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Optimization of the extraction process of bioactive compounds from *Rhamnus alaternus* leaves using Box-Behnken experimental design

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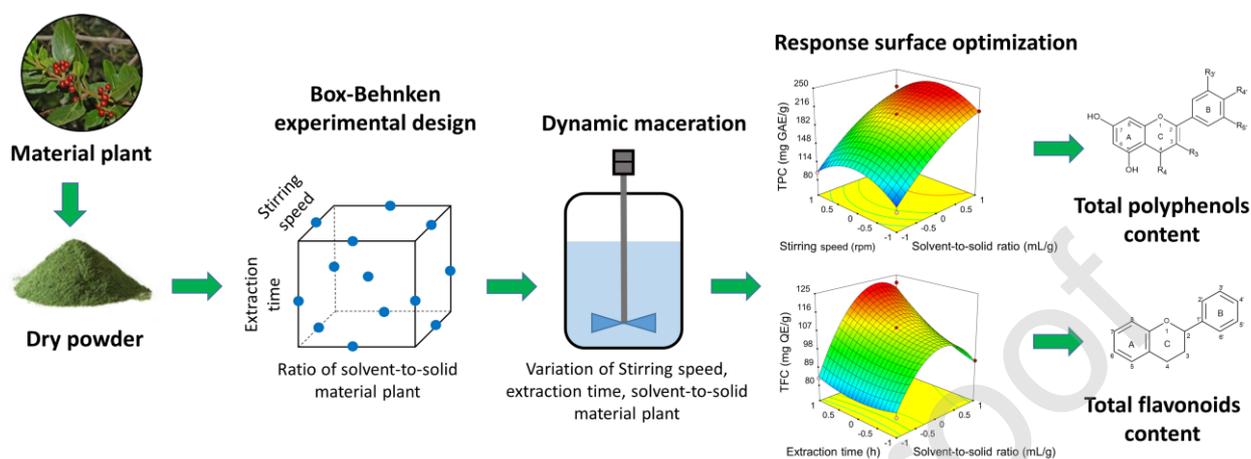
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Graphical abstract

Optimization of extraction of phenolic compounds from *Rhamnus alaternus*



Highlights

- Dynamic maceration is a good method to extract bioactive compounds from *Rhamnus alaternus* leaves.
- Optimization of dynamic maceration method by Box-Behnken as a cost-effective process.
- Determination of optimal parameters for bioactive compounds extraction from *Rhamnus alaternus*.
- Significant correlation between total phenolic content and the antioxidant activity.
- The quadratic regression model shows a predictive capacity of 97 %.

Abstract

The *Rhamnus alaternus* plant is a rich source of excellent bioactive compounds including polyphenols and flavonoids. Yet, the improvement of the extraction yield of total polyphenol content (TPC) and total flavonoid content (TFC) from this plant has never been inspected in depth. Hence,

using a 15-run Box-Behnken design, a dynamic maceration process was optimized for the extraction of TPC and TFC in order to investigate the antioxidant capacity of the various fractions by DPPH assay. Three factors (*i.e.*, ratio of solvent-to-solid material plant, stirring speed and extraction time), were investigated and optimized for establishing a cost-effective process in order to produce extracts rich in phenolic components. With a desirability value of 0.970, the optimal extraction parameters of polyphenols and flavonoids were as follows: a ratio of solvent-to-solid of 10:1 mL/g, a stirring speed of 518 rpm, and 24 h of extraction. For such optimized conditions of dynamic maceration, the highest total polyphenols and total flavonoids contents were experimentally determined at 230.9 mg GAE/g and 121.1 mg QE/g of extract, respectively with an IC₅₀ value of 22.5 µg/mL, and were in agreement with the obtained model values.

Keywords: *Rhamnus alaternus*, Extraction, Optimization, Box–Behnken design, Antioxidant activity, Polyphenols.

1. Introduction

In recent years, the pharmaceutical sector has expanded the vision about the developments in medicinal plant industries. As well, health issues awareness is increasing with consumers demands, more interested in bioactive products. To date, folk medicine still plays an important role for treating

several diseases, and recently, the traditional Chinese medicine has effectively been used against the COVID-19, especially for patients presenting pneumonia symptoms (National Health Commission of the People's Republic of China, 2020). This treatment was based on many medicinal plants, known for their high amount of bioactive compounds such as polyphenols (Yang et al., 2020). Plants contain various classes of natural products including flavonoids, one of the most important polyphenolic compounds (Chen et al., 2016). Recent studies reported the wide spectrum of biological and pharmacological characteristics of flavonoids including antioxidant, anti-inflammatory, antibacterial and anti-cancer properties (Benavente-García et al., 2000; Jin et al., 2014; Yu et al., 2005).

R. alaternus is a Mediterranean plant, that belongs to the *Rhamnaceae* family and is usually called "Imlilisse". This latter one has been largely used in traditional medicine as hypotensive, purgative, laxative and also for treating dermatological complications (Ben Ammar et al., 2007). The most important secondary metabolites in *R. alaternus* are polyphenolic compounds, flavonoids (Moussi et al., 2015), coumarins, anthocyanins (Longo et al., 2005), and anthraquinones (Kosalec et al., 2013). Related to the presence of such biomolecules, this plant is empirically used for its biological properties including antioxidant, antigenotoxic (Bhouri et al., 2011), antihyperlipidemic (Tacherfiout et al., 2018) and antimutagenic activities (Ben Ammar et al., 2005). Besides, other biological effects including antibacterial activity were also mentioned in the literature (Ben Ammar et al., 2007; Nekkaa et al., 2021).

Several extraction techniques for recovering bioactive compounds from *R. alaternus* were reported, notably maceration and decoction (Boussahel et al., 2015), soxhlet extraction (Ben Ammar et al., 2009), ultrasonic assisted extraction (Kosalec et al., 2013) and hydrodistillation (Berka et al., 2008). To obtain a higher yield of phytochemical-rich products with a low cost, the manufacturers of bioactive compounds produced from raw materials plant applied appropriate strategies for optimizing extraction processes (Pompeu et al., 2009). From previous studies, the modification of

various extraction conditions such as the applied solvent-to-solid ratio, the temperature, the extraction time and the solvent composition, among other parameters, significantly influenced the extraction of natural compounds (Bucić-Kojić et al., 2009; Pinelo et al., 2005).

