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Phytochemical Analysis and Effect of Extraction Conditions on Antioxidant and Antibacterial Activities of *Cytisus villosus* Pourr.

Abdelhalim Boussaa*[a, b], Imane Bouhanna [c], Daniela Rigano[d], Maria Maisto[d], Adriana Basile[e], Fatiha Bedjou[a]

(a) : Laboratoire de Biotechnologie Végétale et d'Ethnobotanique, Faculté des Sciences de la Nature et de Vie, Université Abderrahmane Mira, 06000, Bejaia, Algeria (boussaa.abdelhalim@univ-khenchela.dz)

(b) : Département de Biologie Moléculaire et Cellulaire, Faculté des Sciences de la Nature et de Vie, Université Abbes Laghrour, 40000, Khenchela, Algeria

(c): Institut National de l'Alimentation, la Nutrition et des Technologies Agro-Alimentaires (INATAA), 25000, Constantine, Algeria

(d) : Department of Pharmacy, School of Medicine and Surgery, University of Naples Federico II, 80131 Naples, Italy.

(e): Department of Biology, University of Naples "Federico II", 80126 Naples, Italy.

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Abstract

The aim of this study is to assess and optimize *Cytisus villosus* Pourr. biological activities using response surface methodology (RSM). Antioxidant (DPPH, FRAP, ABTS and molybdenum assays) and anti microbial (antifungal and antibacterial) activities of different *C. villosus* extracts have been determined. Central composite design (CCD) was used to investigate the effect of temperature, extraction time, Liquid/Solid ratio, and solvent concentration on total phenolic yield (TPY), Anti-*Staphylococcus aureus* (ASA), Anti-*Escherichia coli* (AEC) and DPPH radical scavenging activities (DRS). The used model has a good fitting with all dependent parameters ($p<0.01$). By applying optimal extraction conditions, maximum antioxidant and antibacterial activities have been found (20 mm, 12mm, and 87.01% for ASA, AEC and DRS, respectively). The results of HPLC/diode-array detector (DAD) analysis of *C. villosus* methanolic extract showed that rutin, quercetin and catechin are the most abundant compounds, which can explain the remarkable antioxidant and antimicrobial activities showed by this extract.

Keywords: *Cytisus villosus*, antioxidant activity, antimicrobial activity, response surface methodology, phenolic compounds

Introduction

Cytisus villosus Pourr. (Hairy Broom, syn *Cytisus triflorus* L'Her) is a shrub of Fabaceae family.[1] It is a perennial plant of one to two meters high and flowers by 1-3 in the axils of higher leaves, native of the Mediterranean region, which colonizes hills, mountains and woods in shady and cool areas.[2] In Algeria, it is abundant in eastern North of the country (Algero-Constantine mountain areas), and rare in Telemcen, Oran and M'sila regions.[3]

The most known species of *Cytisus* genus are scotch (*C. scoparius*), white (*C. multiflorus*) and Portuguese brooms (*C. striatus*). They have been used in European folk medicine for different health problems such as hypertension, hypercholesterolomia, rheumatism, headaches, arthritis and diabetes. Some of them have been used for their sedative, hypnotic, diuretic, and hepatoprotective properties.[4] Antioxidant,

anti-inflammatory and antibacterial activities of *Cytisus* species have been related to their high contents of phenolic compounds.[5] The chemical composition of different extracts of these species has been deeply studied and revealed a variety of compounds (phenolic acids, flavonoids, alkaloids) responsible of different biological activities. [6][7][8]

In Algeria, the most representative among the *Cytisus* species is *C. villosus*. It is traditionally used for treating haemorrhoids, gastrointestinal troubles, and for healing superficial wounds. Increasing interest has been given to *C. villosus* in recent research papers, which show promising bioactive potential of different extracts. These biological properties, such as the antioxidant activity, are related to the high concentration of phenolic compounds and flavonoids. [9], [10], [11] Also the essential oil, whose chemical composition changes according to phenological stage, has been recently studied.[12] Despite the remarkable biological properties, unlike other species of *Cytisus* genus (*C. scoparius*, *C. multiflorus* and *C. striatus*) *C. villosus* is still relatively poorly studied.

The aim of this paper is to deepen our knowledge about *C. villosus* potential activities by studying four extracts (ethanolic, methanolic, acetonic and aqueous) using different in vitro methods to assess antioxidant (Ferric reducing power, Total antioxidant capacity, DPPH and ABTS scavenging activity) and antimicrobial activities (antibacterial and antifungal). Furthermore, optimal conditions for the extraction of bioactive compounds from *C. villosus* leaves using surface response methodology were investigated. Finally, a HPLC/diode-array detector (DAD) analysis of *C. villosus* methanolic extract was carried out.

Results and Discussion

Phytochemical Analysis

Four different extracts of *C. villosus* leaves were obtained by using four different solvents: ethanol, methanol, acetone, and water. The extraction yield had a range of 10-20% (Table 1). First, the extracts were evaluated for their total phenolic content (TPC), total flavonoid content (TFC) and total tannins content (TTC). As we can see from Table 1, TPC ranged between 24.47 and 87.55 mg GAE/g for the four extracts. The highest polyphenols content was obtained with methanolic extract ME (87.55±0.15) followed by aqueous WE (80.66±1.13) and ethanolic extracts EE (72.76±1.51). On the other hand, observing TFC values, no significant difference ($p>0.05$) has been noticed between ME, EE and WE in term of flavonoid content. Moreover, water extract was the richest in tannins (13.66±1.41 mg TAE/g DM), followed by acetone and ethanol extracts (Table 1).

