estudo foi, por meio de um delineamento I-ótimo, aumentar a extração do conteúdo fenólico total (TPC) e dos compostos flavonoides totais (TFC) das folhas de Myrcia fallax (A. Rich) DC a partir de uma mistura de três solventes: etanol, metanol e água (máximo de 50%) para obter os melhores resultados de rendimento, antioxidantes e fitotóxicos. O metanol proporcionou maiores rendimentos percentuais de extração, enquanto para o conteúdo fenólico total (TPC) e o conteúdo flavonoide total (TFC), os melhores valores foram atribuídos ao metanol-água. Além disso, o etanol puro apresentou melhores resultados em relação à atividade antioxidante usando os métodos DPPH e ABTS. Para a avaliação da atividade fitotóxica, os extratos de metanol-água foram indicados devido à sua capacidade de inibir o comprimento da radícula e estimular o crescimento do hipocótilo em comparação ao controle. Então, o delineamento I-ótimo nos permitiu correlacionar os extratos de Myrcia fallax com suas atividades biológicas a partir de misturas de diferentes polaridades de solventes.
Palavras-chave: *M.fallax*; I-optimal; Atividade antioxidante e fitotoxicidade.

Resumen
El objetivo de este estudio fue, a través de un diseño I-óptimo, mejorar la extracción del contenido fenólico total (CPT) y los compuestos flavonoides totales (CFT) de las hojas de Myrcia fallax (A. Rich) DC a partir de una mezcla de tres solventes de etanol, metanol y agua (máximo 50%) para obtener los mejores resultados de rendimiento, antioxidantes y fitotóxicos. El metanol proporcionó mayores rendimientos porcentuales de extracción, mientras que para el contenido fenólico total (CPT) y el contenido flavonoides totales (CFT), se dieron mejores valores al metanol-agua. Además, el etanol puro tuvo mejores resultados con respecto a la actividad antioxidante utilizando los métodos

DPPH y ABTS. Para la evaluación de la actividad fitotóxica, se indicaron extractos de metanol-agua debido a su capacidad para inhibir la longitud de la radícula y estimular el crecimiento del hipocótilo en comparación con el control. Luego, el diseño I-óptimo nos permitió correlacionar los extractos de Myrcia fallax con sus actividades biológicas a partir de mezclas de solventes de diferente polaridad.
Palabras clave: *M.fallax*; I-optimal; Actividad antioxidante y fitotoxicidad.

1. Introduction

The search for a natural and healthy lifestyle has increased interest in active compounds from natural sources such as plants. These compounds are used in pharmaceuticals, nutraceuticals, and cosmetics since they have antioxidant and anti-inflammatory properties and can treat cardiovascular and neurodegenerative illnesses and cancer. (Santos et al., 2018; Ozen et al., 2011).

In this context, phenolic compounds with antioxidant activity are one of the most promising groups since they are found in plants. The generally accepted mechanism for their antioxidant activity is the elimination of free radicals, contributing to reducing oxidative stress (Yashin et al., 2017). These radicals have been associated with the progression of several chronic diseases in humans. Some reports show that high levels of reactive oxygen species (ROS), responsible for oxidative stress, can induce the oxidation of proteins, lipids, and DNA, leading to changes in their normal functions (Afzal et al., 2023; Jomova et al., 2023).

Among the plant families rich in active phenolic compounds, the Myrtaceae family stands out as one of the most important in South America, Australia, and Tropical Asia. In Brazil, 23 genera and approximately 1034 species are distributed in all country regions (Costa et al., 2016). The genus *Myrcia* spp includes shrubs and small trees, where the isolated non-volatile compounds are generally flavonoids, tannins, acetophenone derivatives, and triterpenes (Cascaes et al., 2015; Santos et al., 2018).

Studies carried out by our research group correlated the different phenolic compositions of *Myrcia fallax* extracts with their antioxidant and antimicrobial activities (Santos et al., 2018). However, its phytotoxic activity (allelopathy) has scarcely been studied. This activity consists of biotic interference, where the donor plant releases bioactive metabolites (allelochemicals) into the environment, affecting, positively or not, the growth of adjacent vegetation (Li et al., 2010; Shan et al., 2023). Allelochemicals affect several metabolic processes in organisms, as they prevent cell division (Teerarak et al., 2010, 2012), affect enzymatic activity (Singh et al., 2009), modify membrane permeability (Imatomi et al., 2013), and inhibit the transport of electrons in photosynthesis and respiration (Abrahim et al., 2000).

Among polyphenols, flavonoids like quercetin and myricetin found in *M. fallax* stand out because they are phytotoxic, hypoglycemic, anti-hemorrhagic, and they have antioxidant activities (Cascaes et al., 2015; Imatomi et al., 2013; Souza Filho et al., 2006). These phenolics were correlated to phytotoxic activities since they act inside communication between cell membranes, regulating cell growth and inhibiting germination and seedling growth (Santos et al., 2018; Franco et al., 2015).

Polyphenols have a wide structural variety, so it is difficult to establish a standard extraction method for these compounds (Bucić-Kojić et al., 2011). Furthermore, the improvement of quality and the amount of biologically active phenolics in extracts is a challenging mission for pharmaceutical, cosmetic, and food industry products (Ćujić et al., 2016; Ribeiro De Souza et al., 2009; Zhang et al., 2018).

In recent years, statistical models have been successfully applied to minimize the number of experiments and identify the effect of interactions between experiments. These models provided satisfactory results for optimizing extraction procedures of secondary metabolites from plant leaves and fruits (Ćujić et al., 2016; DiCiaula et al., 2014). It is a useful tool, as it requires less time, labor, and resources compared to univariate procedures and enables access to a large amount of information (Candioti et al., 2014; Ferreira et al., 2007). It also allows the observation of interactions between variables and the prediction

of responses of the study system in a condition that has not been tested in practice, while minimizing the number of experiments. For this reason, it has been widely used in industry (Santos et al., 2020).

These statistical methods have been used to select solvents to enhance the extraction of biologically active compounds from plants, using different solvent systems and mixtures (binary or ternary). However, their extraction effectiveness depends on the solvents' polarity and affinity for the target metabolites (DiCiaula et al., 2014; Garcia-Salas et al., 2010).

Therefore, as explained above, the objective of this study was, through an *I-optimal* experimental design, to enhance the extraction of total phenolic and total flavonoid compounds from *Myrcia fallax* (A. Rich) DC leaves from a mixture of three solvents (water, ethanol, and methanol), to get extracts with the best antioxidant and phytotoxic activities.

2. Methodology

An experimental research of a quantitative nature was conducted (Pereira et al., 2018), using the statistical treatment of data with mean values and standard deviation (Shitsuka & Rabbith, 2024).

Drugs and chemicals

Analytical standards, such as gallic acid and quercetin, as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH), ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and Trolox (6-Hidroxi-2,5,7,8-tetrametilchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other analytical-grade reagents were purchased locally. Lettuce seedlings were provided by Isla Sementes Ltda (Crespa Grand Rapids).

Plant collection

Myrcia fallax leaves were collected by Catarina dos Santos in December 2015 at the Instituto Florestal e Estações Experimentais—Floresta Estadual de Assis at 22°35′ 51.6468" Lat O – 50°23′ 1.9661" Long W, Assis, State of São Paulo, Brazil. Dr. Antonio C. G. Melo identified the specimen, and a voucher (no. 43.522) was deposited in the Herbarium D. Bento Pickel, São Paulo, SP, Brazil, in compliance with legal norms and registered as Cotec 206108-005.298/2009.

Extract preparation

From 5 g of dried and crushed leaves, 50 mL of a solvent mixture was added according to the solvent proportions defined by the I-optimal design from Design Expert software (Design Expert version 23.1.5, Stat-Ease Corporation, Minneapolis, MN, USA).

Ethanol and Methanol were studied in proportions ranging from 0 to 100% and water from 0 to 50%, including 15 experiments (Table I). This solution was kept in dynamic maceration for two hours and then filtered. From the residue, the same procedure was repeated two more times. After filtration, these combined solutions were concentrated in a rotary evaporator (Heidolph, Hei Vap precision, Germany), followed by freeze-drying (Liotop, L-101, Liobras, Brazil) of the aqueous residue to provide the extracts that are going to be used in the analysis of the total phenol and flavonoid content, antioxidant activity, and allelopathic activity.

Polyphenol (TPC) and Flavonoid Content (TFC)

The total polyphenol content (TPC) was determined using Folin-Ciocalteu's assay. Thus, Folin–Ciocalteau (2.5 mL, 10% v/v) and sodium carbonate (2.0 mL, 4% w/v) solutions were added to 0.5 mL of ethanolic solutions of *M. fallax* extracts (1.0 mg/mL w/v), followed by thorough mixing, keeping for 120 min in the dark, at room temperature (rt), and measure of

absorbance at 750 nm. A calibration curve (0.5–40 µg/mL) of gallic acid was used to express the results as mg gallic acid equivalent (GAE)/g dry extract (Kalia et al., 2008). The aluminum chloride colorimetric assay determined the total flavonoid (TFC) content. 1.5 mL of absolute ethanol, 0.1 mL 10% (w/v) aluminum chloride, 0.1 mL potassium acetate 1M, and 8.0 mL distilled water were added to 0.5 mL ethanolic solution of *M. fallax* extracts (1.0 mg/mL). After homogenization, the solution was kept for 30 minutes in the dark at rt. So, the absorbance was measured at 425 nm. Quercetin was used as the standard, and a calibration curve (0.5–40 µg/mL) was used to express the results as mg of quercetin equivalent (QE)/g of dry extract (Kalia et al. 2008). All experiments were carried out in triplicate.

