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Original Paper

Application of response surface methodology for optimizing flavonoid extraction and antimicrobial activity from *Pistacia terebinthus* male flowers

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Abstract

Pistacia terebinthus is a Mediterranean dioecious shrub rich in flavonoids and exhibits various biological activities, including antimicrobial effects. While the leaves and fruits of this species have been studied extensively, its flowers remain underexplored. This study aims to optimize the extraction conditions for total flavonoid content from *P. terebinthus* male flowers and assess their antimicrobial activity against selected bacterial and fungal strains. The optimization was carried out using response surface methodology with a Box-Behnken design, evaluating four parameters: extraction temperature, time, methanol concentration, and solid-to-liquid ratio. The optimal extraction conditions were: 60% methanol, a solid-to-liquid ratio of 1:19.5, and extraction at 32.6 °C for 63 min, yielding a total flavonoid content of 953.27 mg quercetin equivalents 100 g^{-1} dry mass. The extract showed significant antimicrobial activity, particularly against *Aspergillus niger* (19.12 mm inhibition zone), followed by *Bacillus cereus* (16.53 mm). Fungal strains were generally more sensitive than bacterial strains. ANOVA confirmed the significance and predictive power of the quadratic models used for optimization. The findings support the potential use of male flower extracts in the development of natural antimicrobial agents, and underscore the effectiveness of response surface methodology with a Box-Behnken design in optimizing extraction conditions.

Key words: antimicrobial activity, Box-Behnken design, flavonoids, male flowers, *Pistacia terebinthus*, response surface methodology.
Abbreviations: ATCC, American Type Culture Collection; CFU, colony forming units; DM, dry mass; DMSO, dimethyl sulfoxide; MeOH, methanol; QE, quercetin equivalent; TFC, total flavonoid content.

Introduction

Flavonoids are a highly diverse group of plant-derived secondary metabolites, including the well-known pigments responsible for the colouration of fruits and flowers (Albert et al. 2023). They are widespread among various plant species and play critical roles in their defense mechanisms, particularly in protection against microbial attacks (Cushnie, Lamb 2005). Flavonoids and other polyphenols are naturally present in plant systems, and they can accumulate as phytoalexins under microbial attacks (Ramaroson et al. 2022). On top of their role in plant defense mechanisms, flavonoids have been explored for their potential therapeutic value to human health, especially by considering their antifungal and antiviral efficacy (Badshah et al. 2021; Salatin et al. 2022). Besides their antimicrobial properties, flavonoids contribute to plant anti-inflammatory, antioxidant, and cytotoxic activities, enhancing their therapeutic potential.

Pistacia terebinthus L., a notable species within the Anacardiaceae family, is particularly recognized for its abundant flavonoid and phenolic content, which contribute to its antioxidant and anti-inflammatory properties. This shrub is widely distributed across Mediterranean countries, including Algeria, Morocco, Tunisia, and Turkey, where it thrives in diverse climatic conditions (Topçu et al. 2007). Beyond traditional uses in herbal medicine, various parts of the terebinth tree, such as its resin, leaves, and fruits, are central to local culinary practices, enhancing dishes with unique flavors and nutritional benefits (Bozorgi et al. 2013; Batovska, Inbar 2024; Batovska 2025).

Extensive scientific research has underscored the diverse biological activities linked to *P. terebinthus*, indicating its potential therapeutic applications. For instance, studies

have revealed its antimicrobial properties, effective against various pathogens, and its antiviral activity, which may help combat viral infections. Furthermore, the plant exhibits antidiabetic effects by modulating blood sugar levels and reducing insulin resistance (Akyuz et al. 2022). Its antihyperlipidemic properties are also noteworthy, aiding in the management of cholesterol levels. Research has shown that *P. terebinthus* has antiatherosclerotic effects, which can help mitigate the risk of cardiovascular diseases, while its hepatoprotective properties support liver health by safeguarding against toxic damage. Additionally, investigations have highlighted its gastrointestinal and neuroprotective effects, suggesting a role in gut health and function. Emerging studies are indicating the potential anticancer effects of the plant, which are being explored for their capacity to inhibit tumour growth and promote apoptosis in cancer cells (Najibullah et al. 2022; Uysal et al. 2022; Fidan et al. 2023; Firat et al. 2024; Ozgolet et al. 2024).

Despite extensive research on *P. terebinthus*, the male flowers remain underexplored. These flowers, abundant in flavonoid content, present a promising yet untapped resource for bioactive compounds. Unlike female flowers, which develop into fruits, male flowers are typically discarded, making them a sustainable and novel material for extraction. Preliminary studies indicate their significant antimicrobial activity, likely due to high flavonoid concentrations. However, the extraction of these compounds poses scientific challenges regarding optimizing yield and preserving bioactivity under varying conditions.

This study focuses on identifying and optimizing extraction parameters, such as solvent type, temperature, and duration, to maximize total flavonoid content (TFC) from *P. terebinthus* male flowers. Using response surface methodology (RSM) and Box-Behnken design (BBD), we aimed to evaluate the antibacterial and antifungal efficacy of the extracts, addressing gaps in both methodology and application. By leveraging an underutilized resource, this research seeks to advance the sustainable production of bioactive flavonoids while exploring their therapeutic potential.