Table 1: Phytochemical analysis and antioxidant activity of *C.villosus* leaves extracts

Extract	Extraction yield (%)	TPC mg GAE/g	TFC mg QE/g	TTC mg TAE/g	EC50 (mg/ml)			
					DPPH	ABTS	FRAP	TAC
EE	10±0.21^{c}	72.76±1.51^{c}	39.94±1.99^{a}	2.40±0.26^{c}	0.50±0.03^{c}	0.67±0.01^{a}	9.38±0.06^{d}	0.30±0.02^{b}
ME	10±0.12^{c}	87.55±0.15^{a}	41.19± 2.28^{a}	0.67±0.12^{c}	0.14±0.04^{a}	0.45±0.02^{a}	0.42±0.05^{a}	0.14±0.02^{a}
AE	18.42±1.20^{b}	24.47±1.80^{d}	14.78±0.99^{b}	6.75±2.70^{b}	2.09±0.01^{d}	11.29±0.22^{b}	3.6±0.02^{c}	0.56±0.01^{c}
WE	20.10±0.57^{a}	80.66±1.13^{b}	40.66±1.36^{a}	13.66±1.41^{a}	0.41±0.01^{b}	0.51±0.03^{a}	1.11±0.01^{b}	0.54±0.01^{c}

TPC: total phenolic content, TFC: total flavonoid content, TTC: total tannins content, DPPH: DPPH radical scavenging activity, ABTS: ABTS cation scavenging activity, FRAP: ferric reducing antioxidant power, TAC: total antioxidant capacity, GAE: gallic acid equivalent, QE: quercetin equivalent, TAE: tannic acid equivalent. ME: Methanol extract, EE: Ethanol extract, AE: Acetone extract, WE: Water extract. a,b, c: non homogenous groups by ANOVA with post hoc HSD test at the level $\alpha = 0.05$.

To the best of our knowledge, so far there are no studies dealing with total phenolic, flavonoid or tannins content of *C. villosus* crude extracts. The results of total phenolic yield (TPY) ranged those of methanolic extracts of *C. scoparus* and *C. multiflorus* previously reported by [7] and [13]. Furthermore, the methanolic extract of *C. multiflorus* has similar flavonoids content to that of *C. villosus* [7]. As for the tannins, our results show that they are lesser than those recorded for methanolic extract of *C. multiflorus* [14].

In general, Hairy broom can be considered rich in polyphenols and flavonoids compared to well-known Mediterranean medicinal plants such as lavender (*Lavandula angustifolia*, 50.6±3.16, 27.6±3.42), oregano (*Origanum vulgare*, 67.8±3.41, 31.6±4.25) [15], Rosemary (*Rosmarinus officinalis* 16.67 ± 0.40, 38.64) [16], Thyme (*Thymus vulgaris*, 8.10±2.00, 15±1.00) and sage (*Salvia officinalis*, 5.95 ±2.56, 0.923±0.12 mg) [17].

Antioxidant Activity

The antioxidant activity of the four extracts was evaluated by means of different *in vitro* methods, such as DPPH, FRAP, ABTS and molybdenum assays. As regards DPPH scavenging activity, results have shown high antioxidant capacity of *C. villosus* extracts, reaching 92% for methanolic extract with a low EC50 (0.14mg/ml) (Table 1, Figure 1). These results are higher than those found for other *Cytisus* species such as *C. multiflorus* ethanolic (65.43±2.47mg/ml) and aqueous (120.42± 5.33mg/ml) extracts [14]. *C. villosus* ME has remarkably showed a higher DPPH radical scavenging activity compared to rosemary (IC50=0.199 mg/ml) [18].

Similarly, the maximal value of ABTS inhibition was obtained with methanol extract (EC50= 0.45 mg/ml). This finding is slightly higher than that reported by [19] for *C. multiflorus* aqueous and ethanolic extracts (1.66 and 1.70mg/ml respectively). It is to be noticed that WE has ABTS inhibition capacity (75%) similar to that of oregano water extract reported by [20].