In order to optimize the extraction of bioactives compounds from plants, Response Surface Methodology (RSM) is a promising tool. Indeed, this approach, which combines statistical and mathematical techniques, enables to structure the experimental models, examine the effects of various parameters and screen the optimum process variables for obtaining advantageous surfaces (Bezerra et al., 2008; Luo and Chen, 2010). RSM was successfully used to establish and validate the model equations and determine the optimal experimental values of extraction conditions, influenced by diverse independent parameters (Rai et al., 2012).

To the authors' knowledge, RSM has never been applied to optimize the extraction of polyphenols and flavonoids from *R. alaternus*. The objective of this study was not only to investigate the influence of combined parameters such as the solvent-to-solid ratio, the stirring speed and the extraction time on extraction yield, but also to optimize the extraction process of antioxidant compounds from *R. alaternus*. This study was carried out by using a Box–Behnken response surface design, in order to optimize an economic and efficient dynamic maceration technique, enabling to obtain the maximum of bioactive compounds from *R. alaternus*. We were mainly interested in total polyphenols and total flavonoids, used in pharmaceutical and food industries.

2. Material and methods

2.1. Plant material and Chemicals

The *R. alaternus* leaves were collected from Beni Ourthilane region – Algeria (36°19'57"N,5°5'19"E), in June 2018. The plant material was identified in the laboratory of pharmacognosy, in the pharmacy department of the University of Constantine 3 (Constantine, Algeria). These leaves were dried and stored in a room without exposing to sunlight at temperature

between 18 and 21 °C. After one month of storage, the leaves were ground using (SM100 RETSCH) cutting mill and sifted in (AS400 sieve shaker) with 630 µm of mesh openings to obtain a powder.

Methanol and ethanol (Sigma-Aldrich) were of analytical grade. Deionized water was purified by NUWS-100 water purification system (Labnics Equipment Ltd, Glasgow, UK). Folin-Ciocalteu phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, quercetin, aluminium chloride, ascorbic acid and sodium carbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. *Dynamic maceration*

Experimentally, the extraction procedure was carried out as follows: The *R. alaternus* powder was put into a 250 mL Erlenmeyer conical flask, and added with a 2:8 (v/v) water:ethanol mixture. Various solvent-to-solid ratio (5:1 - 10:1), stirring speeds (*i.e.*, 100 - 1200 rpm) and extraction times (*i.e.*, 12 - 24 h) were investigated for maceration according to the formerly defined Box-Behnken design (Table 2). For varying these two last parameters, a multipoint magnetic stirring was carried out at 25 °C with a digital speed control (Merck, 2mag MIX 15).

Then, the collected extracts were filtered through a filter paper (Whatman n°1) and evaporated under vacuum by a rotary evaporator (Büchi, Rotavapor® R-100) at 45 °C. Finally, the obtained concentrated solutions were diluted in five volumes of methanol and stored at 4 °C until further use.

2.3. *Determination of the total phenolic content*

The total phenolic content present in *R. alaternus* extracts was measured by Folin–Ciocalteu method (Singleton and Rossi, 1965) with minor modifications. Each diluted sample (0.5 mL) was mixed with 2.5 mL of 10% (v/v) Folin-Ciocalteu's phenol reagent in Eppendorf tube and vortexed. After 5 minutes, the mixture was added with 2 mL of aqueous sodium carbonate (7.5% w/v), vortexed, and incubated for 60 min in the dark at ambient temperature. Finally, the absorbance of the mixture was read at a wavelength of 765 nm using a spectrophotometer (UV 1800, Shimadzu, Japan). The concentration of the total polyphenols compound was quantified in each investigated extract using a

calibration curve performed with gallic acid as reference from 5 to 30 $\mu\text{g/mL}$ ($R^2 = 0.998$). The results were expressed as mg gallic acid equivalents mg GAE per gram of extract. All tests were triplicated.

2.4. Determination of total flavonoid content

Total flavonoid content was determined using the aluminum chloride assay, adapted from (Woisky and Salatino, 1998) with slight modifications. Briefly, 2 mL of each extract tested was mixed with 2 mL of AlCl_3 (2% w/v) into Eppendorf tube and then vortexed. After 15 minutes of incubation at ambient temperature, the absorbance of the mixture was measured at a wavelength of 430 nm against a blank, using a spectrophotometer (UV 1800, Shimadzu, Japan). Total flavonoid content was quantified from the analytical calibration curve using quercetin as reference from 5 to 25 $\mu\text{g/mL}$ ($R^2 = 0.997$). The results were expressed as mg quercetin equivalents (mg QE) per gram of extract. All tests were carried out in triplicate.

2.5. Evaluation of antioxidant capacity using DPPH assay

The free radical-scavenging capacity of *R. alaternus* extracts was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) *in vitro* assay as described by Brand-Williams et al. (1995) with some modifications. Briefly, the 15 extracts solutions were diluted in series in methanol to obtain various final concentrations (*i.e.*, 10, 30, 50, 70, 90, 110, 130, and 150 $\mu\text{g/mL}$). The DPPH solution was first daily prepared in methanol at 40 $\mu\text{g/mL}$. Then, this solution (2 mL) was mixed with each former diluted extract (2 mL) and the mixture was shaken vigorously. After 30 minutes of incubation in the dark at ambient temperature, the absorbance of the resulting solutions was measured in triplicate at a wavelength of 517 nm against the absorbance of the DPPH radical using UV-Vis spectrophotometer (UV 1800, Shimadzu, Japan). Ascorbic acid was used as reference (from 10 to 100 $\mu\text{g/mL}$). The radical scavenging activity expressed in percentage was calculated as follows:

$$\text{Radical Scavenging Activity (\%)} = \frac{A_{517}(\text{control}) - A_{517}(\text{sample})}{A_{517}(\text{control})} \times 100 \quad (1)$$

where A_{517} (control) was the absorbance of the control in the absence of sample (methanol), and A_{517} (sample) the absorbance of the plant extracts or of the reference (Ascorbic acid), all mixed with DPPH reaction solution.

