Industrial Crops & Products 144 (2020) 112054

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Intra-combined antioxidant activity and chemical characterization of three fractions from *Rhamnus alaternus* extract: Mixture design

Ikrame Zeouk[a,b,*], Wessal Ouedrhiri[c], Ignacio A. Jiménez[d], Jacob Lorenzo-Morales[a], Isabel L. Bazzocchi[d], Khadija Bekhti[b]

[a] Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de la Laguna. Tenerife, Spain
[b] Departement of Biology, Sidi Mohamed Ben Abdellah University, Faculty of Sciences and Techniques, Laboratory of Microbial Biotechnology, PB 2202, Fez, Morocco
[c] National Agency of Medicinal and Aromatic Plants, University Sidi Mohamed Ben Abdellah, BP 159, Taounate, Morocco
[d] Instituto Universitario de Bio-Orgánica Antonio González, Departamento de Química Orgánica, Universidad de La Laguna, Avenida Astrofísico Francisco Sánchez 2, 38206 La Laguna, Tenerife, Spain

ARTICLE INFO

Keywords:
Antioxidant
Rhamnus alaternus
Flavonoids
Anthraquinones
Mixture design
Synergism
Formulation

ABSTRACT

The look for benefits antioxidant agents has attracted much attention from consumers, health and food industries. Certainly, the interactions occurring between different compounds affect the antioxidant efficiency. The current study aims the investigation of the antioxidant combined effect of *Rhamnus alaternus* fractions using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay. The three most active fractions were subject for binary and ternary combinations using the augmented simplex-centroid design. Two known compounds emodin (1) and kaempferol (2) were identified from the ethanolic extract of the leaves of *Rhamnus alaternus*. Their structures were elucidated using spectroscopy data. The antioxidant potent of the crude extract and the mixture was compared with the antioxidant activity of the fractions separately. Important synergetic effects were observed in binary mixture between the most active fractions, besides the response optimizer allowed us to optimize the mixture presenting the highest antioxidant activity at 82.54 %. The results from the present work could have interesting applications in both medical formulations and functional food design.

1. Introduction

The use of antioxidants is a widespread interest in both pharmaceutical and food science. However, this use requires deeply studies and measurements of the antioxidant capacities (Shahidi and Zhong, 2015) -whatever their source- knowing that antioxidant agents may occur in plants (Embuscado, 2015), animals (Rocha et al., 2018), microorganisms (Guo et al., 2010) and marine organisms (Cavalcante Alencar et al., 2019) or may be synthesized chemically (Xue et al., 2018). These antioxidants have numerous biological and pharmacological benefits as combating oxidative stress (Zheng and Wang, 2001) which is a major challenge for many fields. The oxidative stress could be defined as the lack of balance between the occurrence of ROS or RNS (Reactive Oxygen or Nitrogen species) and the organism's capacity to act contrary to their action by the antioxidant defenses system (Poprac et al., 2017) leading to the development of many diseases because the oxidative stress can initiate a disease, and be an important but secondary factor or a major contributing factor in the development of the disease (Spector, 2000). The oxidative damage can be generated by free radicals known as molecules or molecular fragments containing one or more unpaired electrons or ROS/RNS which are presented by both free radical and non-free radical oxygenated molecules (Sies, 1997), this damage makes the look for efficient antioxidants a necessity. Higher plants and their compounds present a rich source of natural antioxidants (Larson, 1988), *Rhamnus alaternus* belonging to Rhamnaceae family is an interesting representative example for the identification and characterization of antioxidant substances (Ben Ammar et al., 2009; Moussi et al., 2015), it is a good example of plant species having a high phytochemical value due to diversity of its active metabolites (Zeouk et al., 2019). Nowadays, a new conception becomes to be a trend in antioxidant studies, due to its spectacular results, which is combining effect that affect the characterization and subsequent medicinal activities in many drug combination studies (Chou, 2007) and nutrition fields (Çelik et al., 2018). The evaluation of the effect of such mixtures may provide different outcomes: an additive, a synergistic or an antagonistic effect. These different effects are related to the types, natures and

* Corresponding author at: Departement of Biology, Sidi Mohamed Ben Abdellah University, Faculty of Sciences and Techniques, Laboratory of Microbial Biotechnology, PB 2202, Fez, Morocco.
E-mail address: ikramezeouk20@gmail.com (I. Zeouk).

