INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
25–09/2025/34:340101
http://www.fspublishers.org

Full Length Article

Optimization of Total Flavonoid Content and Antimicrobial Activity from *Pistacia terebinthus* Flowers using Response Surface Methodology

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Received 03 January 2025; Accepted 19 February 2025; Published online 12 March 2025

Editor: Abdul Wahid

Abstract

The flowers of *Pistacia terebinthus* are a source of extracts with significant antimicrobial activity, mainly due to their flavonoid content. The present study used Box-Behnken Design (BBD) and Response Surface Methodology (RSM) to identify and optimize the main extraction parameters affecting the total flavonoid content (TFC) and the antimicrobial activity of the extract from *P. terebinthus* flowers. Results showed that solid-to-liquid ratio (g/mL), extraction time (min), and solvent concentration (%) were the most influential factors, in that order. The predicted optimal conditions were a solid-to-liquid ratio of 1:19.5 g/mL, an extraction time of 63 min, a solvent concentration of 60% and an extraction temperature of 32.6°C, yielding a TFC of 953.27 mg QE/100 g DM. Under these conditions, the extracts showed remarkable antimicrobial activity, the inhibition zones were 16.53 mm against *Bacillus cereus*, 12.49 mm against *Staphylococcus aureus*, 12.36 mm against *Listeria monocytogenes*, 11.24 mm against *Cladosporium* sp., and 19.12 mm against *Aspergillus niger*. The validation tests confirmed strong agreement between the experimental- and model-predicted results, which attested to the reliability and accuracy of the model in estimating TFC and antimicrobial activity.

Keywords: *Pistacia terebinthus*; Flavonoids; Antimicrobial activity; Box-behnken design; Response surface methodology

Introduction

Flavonoids are a highly diverse group of plant-derived secondary metabolites, including the well-known pigments responsible for the coloration 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 and Lamb 2005). Flavonoids and other polyphenols are naturally present in plant systems, they could however, also 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 values 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 plants' anti-inflammatory, antioxidant and cytotoxic activities, enhancing their therapeutic potential.

Pistacia terebinthus (Anacardiaceae), well recognized for its high flavonoid and phenolic content, is widely distributed in Mediterranean countries like Algeria, Morocco, Tunisia and Turkey (Topçu *et al*. 2007). Aside from its multiple traditional uses, various parts of this shrub are also of culinary importance (Bozorgi *et al*. 2013; Batovska and Inbar 2024). Similarly, scientific studies have shown that terebinth has a wide spectrum of biological activities, including among others antimicrobial, antiviral, antidiabetic, antihyperlipidemic, antiatherosclerotic, hepatoprotective, gastrointestinal neuroprotective and anticancer properties (Durak and Uçak 2015; Akyuz *et al*. 2022; Najibullah *et al*. 2022; Uysal *et al*. 2022; Fidan *et al*. 2023; Fırat *et al*. 2024; Ozgolet *et al*. 2024).

To cite this paper: Rahal K, R Arhab, A Fercha, A Zeraib, Z Bouziane, L Douaouya, M Tahirine, A Foughalia (2025). Optimization of total flavonoid content and antimicrobial activity from *Pistacia terebinthus* flowers using response surface methodology. *Intl J Agric Biol* 34:340101. https://doi.org/10.17957/IJAB/15.2336

Terebinth (turpentine tree) is a perennial dioecious woody shrub growing in Algeria on dry rocky slopes close to coniferous forests (Louzabi *et al*. 2016; Hamlat *et al*. 2019). The plant features sturdy, shiny, narrow leaves (8-10 cm) with resinous flavors, reddish-purple flowers, and spherical drupes (4-7 mm) that turn dark greenish at maturity (Álvarez *et al*. 2008). Terebinth produces very small wind-pollinated flowers. Inflorescences of circa 50 flowers are grouped always in panicles; one panicle may contain approximately 20 inflorescences and as many as 800 flowers. A branch may bear of total of up to 10 panicles, sometimes grouped in several distal clusters (Traveset 1994). While the female flowers turn into fruits and seeds, the male flowers remain largely unexploited. Although no previous studies have explored their potential, preliminary tests have revealed that terebinth flowers serve as a valuable source of antimicrobial compounds, particularly flavonoids. Considering the impact of extraction conditions on flavonoid content and consequently, the antimicrobial properties of extracts, the present study was conducted to identify and optimize the extraction parameters most relevant for maximizing the yield of total flavonoid content (TFC) from *P. terebinthus* flowers. Additionally, the study aimed at the evaluation of the antibacterial and antifungal activities of the extracts by applying response surface methodology (RMS) based on Box-Behnken design (BBD) for analysis.