The standard calibration curve carried out with ascorbic acid was linear between 10 to 100 $\mu\text{g/mL}$.

The half inhibitory concentrations - the so-called IC_{50} value - was defined as the concentration ($\mu\text{g/mL}$) that scavenges 50% of DPPH free radicals. For each assay, IC_{50} was determined from the linear part of the curve and estimated using the linear regression equation.

2.6. *Single-factor experiments*

In the first part, single-factor experiments were carried out to investigate the influence of solid-to-solvent, stirring speed and extraction time on the production of total polyphenols and flavonoids content using dynamic maceration. Therefore, the extraction process was first evaluated with various values of solvent-to-solid plant material ratios (2.5:1, 5:1, 7.5:1, 10:1 and 12.5:1 mL/g), with extraction stirring speed and extraction time set at 650 rpm and 18 h, respectively. Also, stirring speeds was investigated (100, 375, 650, 925 and 1200 rpm). For this second series of experiments, the solvent-to-solid ratio was set at 7.5:1 mL/g and the extraction time lasted 18 h. Finally, the extraction time parameter was investigated for periods of 6, 12, 18, 24 and 30 h, respectively. In this latter experiment, the solvent-to-solid ratio was set at 7.5:1 mg/g, and stirring speed was of 650 rpm. On the basis of these single-factor experimental results, levels of parameters were selected and applied in the response surface method design.

2.7. *Experimental design applied for the dynamic maceration extraction*

2.7.1. *Box-Behnken design*

First, a Box–Behnken experiment design was selected to establish a second order polynomial model of extraction of polyphenols and flavonoids from *R. alaternus* using dynamic maceration process, in link with their antioxidant properties. Indeed, this design is easy to implement compared with other optimization methods, with the advantage of time saving by reducing the number of experiments.

Three responses were studied: (1) the total phenolic content (TPC), (2) the total flavonoid content (TFC) and (3) the antioxidant capacity (see part 2.3). All these three responses were evaluated as a function of the combined effect of three parameters (X_i) *i.e.*,: the solvent-to-solid ratio (X_1), the stirring speed (X_2), and the extraction time (X_3), each one being studied at three levels, coded between -1, 0 and +1 and corresponding to the low, middle and high level, respectively. The variables and experimental design levels used in the Box-Behnken design are reported in Table 1.

[Table 1]

The three variables were coded according to the following equation (Ghellab et al., 2019):

$$x_i = \frac{z_i - z_{i0}}{\Delta z_i} \quad (2)$$

Where x_i , is the dimensionless value of an independent variable; z_i , is the real value of variable; z_{i0} , is the real value of an independent variable at the center point; Δz_i , is the step change of the real value of the variable i corresponding to a variation of a unit for the dimensionless value of the variable i .

On total, the Box-Behnken matrix was designed with these three independent variables (Table 2) and involved 15 experiments, including a triplicate at the central point.

[Table 2]

2.7.2. Statistical analysis

In this study, the Design Expert 12 software (Trial version, Stat-Ease Inc) was used to analyze the response surface. This approach enables to evaluate the factors effects, and optimize the influence of independent variables on extraction process of bioactive compounds of *R. alaternus*. After performing the experimental extraction, the data were analyzed by multiple regressions in order to predict the optimized values of solvent-to-solid ratio, stirring speed and extraction time. A second-order polynomial model equation was used to fit the generalized form of mathematical quadratic response surface. The model can be expressed by the following equation (Bezerra et al., 2008):

$$y = \beta_0 + \sum_{j=1}^k \beta_j x_j + \left\{ \sum_{j=1}^{k-1} \sum_{i=j+1}^k \beta_{ij} x_i x_j \right\} + \sum_{j=1}^k \beta_{jj} x_j^2 + \varepsilon \quad (3)$$

where y is the response value; k is the number of variables, for three independent variables investigated in this experimental design $k = 3$; β_0 is defined as the constant effect; β_j represents linear regression coefficient, β_{jj} is the quadratic regression coefficient, β_{ij} is the interaction regression between the parameters, while x_i and x_j are represented by the levels of the independent coded variables (i and j range from 1 to k); ε is the error.

Analysis of variance was performed to test the significance and adequacy of model. The adequacy of the model was assessed using the R-squared values, the coefficient of determination (R^2), the adjusted R-squared (R_{adj}^2), and the probability value as well. The linear determination coefficients between the phenolic or the flavonoid contents and the DPPH scavenging activities of the 15 extracts were calculated using IBM SPSS Statistics V21. P-values ≤ 0.05 were considered statistically significant.

2.7.3. Optimization using the Desirability Function

The dynamic maceration method used for the extraction of bioactive compounds from plants have many qualities responses, which need usually to be optimized. In this study, Derringer's desirability prediction was used in synchronous optimization of diverse responses in order to determine the optimum conditions of the extraction process of total polyphenols, flavonoids content and the antioxidant capacity from *R. alaternus*, with the best for maximum or minimum of studied parameters. In this approach, the values of all responses were converted to the scale value among 0 and 1 (Hu et al., 2008), which were formed with an estimate maximum or minimum value of factors to obtain the optimal responses.