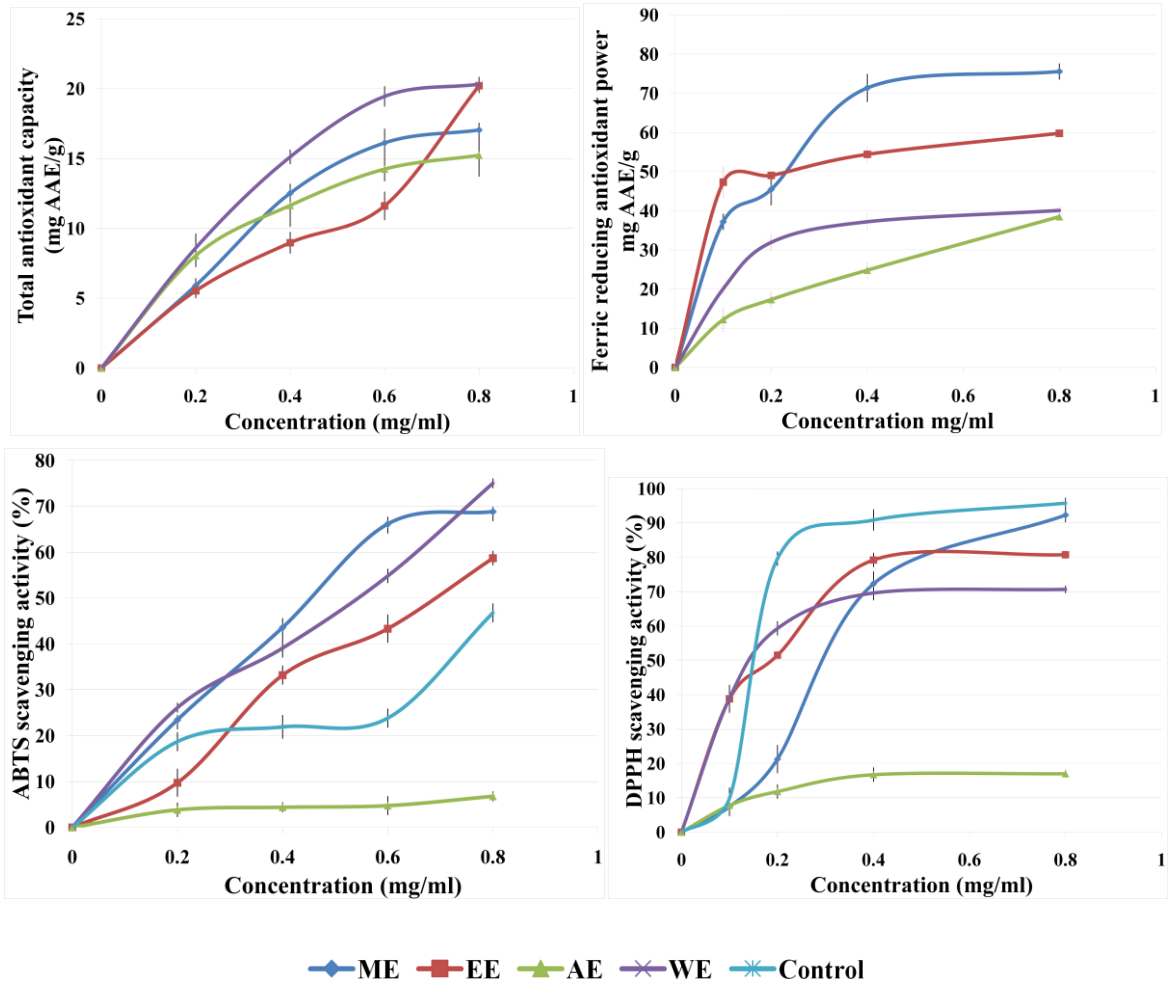


Figure 1. Results of antioxidant activity of *C. villosus* leaves extracts. ME: Methanolic extract, EE: Ethanolic extract, AE: acetone extract, WE: water extract.

In the same way, *C. villosus* methanolic extract has achieved ≈2mg EAA/g as the highest FRAP value followed by ethanolic extract (0.59 mg EAA/g). These results are comparable to those of *C. multiflorus* aqueous (1.20 mg EAA/g) and ethanolic (0.76 mg EAA/g) extracts [19].

Concerning Total antioxidant capacity (TAC), all extracts have shown remarkable activity that attained a maximum value of ≈20 mg EAA/g with WE and EE succeeded by ME (17.04 mg EAA/g) (Figure 1). These results are greater than those found by [21] for 40% methanol extracts of lavender, oregano, sage, thyme, and rosemary. In general, *C. villosus* has showed a remarkable antioxidant activity comparable to that of medicinal plants typically used worldwide for their antioxidant properties.

Antimicrobial Activity

The anti-microbial (antifungal and antibacterial) activities have been determined for the four extracts against eight bacterial and four fungal strains (Table 2). The results revealed that *C. villosus* leaves extracts (excepting aqueous extract) exerted an antibacterial effect against all tested strains, and inhibition zones diameter varied from 6.66±0.57 to 18.33±1.52 mm. It is to be noticed that ME have shown the highest antimicrobial activity against most tested strains ($p<0.05$) followed by EE. Similar results has been recorded for lavender, thyme, rosemary, sage and oregano by Kozlowska et al., [22] who showed that methanol and ethanol extracts were the most active against both Gram-positive and Gram-negative bacteria.

K. oxytoca and *S. aureus* were the most susceptible among Gram-negative and Gram-positive bacteria respectively (Table 2). In general, Gram-positive bacteria are more sensible to phenolic plant extract and no significant difference has been detected ($p>0.05$), which corresponds to a broad-spectrum antibacterial activity of *C. villosus* phenolic extract. Best MICs has been registered for ME (25-50 mg/ml); these results are very similar to those of lavender for *E. coli*, *Klebsiella pneumonia*, *S. aureus* and *Bacillus cereus* [23].

Table 2. Results of antimicrobial activity test of *C. villosus* leaves extracts.