Materials and Methods

Plant sample preparation

The flowers of *P. terebinthus* were collected from El Hamma, Khenchela province, Algeria (35° 26′ 25″ N, 7° 05′ 04″ E; 1058 m asl.) and the voucher specimen stored in the herbarium at the University of Abbès Laghrour, Algeria. The species was taxonomically authenticated by Dr. A Zeraib using Flora of Algeria (Quezel and Santa 1962). The samples were air-dried and then ground into a fine powder and stored at 4°C until analysis. To determine their total flavonoid content and antimicrobial activity, *P. terebinthus* flowers were subjected to extraction by maceration with methanol, employing various extraction parameters informed by preliminary assays and relevant literature data (see below).

Determination of the total flavonoid content

The total flavonoid content (TFC) of dried flowers was determined using aluminum colorimetric assay (Do *et al*. 2014). The diluted extract sample (100 μg/mL) or quercetin (2 mL) was mixed with $AlCl_3$ solution (0.1 mL, 10% w/v) and potassium acetate (0.1 mL, 0.1 m*M*). The absorbance was recorded after 30 min of incubation at room temperature using a Shimadzu UV-1800 spectrophotometer at 415 nm. The TFC was represented as mg quercetin equivalent per 100 g dry material (mg QE/100 g DM) based on the quercetin calibration curve.

Antibacterial and antifungal activity

The antibacterial and antifungal activity of the terebinth flower extracts was assessed using the disk diffusion method as described by Pfaller and Herwaldt (1997). The degree of microbial growth inhibition was evaluated by measuring the diameters of the clear zones around disks impregnated with 10 μL of samples, following a 24 h incubation period at 37°C on Petri dishes filled with 20 mL of Mueller Hinton Agar (MHA). Inhibition zones were calculated using ImageJ software (https://imagej.net/ij/).

The study included strains of three bacterial species from the ATCC collection: *Staphylococcus aureus* (ATCC25923), *Listeria monocytogenese* (ATCC7644) and *Bacillus cereus* (ATCC11778). To ensure experimental consistency, the bacterial inoculum size was standardized to approximately 1×10^8 CFU/mL by adjusting turbidity equivalent to 0.5 McFarland turbidity standards. DMSO was used as the negative control. Bacterial susceptibility to the extracts was categorized as follows, based on EUCAST guidelines: Susceptible: inhibition zone diameter (Ø) $\geq$ 11 mm; Intermediate: 5 mm < Ø < 11 mm; Resistant: Ø $\leq$ 5 mm.

The antifungal assay was performed as described below. Following inoculation of the target fungal strains (*Aspergillus niger* and *Cladosporium* sp.), the petri dishes were incubated at 37°C for a period of five days. Following incubation, 10 mL of the distilled water containing Tween 20 was distributed over the surface of the Petri dish, after which the spore suspension was carefully collected in sterilized test tubes. The inoculum is adjusted to a concentration of 1-2 $\times 10^5$ spores/mL through cell counting using a Malassez cell counter. The inoculation on petri dishes and the results recovering were the same as the antibacterial assay.

Experimental design and RSM modeling

The experiment was conducted by Box-Behnken design (BBD) using Design-Expert software 13.0.5.0 (Box and 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 next equation:

$$x_i = (X_i - X_0)/\Delta X_i \qquad (1).$$

Where, x_i is the coded value of X_i, X_o 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:

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

Where, Yi is the outcome variable (TFC and antimicrobial activity); Xi 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, linear, quadratic and interaction regression coefficients, respectively. These regression coefficients were calculated using the ordinary least-squares method.