https://doi.org/10.1016/j.indcrop.2019.112054
Received 7 January 2019; Received in revised form 27 November 2019; Accepted 16 December 2019
Available online 24 December 2019

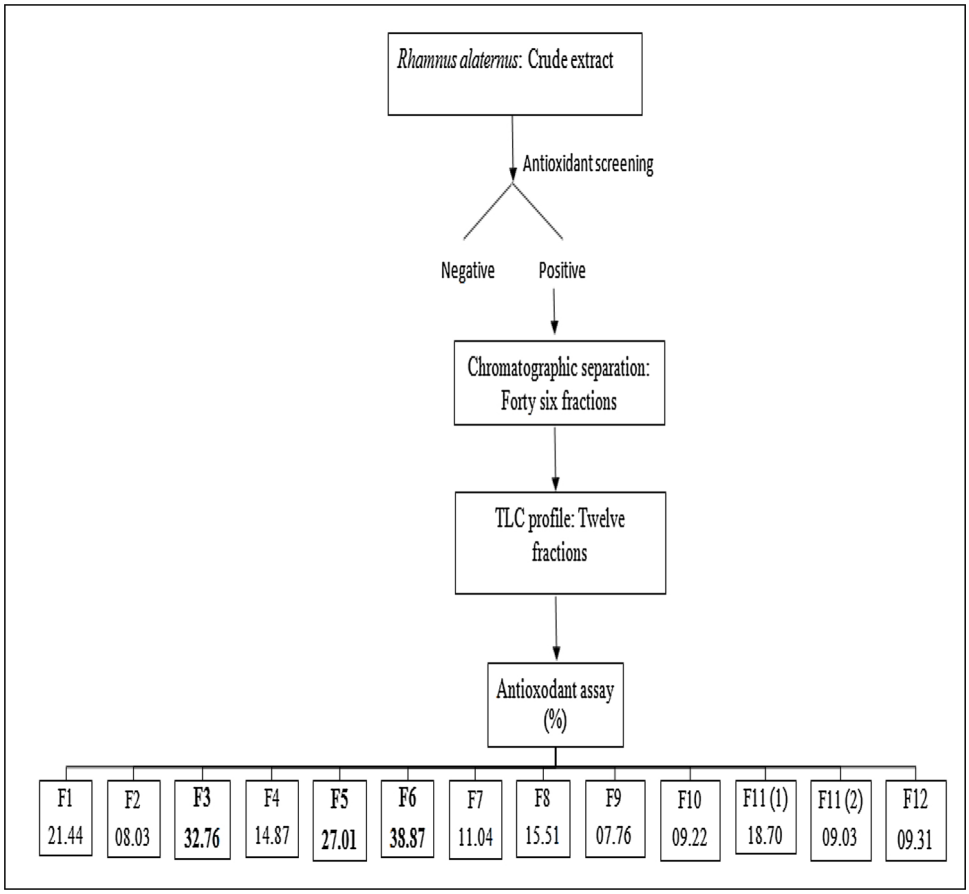

Fig. 1. Diagram of the fractionation process of *R. alaternus* leaves ethanolic extract. Antioxidant assay; Positive: interesting antioxidant potent- Negative: no antioxidant has been observed.

concentration of various natural compounds as well as their interaction or co-existence in different plants or either in the same plant species.

Among methods used to carry out these studies, no one has been standardized, but they were almost statically significant. We cite the most known: Checkerboard assay, where the interaction is identified using simple linear regression (Bag and Chattopadhyay, 2015), and mixture design method using a multiple linear regression (Baj et al., 2018). The mixture design is a class of the response surface experiments in which the independent variables are the proportions of the components under investigation (Myers et al., 1989). The mixture design studies aims are creating in the first time a general conception about responses and interactions between independent factors, there, developing efficient formulations providing optimal requests (Maia et al., 2011).

In this paper, we have evaluated the antioxidant activity of the crude ethanolic extract of *Rhamnus alaternus* and twelve fractions of it. Then, the combination of the most three active fractions have been analyzed using the mixture design method to identify the optimum ratio. Additionally, the phytochemical profile of the three active fractions of *R. alaternus* was investigated and the molecules structure was elucidated.