3. Results and discussion

3.1. Effect of investigated parameters on total polyphenols and flavonoids yield

3.1.1. Effect of solvent-to-solid plant material ratio

The effect of single factor on dynamic maceration was investigated in various solvent-to-solid ratios varying from 2.5:1 to 12.5:1 mL/g, while other variables were set as 650 rpm and 18 h. As shown in Fig. 1a, the total polyphenols yield increases with higher solvent-to-solid ratio, up to reach a maximum of polyphenols content of 247.2 mg GAE/g for a ratio at 12.5:1 mL/g. Furthermore, the solvent-to-solid ratio also affects the flavonoids extraction significantly. The content of total flavonoids extracted is higher while increasing this ratio. The maximum value of flavonoids extracted (108.8 mg QE/g) is obtained for a solvent-to-solid material ratio of 7.5:1 mL/g. After this optimum value, the content of total flavonoids extracted maintains a dynamic balance and then declines greatly between 10:1 and 12.5:1 mL/g. This may be due to the mass transfer and to the solvent solubility of each compound at higher ratio values. Indeed, the increase of solvent quantity-to-raw material plant ratio might increase the penetration of the solvent into the vegetal cells, which would favor the cell release of polyphenols and flavonoids, until reaching the mass transfer equilibrium. In agreement with the literature (Spigno et al., 2007; Zheng et al., 2009), the selection of an appropriate solvent-to-solid ratio (v/w) is an important parameter to consider for the extraction process, especially since its high level increases the cost of the process. Inversely, for a low quantity of solvent-to-raw material plant ratio (v/w), the total polyphenols and flavonoids content cannot be totally extracted up from the plant. Consequently, an appropriate solvent-to-raw material plant ratio has to be selected for the extraction of targeted total polyphenols and flavonoids. Therefore, in this study the solvent-to-solid plant material ratio was set at 7.5:1 mL/g and chosen as the center point for further experiments.

[Fig. 1]

3.1.2. Effect of the stirring speed

The effect of different stirring speed was investigated from 100 to 1200 rpm on extraction of TPC and TFC (Fig. 1b), with the two other factors (solvent-to-solid ratio and extraction time) fixed at 7.5:1 mL/g and 18 h, respectively. The results show the extraction of both total polyphenols and flavonoids yields increase with the stirring speed, up to reach a maximal amount of 203.0 mg GAE/g and 108.7 mg QE/g, respectively. Above 650 rpm, the total polyphenols and flavonoids yields decrease, which might be due to the evaporation loss of the organic solvent at very high stirring speed, and lead to the decrease of total polyphenols and flavonoids contents extraction efficiency, in agreement with previous works carried out on the influence of stirring speed on extraction method (Luo et al., 2016). Besides, the mass transfer was limited for a stirring speed of 650 rpm or above, which seems to be the optimal value in this extraction process.

3.1.3. Effect of extraction time

In order to investigate the effect of extraction time on total polyphenols and flavonoids extraction yield, the extraction time was studied from 6 to 30 h. In this series of experiments, the solvent-to-solid ratio and stirring speed were fixed at 7.5:1 mL/g and 650 rpm, respectively. As shown in Fig. 1c, the yield of total polyphenols increases with the extraction time, with a maximum value of 203.0 mg GAE/g for 18 h of extraction. Beyond this value, the total polyphenols extraction yield begins to decline. Indeed, some bioactive compounds may be degraded during the prolonged extraction time as previously reported by Kumar et al. (2019). Concerning the yield of total flavonoids, this one increases with the extraction time during the experiments without reaching any optimal value. Our results are in agreement with the study of Butsat and Siriamornpun. (2016). Indeed, former authors evidenced that the extraction time was a critical parameter, affecting both the total polyphenols and flavonoids extraction yield. These authors studied the maceration of *Amomum chinense* leaves using various extraction solvents (*i.e.*, methanol, ethanol and acetone) and observed an increased polyphenols and flavonoids extraction yields for extraction time above 24 h. Therefore, a period of 18 h was selected as the center point of extraction time in the present work.

3.2. Optimization of the procedure

3.2.1. Model fitting and statistical analysis

The extraction of total polyphenols and flavonoids content from *R. alaternus* was optimized using response surface methodology. Table 2 summarizes the Box-Behnken matrix design used in this study, with the three independent variables X_i (*i.e.*, solvent-to-solid ratio, stirring speed and extraction time) and the results concerning the three studied responses (*i.e.* yields of total polyphenols and total flavonoids, and IC_{50} value). This represents a total of 15 experiments. By applying a multiple regression analysis on the obtained experimental results, a model was deduced for each bioactive compound and estimated using RSM, eliminating all insignificant terms. The ultimate predictive models of total polyphenols (y_{TPC}) and total flavonoids (y_{TFC}) yields are described hereafter, in Eq. 4 and 5, respectively:

$$y_{TPC} = 203.04 + 56.28x_1 + 17.60x_3 + 13.90x_2x_3 - 17.10x_1^2 - 35.05x_2^2 - 23.02x_3^2 \quad (4)$$

$$y_{TFC} = 108.75 + 10.59x_1 + 6.81x_3 - 5.85x_1x_2 + 6.49x_1x_3 - 17.43x_1^2 - 6.43x_2^2 \quad (5)$$

where x_1 , x_2 and x_3 correspond to the solvent-to-solid ratio, stirring speed and time of extraction variables, respectively.

The results of the statistical analysis are summarized in Table 3, considering a variable as non-significant when its p-value is higher than 0.05. Concerning the total polyphenols yield (y_{TPC}), our results indicate that the linear coefficients such as the solvent-to-solid ratio (x_1) and the extraction time (x_3) are significant but also, the interaction between the stirring speed and the extraction time (x_2x_3) and the quadratic terms of the three studied factors (x_1^2 , x_2^2 , and x_3^2). On the contrary, the linear coefficient of stirring speed (x_2), the interaction between the solvent-to-solid ratio and the stirring speed (x_1x_2), and also the interaction between the solvent-to-solid ratio and the extraction time (x_1x_3) are not significant (p-value > 0.05). Besides, the regression coefficient ($R^2 = 0.986$) is

satisfying for the model obtained from the regression analysis concerning the total polyphenols content (see Eq. 4), the adjusted R-squared value of 0.973, meaning that the model explains about 97.30% of the variance. This result suggests that the established model does fit the experimental information satisfactorily and could be used for predicting responses according to the criteria defined in other works (Song et al., 2019).