Results

Optimization of flavonoid extraction conditions from terebinth flowers

The results of RSM-BBD and ANOVA are shown in Table 2-3. A total of 27 experimental points are designed and implemented. The simplified quadratic polynomial equation for flavonoid extraction in terms of actual factors is expressed as follows:

$$TFC = 714.27 - 53.43\ X_2 + 226.53\ X_3 + 40.15\ X_4 + 60.23\ X_1X_4 + 109.75\ X_2X_3 + 60.29\ X_4^2 \quad (3).$$

The regression coefficient of the model $R^2 = 0.9701$, and CV = 6.49%, which is very low and indicates that the model fits well and with high confidence. The model's *P*-value is less than *0.0001*, and the Lack of fit term is not significant (*P = 0.1804*). The results demonstrated that the model is highly significant and may be used for the prediction of flavonoid yield under varying extraction circumstances.

According to these results, the linear terms X_2 (extraction time), X_3 (solid-to-liquid ratio), and X_4 (MeOH concentration) are significant ($P < 0.05$), whereas the linear term X_1 (extraction temperature) is not significant ($P > 0.05$). Based on the F-values of the linear terms, the factors influencing the extraction of TFC from terebinth flowers were ranked in order of their relative impact as follows: solid-to-liquid ratio (X_3) > extraction time (X_2) > MeOH concentration (X_4) > temperature (X_1). This ranking highlights the dominant role of the solid-to-liquid ratio in optimizing the extraction of flavonoids.

Fig. 1 illustrates the response surface plots generated by the model. The three-dimensional (3D) response surface plots (A-F) show the interaction effect of the independent variables on TFC yield. However, it is useful to note that a steeper slope in these plots indicates a stronger influence of the corresponding independent variable on the extraction yield of flavonoids. In contrast, a gentler slope indicates a lesser impact of the independent variable on the extraction rate of flavonoids. As shown in Fig. 1A, 1C and 1D, the 3D plots were flat, indicating that the interaction between the factors (temperature *vs*. time; temperature *vs*. MeOH concentration and time *vs*. MeOH concentration) had an insignificant influence on the extraction yield of total flavonoids. However, as shown in Fig. 1B, 1D and 1F, the 3D plots were steep, and as the solid-to-liquid ratio increased, the yield of the flavonoid content correspondingly increased. As the yield reached its peak, the increase decelerated, indicating that the interaction between these two independent variables had a significant influence on the yield of flavonoid extraction. These findings align with ANOVA results presented in Table 3.

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

Independent variables	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

Table 2: Four level Box-Behnken Design and responses for TFC (mg QE/100 g DM)

Run	Temperature (°C), X_1	Time (min), X_2	Solid-Liquid ratio (g/mL), X_3	MeOH concentration (%), X_4	TFC (n = 3)
1	0	0	1	1	1054
2	-1	0	-1	0	460
3	0	0	0	0	715.152
4	1	1	0	0	610.606
5	0	-1	0	-1	840.909
6	1	0	0	-1	727.273
7	0	1	0	1	786.364
8	0	1	0	-1	689.394
9	0	0	-1	-1	472
10	-1	0	0	-1	710.606
11	1	0	0	1	909.091
12	0	-1	0	1	850
13	0	1	1	0	1002
14	-1	-1	0	0	696.97
15	0	0	-1	0	502.061
16	1	0	-1	0	525
17	-1	0	1	0	904
18	0	0	-1	1	577
19	-1	1	0	0	689.394
20	-1	0	0	1	651.515
21	0	0	1	-1	906
22	1	-1	0	0	778.788
23	0	1	-1	0	260
24	0	-1	1	0	910
25	0	-1	-1	0	607
26	0	0	0	0	699.091
27	1	0	1	0	872

Optimization of Antimicrobial activity of terebinth flower extracts

Twenty-seven extracts obtained from the experimental runs of the BBD were assessed for their antimicrobial activity against a range of bacterial and fungal strains, as antimicrobial efficacy depends on both the quality and quantity of the metabolites. Accordingly, the influence of the independent variables used for total flavonoid content (TFC) extraction was also investigated for antibacterial and antifungal activities. The corresponding results are illustrated in Fig. 2-3 and Table 4.

As expected, the effects of the independent variables on microbial strains differed from their impact on TFC.