Materials and methods

Plant sample preparation

Fresh male flowers of *Pistacia terebinthus* were collected from El Hamma, Khenchela province, Algeria (35°26'25"N, 7°05'04"E; 1058 m above sea level) during the flowering season. A voucher specimen (No. PT-2023-01) was deposited in the herbarium at Abbès Laghrour University, Algeria, and taxonomically authenticated by Dr. A. Zeraib using Flora of Algeria (Quezel, Santa 1962). The samples were air-dried in the shade at 25 °C for 7 days, ground into a fine powder (particle size < 0.5 mm), and stored at 4 °C in airtight containers until extraction.

Extraction procedure

The powdered flowers (5 g) were subjected to maceration in methanol (MeOH) at varying concentration (60 to 100%), solid-to-liquid ratio (1:20 to 1:10 g/mL), temperature (30 to 70 °C), and duration (30 to 120 min), as per the Box-Behnken design (BBD). After extraction, the mixtures were filtered through Whatman No. 1 filter paper, and the solvent was evaporated under reduced pressure at 40 °C using a rotary evaporator (Büchi R-300). The dried extracts were reconstituted in dimethyl sulfoxide (DMSO) to a final concentration of 100 mg mL^{-1} for subsequent analyses. All extractions were performed in triplicate to ensure reproducibility.

Determination of the total flavonoid content

TFC of dried flowers was quantified using aluminum colourimetric assay (Do et al. 2014) with minor modification. Briefly, 2 mL of the diluted extract (100 µg mL^{-1} in MeOH) or quercetin standard (0 to 100 µg mL^{-1}) was mixed with 0.1 mL of 10% $AlCl_3$ solution (w/v) and 0.1 mL of 0.1 M potassium acetate. After 30 min of incubation at 25 °C, absorbance was recorded at 415 nm using a Shimadzu UV-1800 spectrophotometer. TFC was expressed as mg quercetin equivalent per 100 g dry material [mg quercetin equivalents (QE) 100 g^{-1} DM] based on the quercetin calibration curve (R^2 = 0.988). Each sample was analyzed in triplicate.

Antibacterial activity

The disk diffusion method (Pfaller, Herwaldt 1997) was employed to assess the antibacterial activity of terebinth flower extracts against three bacterial strains: *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 7644), and *Bacillus cereus* (ATCC 11778). Bacterial suspensions were adjusted to 1×10^8 CFU mL^{-1} (0.5 McFarland standard). Extract-impregnated 6 mm diameter disks (10 µL of extract at 100 mg mL^{-1} in DMSO) and control disks (DMSO for negative control and 10 µg mL^{-1} gentamicin for positive control) were placed on Petri dishes filled with 20 mL of Mueller-Hinton agar. After 24 h incubation at 37 °C, inhibition zone diameter was measured using ImageJ software (https://imagej.net/ij/). Bacterial susceptibility to the extracts was assigned as follows according to EUCAST guidelines: susceptible (inhibition zone diameter ≥ 11 mm), intermediate (6 to 11 mm), and resistant (≤ 6 mm).

Antifungal activity

Fungal strains [*Aspergillus niger* (ATCC 16404) and uncharacterized *Cladosporium* sp., which was locally isolated from the Biskra province, Algeria, and provided by the Scientific and Technical Research Center for Arid Areas, Algeria) were cultured on potato dextrose agar for 5 days at 37 °C. Spore suspensions ($1 - 2 \times 10^5$ spores mL^{-1}) were prepared in sterile deionized water with 0.01% Tween

20 and spread on potato dextrose agar plates. Extract-impregnated (10 µL, 100 mg mL^{-1}) and control disks (DMSO or 10 µg mL^{-1} amphotericin B) were applied, and zones of inhibition were measured after 5 days. The results obtained were analyzed and categorized as those of the antibacterial assay.

Experimental design and RSM modeling

The experiment was conducted according to Box-Behnken design (BBD) using Design-Expert software 13.0.5.0 (Box, Behnken 1960). In this study, four independent variables (MeOH concentration, solid-to-liquid ratio, extraction time and temperature) at three levels (high, intermediate and low) were chosen to optimize the total flavonoid content and their antimicrobial activity. The variables were coded following the equation:

$$x_i = (X_i - X_0) / \Delta X_i,$$

where x_i is the coded value of X_i, X_0 is the real value of X_i at the center point value, and ΔX_i is the step change value (Table 1).

A second-order polynomial model was used in the optimization process and linked the response variables with the selected independent ones, using the multiple regression equation as follows:

$$Y_i = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=1}^{4} \beta_{ij} X_i X_j ,$$

where Y_i is the outcome variable (TFC and antimicrobial activity); X_i is the coded independent variable (MeOH concentration, solid-to-liquid ratio, extraction time and temperature); β_0, β_i, β_{ii}, β_{ij} are the intercept, and linear, quadratic and interaction regression coefficients, respectively. These regression coefficients were calculated using the ordinary least-squares method.