Extract / Microbial strains	ME		EE		AE		WE
	Ø	MIC	Ø	MIC	Ø	MIC	
E. coli	14.16±0.57^{a}	25	8.66±0.57^{c}	25	12±0^{b}	25	na
B. subtilis	11.66±0.57^{a}	25	10.66±1.52^{a}	50	11.33±1.66^{a}	50	na
P. aeruginosa	12.5±1.5^{a}	50	9±0.00^{b}	50	12.33±1.15^{a}	50	na
Salmonella sp.	18.5±3.5^{a}	25	13.83±0.28^{a}	50	14±2.65^{a}	25	na
K. oxytoca	18.33±1.52^{a}	25	14.66±1.52^{b}	25	12.33±0.57^{b}	50	na
L. monocytogenes	17.33±1.33^{a}	50	13±3.60^{a}	50	10.33±4.04^{a}	50	na
A. baumannii	13±3.00^{a}	25	8.66±57^{a}	25	10.66±2.08^{a}	25	na
S. aureus	15.16±4.53^{a}	25	8.33±0.57^{a}	25	11±3.60^{a}	50	na
A. niger	13.66±0.47^{a}	50	8±0.81^{b}	25	9.33±2.31^{b}	25	na
P. notatum	15±1.41^{a}	50	11±2.16^{a}	25	12±2^{a}	50	na
A. flavus	15±0.81^{a}	25	9.66±0.47^{b}	25	6.66±0.57^{c}	50	-
F. oxysporum	18.66±0.47^{a}	25	14±1.41^{b}	25	12.66±0.81^{b}	50	-

Ø: Diameter of inhibition zones (mm), MIC: Minimal inhibitory concentrations (mg/ml), ME: Methanol extract, EE: Ethanol extract, AE: Acetone extract, WE: Water extract, na: no activity detected, ^{a,b,c} : Non homogenous groups by ANOVA with post hoc HSD test at the level $\alpha = 0.05$.

Furthermore, our study clearly shows the antifungal activity of *C. villosus* leaves. ME was the most active extract ($p<0.05$), and *F. oxysporum* was the most sensible among fungal strains (18.66±0.47 mm, 25 mg/ml), as regards inhibition zone diameter and MIC respectively (Table 2).

Comparing MIC with MBC and MFC has given us a clear idea about the bactericidal and fungicidal nature of *C. villosus* extracts, as the ratios MBC/MIC and MFC/MIC equal one in most cases and never exceed two with all microbial strains [24].

Effect of Extraction Parameters

Model Fitting

Central composite design (CCD) was used to investigate the effect of temperature, extraction time, Liquid/Solid ratio, and solvent concentration on total phenolic yield (TPY), Anti-*Staphylococcus aureus* (ASA), Anti-*Escherichia coli* (AEC) and DPPH radical scavenging activities (DRS).

Multiple regression has shown high significance of the model ($p<0.01$) through all independent variables (Y_1, Y_2, Y_3, and Y_4). In the other hand, coefficients of determination R2 were between 0.89 and 0.95, and the lack of fit was significant for all four responses ($p = 0.09$; 0.35; 0.48 and 0.42 for Y_1, Y_2, Y_3 and Y_4 in that order) (Table 3).

Table 3. Results of fitting models and the effect of explanatory variables on responses variables.

Source	p-values			
	Y1	**Y2**	**Y3**	**Y4**
Intercept	<.001*	<.001*	<.001*	<.001*
X_1	0.431	0.745	0.108	0.001*
X_2	0,006*	0.094	0.909	0.413
X_3	0,002*	0.003*	0.431	<.001*
X_4	<.001*	<.001*	<.001*	<.001*
X_1X_2	0.421	0.057	0.242	0.316
X_1X_3	0.546	0.494	0.473	0.143
X_2X_3	0.554	0.005*	0.473	0.892
X_1X_4	0.011*	0.311	0.242	<.001*
X_2X_4	0.415	0.311	0.809	0.241
X_3X_4	0.003*	0.009*	0.809	0.764
X_1^2	0.191	0.203	0.002*	0.089
X_2^2	0.028*	0.448	0.422	0.056
X_3^2	0.147	0.168	0.034*	0.235
X_4^2	0.001*	0.737	0.079	0.01*
Model	<.001*	0.002*	<.001*	<.001*
Lack of fit	0.094	0.360	0.490	0.426
R2	0.96	0.89	0.93	0.94

X1: Temperature, X2: Extraction time, X3: Liquid/solid ratio, X4: Methanol concentration, Y1: Total Phenolic Yield, Y2: Anti *S.aureus* activity, Y3: Anti E.coli activity, Y4: DPPH radical scavenging activity ; *Significant effect

Insignificant lack of fit indicated good fitting of the model with studied parameters, and it insures that the chosen model can represent all the experimental data including points out of the regression [25]. In our study R2 values are close to one which represents a satisfactory correlation between experimental and predicted data [26].

Relation between explanatory variable and each response has been determined using polynomial second order equations as follows:

$$Y_1 = 115.51 - 9.87X_2 + 11.66X_3 + 27.46X_4 - 9.38X_1X_4 + 11.83X_3X_4 - 19.35X_2^2 - 41.30X_4^2 \quad (1)$$

$$Y_2 = 16.76 - 1.27X_3 - 2.05X_4 + 1.25X_2X_3 - 1.12X_3X_4 \quad (2)$$

$$Y_3 = 8.08 + 4.88X_4 - 4.94X_1^2 + 1.05X_2^2 + 3.05X_3^2 - 2.44X_4^2 \quad (3)$$