Concerning the total flavonoids yield (y_{TFC}), the results of the analysis of variance and the adequacy of the model (Table 3) indicate that the linear terms of solvent-to-solid ratio (x_1), the extraction time (x_3), the interaction terms between the ratio of solvent-to-solid and stirring speed (x_1x_2), and between the ratio of solvent-to-solid and extraction time (x_1x_3) are significant (p -value ≤ 0.05). In addition, the quadratic terms of solvent-to-solid ratio (x_1^2) and stirring speed of extraction (x_2^2) are significant as well ($p \leq 0.05$). Inversely, the linear coefficient of stirring speed (x_2), the interaction between the stirring speed and the extraction time (x_2x_3) and the quadratic term of extraction time (x_3^2) are not significant (p -value > 0.05), showing no effect on the flavonoid's extraction yield. The regression Eq. 5 represents the model for the total flavonoids content extraction. With an R-squared value of 0.983 and an adjusted R-squared value of 0.961, the model can explain about 96.1% of the variance. Hence, the model obtained is highly explainable by the analyzed variables.

Both assays presented in Table 3 exhibited a significant lack of fit, nevertheless this result does not invalidate the models (Lebron et al., 2020) since there is a high level of models' significance (p -value < 0.001), high values of R^2 and R^2 adjusted, adequacy precision are greater than 4, and a low coefficient of variation values (C.V. $< 10\%$), which explain the low deviations between the experimental and predicted values.

[Table 3]

3.2.2. Analysis of response surface

The Fig. 2 presents the response surface plots concerning the total polyphenols content (panels a, b, and c) and the total flavonoids content (panels d, e and f), respectively. Such graphical representations show the effects of two factors on the investigated response, while the third factor remains set at the center of the design. More useful than the traditional single effect optimization and in an optic of industrial valorization, the responses surface plots enable to understand the interaction effects between two parameters or more, and to determine the optimal value of each variable in order to minimize or maximize a given response, as reported in the literature (Yin and Dang, 2008).

Fig. 2 represents the combined effects of solvent-to-solid ratio and stirring speed on the extraction yields of total polyphenols (panel a) and of total flavonoids (panel d), respectively, the extraction time being set at 18 h. With an increase in stirring speed, the yields of both total polyphenols and total flavonoids slightly increase when the stirring speed is kept under zero levels, *i.e.*, 650 rpm. Above this value, the total polyphenols content extracted slightly decreases, while the total flavonoids content extracted still slightly increases. Therefore, a great linear increase in the yield of total polyphenol is observed when the solvent-to-solid ratio increases from 5:1 to 10:1 mL/g. Concerning the yield of total flavonoids, this one increases with the solvent-to-solid ratio until 7.5:1 mL/g, but decreases beyond this value. Hence, the solvent-to-solid ratio is an important parameter for improving the extraction yield of both total polyphenols and flavonoids.

The combined effect of the extraction time and the solvent-to-solid ratio on the yield of total polyphenols and flavonoids was investigated (Fig. 2b and e, respectively). The extraction time has a weak effect on total polyphenols yield when the solvent-to-solid ratio is kept at lower levels. As the ratio increases to higher values, the total polyphenols content increases greatly with the extraction time. Concerning the flavonoids, the lower values of solvent-to-solid ratio results in lower total flavonoids yield. The flavonoids content increases greatly with the solvent-to-solid ratio from 7.5:1 to 10:1 mL/g, but the yield decreases beyond. Also, the extraction time has only a weak effect on total flavonoids yield when the solvent-to-solid ratio is low. As the solvent-to-solid ratio increases

from 7.5:1 to 10:1 mL/g, the total flavonoids yield increases with the extraction time. Hence, an appropriate extraction time influences both the total polyphenols and the total flavonoids yields of extraction.

The combined effects of extraction time and stirring speed on the yield of total polyphenols and flavonoids is presented in Fig. 2c and f, respectively. Contrarily to the stirring speed, the extraction time shows a significant effect on total polyphenols yield. The total polyphenols could be significantly increased with the stirring speed until 650 rpm. Beyond this value, both the total polyphenols and flavonoids content strongly decreases. Also, the yield of total polyphenols increases with the extraction time until 20 h, and decreases beyond. It is interesting to note that the yield of total flavonoids increases greatly with the extraction time, especially at elevated values, indicating that more than 20 h are required for a maximum yield.

[Fig. 2]

3.2.3. Antioxidant capacity

Rumbaoa et al. (2009) evidenced different health benefits of phenolic compounds, especially flavonoids. Phenolic and flavonoid are important antioxidant compounds, which are able to donate hydrogen atoms to free radicals and finally, stabilize them. The antioxidant capacity was determined by DPPH test, which is considered as a valid accurate assay and an economic method to evaluate radical scavenging activity (Kedare and Singh, 2011). IC_{50} values are shown in Table 2.

Simple linear regression analyses were performed to investigate the relationship between total polyphenols content or total flavonoids content and the antioxidant activity.

For total polyphenols content, a significant regression equation was found (Fisher's test p-value = 6.60×10^{-7}), with a determination coefficient ($R^2 = 0.860$). Therefore, 86.0 % of the variance of the antioxidant activity expressed as IC_{50} is explained by total polyphenols content. Also, a negative correlation between the polyphenol content and IC_{50} was indicated. Thus, the increase of total polyphenols content will improve the antioxidant activity (Fig. 3).

For total flavonoids content, we observed from the Fisher's test a significant regression equation (p -value = 0.025), yet with a low correlation between flavonoids content and antioxidant activity ($R^2 = 0.332$).

Our results are in agreement with the study of Fitriansyah and colleagues (Fitriansyah et al., 2017), who reported a linear correlation of total polyphenols and flavonoids content from *Sesbania sesban* (L. Mr) leaves with antioxidant capacity. By comparing the determination coefficients (R^2 values), the polyphenolic compounds seem to be the major contributors in antioxidant activity by DPPH method, in agreement with previous works on Mate tea and seventeen common commercial herbs (Grujic et al., 2012; Yoo et al., 2008).