Statistical analysis

All experiments were conducted in triplicate. Results are presented as the mean ± SD. Differences between means were analyzed using one-way ANOVA followed by a regression test ($p < 0.05$). Model suitability was evaluated through predicted R^2, adjusted R^2, and lack-of-fit tests. To validate the optimal extraction conditions for phenolic compounds, regression models were used to predict TFC and antimicrobial activity at grid points within the optimal response region, based on three independent experimental trials.

Results

Optimization of flavonoid extraction conditions from P. terebinthus *male flowers*

The extraction conditions for flavonoids were optimized using RSM with a BBD (Fig. 1). A total of 27 experimental runs were conducted. The results and ANOVA analysis are summarized in Tables 2 and 3. The simplified quadratic polynomial equation for total flavonoid content (TFC) in terms of actual factors was derived as follows:

$$TFC = 714.27 - 53.43\, X_2 + 226.53\, X_3 + 40.15\, X_4 + 60.23\, X_1X_4 + 109.75\, X_2X_3 + 60.29\, X_4^2.$$

The model exhibited a high regression coefficient ($R^2 = 0.9701$) and a low coefficient of variation (CV = 6.49), indicating excellent fit and reliability. The model's significance was confirmed by a p-value less than 0.0001 and a non-significant lack of fit ($p = 0.1804$). These results demonstrated that the model was highly significant and suitable for predicting flavonoid yields under varying extraction conditions.

Optimization of antimicrobial activity of P. terebinthus *male flower extracts*

The twenty-seven extracts obtained from the BBD experimental runs were evaluated for antimicrobial activity against bacterial (*S. aureus, L. monocytogenes* and *B. cereus*) and fungal (*A. niger* and *Cladosporium* sp.) strains. The effects of extraction parameters on antimicrobial activity differed from their effects on TFC. The corresponding results are illustrated in Figs. 2 & 3 and Table 4.

For *L. monocytogenes, Cladosporium* sp., and *A. niger*, most independent variables exhibited a negative linear effect (Table 4). In contrast, *S. aureus* showed no significant response, except for the solid-to-liquid ratio ($p = 0.022$), which had a weak linear influence ($\beta = 0.69$). For *B. cereus*, quadratic and interaction effects predominated, indicating a non-linear relationship between extraction parameters and antimicrobial activity. The prevalence of quadratic effects across most microbial strains suggests a curved rather than linear pattern in the response. Each bacterial and fungal strain displayed distinct sensitivity profiles to *P. terebinthus* male flower extracts, highlighting pathogen-specific bioactivity.

Overall, fungal strains displayed greater sensitivity to the extracts than bacterial strains. Among the fungi, *A. niger* demonstrated the highest susceptibility, with an

Table 1. Independent variables, their coded and actual values used for the optimization study

Independent variable	Unit	Symbol	Coded level		
			–1	0	+1
Temperature	°C	X_1	30	50	70
Time	min	X_2	30	75	120
Solid-to-liquid ratio	g/mL	X_3	1:20	1:15	1:10
MeOH concentration	%	X_4	60	80	100