Table 3: ANOVA of the Quadratic model for TFC

Source	Sum of Squares	Mean Square	df	*F*-value	*P*-value
Model	8.468E+05	60485.12	14	27.76	*< 0.0001***
X_1-Extraction temperature	8022.43	8022.43	1	3.68	*0.0791*
X_2-Extraction time	34766.55	34766.55	1	15.96	*0.0018***
X_3-Solid-to-liquid ratio	6.576E+05	6.576E+05	1	301.82	*< 0.0001***
X_4-MeOH concentration	19343.30	19343.30	1	8.88	*0.0115**
X_1X_2	6448.58	6448.58	1	2.96	*0.1110*
X_1X_3	2352.25	2352.25	1	1.08	*0.3193*
X_1X_4	14509.30	14509.30	1	6.66	*0.0241**
X_2X_3	48180.25	48180.25	1	22.11	*0.0005***
X_2X_4	1930.67	1930.67	1	0.8862	*0.3651*
X_3X_4	462.25	462.25	1	0.2122	*0.6533*
X_1^2	1798.80	1798.80	1	0.8256	*0.3814*
X_2^2	64.30	64.30	1	0.0295	*0.8665*
X_3^2	890.82	890.82	1	0.4089	*0.5346*
X_4^2	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*^{ns}
Pure Error	128.97	128.97	1		
Cor Total	8.729E+05		26		

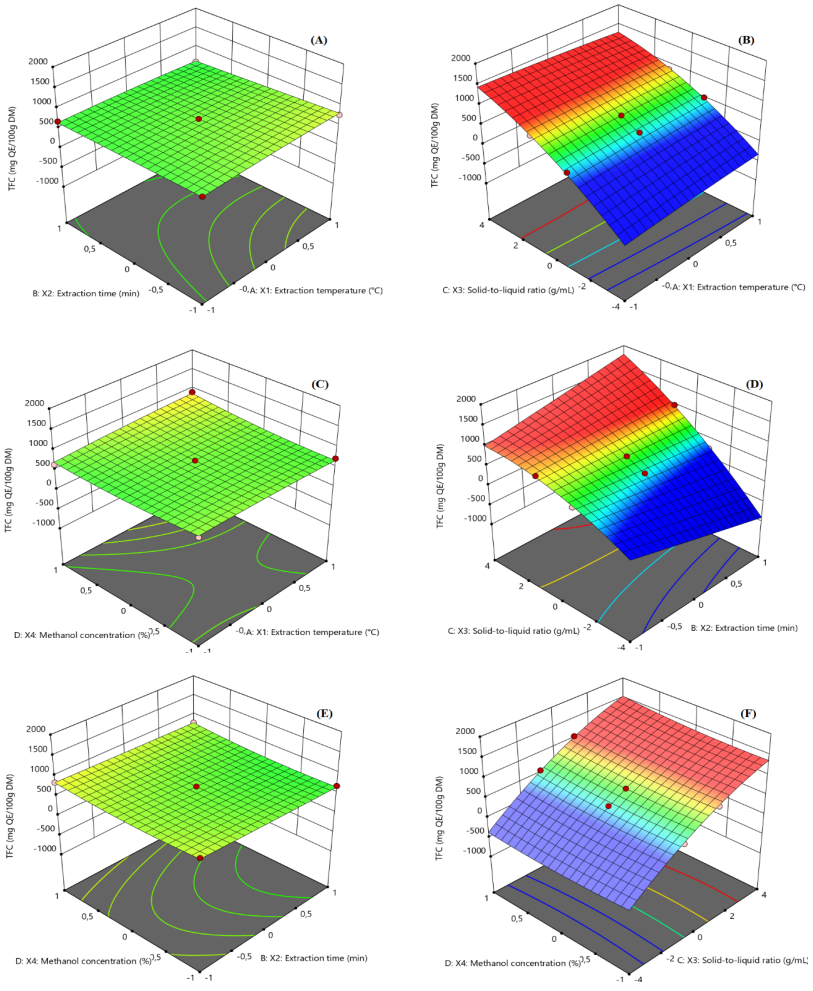

Fig. 1: Three-dimensional surface plots showing the effects of interaction between the independent factors, namely, extraction temperature, extraction time, solid-to-liquid ratio and MeOH concentration, on the extraction yield of flavonoids from terebinth flowers

Most independent variables exhibited a negative linear effect (Table 4) on *L. monocytogenes*, *Cladosporium* and *Aspergillus*. For *S. aureus*, no significant effect was observed except for the solid-to-liquid ratio ($P = 0.022$), which demonstrated a weak linear influence ($\beta = 0.69$). Conversely, for *Bacillus* species, no linear effects were detected;