2. Materials and methods

2.1. Chemicals and reagents

All solvents used were analytical grade from Sigma-Aldrich. Silicagel 60 F_{254} (Merck -Darmstadt, Germany) plates (20 × 20 cm) supported on aluminium sheet was used for thin-layer chromatography (TLC) separations. Silicagel 60 [Fluka-chemie GmbH] for Column chromatography was obtained from Sigma-Aldrich. 2.2-dyphenyl-1-picrylhydrazyl (DPPH) and Butylhydroxytoluene (BHT) were purchased from Sigma-Aldrich and used without further purification.

For the purification procedure, Silica gel 60 (particle size 15–40 and 63 – 200 μm, Macherey-Nagel) was used for column chromatography (CC), while silica gel 60 F254 was used for analytical TLC. The developed TLC plates were visualized by UV light and then spraying with HOAc-H_2SO_4-H_2O (80:16:4) system, followed by heating at 100 °C during 3 min. ^{1}H NMR spectrum was carried out on a Bruker Avance 600 spectrometer, with the pulse sequences given by Bruker. The electron impact mass spectrometry (EIMS) and high-resolution electron ionization mass spectrometry (HREIMS) were measured on an LCT Premier XE Micromass Electrospray Spectrometer.

2.2. Plant material identification and extraction

R. alaternus leaves were collected in the Atlas Mountains of Imouzzer region-Morocco in July 2017 (34°1'8"N, 5°0'26"W). The plant material was identified by Pr EL OUALIDI. J. and Pr IBN TATTOU. M. A voucher specimen (RAB107343) has been deposited in the Herbarium of the Scientific Institute, Rabat, Morocco.

To obtain the crude extract of *R.alaternus*, cleaned leaves were shade dried at room temperature and subsequently milled with a simple commercial electric grinder. Powdered material was extracted by maceration in ethanol (1:10 w/v) for 6 h at room temperature with continuous stirring at 500 rpm (Revolutions per minute). The resulting mixture was filtered through Whatman filter n°1 and the solvent concentrated under vacuum in a rotary evaporator at 45 °C, dried extracts were stored in a refrigerator at 4 °C until further use.

2.3. Fractionation of R. Alaternus leaves

The active extract was fractionated by column chromatography using different mixtures of solvents selected from a preliminary study of polarity to get the optimal eluent system, and then a TLC was checked up on chromatogram under visible-ultraviolet (UV) to look for conditions that give the optimal separation. The best separation was obtained with a mixture of (ethyl acetate: methanol: distillated water) (8:1:1). Consequently, 8 g of the crude extract were subjected to silica gel column chromatography eluted with gradients of the selected solvents. Forty six fractions were collected, and combined into twelve fractions on the basis of the TLC profiles, coded from F1 to F12. The fractionation process is illustrated in Fig. 1. After that, all the fractions obtained were submitted to antioxidant screening.

2.4. Evaluation of antioxidant activity using DPPH assay

The scavenging activity of the studied extract was evaluated using 2.2-dyphenyl-1-picrylhydrazyl (DPPH) assay (Blois, 1958) (Fumio Nanjo et al., 1996). The method described by (Ouedrhiri et al., 2015) was performed with slight modifications. Briefly, the crude extract and the twelve fractions were serially diluted (62.50, 125, 250, 500 and 1000 µg/mL (w/v)) in methanol, and a solution of DPPH (0.004% (w/v)) was prepared in the same solvent. Then 2 mL of each dilution were mixed with 2 mL of DPPH solution. Absorbance of the reaction mixture was measured after 30 min incubation in the dark at room temperature at 517 nm. The absorbance was measured in triplicate at 517 nm using BK-UV 1000 UV/VIS Spectrophotometer. BHT was used as standard. The antioxidant activity was calculated as follow:

Antioxidant activity % = [(Abs (control) − Abs (sample)) / Abs (control)] × 100

The half inhibitory concentrations (IC_{50}) were determined graphically.

2.4.1. Screening of the antioxidant activity of the fractions

To screen the most active fractions, the antioxidant activity of the studied fractions was tested at the same IC_{50} of the crude extract (Table 1 and 2). Fractions F3, F5 and F6 with the most considerable antioxidant activity were subjected to a binary and ternary antioxidant combined effect using mixture design analysis and a phytochemical identification in order to elucidate the structures of the active molecules.