Antioxidant Activity

Antioxidant activity was measured through the scavenging ability of radical DPPH (2,2-diphenyl-1-picrylhydrazyl) according to Enujiugha et al., with modifications (Enujiugha et al., 2012). To 2.0 mL of the ethanolic solution of *M. fallax* extracts (1.56 to 200 µg /mL) was added 0.5 mL DPPH 0.03% in methanol, in triplicate. After that, the samples were stirred and kept in darkness at rt for 30 min, and the absorbance was determined at 517 nm. Methanol was the negative control, and quercetin and gallic acid were the positive controls. The percentage of DPPH radical scavenging activity of the extract was calculated according to the following Equation (1):

$$\%\ \text{Inhibition} = \left(1 - \frac{Abs_{sample}}{Abs_{control}}\right) * 100 \quad (1)$$

Abs_{sample} is the absorbance of the extracts measured in different concentrations, and $Abs_{control}$ is the absorbance of DPPH in methanol (blank).

Equation 1 results were plotted as a graphic of % DPPH inhibition versus concentration using MATLAB (R2012, The MathWorks, Inc., Boston, MA, USA). From this graphic, the extract concentration necessary to decrease the initial concentration of the DPPH radical by 50% (EC_{50}) was measured and expressed in µg/mL.

The antioxidant activity of the extracts was also measured through the $ABTS^{*+}$(2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) scavenging ability radical, according to Ruffino et al. (Rufino et al., 2010). Thus, the $ABTS^{*+}$ radical was prepared by mixing 0.88 µL of 140 mM Potassium Persulfate with 5 mL of ABTS 7 mM solution, undergoing vigorous agitation, and kept in the dark for 16h at room temperature. After this period, 1 mL of the radical mixture was diluted in distilled water until the absorbance reached 0.7 ± 0.05 nm at the wavelength of 734 nm. Then, 3 mL of this radical solution was added to test tubes with 0.30 mL of solutions (25 to 400 µg/mL) of the extracts of the determined design. The mixture was stirred vigorously and maintained for 6 minutes in the dark and at room temperature. The new absorbances were measured at the same wavelength. Ethanol was used as the negative control, and Trolox was used as the positive control. All tests were performed in triplicate, and the results are expressed in µM Trolox/mg dry extract.

Assessment of phytotoxic activity

The phytotoxic bioassay was performed according to Silvestre et al., with small modifications (Silvestre et al., 2013). Petri dishes (6 cm in diameter) were previously disinfected with 2% sodium hypochlorite (v/v), rinsed with 70% ethanol (v/v), washed with distilled water, and dried in an oven for two hours at 105 ºC. Then, the plates were coated with two sheets of filter paper and moistened with 2.0 mL of 5 mg extract/mL ethanol each. After solvent drying, the sheets of filter paper were moistened with 2.0 mL of distilled water, and ten lettuce seedlings were placed on each plate. Distilled water was used as a negative control. The experiments were conducted in a seed germinator (Solab, model SL-224) at a temperature of 25±1°C and

a photoperiod of 12 hours of light/dark cycle. The effect of the extracts on the radicle length and hypocotyl growth was evaluated four days after the experiment. The solvents had no significant effect on germination or development compared to the negative control ($p>0.05$, data not shown). The experimental design was completely randomized, and each treatment was applied in quadruplicate.

Statistical Analysis

The solvent mixtures of various water, methanol, and ethanol proportions were prepared using an I-optimal design (Table I). Each component of the mixture was measured in a proportion of 0 to 100%, with a restriction of 50% for water solvent, including 15 experiments, among which there were three repetitions and three combinations to perform lack-of-fit tests for models associated with the response variables, according to Design Expert Software (version 12, Stat-Ease, Inc., Minneapolis, MN, USA). For each of the responses evaluated, the most appropriate model was adopted. The statistical significance tests of the model, its parameters, and the lack-of-fit test were performed with a significance level of 95% ($\alpha=0.05$). For statistical analyses involving the allelopathic activity of each of the extracts concerning the control (water), ANOVA - One Way (Dunnett's Test) was used in the Minitab® software (version 18.1 demo, State College, Pennsylvania, USA).

3. Results and Discussion

Fitting the Experimental Results with the Model

The extraction of substances with potential biological activity in plants is influenced by their chemical complexity and the polarity of the solvent used for extraction. Applying design modeling and multivariate analysis of solvents and their mixtures could achieve good selectivity in the extraction of these molecules (Mendes et al., 2019). The interactions among extractive solvents, provided by the I-optimal design, had important implications for understanding the effects of these mixtures of solvents on the analyzed parameters, since the aim of modeling data was to identify the terms that explain the relationship between the factors and the response. The results of the solvent mixture interaction with the response variable are stated in Table 1.

Table 1. Solvents and results of the experimental matrix of the *Myrcia fallax* I-optimal design.

Extract	Solvent [a] (v/v/v)	Yield (%)[b]	TPC (mgGAE/g)[c]	TFC (mgQ/g)[d]	EC_{50} (mg/ml)[e]	ABTS (μM_{Te}/g)[f]	Radicle (cm)[g]	Hypocotyl (cm)[h]
1	73.8:0.0:26.2	17.68	280.51±0.53	18.30±0.58	10.39±5.24	2.80±0.23	3.22±0.94	0.55±0.10
2	34.1:40.0:25.9	21.06	203.70±0.89	16.57±0.35	10.61±5.53	3.08±0.07	3.18±1.01	0.58±0.10
3	16.7:33.3:50.0	8.40	520.36±1.65	16.78±0.15	8.64±4.07	2.67±0.05	3.83±0.63	0.67±0.11
4	100.0:0.0:0.0	21.45	232.68±1.17	11.58±0.16	8.65±3.72	2.96±0.28	1.89±0.50	0.56±0.10
5	56.3:21.9:21.8	17.43	226.16±0.25	15.92±1.44	11.35±6.54	3.85±0.11	2.94±0.96	0.59±0.09
6	73.8:0.0:26.2	17.53	260.94±0.48	20.04±0.22	6.69±3.68	3.01±0.06	2.72±0.67	0.64±0.13
7	0.0:100.0:0.0	24.81	209.49±0.40	22.24±0.32	14.16±5.45	2.84±0.16	1.71±0.57	0.50±0.09
8	77.6:22.4:0.0	24.86	223.99±0.37	20.42±0.11	9.72±5.39	2.86±0.12	1.75±0.49	0.52±0.08
9	22.2:70.8:7.0	19.36	435.58±1.85	18.74±0.56	10.04±5.39	2.68±0.08	2.11±0.56	0.56±0.10
10	34.1:40.0:25.9	15.43	398.62±0.63	19.21±0.70	11.89±7.31	2.49±0.16	1.33±0.42	0.62±0.11
11	51.5:48.5:0.0	26.12	249.35±0.58	19.46±0.37	8.58±3.95	2.88±0.21	1.08±0.32	0.54±0.13
12	0.0:58.3:41.7	24.50	367.46±0.51	9.90±1.79	12.43±7.62	2.92±0.07	1.41±0.56	0.66±0.16
13	0.0:58.3:41.7	18.00	319.64±3.14	10.21±0.18	10.65±6.16	3.79±0.09	1.15±0.28	0.68±0.14
14	34.1:40.0:25.9	16.99	310.22±0.44	18.09±0.18	11.15±5.57	2.41±0.08	1.86±0.38	0.55±0.10
15	41.1:8.9:50.0	10.42	334.86±2.09	14.45±0.39	13.60±2.34	2.68±0.13	2.46±0.57	0.60±0.12

a.EtOH:MeOH:H_2O b.(Relative yield: g extract/g *M.falllax* leaves) c.Gallic acid (mg GAE/g) d. Quercetin (mgQ/g) e.amount of antioxidant required to reduce the initial DPPH concentration by 50% f. mM de Trolox/mg extract g, h. Negative control water (radicle 4.38±0.57 cm) (hypocotyl 0.49±0.08 cm). Source: Authors.

Analysis of variance (ANOVA) was used to evaluate the fitted mathematical model, with a confidence interval of 95% (Table 2-3). ANOVA analysis was done to choose the best model for yield, TPC, TFC, antioxidant activity (measured EC_{50} through the scavenging ability inhibition of DPPH radical, ABTS), and phytotoxicity (radicle length and hypocotyl growth). The regressions of the ANOVA model were evaluated using F statistics and a lack-of-fit test. A model was considered significant if the F-value is greater than the tabulated F-value ($p<0.05$) and the lack of fit is insignificant with a high p-value ($p>0.05$). An insignificant lack of fit means that the variation of the design points about their predicted values is much smaller than that of the replicates about their mean values.

Another guide for the good-fit model was the R^2 (coefficient of determination). R^2 measures the variation of a regression model, i.e., its suitability (Barros-Neto et al., 2022; Sapra, 2014). The closer the R^2 value is to unity, the better and more significant the model fits the real data; in other words, higher values indicate that the model explains more variation in the data or predictions. A good fit is considered R^2=70-90% (the model explains 70% to 90% of the variability), a moderate fit ($50\%<R^2<70\%$), and a poor fit $R^2<50\%$. Moreover, a low predicted R^2 means more unexplained variation in the system. However, using R^2 as the only indicator can be deceiving, even with high values, especially if the model is overfitted or there are issues such as multicollinearity, heteroscedasticity, or non-linearity (Shrestha, 2020).