[Fig. 3]

3.3. Optimization of extracting parameters and validation of the model

In this study, the conditions for the extraction by dynamic maceration of bioactive compounds from *R. alaternus* were optimized in order to extract the maximum of total polyphenols and of total flavonoids contents, with the highest antioxidant capacity. The Derringer's desirability prediction method was used in order to acquire the particular optimum conditions of parameters (Table 4). The optimum values for the independent variables investigated are as follows: solvent-to-solid ratio (10:1 mL/g), stirring speed (518 rpm) and extraction time (24 h), respectively (Table 4). For these former optimum parameters, the predicted values of total polyphenols content, total flavonoids content and antioxidant capacity values were calculated by Design Expert 12 software and determined as 231.5 mg GAE/g, 119.9 mg QE/g and IC_{50} value of 22.9 μ g/mL, respectively, with a desirability value of 0.970.

Then, those predicted values were compared with the experimental ones determined in the same conditions *i.e.*: solvent-to-solid ratio (10:1 mL/g), stirring speed (518 rpm) and extraction time (24 h), respectively. The experiment of dynamic maceration was triplicated under these optimum values. In such conditions, a total phenolic content of 230.9 mg GAE/g, a total flavonoids content of 121.1

mg QE/g and an IC₅₀ equals to 22.5 µg/mL for the antioxidant DPPH test are experimentally determined (Table 4). These experimental values lay inside a 95 % average confidence interval of the predicted values, which indicate a good agreement between the experiment and the predicted values using the established models. Hence, the developed quadratic models based on Box–Behnken design are very adequate and significant.

[Table 4]

4. Conclusion

In the present study, for the first time a dynamic maceration method was performed for optimizing the extraction of natural antioxidant components including total polyphenols and flavonoids content from *R. alaternus* and for evaluating their antioxidant capacity using DPPH *in vitro* assay. The extraction process was successfully optimized and established using a Box–Behnken response surface design on three parameters (*i.e.*, the solvent-to-solid ratio, the stirring speed and the extraction time). The statistical analysis of the obtained model specified that, this one was appropriate and adequate with the experimental results. This study shows that a solvent-to-solid material ratio of 10:1 mL/g, a stirring speed of 518 rpm and an extraction time of 24 h are the best conditions to extract *R. alaternus*' total polyphenols and flavonoids with high antioxidant capacity. At these optimal conditions of extraction, we experimentally determined the total polyphenols and flavonoids content equal to 230.9 mg GAE/g and 121.1 mg QE/g, respectively, with an IC₅₀ value of 22.5 µg/mL. More, a rising positive correlation is confirmed between the total phenolic content and the DPPH scavenging activities of *R. alaternus* extracts. Therefore, the use of dynamic maceration can be recommended as a simple and cost-effective process in the pharmaceutical industry in order to extract the antioxidant compounds from *R. alaternus*.

Declaration of interest statement

All the authors confirm that there are no known conflicts of interest.

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Figure captions

Fig. 1. Effect of variable factors on total polyphenol and total flavonoid content of *R. alaternus*: (a) solvent-to-solid ratio (mL/g), (b) stirring speed (rpm), and (c) extraction time (h).

Fig. 2. Response surface plots (a,b,c,d,e and f) showing the interactive effects of solvent-to-solid ratio (X_1), stirring speed (X_2) and extraction time (X_3) on extraction yield of total polyphenol and flavonoid contents from *R. alaternus* by dynamic maceration.

Fig. 3. Antioxidant activity (IC_{50}) versus total polyphenol content of maceration extracts obtained from *R. alaternus*.