2.5. Purification of the active sub-fractions

In order to isolate and then identify the bioactive compounds, the active sub-fractions F3, F5 and F6 were further chromatographed, using column chromatography on silica gel eluted with CH_2Cl_2/ Me_2CO of increasing polarity. Sub-fraction F3A yielded compound 1, sub-fraction F5B yielded compound 1 and F5E yielded compound 2. F6B yielded compound 2, which were identified as emodin (1) and kaempferol (2) by comparison of their spectroscopy and spectrometric data with those previously reported.

Table 1
Antioxidant activity of the ethanolic crude extract of *R. alaternus* leaves.

R. alaternus leaves	IC_{50} (µg/ml)
Crude ethanolic extract	58.00 ± 0.007
BHT	31.00 ± 0,003

Antioxidant screening using DPPH assay.
In comparison with the standard BHT: Butylated hydroxytoluene.

Table 2
Preliminary screening of the antioxidant activity of *R. alaternus* fractions.

R. alaternus fractions	AA% of fractions at IC_{50} of the crude extract of *R. alaternus*
F1	21.44
F2	08.03
F3	32.76
F4	14.87
F5	27.01
F6	38.87
F7	11.04
F8	15.51
F9	07.76
F10	09.22
F11 (1)	18.70
F11 (2)	09.03
F12	09.31

% of the antioxidant screening of the obtained fractions.
IC_{50} is expressed in %.

Table 3
Original components of the design matrix and experimental responses for the three analyzed fractions.

N° Exp	F3	F5	F6	CI_{50} (µg/ml)
1	1.0000	0.0000	0.0000	45.00 ± 0.003
2	0.0000	1.0000	0.0000	164.00 ± 0.039
3	0.0000	0.0000	1.0000	36.00 ± 0.003
4	0.5000	0.5000	0.0000	58.00 ± 0.005
5	0.5000	0.0000	0.5000	41.00 ± 0.001
6	0.0000	0.5000	0.5000	48.00 ± 0.000
7	0.3333	0.3333	0.3333	53.00 ± 0.004
8	0.6667	0.1667	0.1667	38.00 ± 0.001
9	0.1667	0.6667	0.1667	57.00 ± 0.007
10	0.1667	0.1667	0.6667	38.00 ± 0.001

Matrix of the three most active fractions.
using antioxidant assay through 10 experiments.

2.6. Mixture design and statistical analysis

To evaluate the interaction effects between *R. alaternus* fractions, augmented simplex-centroid design was performed as described previously (Ouedrhiri et al., 2016). Based on Table 3, the complete experimental design for fraction consisted on 10 experiments in triplicate. The experiments were performed in random order (Table 3). Fig. 2 shows the points of designed experiments. The vertices of the triangle (1, 2, 3) correspond to the three fractions alone. The midpoints of the three sides of the triangle (4, 5, 6) consist of the binary combinations

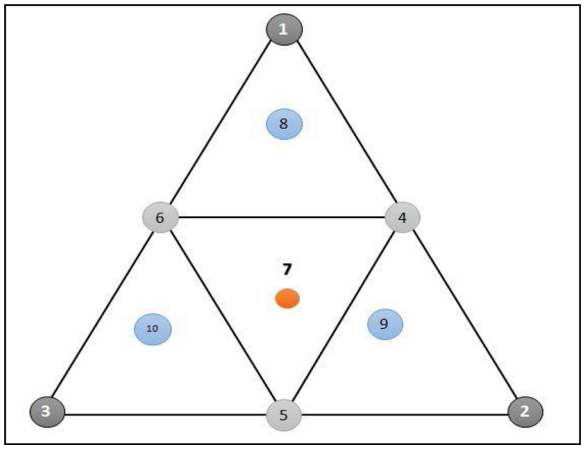

Fig. 2. An overview of the augmented simplex-centroid design for a three-component mixture.

and the central point (centroid) and the three augmented points (8, 9, 10) ascribed to the ternary combinations.

The factors X1, X2, and X3 represent fractions F3, F5 and F6 respectively in the mixtures, which range from 0 to 1 without constraints.