So, for the reasons pointed out above, the Adjusted R^2 and Predicted R^2 values were also used to evaluate how well a regression model predicts responses for new observations. Adjusted R^2 can provide an accurate model that fits the current data, and the Predicted R^2 determines how likely it is that this model will be accurate for future data. Adjusted R^2 increases if the new predictor improves the model more than would be expected by chance (Miles, 2014). A good model was considered when the difference between the predicted R^2 value and the Adjusted R^2 value was less than 0.2 points, indicating no need to test other models (Barros-Neto et al., 2022).

Multicollinearity among the variables is examined using the coefficient of variation (CV). CV is a measure of standard deviation divided by the mean value, indicating the precision and repeatability of an assay. Expressed as a percentage, the ideal CV must always be the smallest possible, assuming that the variation is small and the data set is homogeneous. However, the higher the CV, the greater the dispersion in the variable. It is usual to consider CV<10% to indicate a reliable and precise model, indicating that the data are relatively homogeneous, 10-20 is good, 20-30 is acceptable, and CV>30 is not

acceptable (Pélabon et al., 2020). Finally, Adequate Precision was used to indicate the signal-to-noise ratio. It compares the predicted values at the design points to the average prediction error. Ratios above 4 indicate an acceptable model differentiation (Barros-Neto et al., 2022).

The statistical analysis by ANOVA showed a significant linear regression model for yield, TPC, and TFC (Yield: F=22.63, p=0.0001; TPC: F=13.50, p=0.0027; TFC: F=14.22, p=0.0009) at the 95% confidence level. The lack of fit was also insignificant (Yield: 1.08, p=0.5275, TPC: F=1.23, p=0.5142; TFC: F=3.66, p=0.1162). The coefficient of determination R^2 (Yield: 0.8045, TPC: 0.7715; TFC: 0.8258) indicated a good correlation between the experimental and predicted response values.

Table 2. ANOVA results for response surface models used in optimizing yield, TPC, and TFC on the parameters from *M. fallax* leaves extraction.

	Yield		**TPC[a]**		**TFC[b]**	
	Linear Model	**Lack of Fit**	**Linear Model**	**Lack of Fit**	**Reduced Quadratic Model**	**Lack of Fit**
F	22.63	0.7113	13.50	1.23	14.22	3.66
p	0.0001	0.5275	0.0027	0.5142	0.0009	0.1162
R^2	0.8045		0.7715		0.8258	
Adj-R^2	0.7689		0.7143		0.7677	
Pred-R^2	0.6800		0.5001		0.6478	
Adeq-R^2	12.3286		8.5591		11,2645	
% CV	13.19		10.19		10.20	

a. Total Phenolic Content b. Total Flavonoid Content. Source: Authors.

The difference between the Predicted R^2 and the Adjusted R^2 values was less than 0.2 points for all tested parameters. Also, Adequate Precision values had an adequate signal since a ratio greater than 4 (Table 2). The CV value is slightly greater than the ideal for yield (13.19), meaning there is some data dispersion. In general, the results above demonstrated that the variability of the models was compatible with the variability that occurred experimentally. So, the proposed models in Table II can be used to understand the effects of solvents on extracted yield, TPC, and TFC.

A better model's performance adequacy for R^2 would be more desirable for yield, TPC, and TFC. However, Adjusted R^2, Predicted R^2, Adequate Precision, and % CV presented good results; the model was considered valuable. In this case, even though accurate predictions cannot be obtained, they would clarify the factors that significantly influence a response, guiding subsequent, more detailed investigations.

For antioxidant activity (EC_{50} measured by DPPH radical), the linear model was significant (F=13.72, p=0.0018) with an insignificant lack of fit test (F=0.7108, p=0.6705). For antioxidant activity measured by ABTS radical, a reduced special cubic was the significant model (F=21.38, p=0.0004) with an insignificant lack of fit (F=0.2175, p=0.9385). For phytotoxicity analysis, radicle length (F=7.93, p=0.0088) with a reduced quadratic model and hypocotyl growth (F=11.21,= p=0.0036) with a linear model proved significant. Both parameters showed an insignificant fit (F=1.85, p=0.3243; F=0.2978, p=0.9050, respectively) (Table 3).

All tested parameters presented a difference between Adjusted and Predicted R^2 values of less than 0.2 points. Also, adequate precision values had an adequate signal since the ratio was greater than 4. For both antioxidant activities, the CV<10% demonstrated that the variability of the models was compatible with the variability that occurred experimentally. The CV value is good for radicle (16.64) but not acceptable for hypocotyl (30.23), meaning there is important data dispersion for this parameter (Table 3). These dispersions can be caused by the inhibitory and/or stimulating effects of the extracts on the radicle and hypocotyl compared to the negative control (water).

Table 3. ANOVA results for response surface models used in optimizing antioxidant and phytotoxicity on the parameters from *M. fallax* leaves extraction.

	EC_{50}[a]		ABTS		Radicle		Hypocotyl	
	Linear Model	Lack of Fit	Reduced Special Cubic Model	Lack of Fit	Reduce Quadratic Model	Lack of Fit	Linear Model	Lack of Fit
F	13.72	0.7108	21.38	0.2175	7.93	1.85	11.21	0.2978
p	0.0018	0.6705	0.0004	0.9835	0.00088	0.3243	0.0036	0.9050
R^2	0.7530		0.8891		0.7483		0.7135	
Adj-R^2	0.6981		0.8475		0.6539		0.6499	
Pred-R^2	0.5125		0.7768		0.6132		0.4589	
Adeq-R^2	9.4195		11,1411		7.7472		8.1323	
% CV	7.49		2.61		16.64		30.23	

a. concentration required to obtain a 50% antioxidant effect measured by DPPH radical. Source: Authors.

Effects of the solvent system on the response variable

Yield, Total phenol content (TPC) and total flavonoid content (TFC)

The selection of plant materials and the appropriate solvents and their combinations can effectively facilitate the extraction of bioactive compounds (Alara et al., 2021; Yan et al., 2022). Usually, a higher phenolic yield in extracts is expected when phytochemicals have a good binding with solvents. Water, methanol, and ethanol (polar solvents) have higher extract yields than nonpolar solvents (acetone and chloroform), especially for phenolic recovery (Ghaffar & Perveen, 2024). Also, these solvents, plus acetone and isopropyl alcohol, with different proportions of water, are classified as Generally Recognized As Safe Solvents (GRAS) for extraction applications (Keerthiga & Sridhar, 2022). Moreover, the extraction efficiency of phenolic compounds, including flavonoids, is increased by using a mixture of water and organic solvents such as methanol and ethanol (Bhadange et al., 2022). Also, hydroethanolic extraction is frequently stated as the most appropriate alternative to recover a higher yield of phenolic compounds from plants (Santos et al., 2020). In this study, polar solvents (ethanol, methanol, and water) were chosen for an I-optimal design with a water restriction of 50%. This restriction was established because most phenolic extraction includes 50 or 70% water and methanol or ethanol. The yield, the first parameter analyzed, is represented by the linear model in polynomial Equation 2 and Figure 1 (coded terms: e=EtOH, m=MeOH, w=H_2O):

"Yield%" = 23,11*e + 25,17*m – 1,28*w (2)

From Equation 2, it can be noted that the yields were better for solvent systems richer in methanol (+25.17), followed by ethanol (+23.11). These coefficients presented a positive sign, i.e., a positive effect (red zone, Figure 1a). For water, a negative effect (-1.28) was noted (blue zone, Figure 1a). From Table 1, the extracts that had a higher proportion of water, such as extract 3 (EtOH:MeOH:H_2O 16.5:33.5:50.0; 8.40%) and extract 15 (EtOH:MeOH:H_2O 41.0:9.0:50.0;10.42%), showed lower yields. Moreover, the yield increases when water is absent, such as in extract 11 (EtOH:MeOH:H_2O 51.5:48.5:0.0; 26.12%). Compared with existing literature, water proved to be an ineffective solvent for cold extraction at room temperature. Hot water (90°C) showed slightly better extraction yields for *Myrcia tomentosa, M. bella,* and *M. splendens* leaves (from 18.0% to 19.4%) compared to the results in Table I (Takao et al., 2015).