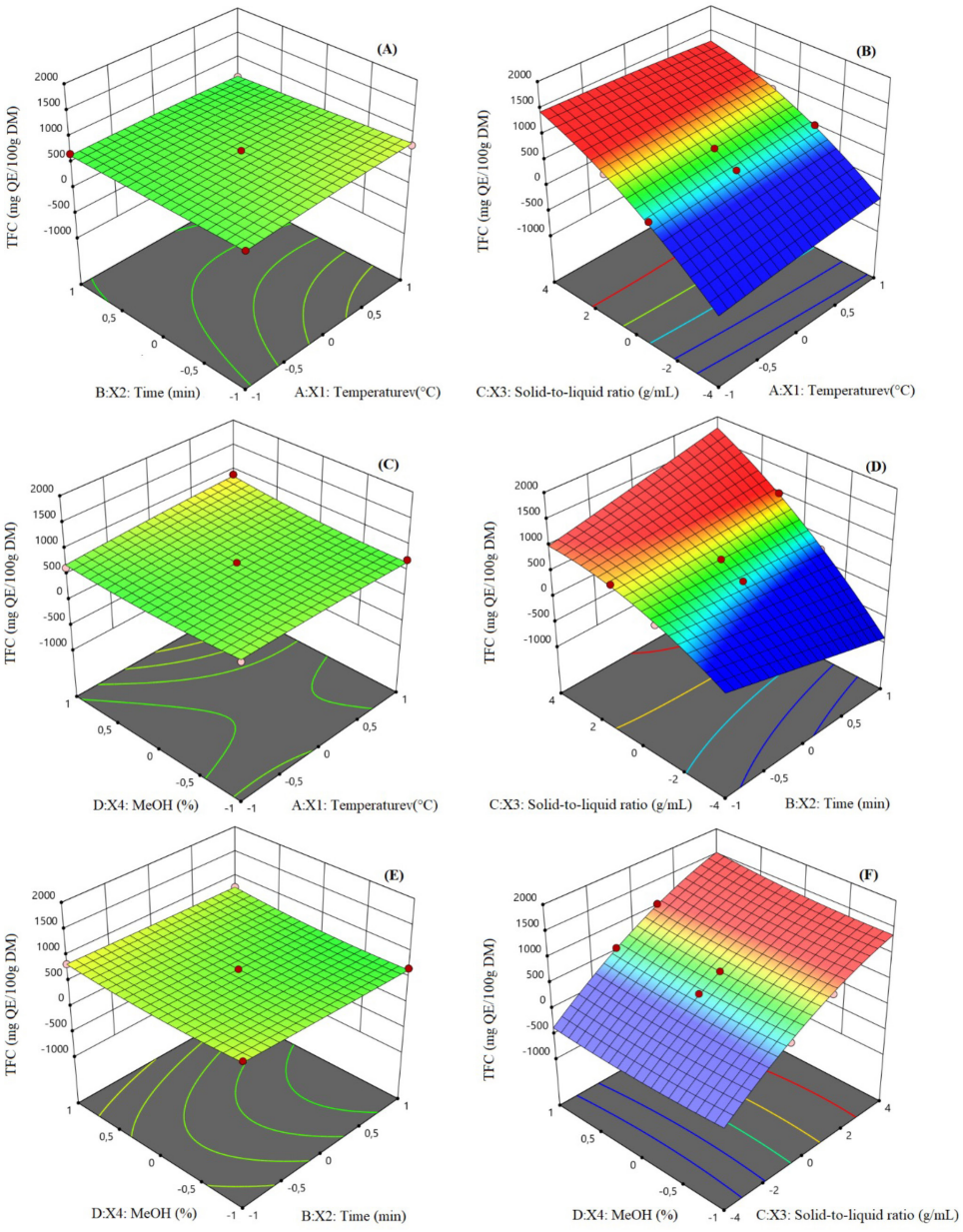

Fig. 1. Three-dimensional surface plot showing the effects of interaction between the independent factors, namely, extraction temperature, extraction time, solid-to-liquid ratio, and MeOH concentration, on the extraction yield of flavonoids from terebinth male flowers.

inhibition zone diameter reaching 25 mm, followed by *Cladosporium*, which showed a maximum inhibition zone diameter exceeding 18 mm. Among the bacterial strains, *L. monocytogenes* was the most sensitive, with inhibition zone diameters exceeding 16 mm.

The regression coefficients presented in Table 4 further support these observations. A stronger linear effect of an independent variable indicates a greater impact of the extract on the corresponding microbial strain. These findings underscore the importance of optimizing extraction parameters to maximize the antimicrobial efficacy of *Pistacia* extracts.

In this study, the linear effects of the independent variables had a more significant influence on the extract compared to the interaction and quadratic effects, and consequently on their activity against different microbial strains. The extracts exerted a stronger effect on fungal strains than on bacterial strains, as evidenced by the more pronounced linear effects. This was illustrated by the following reduced regression equations:

$$Y_{\text{Bacillus}} = 7.79 + 2.69\,X_1X_2 + 2.25\,X_1X_3 + 1.87\,X_1X_4 - 1.68\,X_2X_3 + 2.35\,X_2X_4 - 1.80\,X_3X_4 + 2.59\,X_1^2 + 1.54\,X_2^2 + 2.14\,X_3^2 + 1.44\,X_4^2,$$

Table 2. Four level Box-Behnken design and responses for total flavonoid content (mg QE 100 g^{-1} DM)

Run	Temperature (°C), X1	Time (min), X2	Solid-liquid ratio (g/mL), X3	MeOH concentration (%), X4	TFC (n = 3)
1	0	0	1	1	1054
2	–1	0	–1	0	460
3	0	0	0	0	715
4	1	1	0	0	611
5	0	–1	0	–1	841
6	1	0	0	–1	727
7	0	1	0	1	786
8	0	1	0	–1	689
9	0	0	–1	–1	472
10	–1	0	0	–1	711
11	1	0	0	1	909
12	0	–1	0	1	850
13	0	1	1	0	1002
14	–1	–1	0	0	697
15	0	0	–1	0	502
16	1	0	–1	0	525
17	–1	0	1	0	904
18	0	0	–1	1	577
19	–1	1	0	0	689
20	–1	0	0	1	652
21	0	0	1	–1	906
22	1	–1	0	0	779
23	0	1	–1	0	260
24	0	–1	1	0	910
25	0	–1	–1	0	607
26	0	0	0	0	699
27	1	0	1	0	872

Table 3. ANOVA of the quadratic model for total flavonoid content. * significant at 5%, ** significant at 1%