Table 4: Coefficients of regression and *p*-values of the all-response variables

Term	Model *P*	Lack of fit	Intercept	X_1	X_2	X_3	X_4	X_1X_2	X_1X_3	X_1X_4	X_2X_3	X_2X_4	X_3X_4	X_1^2	X_2^2	X_3^2	X_4^2
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*

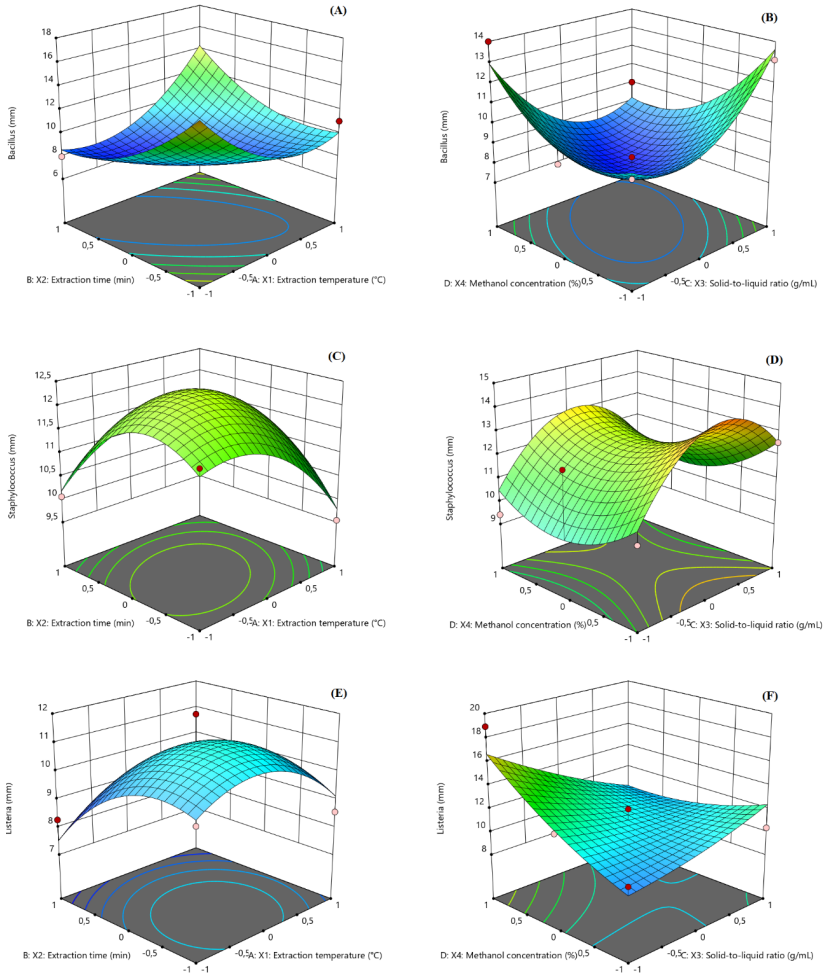

Fig. 2: Three-dimensional response surface plots of antibacterial activity of terebinth flower extract against *Bacillus* (A, B), *Staphylococcus* (C, D) and *Listeria* (E, F) strains

instead, quadratic effects and interaction effects were noted for all independent variables. The observed quadratic effects across most microbial strains indicate a non-linear relationship, where the response follows a curved pattern rather than a simple linear trend. Each bacterial and fungal strain exhibited distinct sensitivity profiles against the extracts of *P. terebinthus* flowers.

Overall, fungal strains exhibited greater sensitivity to the extracts compared to bacterial strains. Among the fungi, *A. niger* demonstrated the highest susceptibility, with an inhibition zone diameter reaching 25 mm, followed by *Cladosporium*, which showed a maximum inhibition zone exceeding 18 mm. In contrast, among the bacterial strains, *L. monocytogenes* was found 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