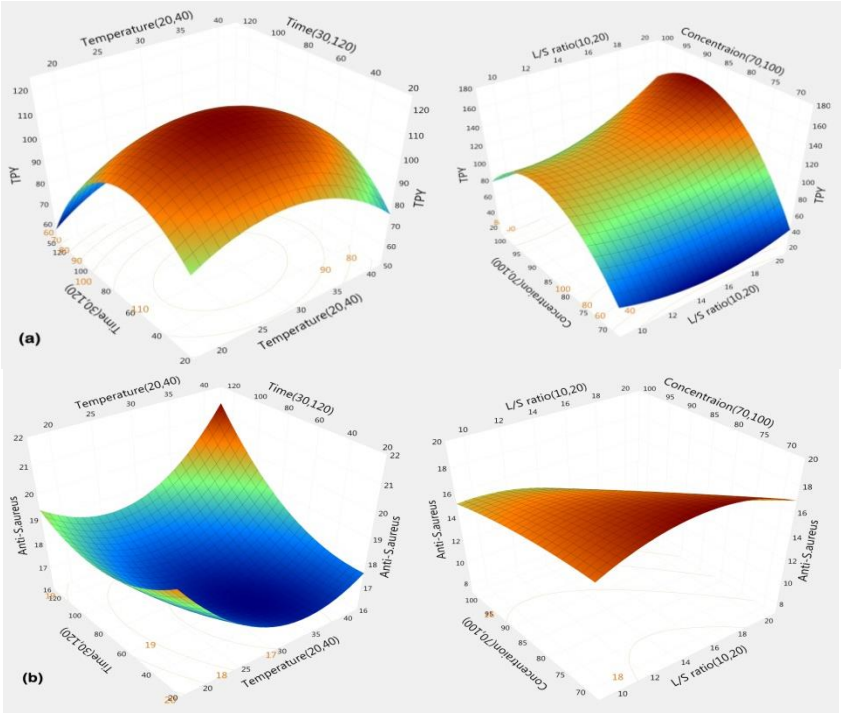
$$Y_4 = 88.10 + 1.54X_1 + 2.57X_3 - 1.81X_4 + 1.87X_1X_4 - 3.15X_4^2 \quad (4)$$

Effect of Temperature

Temperature has demonstrated a highly significant effect ($p<0.01$) on DRS, TPY and ASA, with an optimal at 35°C (Table 3). However, it had no significant effect on AEC.

Temperature is one of the most influencing factors when extracting phenolic compounds [27], in fact it increases extraction yield by both desorbing phenolic compounds from other substances and reducing solvent viscosity and surface tension [28, 29]. In addition, temperature enhances selectivity and efficiency when using methanol as a solvent [30].

Response surface plots showed that TPY and DRS decreased noticeably when temperature exceeded 35°C (Figure 2). Several authors have reported that temperature is the major factor causing degradation and conversion of bioactive compounds during extraction.[29, 31, 32]



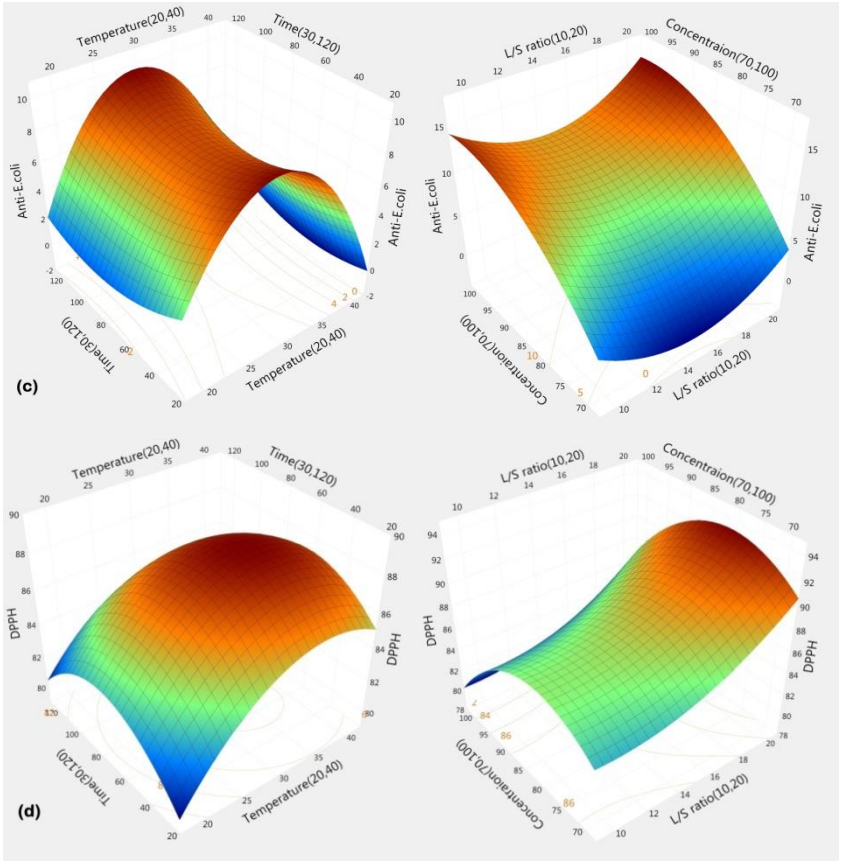


Figure 2. Tridimensional representation of second order polynomial models. (a): TPY, (b) : ASA, (c): AEC, (d): DRS.

On the other hand, antibacterial activity of *C. villosus* extract was insensitive to temperature augmentation. We suggest that altering antioxidant activity of bioactive compounds doesn't include inevitably the alteration of antibacterial capacity as well. In all cases, it's preferable not to exceed 35°C when extracting *C. villosus* polyphenols, to prevent losing bioactivity of thermo-sensitive compounds.

Effect of Extraction Time

Extraction time was another effective parameter on extraction efficiency; it displayed a highly significant effects on TPY (as X2 and X22) and ASA (in interaction with X3) (Table 3). Oppositely, when extraction time reaches 90 min and above, a negative influence appeared on all responses (Figure 2).