Phenolic compounds are secondary metabolites in plants characterized by at least one hydroxyl group attached directly to an aromatic ring. The yield and content of phenolic compounds vary due to the extraction parts and solvent polarity. These compounds' amounts and structures also change based on the harvest season (Dai & Mumper, 2010; Yan et al., 2022). Therefore, removing all phenolic classes from plants is not an easy job, and the solvent choice must always be made to get the

best phenolic extraction performance with maximum biological activity and minimal toxicity. Considering only the significant effects, the linear model for TPC is represented by polynomial Equation 3 and Figure 1(coded terms):

"TPC (mg GAE/g extract)" = 214.95*e + 236.29*m + 460.85*w (3)

All terms were positive; therefore, all solvents positively affected the recovery of phenolic compounds. Water showed the highest coefficient value from Equation 3 (+460.85) for phenolic extraction, followed by methanol (+236.29) and ethanol (+214.95). In Fig. 1b, it can also be seen that water-methanol resulted in a higher quantity of phenolic compounds (red zone). From Table I, extracts 12 and 13 (EtOH:MeOH:H_2O 16.7:33.3:50.0) showed 367.46±0.51 and 319.64±3.14 mg GAE/g extract, respectively. On the other hand, water-ethanol had a smaller positive effect for phenolic recovery compared to the methanol-water, as can be seen on the extract replicates 1 and 6 (EtOH:MeOH:H_2O 73.8:0.0:26.2) with 280.51±0.53 and 269.94±0.48 mg GAE/g extract, respectively. These results are superior to the previously published for the hydroethanolic extracts of *M. fallax* (EtOH:H_2O 70:30; 218.19±0.81 mg GAE/g extract) (Santos et al., 2018). A synergistic alcohol effect was observed in extract 3 (EtOH:MeOH:H_2O 16.7:33.3:50.0) with a 520.36±1.65 mg GAE/g extract recovery. The predominant positive effect of water and methanol may be due to the presence of polar substances such as phenolic glycosides: gallic and quinic acids, flavonoids as quercetin and myricetin glycosides, and tannins as HHDP derivatives (Santos et al., 2018)

The synergistic effect of alcohol or alcohol-water mixtures was already described in other Myrtaceae. In *Syzygium cumini* L. bark (Myrtaceae), TPC was tested using different ratios of ethanol or methanol mixed with water. For a similar ratio (70:30 v/v), the methanol yields higher TPC (47.22 ± 3.35 mg GAE/g) than ethanol (17.5 ± 4.5 mg GAE/g (Keerthiga & Sridhar, 2022). In *Eugenia punicifolia* leaf extraction, the ethanolic extraction enabled more phenolic compounds than ethanol-water mixtures (Santos et al., 2020).

Polar solvents, such as alcohols, are selected for flavonoid extraction due to their higher dielectric constants than nonpolar solvents, making them more suitable. Methanol and ethanol, being more polar, exhibit a greater affinity for extracting these phenolics (Bhadange et al., 2022). The reduced quadratic model best described the TFC coded values, as shown in Equation 4 and Figure 1:

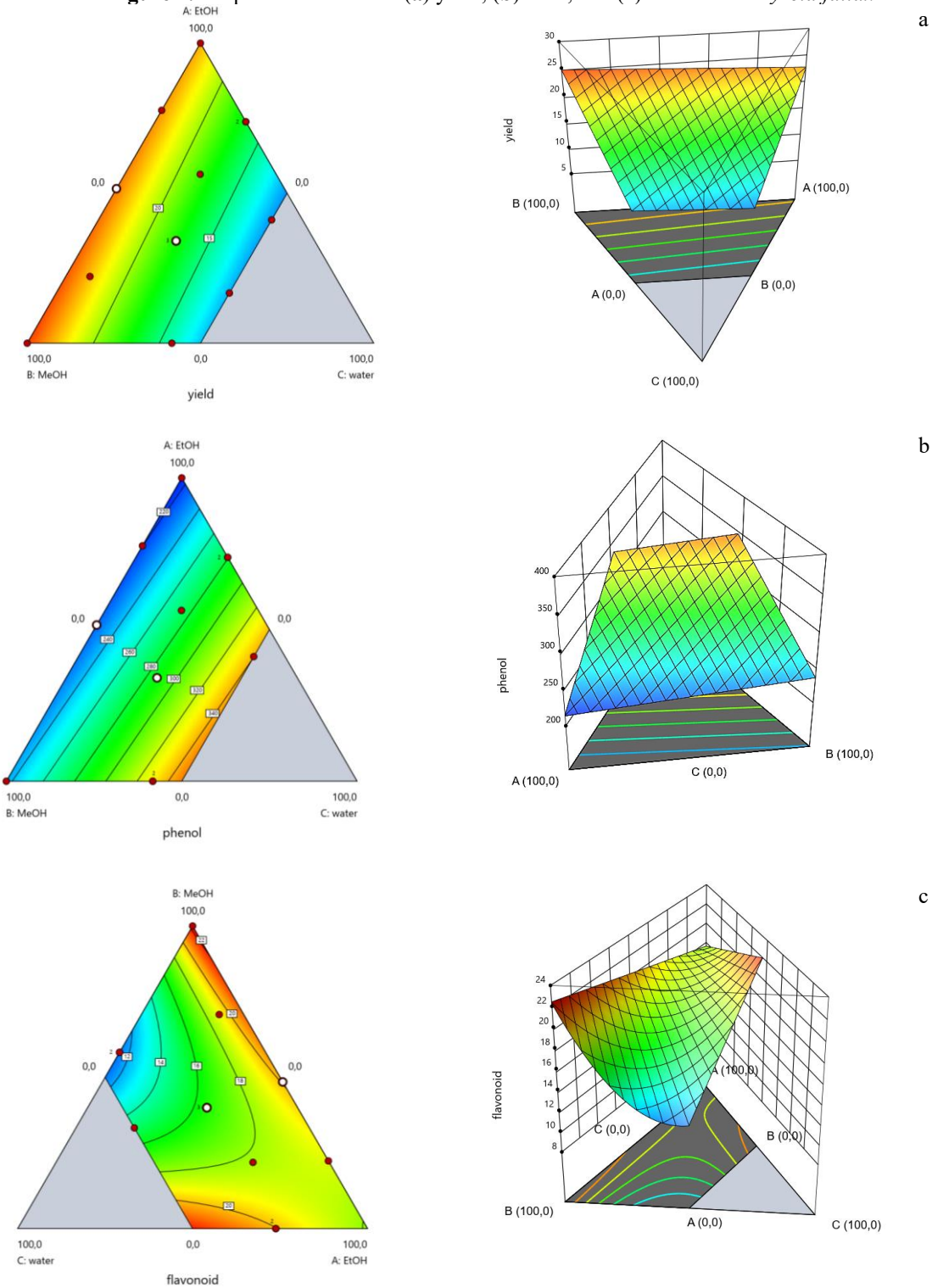
"TFC (mgQ/g extract)" = 17.89*e + 22.46*m +26.13*w -51.86*mw (4)

The coefficients for TFC showed a positive effect for ethanol (+17.89), methanol (+22.46), and water (+26.13), while for the binary interactions, methanol-water (-51.86) had a negative effect. From Figure 1c, it can be observed that MeOH 100% is a better solvent for the extraction of flavonoids (22.24±0.32 mg Q/g), while extract 12 (EtOH:MeOH:H_2O 0.0:58.3:41.7) presented the lowest content obtained for TFC. From Figure 1c, the best results for TFC are shown for the regions richer in pure methanol and ethanol-water (red color) than those richer in water (blue color).

Their structure and configuration influence the solubility of flavonoids. A solvent with a medium dielectric constant is generally the most effective for extraction due to varying polarities among flavonoids (Dai & Mumper, 2010). These compounds exhibit low solubility in water due to their low capacity to form hydrogen bonds with the surrounding solvent. The most commonly used solvents for extracting flavonoids are ethanol or methanol, as well as their mixtures with water. These solvents were chosen based on their effectiveness in solubilizing moderately polar flavonoids (Chebil et al., 2007; Dong et al., 2023). Flavonoids that possess low solubility in water are rutin (0.12 g/L), naringin (0.5g/L), quercetin (0.01g/L), and neohesperidine dihydrochalcone (0.4g/L) (Chebil et al., 2007; Tang et al., 2025).

For *Myrcia* spp, the hydroethanolic extracts (EtOH:H_2O 96:4 v/v) of *Myrcia splendens* and *Myrcia palustris* achieved different values of total flavonoid content 16.43±0.69 and 59.17 mg of Q/g), respectively (Moresco et al., 2014). In *M. fallax*, the predominant positive effect of water, methanol, and their mixtures proved to be the most effective for extracting glycosylated flavonoids, mainly quercetin and myricetin glycosylated derivatives, in the range of 9.90±0.53 to 22.24±0.32 mg of Q/g.

Figure 1. Response surfaces for (**a**) yield, (**b**) TPC, and (c) TFC of the *Myrcia fallax* extracts.



Source: Authors.

Antioxidant activity

An antioxidant is a molecule that can inhibit or prevent the oxidation of other molecules. These compounds reduce macromolecule mutations by neutralizing Reactive Oxygen Species (ROS) and reducing ROS-induced oxidative damage. Such oxidative damage is responsible for Alzheimer's disease, arthritis, inflammation, Parkinson's disease, and atherosclerosis. As a result, antioxidants have been utilized to treat numerous conditions and prevent oxidative damage (Moon & Shibamoto, 2009). To assess the antioxidant activities of the *M. fallax* extracts, two in vitro methods were tested: scavenging of DPPH and ABTS. Both assays are commonly used to evaluate the antioxidant capacity of plant extracts, foods, and biological samples, and they often show a correlation to the total phenolic content (TPC).

For DPPH radical scavenging, a lower EC_{50} value (concentration of extract necessary to reduce the total radicals by half) indicates a greater antiradical capacity by reducing the DPPH radical. Equation 4 describes the predictive function for solvent mixtures with the most effective EC_{50} values (Figure 2).