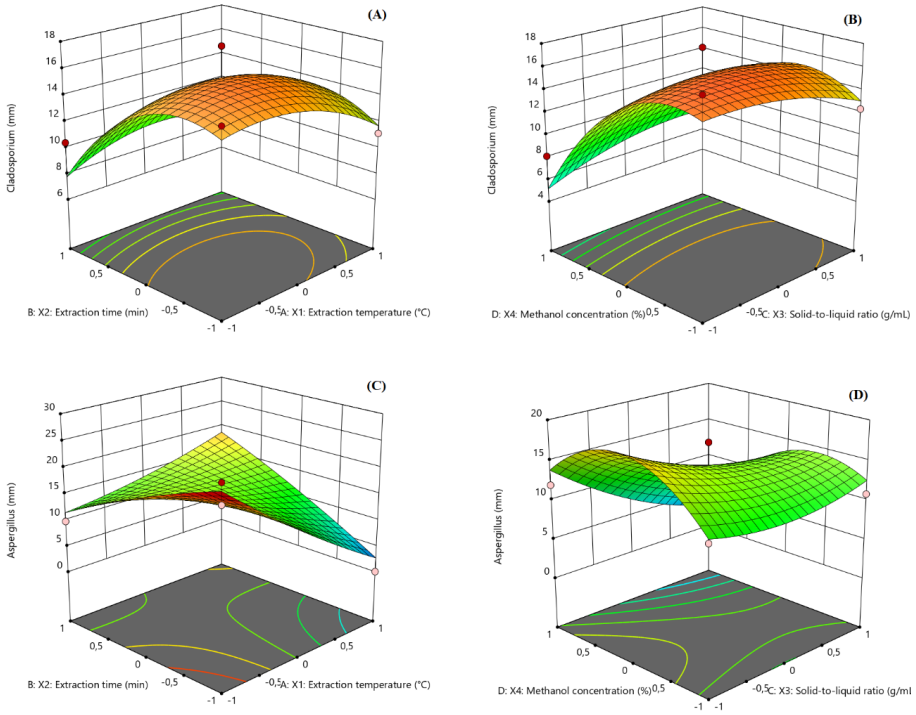

Fig. 3: Three-dimensional response surface plots of antifungal activity of terebinth flower extract against *Cladosporium* (A, B) and *Aspergillus* (C, D) strains

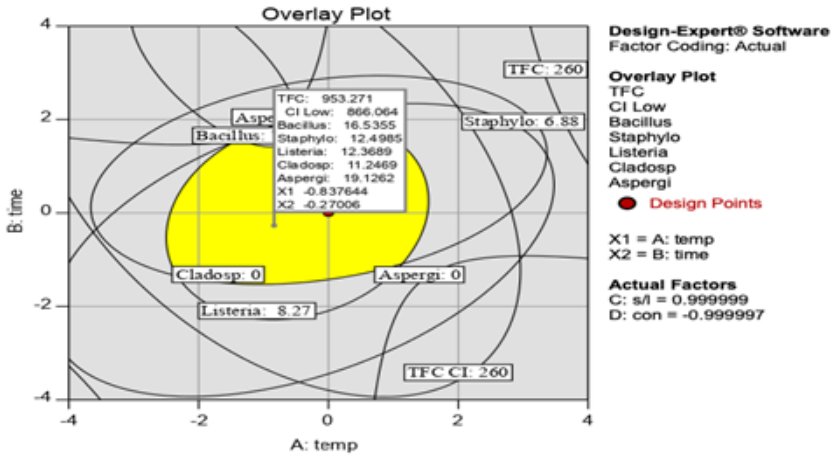

Fig. 4: Optimization of extraction of total flavonoid content and antimicrobial activity of terebinth flowers based on the superimposing method

extract on the respective microbial strain. These findings underscore the critical role of optimizing extraction parameters to maximize the antimicrobial efficacy of *Pistacia* extracts.

In this work, the linear effects of the independent variables exhibit a more significant effect on the extract, compared to the interaction and quadratic effects, and consequently on its activity against the different microbial strains. The extract showed a more significant effect on the fungal strains compared to the bacterial strains, as proven by the more pronounced linear effects, as illustrated by the following reduced regression equations:

$$Y_{Bacillus} = 7.79 + 2.69\ X_1X_2 + 2.25\ X_1X_3 + 1.87\ X_1X_4 - 1.68\ X_2X_3 + 2.35\ X_2X_4 - 1.80\ X_3X_4 + 2.59\ X_1^2 + 1.54\ X_2^2 + 2.14\ X_3^2 + 1.44\ X_4^2 \quad (4).$$

$$Y_{Staphylococcus} = 11.88 + 0.69\ X_3 + 1.43\ X_2X_4 - 2.26\ X_3^2 + 1.54\ X_4^2 \quad (5).$$

$$Y_{Listeria} = 10.66 - 0.88\ X_2 - 0.96\ X_3 + 1.24\ X_4 - 2.34\ X_3X_4 \quad (6).$$

$$Y_{Cladosporium} = 15.46 - 2.09\ X_2 - 3.62\ X_4 + 4.44\ X_2X_3 - 2.05\ X_2X_4 + 2.11\ X_3X_4 - 3.01\ X_2^2 - 4.26\ X_4^2 \quad (7).$$

$$Y_{Aspergillus} = 12.84 - 3.8X_1 - 2.72X_3 - 1.84X_4 + 7.38X_1X_2 - 3X_1X_4 - 3.08X_3X_4 - 4.67\ X_4^2 \quad (8).$$