Extended extraction time can lead to the degradation of bioactive compounds including polyphenols, flavonoid and sugars, particularly with high temperature [33, 34, 35]. One of the aims of optimization is to minimize the extraction time; hence, it would be better not to exceed 90 minutes when extracting *C. villosus* polyphenols.

Effect of Liquid/Solid Ratio

This factor influenced all studied responses significantly as linear, interaction or quadratic term (Table 3).

The extraction of bioactive compounds under higher ratio of solvent on respect to plant material allows more solvent penetration into the plant cells and more phenolic compound permeation [36]. It guarantees a lesser solvent saturation, which provides more solubility to bioactive compounds leading to a higher extraction yield [37].

Effect of Solvent Concentration

Selecting a solvent is a key step in the process of extraction. Our data show that *C. villosus* methanol extract has shown better TPY, TFC, antioxidant and antimicrobial activities on respect to the other three extracts.

Solvent concentration has the highest significant effect ($p<0.001$) among all explanatory variables of the model (Table 3). Excepting AEC, all other responses reached their highest levels at 70-80% methanol (Figure 2). Mixture of water with the solvent enhances polyphenols extraction efficiency, by maintaining a better bioactive compounds permeation to extracellular medium as it is driven by the solvent concentration gradient [38, 39]. It is to be noticed that X_4 affects differently ASA and AEC responses. The highest *S. aureus* inhibition zones values have been registered with 75% methanol. In contrast, best inhibition of *E. coli* was found with pure methanol extract. According to [40] and [41], the presence of water in the mixture enhances the extraction efficiency by augmenting contact surface area and plant tissues inflation. It is better to use alcohol-aqueous mixture when extracting anti-Gram positive molecules from *C. villosus*. Compared to water-methanol mixture, pure methanol allowed the extraction of non polar molecules, which can diffuse easily through Gram-negative bacteria cell wall, whereas the polar molecules can only penetrate using porin channels. Under the light of these results, 73.54% and 100% are the best methanol concentration for the extraction of Anti-*S.aureus* and Anti-*E.coli* bioactive compounds from *C. villosus*.

Model Validation

To check our model adequacy, a new extraction has been performed for each response using obtained optimal levels of each explanatory parameter. The attained values (110 GAE mg/g, 20 mm ,12mm,87.01%) were close to those predicted by the model (117.16 mg GAE/g, 19.12mm,11mm, and 87.47%) for Y_1, Y_2, Y_3 and Y_4, successively. Checking the model adequacy is crucial to any optimization procedure; this prevents obtaining poor or misleading results [31]. Our results demonstrated the adequacy and the prediction power of the chosen model in optimizing bioactive compounds extraction from *C. villosus* leaves.

Phenolic Composition Analysis

Our data show that the the best solvent to extract *C. villosus* phenolic compounds is methanol. Based on this, we decided to study the chemical composition of methanol extract by means of HPLC-DAD analysis. The results show that this extract was rich of interesting bioactive compounds, specially flavonoids such as rutin, a molecule well-known for its pharmaceutical properties. Rutin is the most abundant compound in *C. villosus* leaves extract (34.97 mg/g of DM), followed by quercetin and catechin with values of 5.53 and 3.83 mg/g of DM respectively. The methanolic extract of *C. villosus* leaves also contains quercetin and catechin derivates (quercetin-3-*O*-glucoside and gallocatechin gallate), phenolic acids (caffeic and vanillic acids) and other interesting phenolic compounds such as myricetin, naringenin, apigenin, kaempferol, procyanidin B2 with quantities of less than one mg/g of DM (Table 4).

Table 4. Phenolic composition of *C.villosus* leaves methanolic extract using HPLC-DAD

Phenolic compound	Yield (mg /g dry matter)	Phenolic compound	Yield (mg /g dry matter)
Ferulic acid	nd	Quercetin	5.534
Caffeic acid	0.092	Rutin	34.972
Gallic acid	nd	Myricetin	0.044
Vanillic acid	0.608	Apigenin-7-O-glucoside	0.062
Chlorogenic acid	nd	Naringenin	0.04
Catechin	3.836	Apigenin	0.046
Gallocatechin gallate	1.68	Kaempferol	0.016
Quercetin-3-O-glucoside	1.05	Procyanidin B2	0.186

Various reaserch works involving both animals and humans had shown the wide range of pharmaceutical properties of rutin [42], such as anti bacterial, antifungal, antidiabetic, antitumor, anti-allergic, anti-inflammatory, nephroprotective, neuroprotective, hepatoprotective, skin protective, osteoblast and endothelial function stimulant [43, 44]. Besides its own activity, the presence of rutin can enhance the antibacterial activities of other flavonoids such as quercetin, kaempherol and myricetin [45].