"EC_{50}" = 9.22*e + 8.93*m +16.58*w (4)

Ethanol (+9.22) and methanol (+8.93) showed better antioxidant activity (lower EC_{50}), i.e., these solvents are more effective than water (+16.58) for antioxidant activity. Also, we found ethanol-water as an expressive EC_{50}, such as extract 6 (EtOH:MeOH:H_2O 74.0:0.0:26.0; 6.69±3.68 µg/ml). The variation in the EC_{50} values observed is due to the synergism/antagonism of the different phenolic compounds present in the *Myrcia* spp extracts, such as quercetin and myricetin glycosylated derivatives (Santos et al., 2018).

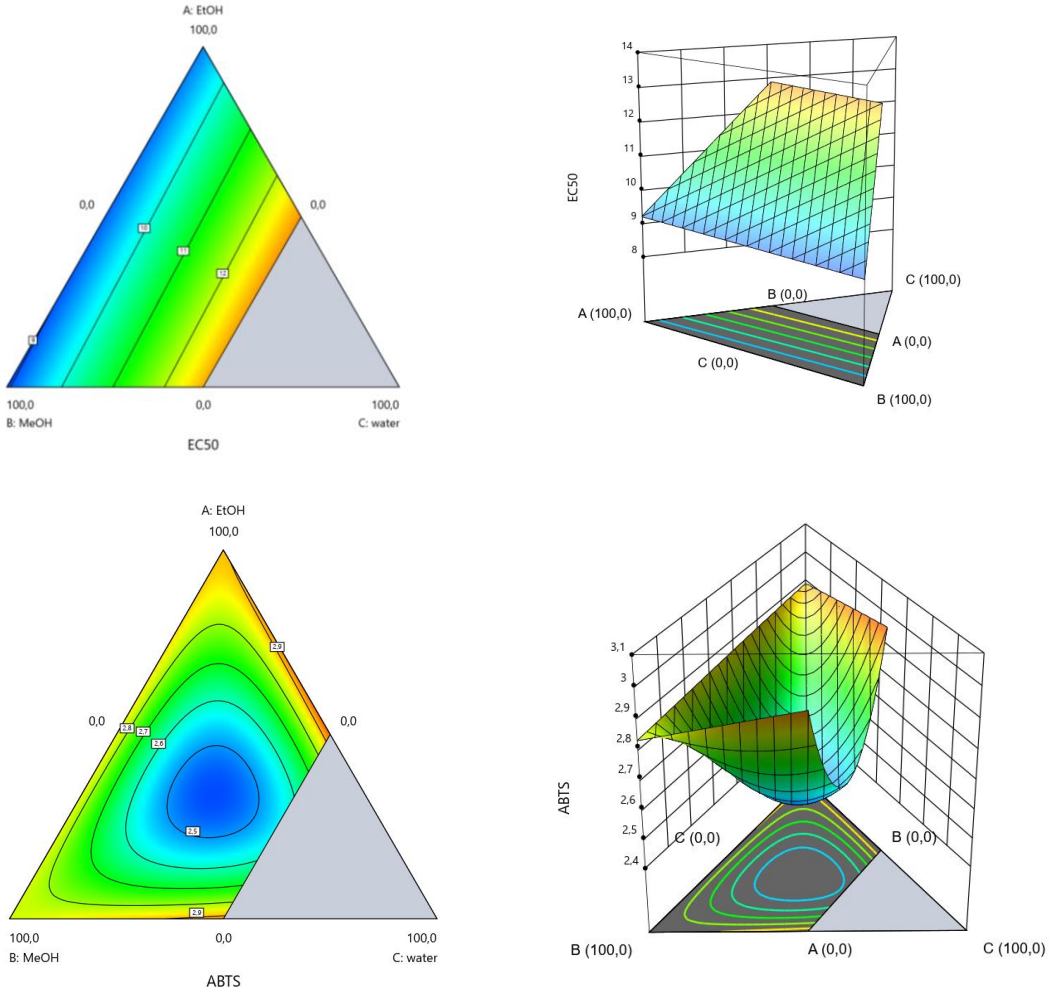
The model that best described the observed values for the antioxidant activity measured by ABTS was the special cubic model, which can be represented by coded factors, by Equation 5, Figure 2:

"ABTS" = 2.90*e + 2.83*m +3.02*w -12.41emw (5)

From Equation 5, it can be noted that for the linear coefficients, ethanol (+2.90), methanol (+2.83), and water (+3.02) have a positive effect on the extraction of compounds with antioxidant activity. In contrast, the ternary interactions of solvents (-12.41) had the opposite effect. For instance, from Table 1, extract 14 presented the lowest value of 2.41±0.08 µMTe/g (EtOH:MeOH:H_2O 34.1:40.0:25.9). Comparatively, extracts from EtOH and MeOH 100% showed higher values (2.96±0.28 and 2.84±0.16 µMTe/g µMTe/g, respectively). However, the best antioxidant values were observed in extracts from ethanol-water solvents (Figure 2c, red zone).

Phenolic compounds, such as phenolic acids, tannins, and flavonoids, as found in *M. fallax*, possess antioxidant activities due to their ability to donate hydrogen atoms or electrons and stabilize free radicals. The hydroxyl groups attached to aromatic rings and their conjugation increase the redox potential of these compounds. Moreover, hydroxyl glycosylation influences antioxidant activity by altering their solubility, stability, and ability to donate hydrogen atoms or electrons to phenolics (Tang et al., 2025). So, the antioxidant activity of these phenolics depends on the number and position of hydroxyl groups and glycosylation. For instance, the antioxidant capacity of 3-glycosylated quercetin was significantly higher than that of quercetin in DPPH measurements. Also, there is a well-known correlation between antioxidant activity and glycosylated HHDP derivatives and phenolic acids (quinic and ellagic acid) in *M. fallax* (Bodoira & Maestri, 2020; Santos et al., 2018).

Figure 2. Response surfaces for antioxidant activities (**a**) DPPH antiradical scavenging activity measured by EC_{50} (**b**) ABTS of the *M. fallax* extracts.



Source: Authors.

Studies have demonstrated a correlation between the antioxidant activity measured by the ABTS assay and the total phenolic content of extracts. The ABTS test (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) measures the ability of antioxidants to scavenge the ABTS•+ radical cation by donating hydrogen atoms or electrons. Since the ABTS assay is also sensitive to the molecular structure of phenolic compounds, this test is also effective in assessing antioxidant potential (Carrillo-Martinez et al., 2024; Prior et al., 2005).

Phytotoxic activity

The extracts were screened using a oassay for the initial development of lettuce (*Lactuca sativa* L.), i.e., the effect of extracts from different solvents on the radicle bi length and growth of hypocotyls. Firstly, the analysis was performed using an I-optimal experimental design. The model that best described the observed values for the radicle length measured was the special reduced quadratic model, which can be represented by coded factors, by Equation 6:

"Radicle" = 2.46*e + 2.60*m +4.13*w -7.23 ew (6)

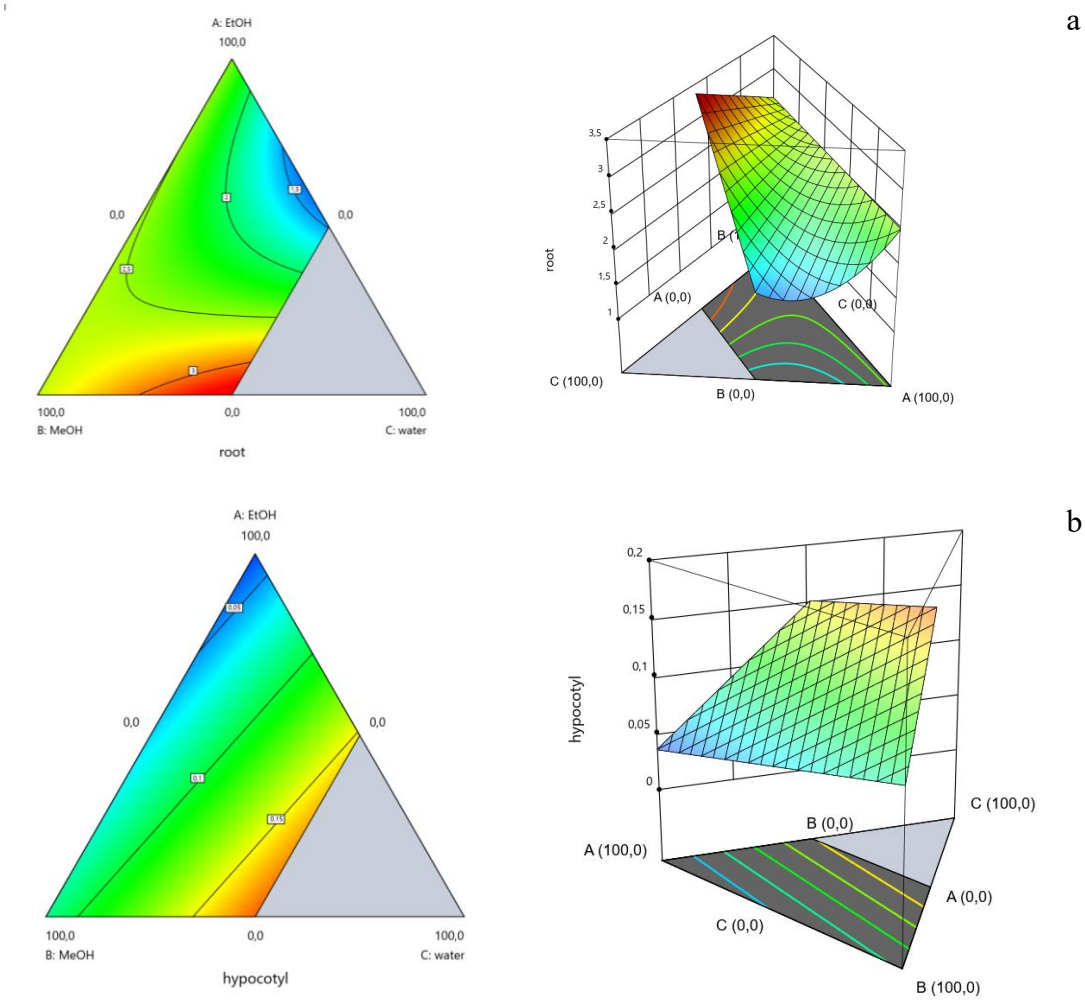
From Equation 6, it can be noted that for the linear coefficients, ethanol (+2.46), methanol (+2.60), and water (+4.13) have a positive effect on the extraction of compounds with phytotoxic effects on radicle length. In contrast, the binary interactions of solvents (-7.23) had the opposite effect. Figure 3a shows that the solvents rich in methanol-water result in better results (red zone), and ethanol-water solvents (blue zone) have negative effects. From Table I, the extracts 11 (EtOH:MeOH:H_2O 51.5:48.5:0.0; 1.08±0.32 cm) and 13 (EtOH:MeOH:H_2O 0.0:58.5:41.5; 1.15±0.28 cm) stood out, as they presented lower radicle growth relative to the control and the other treatments.