Synergistic model of the third degree was undertaken for variation of all studied responses as function of significant interaction effects between variables applying the least squares regression to estimate the unknown coefficients in the following equation:

$$\mathbf{Y} = \mathbf{b}_1X_1 + \mathbf{b}_2X_2 + \mathbf{b}_3X_3 + \mathbf{b}_{12}X_1X_2 + \mathbf{b}_{13}X_1X_3 + \mathbf{b}_{23}X_2X_3 + \mathbf{b}_{123}X_1X_2X_3$$

Where **Y** is the predicted response; **b1**, **b2** and **b3** are the regression coefficients for each linear effect terms; **b12**, **b13** and **b23** are the binary interaction effect terms; **b123** is the ternary interaction term; X1, X2 and X3 are the proportions of the fractions in the mixture. This analysis was performed using NEMRODW software package (Mathieu, Nony and Phan-Tan-Luu, version 2000, Marseille-France).

3. Results and discussion

3.1. Antioxidant activity of R. Alaternus ethanolic extract and fractions

The oxidative stress has been identified as a possible cause of the progression of different diseases. Therefore, discovering new efficient antioxidant agents is of great importance. In the present study, the antioxidant activity of the crude ethanolic extract of *R. alaternus* and its fractions were assessed *in vitro* using DPPH assay. The crude extract has shown a very good antioxidant activity with an IC_{50} of 58.00 ± 07.00 µg/mL (Table 1), this result was encouraging to conduct a fractionation assay in order to isolate more active pure compounds and to optimize the combined effect. Significant differences were observed between the evaluated elements, based on Table 2 the preliminary screening of the antioxidant effects has shown that the IC_{50} of *R. alaternus* leaves fractions varied between 07.67% and 38.87%. The fractions exhibiting the highest antioxidant activity were F3, F5 and F6 with an AA% of 32.76%, 27.01% and 38.87% respectively. As highlighted by previous studies, the crude extract and fractions of *R. alaternus* showed an interesting antioxidant activity (Bhouri et al., 2011; Ben Ammar et al., 2011; Boussahel et al., 2013). Indeed, (Moussi et al., 2015) have demonstrated that the methanol extract and fractions of *R. alaternus* leaves have exhibited antioxidant effect with percentage ranging between 90.81 ± 02.46% and 12.60 ± 01.24% for fractions and 66.83 ± 03.88% for the crude extract. In the current work, this important result was a starting test for further development and optimization of the antioxidant power. Thus, the active fractions were subject to phytochemical characterization, binary and ternary mixture analysis.

3.2. Phytochemistry

The results achieved in the characterization process of the three active fractions lead to the isolation of emodin from F3, kaempferol from F6 (Fig. 3), while F5 has been analyzed as a mixture of emodin as main compound and kaempferol. Their structures have been elucidated on the basis of spectroscopic analysis and mass spectrometry, and comparison with values reported in the literature (Mari et al., 2019) (Demmak et al., 2019). This finding corroborate previous phytochemical investigations of *R. alaternus* which report that the leaves of *R. alaternus* contains active compounds belonging to the anthraquinones and flavonoids families (Ben Ammar et al., 2009). Emodin has been widely reported as the most abundant metabolite in *R. alaternus* with multiple ecological functions such as preventing this plant from biotic stress (Abou-chaar et al., 1982) (Tsahar et al., 2002). As highlighted by numerous researchers emodin is endowed with interesting antioxidant potency in other plant species such as *Rheum emodi* rhizomes (Mishra et al., 2014), *Polygonum cuspidatum* root (Zhuang et al., 2008) and *Cassia tora* L. seed (Kim et al., 2004). Moreover, the ex *vivo* and pharmacokinetics antioxidant potent of emodin has been confirmed (Mishra et al., 2014). Regarding kaempferol, it is too expensive to be used in commercial products. In the present study, we have shown that *R. alaternus* is rich in this compound. Similar to these results, numerous studies have confirmed the abundant of kaempferol in *R. alaternus* species (Moussi et al., 2015) (Boussahel et al., 2013). The interesting antioxidant potency of this species has been also demonstrated (Bhouri et al., 2011) (Ben Ammar et al., 2009).