Quercetin, as well, is endowed with an anti-infective and anti-replicative activity against both Gram-positive and Gram-negative bacteria, especially those infecting gastrointestinal tract and urinary system [46]. Additionally, this flavonoid has diverse pharmacological activities namely, antiviral, antioxidant, anti-Inflammatory, anti-SARS-CoV-2, anticancer, antiaging activities.[47]

The third major phenolic compound of *C. villosus* extract was catechin and its derivate gallocatechin gallate. Many researches have revealed catechin benefits on human health (activation of skin barrier passage, UV protection, anti-allergenic, anti-inflammatory, anti-cancer, promoting cell, anti-oxidant, antiviral and antimicrobial activities), and have led to many applications in different fields (pharmaceutical, cosmetic, food packaging).[48]

To the best of our knowledge, this work is the first report dealing with the composition of methanol extract of *C. villosus*. A previous analysis of ethyl acetate extract of hairy broom collected from other regions of Algeria (Laghouat and Collo cities) had shown the presence of quercetin, kaempferol, catechin, gallocatechin, myricetin-*O*-rhamnoside genistein, chrysin, chrysin-7-O-β-d-glucopyranoside, and 2″-O-α-l-rhamnosylorientin [9, 11]. The presence of a variety of bioactive compounds in *C. villosus* leaves with a large range of biological activities may give to this plant an importance in different fields.

In conclusion, *C. villosus* has shown a remarkable antioxidant and antimicrobial potential that render this plant potentially useful in different contexts. Given these interesting data, an investigation of its biological activities, such as anti-inflammatory activities (in vitro and in vivo), and a deepened analysis of its composition through different techniques (such as NMR), could be the key to a best evaluation of this interesting natural resource.

Experimental Section

Plant Material

The leaves of *C. villosus* were harvested between March and April 2016 (in flowering period), in El-Milia, (Jijel, NE of Algeria 36°75'31 N 6°26'77 E). Plant material has been cleaned and air-dried at for 48 hours at 35°C, then grinded using a blender. Leaves powder was stored at 4°C until analysis of bioactive compounds.

Microbial Stains

Eight bacterial and four fungal strains of American Type Culture Collection (ATCC) has been used to assess the antimicrobial activity of *C.villosus* extracts namely (*Escherichia coli* 25922, *Staphylococcus aureus* 25923, *Pseudomonas aeruginosa* 27853, *Bacillus subtilis* 21332, *Klebsiella oxytoca* 49131, *Acenitobacter baumannii* 19606 , *Listeria innocua* 33090, *Salmonella typhemurium* 13311, *Penicillium chrysogenum* 10106, *Fusarium oxysporum* 11850, *Aspergillus flavus* 9643, *Aspergillus niger* 16404). *E. coli* and *S. aureus* have been used for the optimization of antibacterial compounds extraction.

Chemical Reagents

All solvent and chemical reagents used in this study were of analytic grade. Folin-Ciocalteu reagent, methanol, ethanol, vanillin, chloride acid, acetone, sulfuric acid, sodium phosphate, potassium ferricyanide, iron (III) chloride, tween 20, Ascorbic acids, gallic acids, sodium carbonate (Na_2CO_3) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were from Sigma–Aldrich (Steinheim, Germany).
The standards used for the identification and quantification of phenolic acids and flavonoids were chlorogenic acid, ferulic acid, caffeic acid, ellagic acid, gallic acid, vanillic acid, (+)-catechin, procyanidin B2, quercetin, rutin (quercetin-3-O-rutinoside), isoquercetin (quercetin-3-O-glucoside), Apigenin, apigenin-7-O-glucoside, myricetin, Narigenin and kaempherol (Sigma Chemical Co., St. Louis, MO, USA). Solvents and reagents used for the preparation of mobile phases and stock 2.3. solutions were also purchased from Sigma-Aldrich: water (Chromasolv® for HPLC), methanol (Chromasolv® for HPLC, ≥99,9%) and formic acid (reagent grade, ≥95%).

Extraction Procedure

Extraction of polyphenols from *C. villosus* leaves was carried out by maceration using four different solvents (ethanol, methanol, acetone and water). 10 g of dried leaves powder were mixed with 100 ml of solvent, and let for maceration under agitation over-night at 25°C. Each extract was centrifuged at 5000 rpm/min for 10 min. Supernatants were then filtered through Wattman 04 paper. Finally solvents were dried out using a rotary evaporator at 30°C. Dried extracts were weighted and preserved at 4°C for further experimentations.
Extraction yields were determined as follows:

$$Extraction\ Yield = \frac{Dry\ extract\ weight}{Plant\ material\ weight} \times 100$$

Phytochemical Analysis

Folin-Ciocalteu reagent method was used to investigate total phenolic yield (TPY) in *C. villosus* extracts [49]. TPY values were obtained using gallic acid standard calibration curve. The results were expressed in terms of gallic acid equivalents/g of dry matter (mg GAE/g of DM).
Flavonoids content was assessed using alumina trichloride method according to a quercetin standard curve [50]. Values were indicated in mg equivalents quercetin/g of dry plant material (mg QE/g DM).
A colorimetric method was applied to evaluate tannins content using vanillin reagent as described by [51]. Tannin concentrations were determined using a catechin standard curve and communicated in mg catechin equivalents/g of dry plant material (mg CE/g of DM).

Determination of Antioxidant Activity

Antioxidant activity of *C. villosus* extract was assessed using different methods. DPPH scavenging activity (DRS), based on the capacity of antioxidants to trap DPPH radical, was determined as described by [52]. DRS were calculated as a percentage (%) using the following equation:

$$DRS\ (\%) = \frac{A_C\ - A_S}{A_C} \times 100$$

where AS is the absorbance of the test sample, and AC is the absorbance of the control. Obtained results were compared to those of ascorbic acid as a standard antioxidant.