Initially, for hypocotyl growth measurements, the linear model provided the best fit for the observed hypocotyl and can be represented by coded factors in Equation 7.

"Hypocotyl"= 0.0365*e + 0.0863*m +0.2677*w (7)

Regarding the phytotoxic effects on hypocotyl length, there was an observed positive effect on ethanol (+0.0365), methanol (+0.0863), and water (+0.2677). Figure 3b shows that the solvents rich in methanol-water result in better results (red zone), while ethanol (blue zone) has negative effects. Some extracts presented a significant growth stimulation effect on the hypocotyls (Table 1). The extracts that presented the hypocotyls with the highest growth were: 3 (EtOH:MeOH:H_2O 16.5:33.5:50.0 (v/v/v) 0.67±0.11 cm), 12 (EtOH:MeOH:H_2O 0.0:58.5:41.5 (v/v/v) 0.66±0.16 cm), and 13 (EtOH:MeOH:H_2O 0.0:58.5:41.5 (v/v/v) 0.68±0.14 cm).

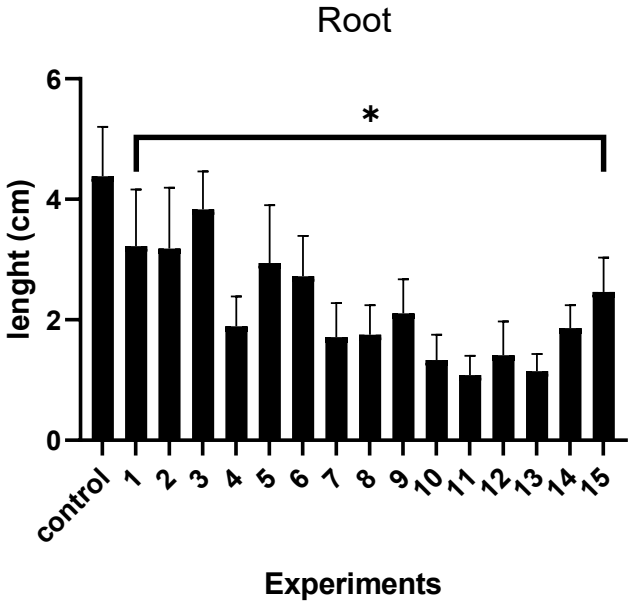
Figure 3. Response surfaces for antioxidant activities (**a**) Radicle length (**b**)Hypocotyl growth of the *Myrcia fallax* extract according to the proportion of solvents.



Source: Authors.

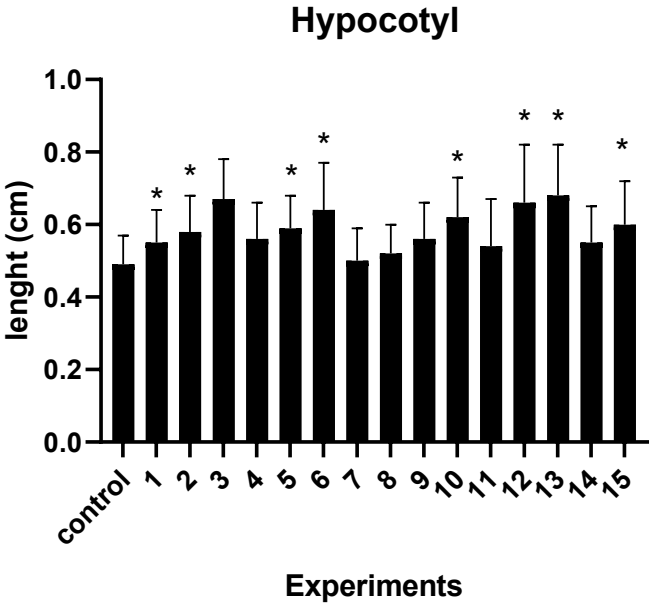
However, % CV for radicle length and hypocotyl growth showed values above 10%, which means great dispersion results (Table 3). So, in addition to the analysis conducted above, the phytotoxic effects of the extracts on radicle length and hypocotyl growth stimulation or inhibition were examined compared to the negative control (water) (Figures 4-5).

Figure 4. Lettuce Radicle Length with *M. fallax* leaf extract treatments with 95% confidence intervals (ANOVA – Dunnett's Test). *Indicates a significant difference from the negative control (water) ($p<0.05$, F 60.02).



Source: Authors.

Figure 5. Lettuce Hypocotyl Growth with *Myrcia fallax* leaf extract treatments with 95% confidence intervals (ANOVA – Dunnett's Test). *Indicates a significant difference from the negative control (water) ($p<0.05$, F 60.02).



Source: Authors.

For Figure 5, *M. fallax* extracts provide significant length inhibition results in radicle length compared to the control, especially for methanol-water solvents. For hypocotyl, the same solvent systems provide an opposite effect; therefore, stimulating hypocotyl elongation causes a decrease in radicle size (Figure 6). In short, the phenolic compounds obtained from methanol-water extraction can cause a decrease in the radicle, and the increase in the hypocotyl occurs in the same proportion.

The phytotoxicity of these extracts was possible because of the synergistic effect of phenolic acids and flavonoids extracted in *M. fallax* methanol-water extracts, which, in turn, affect the development of radicles and hypocotyls in an inhibitory or stimulating manner (Buer & Djordjevic, 2009; Pardo-Muras et al., 2020). Their activity possibly occurs due to water entry through the integument during the germination process, which carries phenolics by mass flow, which begins their physiological activities in radicle growth (De Martino et al., 2012).

Phenolic acids, like carboxylic acids, extracted in more aqueous solvents, cause uncoupling of oxidative phosphorylation and photophosphorylation in mitochondria and chloroplasts. For example, gallic acid, present in *M. fallax* hydroethanolic extracts, did not inhibit germination but can increase cell division and enlargement at low concentrations by accelerating mitosis and cellulose synthesis in maize seedlings (Bravo. et al., 2013; Santos et al., 2018; Mousavi et al., 2021).

Flavonoids are usually extracted in ethanol, methanol, or a mixture of these solvents. Quercetin and kaempferol inhibit germination speed and radicle length (Franco et al., 2015). Some authors claim these compounds can inhibit or promote plant development, interact with auxin transportation, and regulate reactive oxygen species (Bravo. et al., 2013; Buer et al., 2010). In contrast, luteolin, ellagic acid, kaempferol, and naringenin were phytotoxic but only at low concentrations (≤0.1 mM) (Pardo-Muras et al., 2020). In plants such as *Robinia pseudoacacia,* ethanolic leaf extracts were found to contain myricetin and quercetin. These compounds were correlated to the growth inhibition of lettuce radicles and shoots (Nasir et al., 2005).

4. Final Considerations

The bioactivity of the phenolic compounds from *M. fallax* is dependent on the solvents used in the extraction. For a higher yield, pure methanol proved to be the best solvent. When the extraction was done from methanol-water solvent mixtures, the TPC and TFC values were higher. Moreover, this solvent mixture provided extracts with the most interesting effects on the phytotoxic activity. The phenolics extracted showed higher antioxidant activity when the extraction was done from a less polar solvent, such as pure ethanol. TFC can also be extracted in mixtures rich in ethanol-water; however, in this case, the flavonoids extracted from this solvent mixture did not show any biological assays tested in this manuscript. Upon evaluation of phytotoxicity activity, the phenolic compounds extracted in methanol-water were indicated due to their ability to inhibit radicle length and stimulate hypocotyl growth compared to the control, which in turn can be correlated to the presence of phenolic compounds. Then, the I-optimal design allowed us to correlate the *Myrcia fallax* biological activities with the extracts obtained from solvent-different polarity mixtures.