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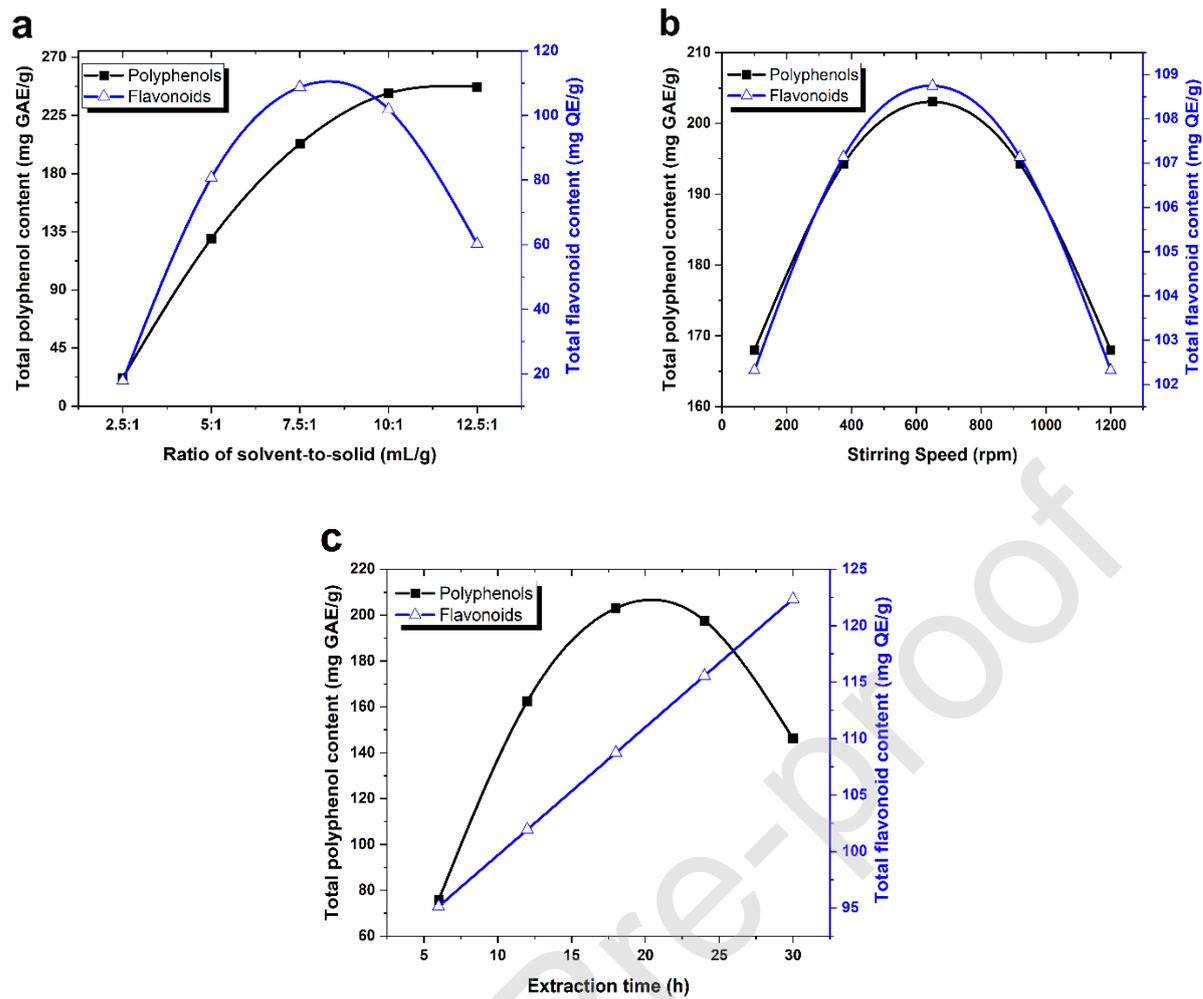
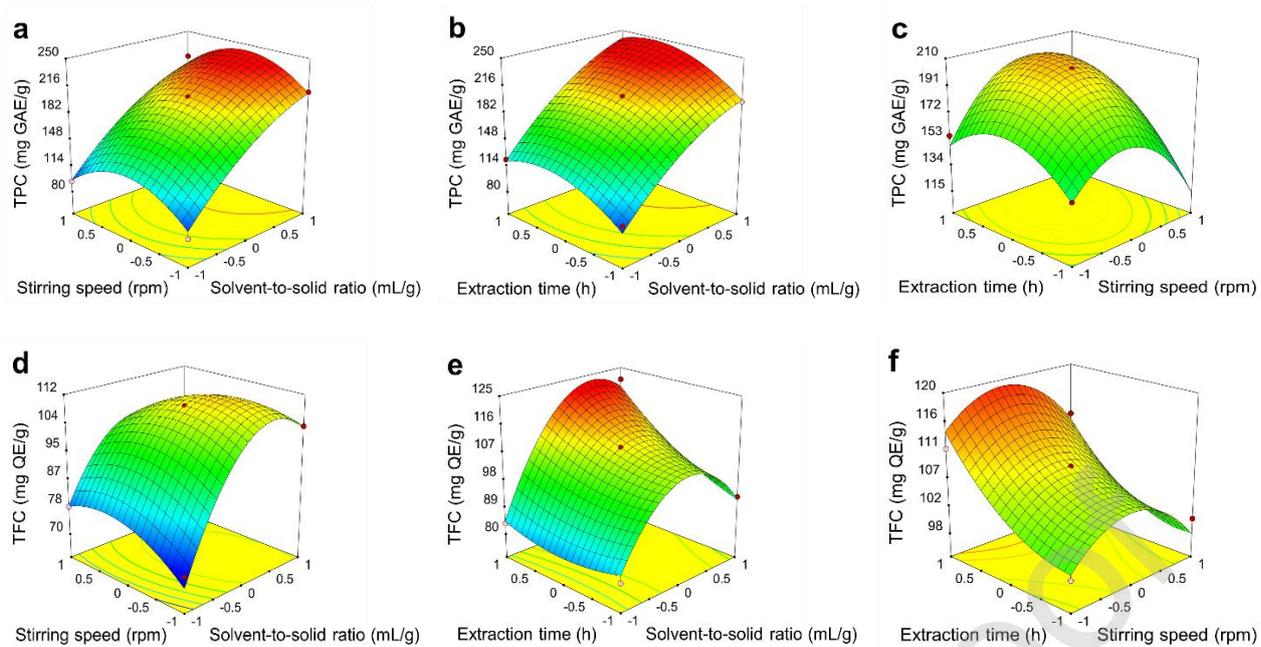


Fig. 1

**Fig. 2**

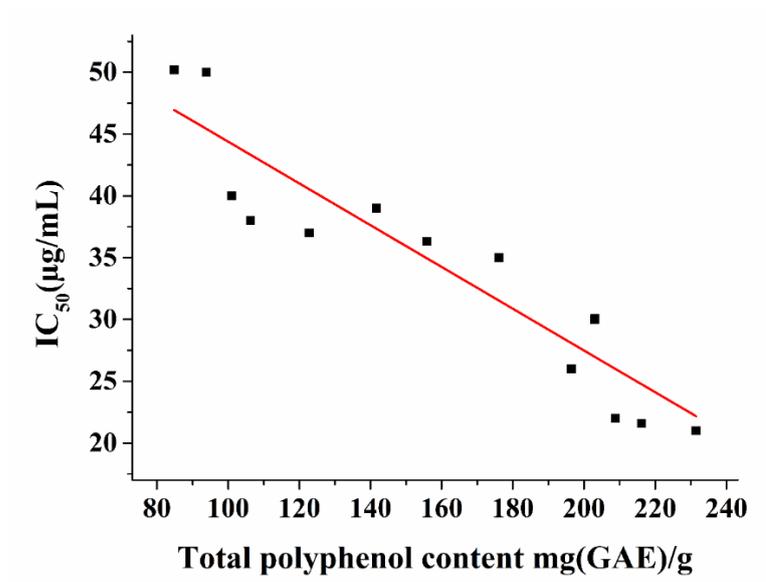


Fig. 3

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Table 1

Variables and their experimental design levels used in Box-Behnken design.