Another method based on the ability of inhibiting cationic free radicals (ABTS radical assay) was employed [53]. Trolox was used as positive control. ABTS scavenging activity was calculated as an inhibition percentage using this equation:

$$Inhibition\ (\%) = \frac{A_C\ - A_S}{A_C} \times 100$$

Total antioxidant capacity (TAC) of *C. villosus* extracts was evaluated by the Phosphomolybdenum method described by [54]. Total antioxidant capacities were determined using ascorbic acid calibration curve and the results were expressed in milligram equivalent of ascorbic acid per gram of dry matter (mg EAA/g DM).

Ferric reducing antioxidant power was assessed according to method of [55]. Ascorbic acid was used as standard antioxidant. The percentage of iron reduction was calculated by the following equation:

$$Iron\ reducing\ power\ (\%) = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

where Ao is the absorbance of $FeCl_3$, A1 is the absorbance of $FeCl_3$ solution in the presence of the extract.

Antibacterial activity Assay

Bacterial inocula have been prepared according to the Clinical Laboratory and Standards Institute recommendation. From 18 hours incubation cultures, 1.5×108 (CFU/mL) suspensions have been set up by adjusting their opacities to 0.5 McFarland standard solution [56].

In the other hand, fungal inocula were obtained as described by [57]. Spores were collected from 5 days incubation cultures by means of 1% Tween 20 solution. Inocula were standardized to 1-2.10^5 spore/ml using Malassez counting chamber.

The antimicrobial activity of *C. villosus* leaves extracts was assessed using paper disk diffusion method [58]. On Muller Hinton (M-H) agar previously inoculated by standardized inocula, 6mmØ Wattman paper N°01 discs received 20 µL of phenolic extract. After 18-24 hours incubation at 37°C, inhibition zones diameters were measured.

Minimal Inhibitory Concentrations (MIC) were determined using the micro-dilution technique with Muller Hinton Broth [56]. In 96-well micro plates, series of dilutions (1/2 - 1/256) were prepared from *C. villosus* extracts stock solutions (100 mg/ml). After inoculating each dilution with 10 µl of standardized inocula, plates were covered and incubated at 37 °C for 24 hours. MIC is defined as the minimum concentration of extract for which no microbial growth is observed.

Minimal Bactericidal and Fungicide Concentrations (MBC and MFC) were determined by the means of classical microbiology method. M-H broth tubes were inoculated by 100 µl of negative wells from MIC assay and incubated at 37 ° C for 48 hours, when broth tubes were observed for any microbial growth [56].

Optimization of Extraction Conditions

Central composite design (CCD) was used to optimize effective parameters on the extraction of phenolic compounds of *C. villosus*. Explanatory variables were temperature (X_1: 20-40°C), time (X_2: 30-120min), Liquid/Solid ratio (X_3: 10-20mL/g) and solvent concentration (X_4: 70-100%). Also four response variables were considered, namely total phenolic yield (TPY, Y_1), anti-Staphylococcus aureus (ASA, Y_2), anti-Escherichia coli (AEC, Y_3) and DPPH radical scavenging activities (DRS, Y_4).

According to CCD essay matrix, a total of 26 extractions were realized based on the combinations between extraction parameters levels programmed by the used design.

To predict optimal points, a second order polynomial function was used. It permits studying relationship between explanatory variables and response variables. General form of second order polynomial equation is:

$$Y_i = \beta_0 \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

With:

Yi : Response variable ;β0 : model constant ;Xi, Xj : explanatory variables ;βi : linear coefficient ; βij : interaction coefficient; βii : Quadratic coefficient.

Active Compounds Analysis

The major polyphenols of ME have been evaluated by HPLC/diode-array detector (DAD) analysis, realized by means of HPLC system Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD, USA) en suite with an automized sampler, a binary gradient solvent pumping system, and a diode-array detector (DAD). The chromatography was run at 35 °C using Synergy Polar-RP C18 column (250 × 4.6 mm I.D., 5 µm particle size, Phenomenex, Torrance, CA, USA) preceded by a Polar-RP security guard cartridge. 0.1% formic acid in water (v/v) and acetonitrile were used as mobile phase A and B respectively. The separation was executed as follows : 0-2 min 90% (A), 2-17 min 40% (A), 17-22 min 40% (A), 22-28 min 90% (A), 28-33 min 90% (A) with a flow rate of 1 mL/min and an injection volume of 20 µL. The DAD detector was set on three different wavelengths: 280, 315 and 360 nm for procyanidin, hydroxycinnamic acids and flavonols correspondingly. The identification of polyphenols was performed using available standards to match up to similar retention time and UV absorption spectra. Standard curves were realized by scheming HPLC peak areas with standard polyphenols concentrations in order to quantify phenolic compounds (mg/L) ($r2 > 0.99$) [59].

Statistical Analysis

All assays were performed in triplicates. Analysis of variance (one-way ANOVA) followed by honestly significant difference (HSD Tukey) test were realized for phytochemistry and biological activities study. For extraction conditions optimization, building and fitting the model was carried out using JMP® 13.2.0 Software (Copy Right © 2016 SAS Institute Inc, USA).The statistical analysis consist of Fischer test, lack of fit, and linear regression of the model. For each response variable, quadratic models were represented using tridimensional response surface plots.

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Author Contribution Statement

Abdelhalim BOUSSAA (Conceptualization, methodology, formal analysis, software, visualization, validation, writing original draft, writing- reviewing and editing).
Imane BOUHANNA (writing original draft, writing- reviewing and editing).
Daniela RIGANO (Methodology).
Maria MAISTO (Methodology).
Adriana BASILE (Methodology).
Fatiha BEDJOU (supervision, writing- reviewing and editing).

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