Acknowledgments

This study was supported by Fapesp - Fundação de Amparo à Pesquisa do Estado de São Paulo - grants number 2015/22528-2 and 2017/15610-0.

Author's Contributions

MM: Experimental execution, data interpretation, statistical tests, data analysis. DS: Contributed expertise in analytical chemistry and performed HPLC analysis. CS: Conceived, designed the work, and wrote the manuscript.

References

Abrahim, D., Braguini, W. L., Kelmer-Bracht, A. M., & Ishii-Iwamoto, E. L. (2000). Effects of Four Monoterpenes on Germination, Primary Root Growth, and Mitochondrial Respiration of Maize. *Journal of Chemical Ecology*, *26*(3), 611–624. https://doi.org/10.1023/A:1005467903297

Afzal, S., Abdul Manap, A. S., Attiq, A., Albokhadaim, I., Kandeel, M., & Alhojaily, S. M. (2023). From imbalance to impairment: The central role of reactive oxygen species in oxidative stress-induced disorders and therapeutic exploration. *Frontiers in Pharmacology*, *14*, 1269581. https://doi.org/10.3389/fphar.2023.1269581

Barros-Neto, B., Scarminio, I. S., & Bruns, R. E. (2022). *Como fazer experimentos: Pesquisa e desenvolvimento na ciência e na indústria*. Bookman.

Bhadange, Y. A., Saharan, V. K., Sonawane, S. H., & Boczkaj, G. (2022). Intensification of catechin extraction from the bark of Syzygium cumini using ultrasonication: Optimization, characterization, degradation analysis and kinetic studies. *Chemical Engineering and Processing - Process Intensification*, *181*, 109147. https://doi.org/10.1016/j.cep.2022.109147

Bodoira, R., & Maestri, D. (2020). Phenolic Compounds from Nuts: Extraction, Chemical Profiles, and Bioactivity. *Journal of Agricultural and Food Chemistry*, *68*(4), 927–942. https://doi.org/10.1021/acs.jafc.9b07160

Bravo., H., Copaja, S., & Lamborot, M. (2013). Phytotoxicity of Phenolic Acids From Cereals. Em A. Price (Org.), *Herbicides—Advances in Research*. InTech. https://doi.org/10.5772/55942

Bucić-Kojić, A., Planinić, M., Tomas, S., Jokić, S., Mujić, I., Bilić, M., & Velić, D. (2011). Effect of Extraction Conditions on the Extractability of Phenolic Compounds from Lyophilised Fig Fruits (Ficus Carica L.). *Polish Journal of Food and Nutrition Sciences*, *61*(3), 195–199. https://doi.org/10.2478/v10222-011-0021-9

Buer, C. S., & Djordjevic, M. A. (2009). Architectural phenotypes in the transparent testa mutants of Arabidopsis thaliana. *Journal of Experimental Botany*, *60*(3), 751–763. https://doi.org/10.1093/jxb/ern323

Buer, C. S., Imin, N., & Djordjevic, M. A. (2010). Flavonoids: New Roles for Old Molecules. *Journal of Integrative Plant Biology*, *52*(1), 98–111. https://doi.org/10.1111/j.1744-7909.2010.00905.x

Candioti, V. L., De Zan, M. M., Cámara, M. S., & Goicoechea, H. C. (2014). Experimental design and multiple response optimization. Using the desirability function in analytical methods development. *Talanta*, *124*, 123–138. https://doi.org/10.1016/j.talanta.2014.01.034

Carrillo-Martinez, E. J., Flores-Hernández, F. Y., Salazar-Montes, A. M., Nario-Chaidez, H. F., & Hernández-Ortega, L. D. (2024). Quercetin, a Flavonoid with Great Pharmacological Capacity. *Molecules*, *29*(5), 1000. https://doi.org/10.3390/molecules29051000

Cascaes, M., Guilhon, G., Andrade, E., Zoghbi, M., & Santos, L. (2015). Constituents and Pharmacological Activities of Myrcia (Myrtaceae): A Review of an Aromatic and Medicinal Group of Plants. *International Journal of Molecular Sciences*, *16*(10), 23881–23904. https://doi.org/10.3390/ijms161023881

Chebil, L., Humeau, C., Anthoni, J., Dehez, F., Engasser, J.-M., & Ghoul, M. (2007). Solubility of Flavonoids in Organic Solvents. *Journal of Chemical & Engineering Data*, *52*(5), 1552–1556. https://doi.org/10.1021/je7001094

Costa, M. F., Jesus, T. I., Lopes, B. R. P., Angolini, C. F. F., Montagnolli, A., Gomes, L. de P., Pereira, G. S., Ruiz, A. L. T. G., Carvalho, J. E., Eberlin, M. N., dos Santos, C., & Toledo, K. A. (2016). Eugenia aurata and Eugenia punicifolia HBK inhibit inflammatory response by reducing neutrophil adhesion, degranulation and NET release. *BMC Complementary and Alternative Medicine*, *16*(1), 403. https://doi.org/10.1186/s12906-016-1375-7

Ćujić, N., Šavikin, K., Janković, T., Pljevljakušić, D., Zdunić, G., & Ibrić, S. (2016). Optimization of polyphenols extraction from dried chokeberry using maceration as traditional technique. *Food Chemistry*, *194*, 135–142. https://doi.org/10.1016/j.foodchem.2015.08.008

Dai, J., & Mumper, R. J. (2010). Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. *Molecules*, *15*(10), 7313–7352. https://doi.org/10.3390/molecules15107313

De Martino, L., Mencherini, T., Mancini, E., Aquino, R. P., De Almeida, L. F. R., & De Feo, V. (2012). In Vitro Phytotoxicity and Antioxidant Activity of Selected Flavonoids. *International Journal of Molecular Sciences*, *13*(5), 5406–5419. https://doi.org/10.3390/ijms13055406

DiCiaula, M. C., Lopes, G. C., Scarminio, I. S., & de Mello, J. C. P. (2014). Optimization of solvent mixtures for extraction from bark of Schinus terebinthifolius by a statistical mixture-design technique and development of a UV-Vis spectrophotometric method for analysis of total polyphenols in the extract. *Química Nova*, *37*(1), 158–163. https://doi.org/10.1590/S0100-40422014000100026

Enujiugha, V. N., Talabi, J. Y., Malomo, S. A., & Olagunju, A. I. (2012). DPPH Radical Scavenging Capacity of Phenolic Extracts from African Yam Bean Sphenostylis stenocarpa. *Food and Nutrition Sciences*, *03*(01), 7–13. https://doi.org/10.4236/fns.2012.31002

Ferreira, S. L. C., Bruns, R. E., Ferreira, H. S., Matos, G. D., David, J. M., Brandão, G. C., Da Silva, E. G. P., Portugal, L. A., Dos Reis, P. S., Souza, A. S., & Dos Santos, W. N. L. (2007). Box-Behnken design: An alternative for the optimization of analytical methods. *Analytica Chimica Acta*, *597*(2), 179–186. https://doi.org/10.1016/j.aca.2007.07.011

Franco, D. M., Saldanha, L. L., Silva, E. M., Nogueira, F. T. S., & Santos, C. (s. d.). *Effects of leaf extracts of Myrcia guianensis (Aubl.) DC.: On growth and gene expression during root development of Sorghum bicolor (L.) Moench*. *35*(2), 237–248.

Franco, D. M., Silva, E. M., Saldanha, L. L., Adachi, S. A., Schley, T. R., Rodrigues, T. M., Dokkedal, A. L., Nogueira, F. T. S., & Rolim De Almeida, L. F. (2015). Flavonoids modify root growth and modulate expression of SHORT-ROOT and HD-ZIP III. *Journal of Plant Physiology*, *188*, 89–95. https://doi.org/10.1016/j.jplph.2015.09.009

Garcia-Salas, P., Morales-Soto, A., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2010). Phenolic-Compound-Extraction Systems for Fruit and Vegetable Samples. *Molecules*, *15*(12), 8813–8826. https://doi.org/10.3390/molecules15128813

Ghaffar, N., & Perveen, A. (2024). Solvent polarity effects on extraction yield, phenolic content, and antioxidant properties of Malvaceae family seeds: A comparative study. *New Zealand Journal of Botany*, 1–11. https://doi.org/10.1080/0028825X.2024.2392705

Imatomi, M., Novaes, P., & Gualtieri, S. C. J. (2013). Interspecific variation in the allelopathic potential of the family Myrtaceae. *Acta Botanica Brasilica*, *27*(1), 54–61. https://doi.org/10.1590/S0102-33062013000100008

Jomova, K., Raptova, R., Alomar, S. Y., Alwasel, S. H., Nepovimova, E., Kuca, K., & Valko, M. (2023). Reactive oxygen species, toxicity, oxidative stress, and antioxidants: Chronic diseases and aging. *Archives of Toxicology*, *97*(10), 2499–2574. https://doi.org/10.1007/s00204-023-03562-9

Kalia, K., Sharma, K., Singh, H. P., & Singh, B. (2008). Effects of Extraction Methods on Phenolic Contents and Antioxidant Activity in Aerial Parts of Potentilla atrosanguinea Lodd. And Quantification of Its Phenolic Constituents by RP-HPLC †. *Journal of Agricultural and Food Chemistry*, *56*(21), 10129–10134. https://doi.org/10.1021/jf802188b