Source	Sum of squares	Mean square	*df*	*F*-value	*P*-value
Model	8.468E+05	60485.12	14	27.76	< 0.0001**
X1-Extraction temperature	8022.43	8022.43	1	3.68	0.0791
X2-Extraction time	34766.55	34766.55	1	15.96	0.0018**
X3-Solid-to-liquid ratio	6.576E+05	6.576E+05	1	301.82	< 0.0001**
X4-MeOH concentration	19343.30	19343.30	1	8.88	0.0115*
X1X2	6448.58	6448.58	1	2.96	0.1110
X1X3	2352.25	2352.25	1	1.08	0.3193
X1X4	14509.30	14509.30	1	6.66	0.0241*
X2X3	48180.25	48180.25	1	22.11	0.0005**
X2X4	1930.67	1930.67	1	0.8862	0.3651
X3X4	462.25	462.25	1	0.2122	0.6533
$X1^2$	1798.80	1798.80	1	0.8256	0.3814
$X2^2$	64.30	64.30	1	0.0295	0.8665
$X3^2$	890.82	890.82	1	0.4089	0.5346
$X4^2$	17068.86	17068.86	1	7.83	0.0161*
Residual	26144.10	2178.68	12		
Lack of fit	26015.13	2365.01	11	18.34	0.1804
Pure error	128.97	128.97	1		
Cor total	8.729E+05		26		

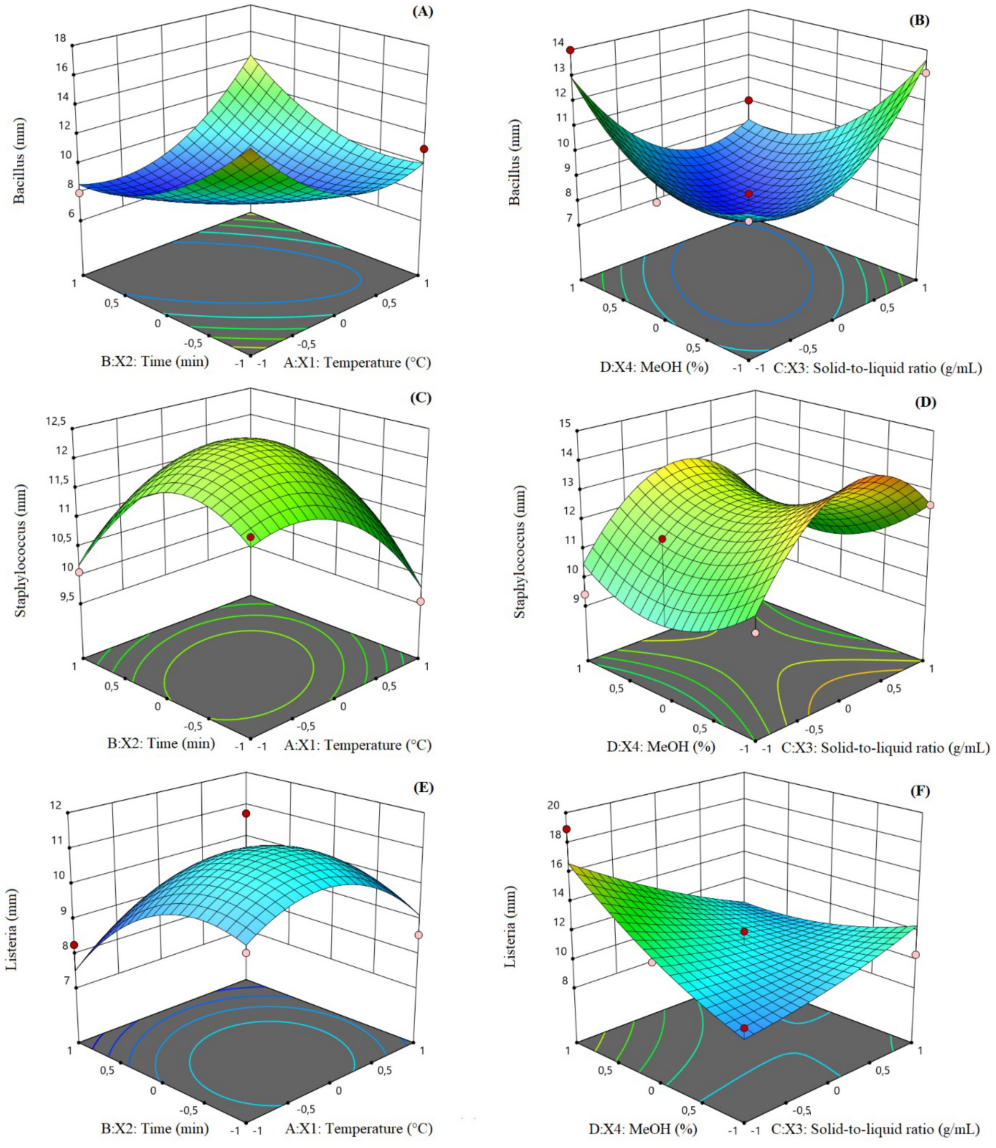

Fig. 2. Three-dimensional response surface plots of antibacterial activity of terebinth male flower extract against *Bacillus* (A, B), *Staphylococcus* (C, D), and *Listeria* (E, F) strains.