| Independent variables | Coded symbols | Levels | | |
|-------------------------------|----------------|--------|--------|------|
| | | -1 | 0 | 1 |
| Solvent-to-solid ratio (mL/g) | X ₁ | 5:1 | 7.5 :1 | 10:1 |
| Stirring speed (rpm) | X ₂ | 100 | 650 | 1200 |
| Extraction time (h) | X ₃ | 12 | 18 | 24 |

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Table 2

Box–Behnken matrix design with three independent variables and the three measured responses.

| Experimental run | X ₁ | X ₂ | X ₃ | Total polyphenol content mg (GAE)/g | Total flavonoid content mg (QE)/g | IC ₅₀ (µg/mL) |
|------------------|----------------|----------------|----------------|-------------------------------------|-----------------------------------|--------------------------|
| 1 | -1 | -1 | 0 | 84.8 | 73.1 | 50.2 |
| 2 | 1 | -1 | 0 | 208.8 | 102.8 | 22.0 |
| 3 | -1 | 1 | 0 | 93.9 | 78.6 | 50.0 |
| 4 | 1 | 1 | 0 | 216.1 | 85.0 | 21.6 |
| 5 | -1 | 0 | -1 | 101.0 | 81.3 | 40.0 |
| 6 | 1 | 0 | -1 | 196.4 | 92.7 | 26.0 |
| 7 | -1 | 0 | 1 | 122.8 | 83.7 | 37.0 |
| 8 | 1 | 0 | 1 | 231.5 | 121.0 | 21.0 |
| 9 | 0 | -1 | -1 | 141.7 | 99.0 | 39.0 |
| 10 | 0 | 1 | -1 | 106.3 | 100.4 | 38.0 |
| 11 | 0 | -1 | 1 | 155.9 | 111.5 | 36.3 |
| 12 | 0 | 1 | 1 | 176.1 | 111.7 | 35.0 |
| 13 | 0 | 0 | 0 | 203.0 | 108.8 | 30.0 |
| 14 | 0 | 0 | 0 | 203.1 | 108.7 | 30.0 |
| 15 | 0 | 0 | 0 | 203.0 | 108.8 | 30.1 |

Ascorbic acid standard: IC₅₀ = 5.0 µg/mL

Table 3. ANOVA analysis for the quadratic model of the total polyphenols and total flavonoids contents.

| Variable | Total polyphenols content | | | | | Total flavonoids content | | | | |
|-------------------------------|---------------------------|----|-------------|-----------|----------|--------------------------|----|-------------|----------|----------|
| | Sum of squares | df | Mean square | F-value | p-value | Sum of squares | df | Mean square | F-value | p-value |
| Model | 35332.08 | 9 | 3925.79 | 44.78 | 0.0003 | 2902.21 | 9 | 322.47 | 32.49 | 0.0007 |
| X ₁ | 25341.76 | 1 | 25341.76 | 289.06 | < 0.0001 | 897.18 | 1 | 897.18 | 90.39 | 0.0002 |
| X ₂ | 0.19 | 1 | 0.19 | 0.00 | 0.9650 | 14.36 | 1 | 14.36 | 1.45 | 0.2828 |
| X ₃ | 2477.38 | 1 | 2477.38 | 28.26 | 0.0032 | 370.74 | 1 | 370.74 | 37.35 | 0.0017 |
| X ₁ X ₂ | 0.69 | 1 | 0.69 | 0.01 | 0.9328 | 137.12 | 1 | 137.12 | 13.81 | 0.0138 |
| X ₁ X ₃ | 43.82 | 1 | 43.82 | 0.50 | 0.5111 | 168.74 | 1 | 168.74 | 17.00 | 0.0091 |
| X ₂ X ₃ | 772.84 | 1 | 772.84 | 8.82 | 0.0312 | 0.38 | 1 | 0.38 | 0.04 | 0.8517 |
| X ₁ ² | 1079.88 | 1 | 1079.88 | 12.32 | 0.0171 | 1122.28 | 1 | 1122.28 | 113.07 | 0.0001 |
| X ₂ ² | 4535.15 | 1 | 4535.15 | 51.73 | 0.0008 | 152.86 | 1 | 152.86 | 15.40 | 0.0111 |
| X ₃ ² | 1956.06 | 1 | 1956.06 | 22.31 | 0.0052 | 41.21 | 1 | 41.21 | 4.15 | 0.0972 |
| Lack of Fit | 438.35 | 3 | 146.12 | 6.262E+05 | < 0.0001 | 49.63 | 3 | 16.54 | 13412.66 | < 0.0001 |
| Pure Error | 0.0005 | 2 | 0.0002 | | | 0.0025 | 2 | 0.0012 | | |
| Total | 35770.43 | 14 | | | | 2951.84 | 14 | | | |
| *R ² | 0.987 | | | | | 0.983 | | | | |
| *R ² . adj | 0.973 | | | | | 0.961 | | | | |
| Adeq. Precision | 19.32 | | | | | 18.95 | | | | |
| C.V. % | 5.75 % | | | | | 3.22 % | | | | |

df: Degree of freedom.

X₁: solvent-to-solid ratio (mL/g); X₂: stirring speed (rpm); X₃: extraction time (h).*R² and *R². adj are calculated for the reduced model.

C.V. % coefficient of variation.

Table 4

Predicted and experimental values of responses at the optimum values of solvent-to-solid ratio (10:1 mL/g), stirring speed (518 rpm) and extraction time (24 h).

| Responses | Predicted value | 95% CI low* | 95% CI high** | Experimental value |
|--------------------------------------|-----------------|-------------|---------------|--------------------|
| Total polyphenols content (mg GAE/g) | 231.5 | 217.5 | 245.5 | 230.9 ± 0.25 |
| Total flavonoids content (mg QE/g) | 119.9 | 113.8 | 126.1 | 121.1 ± 0.20 |
| IC ₅₀ (µg/mL) | 22.9 | 18.8 | 27.1 | 22.5 ± 0.30 |

*95% CI low is the lower limit of the 95% confidence interval.

**95% CI high is the upper limit of the 95% confidence interval.

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