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

Variables	Predicted value	Observed value	Desirability
X_1 (°C)	32.6	32	
X_2 (min)	62,8	63	0.73
X_3 (w/v)	1/19.5	1/20	
X_4 (%)	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	

Discussion

Although the positive impact of the Solid/liquid ratio on the TFC yield can be explained by the increased mass-transfer between the extraction solvent and sample. However, the effectiveness of lower MeOH concentrations in maximizing TFC yield may be attributed to the polarity of the extract's components (Rudić *et al*. 2021). Another potential explanation could be the increase in solubility of flavonoids as well as a decrease in solvent viscosity (Liao *et al*. 2021), which is in line with the effect of the interaction between temperature and MeOH concentration on the TFC yield (Table 4). The negative effect, however, of extraction time on TFC yield indicates that prolonged exposure to extraction conditions increases the risk of thermal degradation even at low temperatures or low operating power (Chaves *et al*. 2020) and induces solvent loss through evaporation, thereby decreasing mass transfer during extraction (Tan *et al*. 2013).

The impact of extraction time on the flavonoid yield varies across different studies and plant matrices. For instance, a study on *Salix babylonica* L. buds found that the TFC yield increased with extraction time, peaking at 30 minutes, after which it declined (Zhang *et al*. 2022). In contrast, research on pine bark indicated that extraction time was not a critical factor for 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).

Antimicrobial activity's results align with those of Benhammou *et al*. (2008), who observed that leaf extracts of *P. lentiscus* and *P. atlantica* possess stronger antifungal activity than antibacterial effects. Their work highlighted the selective antimicrobial properties of *Pistacia* species, particularly their pronounced effectiveness against fungal pathogens. Similarly, our study revealed that terebinth flower extracts exhibit more potent antifungal activity compared to its antibacterial effects. This observation suggests that species within the *Pistacia* genus may share a common molecular profile associated with antimicrobial activity, potentially driven by bioactive molecules such as terpenoids, tannins, and flavonoids. These findings contribute to the growing evidence of the antifungal potential of *Pistacia* species and underscore their relevance in the development of alternative strategies for managing fungal diseases.

Our findings further support the results of previous studies regarding the antibacterial properties of *P. terebinthus* fruit and leaf (Kavak *et al*. 2010; Durak and 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 *Escherichia 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 results reveal that *P. terebinthus* 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.

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: methanol 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 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 conditions were further validated by conducting three successive verification experiments. The experimental and predicted values (Table 5) were consistent and showed no significant variation at $P < 0.05$, confirming the accuracy and reliability of the regression equations derived from the study.

Conclusion

The RMS combined with Box-Behnken Design (BBD), were applied to optimize the extraction parameters (methanol concentration, solid-to-liquid ratio, time, and temperature) impacting the TFC and the antimicrobial activity of *P. terebinthus* flower extract. The optimal conditions included a solid-to-liquid ratio of 1:19.5, an extraction time of 63 min, a methanol concentration of 60%, and an extraction temperature of 32.6°C, yielding a TFC of 953.27 mg QE/100 g DM. Under these conditions, the extract exhibited outstanding antimicrobial activity, the inhibition zones were 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*. The validation tests confirmed strong agreement between experimental and model-predicted results, highlighting the robustness of the RSM-BBD model in estimating TFC and

antimicrobial activity.

Acknowledgements

The authors would like to thank the Ministry of Higher Education and Scientific Research of Algeria for their support.

Author Contributions

KR and RA conceived and designed the experiments, KR, AF, and AZ performed and interpreted the results. KR and AF prepared the manuscript draft. ZB, LD, MT, AFo, contributed by providing critical feedback, assisting with data interpretation, and revising the manuscript.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper

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