$$Y_{\text{Staphylococcus}} = 11.88 + 0.69\ X_3 + 1.43\ X_2X_4 - 2.26\ X_3^2 + 1.54\ X_4^2,$$

$$Y_{\text{Listeria}} = 10.66 - 0.88\ X_2 - 0.96\ X3 + 1.24\ X_4 - 2.34\ X_3X_4,$$

$$Y_{\text{Cladosporium}} = 15.46 - 2.09\ X_2 - 3.62\ X_4 + 4.44\ X_2X_3 - 2.05\ X_2X_4 + 2.11\ X_3X_4 - 3.01\ X_2^2 - 4.26\ X_4^2,$$

$$Y_{\text{Aspergillus}} = 12.84 - 3.8\ X1 - 2.72\ X_3 - 1.84\ X4 + 7.38\ X_1X_2 - 3.00\ X_1X_4 - 3.08\ X_3X_4 - 4.67\ X_4^2.$$

Validation of optimal extraction conditions

To determine the optimal extraction conditions for phenolic compounds from *P. terebinthus* male flowers, regression models were employed to predict TFC and antimicrobial activity at grid points within the region of optimal responses. The optimized extraction parameters, presented in Fig. 4 and Table 5, were validated through three independent experimental trials. The observed values for TFC (933.23 mg QE 100 g^{-1} DM) and antimicrobial activity (e.g., 18.43 mm inhibition zone against *Aspergillus niger*) closely matched the predicted values (953.27 mg QE 100 g^{-1} DM and 19.12 mm, respectively). No statistically significant differences were found ($p < 0.05$), confirming the model's reliability. Furthermore, the desirability index of 0.73 confirmed the adequacy and robustness of the optimization model, reflecting the overall suitability of the predicted values for TFC and antibacterial activity from the male floral extract of *P. terebinthus*, as assessed by the desirability approach described by Zaddem (2014).

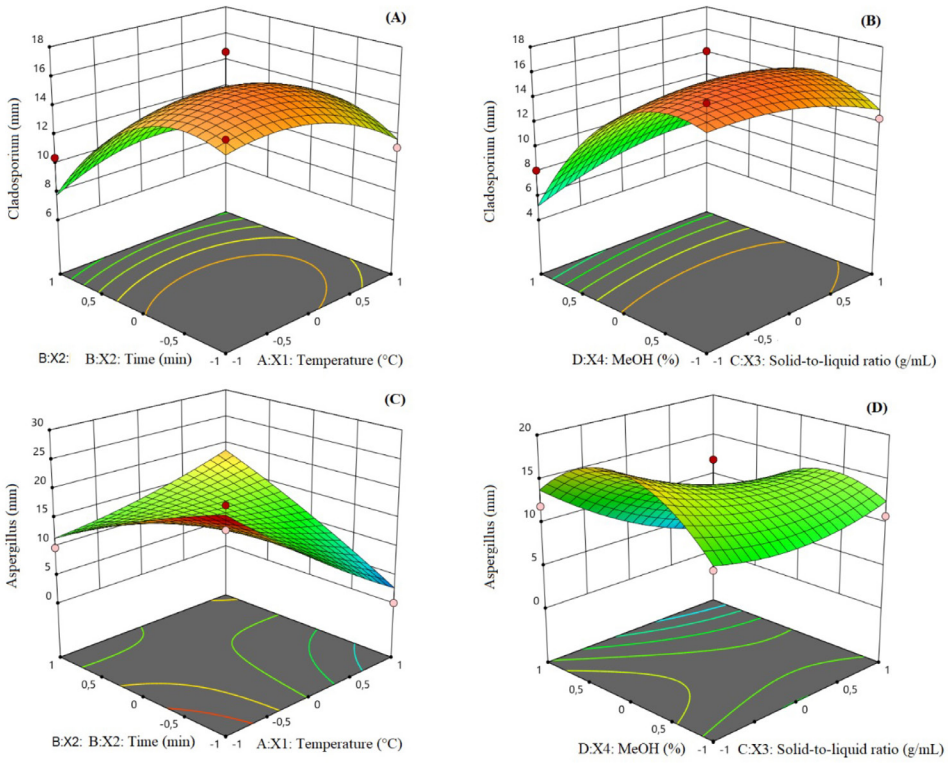

Fig. 3. Three-dimensional response surface plots of antifungal activity of terebinth male flower extract against *Cladosporium* (A, B) and *Aspergillus* (C, D) strains.

Discussion

The extraction efficiency of flavonoids from *P. terebinthus* male flowers was significantly influenced by the solid-to-liquid ratio, MeOH concentration, temperature, and extraction time. The positive correlation between the solid-to-liquid ratio and TFC yield can be attributed to enhanced mass transfer between the solvent and plant matrix (Rudić et al. 2012). Lower MeOH concentrations (60%) maximized TFC yield, likely due to the optimal polarity for flavonoid solubility and reduced solvent viscosity (Liao et al. 2021). This aligns with the observed interaction between temperature and MeOH concentration (Table 4). Conversely, prolonged extraction times negatively impacted TFC, possibly due to thermal degradation of flavonoids (Chaves et al. 2020) or solvent evaporation (Tan et al. 2013).