Keerthiga, G., & Sridhar, A. (2022). Batch extraction kinetics and total phenolic content estimation of *Syzygium Cumini.L* bark. *Indian Chemical Engineer*, *64*(5), 565–575. https://doi.org/10.1080/00194506.2022.2046512

Li, Z.-H., Wang, Q., Ruan, X., Pan, C.-D., & Jiang, D.-A. (2010). Phenolics and Plant Allelopathy. *Molecules*, *15*(12), 8933–8952. https://doi.org/10.3390/molecules15128933

Mendes, M. K. D. A., Oliveira, C. B. D. S., Veras, M. D. A., Araújo, B. Q., Dantas, C., Chaves, M. H., Lopes Júnior, C. A., & Vieira, E. C. (2019). Application of multivariate optimization for the selective extraction of phenolic compounds in cashew nuts (Anacardium occidentale L.). *Talanta*, *205*, 120100. https://doi.org/10.1016/j.talanta.2019.06.100

Miles, J. (2014). R Squared, Adjusted R Squared. Em R. S. Kenett, N. T. Longford, W. W. Piegorsch, & F. Ruggeri (Org.), *Wiley StatsRef: Statistics Reference Online* (1º ed.). Wiley. https://doi.org/10.1002/9781118445112.stat06627

Moresco, H. H., Pereira, M., Bretanha, L. C., Micke, G. A., Pizzolatti, M. G., & Brighente, I. M. C. (2014). Myricitrin as the main constituent of two species of Myrcia. *Journal of Applied Pharmaceutical Science*, 1–7. https://doi.org/10.7324/JAPS.2014.40201

Mousavi, S. S., Karami, A., Haghighi, T. M., Alizadeh, S., & Maggi, F. (2021). Phytotoxic Potential and Phenolic Profile of Extracts from Scrophularia striata. *Plants*, *10*(1), 135. https://doi.org/10.3390/plants10010135

Nasir, H., Iqbal, Z., Hiradate, S., & Fujii, Y. (2005). Allelopathic Potential of Robinia pseudo-acacia L. *Journal of Chemical Ecology*, *31*(9), 2179–2192. https://doi.org/10.1007/s10886-005-6084-5

Ozen, T., Demirtas, I., & Aksit, H. (2011). Determination of antioxidant activities of various extracts and essential oil compositions of Thymus praecox subsp. Skorpilii var. Skorpilii. *Food Chemistry*, *124*(1), 58–64. https://doi.org/10.1016/j.foodchem.2010.05.103

Pardo-Muras, M., Puig, C. G., Souto, X. C., & Pedrol, N. (2020). Water-soluble phenolic acids and flavonoids involved in the bioherbicidal potential of Ulex europaeus and Cytisus scoparius. *South African Journal of Botany*, *133*, 201–211. https://doi.org/10.1016/j.sajb.2020.07.023

Pélabon, C., Hilde, C. H., Einum, S., & Gamelon, M. (2020). On the use of the coefficient of variation to quantify and compare trait variation. *Evolution Letters*, *4*(3), 180–188. https://doi.org/10.1002/evl3.171

Pereira, A. M., Shitsuka, D. M., Parreira, F. B., & Shitsuka, R. (2018). *Metodologia de pesquisa científica*. Uab/Nte/Ufsm.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *Journal of Agricultural and Food Chemistry*, *53*(10), 4290–4302. https://doi.org/10.1021/jf0502698

Ribeiro De Souza, E. B., Da Silva, R. R., Afonso, S., & Scarminio, I. S. (2009). Enhanced extraction yields and mobile phase separations by solvent mixtures for the analysis of metabolites in *Annona muricata* L. leaves. *Journal of Separation Science*, *32*(23–24), 4176–4185. https://doi.org/10.1002/jssc.200900375

Rufino, M. do S. M., Alves, R. E., de Brito, E. S., Pérez-Jiménez, J., Saura-Calixto, F., & Mancini-Filho, J. (2010). Bioactive compounds and antioxidant capacities of 18 non-traditional tropical fruits from Brazil. *Food Chemistry*, *121*(4), 996–1002. https://doi.org/10.1016/j.foodchem.2010.01.037

Santos, C., Galaverna, R., Angolini, C., Nunes, V., de Almeida, L., Ruiz, A., de Carvalho, J., Duarte, R., Duarte, M., & Eberlin, M. (2018). Antioxidative, Antiproliferative and Antimicrobial Activities of Phenolic Compounds from Three Myrcia Species. *Molecules*, *23*(5), 986. https://doi.org/10.3390/molecules23050986

Santos, C., Galaverna, R. S., Angolini, C. F. F., Nunes, V. V. A., De Almeida, L. F. R., Ruiz, A. L. T. G., De Carvalho, J. E., Duarte, R. M. T., Duarte, M. C. T., & Eberlin, M. N. (2018). Antioxidative, Antiproliferative and Antimicrobial Activities of Phenolic Compounds from Three Myrcia Species. *Molecules*, *23*(5), 986. https://doi.org/10.3390/molecules23050986

Santos, C., Mizobucchi, A. L., Escaramboni, B., Lopes, B. P., Angolini, C. F. F., Eberlin, M. N., De Toledo, K. A., & Núñez, E. G. F. (2020). Optimization of Eugenia punicifolia (Kunth) D. C. leaf extraction using a simplex centroid design focused on extracting phenolics with antioxidant and antiproliferative activities. *BMC Chemistry*, *14*(1), 34. https://doi.org/10.1186/s13065-020-00686-2

Sapra, R. L. (2014). Using R2 with caution. *Current Medicine Research and Practice*, *4*(3), 130–134. https://doi.org/10.1016/j.cmrp.2014.06.002

Shan, Z., Zhou, S., Shah, A., Arafat, Y., Arif Hussain Rizvi, S., & Shao, H. (2023). Plant Allelopathy in Response to Biotic and Abiotic Factors. *Agronomy*, *13*(9), 2358. https://doi.org/10.3390/agronomy13092358

Shitsuka, D. M., & Rabbith, I. (2024). *Matemática Fundamental Para Tecnologia*. Erica, Iatria.

Shrestha, N. (2020). Detecting Multicollinearity in Regression Analysis. *American Journal of Applied Mathematics and Statistics*, *8*(2), 39–42. https://doi.org/10.12691/ajams-8-2-1

Silvestre, D. M., Kolb, R. M., Frei, F., & dos Santos, C. (2013). Phytotoxicity of organic extracts of Turnera ulmifolia L. and Turnera diffusa Willd. Ex Schult. In cucumber seeds. *Acta Botanica Brasilica*, *27*(3). https://doi.org/10.1590/S0102-33062013000300003

Singh, A., Singh, D., & Singh, N. B. (2009). Allelochemical stress produced by aqueous leachate of Nicotiana plumbaginifolia Viv. *Plant Growth Regulation*, *58*(2), 163–171. https://doi.org/10.1007/s10725-009-9364-1

Souza Filho, A. P. S., Santos, R. A., Santos, L. S., Guilhon, G. M. P., Santos, A. S., Arruda, M. S. P., Muller, A. H., & Arruda, A. C. (2006). Potencial alelopático de Myrcia guianensis. *Planta Daninha*, *24*(4), 649–656. https://doi.org/10.1590/S0100-83582006000400005

Takao, L. K., Imatomi, M., & Gualtieri, S. C. J. (2015). Antioxidant activity and phenolic content of leaf infusions of Myrtaceae species from Cerrado (Brazilian Savanna). *Brazilian Journal of Biology*, *75*(4), 948–952. https://doi.org/10.1590/1519-6984.03314

Tang, S., Wang, B., Liu, X., Xi, W., Yue, Y., Tan, X., Bai, J., & Huang, L. (2025). Structural insights and biological activities of flavonoids: Implications for novel applications. *Food Frontiers*, *6*(1), 218–247. https://doi.org/10.1002/fft2.494

Teerarak, M., Charoenying, P., & Laosinwattana, C. (2012). Physiological and cellular mechanisms of natural herbicide resource from Aglaia odorata Lour. On bioassay plants. *Acta Physiologiae Plantarum*, *34*(4), 1277–1285. https://doi.org/10.1007/s11738-011-0923-5

Teerarak, M., Laosinwattana, C., & Charoenying, P. (2010). Evaluation of allelopathic, decomposition and cytogenetic activities of Jasminum officinale L. f. Var. Grandiflorum (L.) Kob. On bioassay plants. *Bioresource Technology*, *101*(14), 5677–5684. https://doi.org/10.1016/j.biortech.2010.02.038

Yan, K., Cheng, X., Bian, G., Gao, Y., & Li, D. (2022). The Influence of Different Extraction Techniques on the Chemical Profile and Biological Properties of Oroxylum indicum: Multifunctional Aspects for Potential Pharmaceutical Applications. *Evidence-Based Complementary and Alternative Medicine*, *2022*, 1–17. https://doi.org/10.1155/2022/8975320

Yashin, A., Yashin, Y., Xia, X., & Nemzer, B. (2017). Antioxidant Activity of Spices and Their Impact on Human Health: A Review. *Antioxidants*, *6*(3), 70. https://doi.org/10.3390/antiox6030070

Zhang, Q.-W., Lin, L.-G., & Ye, W.-C. (2018). Techniques for extraction and isolation of natural products: A comprehensive review. *Chinese Medicine*, *13*(1), 20. https://doi.org/10.1186/s13020-018-0177-x