The impact of extraction time on the flavonoid yield varies across studies and plant matrices. For instance, a study on Salix babylonica buds found that TFC yield increased with extraction time, peaking at 30 min, after which it declined (Zhang et al. 2022). In contrast, research on pine bark indicated that extraction time was not a critical factor in maximizing polyphenol extraction (Jerez et al. 2006). These discrepancies highlight the importance of optimizing extraction parameters for each specific plant matrix to achieve maximum efficiency (Kim et al. 2022).

The antimicrobial activity results are consistent with those of Benhammou et al. (2008), who observed that leaf extracts of *Pistacia lentiscus* and *Pistacia atlantica* possess stronger antifungal activity than their antibacterial effects. Their work highlighted the selective antimicrobial properties of *Pistacia* species, particularly their marked efficacy against fungal pathogens. Similarly, our study revealed that terebinth male flower extracts exhibit more potent antifungal activity than their antibacterial effects. This observation suggests that species of the genus *Pistacia* may share a common molecular profile associated with antimicrobial activity, potentially mediated by bioactive molecules such as terpenoids, tannins, and flavonoids. These findings contribute to the growing evidence for the antifungal potential of *Pistacia* species and highlight their relevance in the development of alternative strategies for fungal disease management.

Our findings further support the results of previous studies regarding the antibacterial properties of *P. terebinthus* fruit and leaf (Kavak et al. 2010; Durak, Uçak 2015). For instance, Durak and Uçak (2015) observed a concentration-dependent antibacterial effect, notably against *L. monocytogenes* and *Salmonella typhimurium*, with moderate efficacy against *S. aureus* and *E. coli*. Likewise, Kavak et al. (2010) reported the antimicrobial activity of *P. terebinthus* leaf extracts against the Gram-positive bacterium *S. aureus*. In line with these findings, our

Table 4. Coefficients of regression and P-values of the all-response variables

Term	Model *p*	Lack of fit	Intercept	X1	X2	X3	X4	X1X2	X1X3	X1X4	X2X3	X2X4	X3X4	$X1^2$	$X2^2$	$X3^2$	$X4^2$
TFC	<.0001***	0.1804	714.271	25.8561	−53.82	226.5	40.14	−40.15	−24.25	60.22	109.75	21.96	10.75	−19.57	3.700	−14.27	60.29
P–values				0.0791	0.0018	< 0.0001	0.0115	0.1110	0.319	0.024	0.0005	0.36	0.653	0.381	0.866	0.534	0.016
Bacillus	0.0014**	0.2522	7.795	0.17916	−0.73	−0.06	−0.24	2.69	2.5	1.87	−1.68	2.35	−1.802	2.592	1.540	2.142	1.44
P–values				0.6518	0.083	0.870	0.5333	0.0017	0.0029	0.016	0.027	0.0043	0.0198	0.0013	0.028	0.0059	0.037
Staphylococcus	0.0007**	0.6141	11.88	−0.3108	−0.11	0.69	−0.48	0.55	0.99	0.63	0.54	1.43	−0.20	−0.504	−0.767	−2.267	1.548
P–values				0.2782	0.6817	0.0228	0.1022	0.2643	0.058	0.203	0.27	0.0107	0.67	0.271	0.104	0.0003	0.0041
Listeria	0.0050**	0.4087	10.66	−0.1566	−0.88	−0.96	1.24	0.37	−0.177	0.64	−0.02	−0.83	−2.34	−1.073	−0.94	0.525	0.950
P–values				0.6569	0.0239	0.0132	0.0034	0.5435	0.770	0.303	0.97	0.18	0.0020	0.074	0.109	0.374	0.109
Cladosporium	0.0033**	0.9202	15.46	−0.6483	−2.09	0.61	−3.62	1.002	−1.122	−0.70	4.445	−2.05	2.11	−1.219	−3.013	−0.938	−4.26
P–values				0.2224	0.0013	0.2280	< 0.0001	0.2730	0.222	0.434	0.0003	0.036	0.032	0.155	0.0028	0.282	0.0002
Aspergillus	0.0003***	0.1147	12.84	−3.8066	0.60	−2.72	−1.84	7.38	−2.42	−3	−2.29	1.025	−3.085	1.641	−0.076	2.486	−4.678
P–values				0.0004	0.461	0.0041	0.038	0.0002	0.103	0.049	0.12	0.47	0.044	0.220	0.952	0.083	0.0031

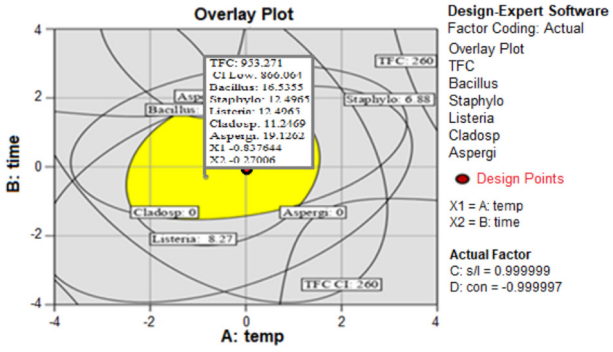

Fig. 4. Optimization of extraction of total flavonoid content and antimicrobial activity of terebinth male flowers based on the superimposing method.

results reveal that *P. terebinthus* male flower extracts exhibit enhanced inhibitory effects against *L. monocytogenes* and *S. aureus*. Together, these findings underline the promising antibacterial potential of *Pistacia* species, highlighting the critical role of plant part and extract concentration in determining efficacy against various microbial strains. Other studies have confirmed strong antibacterial activity of leaf, fruit, and gall extracts of *P. terebinthus* against pathogens including *S. aureus, E. coli, B. subtilis*, and *P. aeruginosa*, often using methanolic extracts and disc diffusion methods. Gall extracts exhibited remarkable effects with inhibition zones up to 20 mm against *S. aureus*, indicating that different plant organs vary in potency but overall reveal bactericidal potential (Bellifa 2021).

The antimicrobial activity of *P. terebinthus* male flowers can be discussed in light of the chemical compositions characterized in other parts of the plant, although direct chemical data on the male flowers is currently lacking. Studies on various organs of *P. terebinthus* show a rich presence of phenolic compounds, flavonoids, and fatty acids, all associated with antimicrobial properties. For example, *P. terebinthus* fruits contain high levels of phenolic compounds such as quercetin and catechin, and fatty acids including palmitic, oleic, and linoleic acids, which contribute to their bioactivity (Özcan 2020). Oils extracted from roasted fruits also show a high total phenolic content despite low β-carotene and lutein levels (Durmaz, Gökmen 2011). Methanol extracts generally contain higher flavonoid and phenolic contents than acetone extracts, with fruit methanol extracts especially rich in total phenols (Topçu 2007; Orhan 2012). Given that phenolic compounds and flavonoids are recognized antimicrobial agents, it is plausible that the antimicrobial activity of male flowers is linked to a unique blend of these compounds, even if their full chemical profile is yet to be elucidated. Thus, integrating the documented presence of these bioactive phytochemicals in other organs supports understanding the antimicrobial efficacy of male flowers, affirming the crucial role of chemical composition diversity across plant parts in determining antimicrobial potential.

Table 5. Experimental and predicted values of response variables under optimal extraction conditions

Variable	Predicted value	Observed value	Desirability
X1 (°C)	32.6	32	
X2 (min)	62.8	63	0.73
X3 (w/v)	1:19.5	1:20	
X4 (%)	60	60	
TFC	953.27	933.23	
$Y_{Bacillus}$	16.53	15.60	
$Y_{Staphylococcus}$	12.49	13.03	
$Y_{Listeria}$	12.36	11.23	
$Y_{Cladosporium}$	11.24	10.25	
$Y_{Aspergillus}$	19.12	18.43	

The extraction procedure was optimized using a desirability criterion to maximize both total flavonoid content and antimicrobial activity. The optimal extraction conditions identified for this study were: MeOH concentration of 60%, solid-to-liquid ratio of 1:19.5 (g/mL), extraction temperature of 32.6 °C, and time of 63 min (Fig. 4). Under these conditions, the flavonoid content was 953.27 mg QE 100 g^{-1} DM, and the antimicrobial activity expressed as inhibition zones of 16.53 mm for *B. cereus*, 12.49 mm for *S. aureus*, 12.36 mm for *L. monocytogenes*, 11.24 mm for *Cladosporium* sp., and 19.12 mm for *A. niger*.

These optimized extraction conditions successfully maximized both flavonoid yield and antimicrobial activity from *P. terebinthus* male flowers, demonstrating the effectiveness of RSM-BBD modeling. The minor deviations (< 10%) between predicted and experimental values for both TFC and inhibition zones validate the model's accuracy and reliability. The superior antifungal activity against *A. niger* (19.12 mm inhibition zone) compared to bacterial strains aligns with previous reports of *Pistacia* species' bioactive profiles, likely due to synergistic effects between flavonoids and other phytochemicals like terpenoids and tannins (Benhammou et al. 2008; Rudic et al. 2021; Fidan et al. 2023). The high desirability score (0.73) confirms that the optimized conditions strike an ideal balance between extraction efficiency and bioactivity.

Conclusions

This study successfully optimized the extraction of flavonoids from *Pistacia terebinthus* male flowers using Response Surface Methodology (RSM) and Box-Behnken Design (BBD), identifying the optimal conditions as 60% MeOH, a 1:19.5 solid-to-liquid ratio, 32.6 °C, and 63 min, yielding a high total flavonoid content (953.27 mg QE 100 g^{-1} DM). The extracts demonstrated significant antimicrobial activity, particularly against fungal strains like *Aspergillus niger* (with an inhibition zone diameter of 19.12 mm), underscoring their potential as natural antifungal agents. The robust predictive power of the RSM-BBD model was validated, highlighting its efficacy for optimizing bioactive compound extraction. The findings also highlight the potential of *P. terebinthus* male flowers as a sustainable resource for developing natural antimicrobials, particularly for fungal control. However, further studies are needed to isolate and characterize the specific compounds responsible for these activities.

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