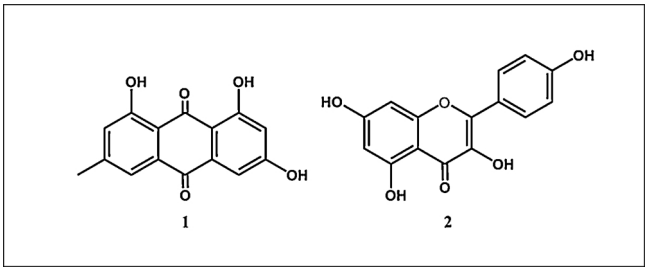

Fig. 3. Chemical structures of the antioxidant compounds isolated from *R. alaternus* ethanolic extract: emodin **(1)**, kaempferol **(2).**

Based on literature, the reported phytochemical studies could confirm the richness of *R. alaternus* in different active compounds, (Bhouri et al., 2012) have isolated two antioxidant flavonoids namely Kaempferol 3-O-beta isorhamninoside and Rhamnocitrin 3-O-beta isorhamninoside which had capacities to transfer electron leading to an attack against free radicals and then combating cellular damage. In a recent study reported by Ben Ammar et al. (2018), one new alaternoside (1,6 dihydroxy-3 methyl 6 [2'-Me (heptoxy)] anthraquinone) and two known isolated compounds (physcion-8-O-rutinoside and Kaempferol-7-methyl-lether) from leaves and bark roots of *R. alaternus* have shown an important antioxidant activity with an IC_{50} of 09.46 µg/ml, 27.68 µg/ml and 02.35 µg/ml respectively. Moreover, the high-performance liquid chromatography with a diode-array detector (HPLC-DAD) analysis of this species confirms the presence of anthraquinones as noticed in the previous cited investigations and other compounds namely luteolin, quercetin-3-rhamnoside, p-coumaric acid, ferulic acid, gallic acid, and rutin. These phytochemical families have been demonstrated to play a role in antioxidant mechanism of action due to their molecular structures (Huang and Frankel, 1997; (Montoro et al., 2005). Flavonoids as putative example were considered as good electron and hydrogen donors, this character brings to the end of radical chain through converting free radicals and ROS to more stable compounds (Kelly, 2010).

3.3. Establishment of response prediction models

Based on Table 3, the coefficient of determination (R^2), the adjusted R^2 values and the statistical significance of the regression models using ANOVA (Analysis of variance) confirm that the selected model is suitable to provide a significant data. As can be seen from Table 4, coefficient values ranges between -0.19 and 0.15. Knowing that the sign plays a role in the factor ability to increase (+) or decrease (-) the response of variable. Obviously, our survey aims to increase the antioxidant combined effect of the three selected fractions, hence the negative coefficient value could present the ability of its associated factor to increase the antioxidant activity. In fact, based on Table 5, F6 was the most efficient fraction with IC_{50} of 36.00 µg/mL followed by F3 with IC_{50} of 45.00 µg/mL and F5 with 164.00 µg/mL ($p < 0.01$). Hence, in comparison to the crude extract (IC_{50} = 58.00 µg/mL), F3 and F6 have noticed more interesting antioxidant effect in opposition with F5 that has shown an IC_{50} of 164.00 µg/mL. The combined antioxidant power of the studied fractions was as follow F3/F5 > F5/F6, while F3/F6 was not significant. F3/F5 has exhibited a significant synergistic antioxidant effect with coefficient of -0.19 representing the low one, followed by that of F6/F5 (coefficient -0.14)

Table 4
Analysis of variance for the different models fitted to response.

Source of variation		Sum of squares	Freedom degree	Mean squares	Ratio	Signif
Linear regression		0.0368	6	0.0061	32.5603	< 0.0***
Residues		0.0061	23	0.0003		
Validity		0.0023	3	0.0008	4.1443	1.94*
Error		0.0038	20	0.0002		
Total		0.0429	29			
R^2	*0.858*					
R^2 Adj	*0.820*					

Level of statistical significance:
Adj: Adjusted.
* P < 0.05.
*** P < 0.001.

Table 5
Coefficients'estimates and statistics of the response.

Elements	Coefficient	Statistical significance
F3	0.0462	< 0.01***
F5	0.1575	< 0.01***
F6	0.0376	0.0106***
F3/F5	−0.1941	< 0.01***
F3/F6	0.0089	81.5
F5/F6	−0.1419	0.155 **
F3/F5/F6	−0.0641	79.9

Level of statistical significance:
*P < 0.05.
** P < 0.02.
*** P < 0.001.

(p < 0.05) which were all statistically significant. However, the binary combination of F3/F6 and the ternary one did not exhibit any significant interaction (p > 0.05) (Table 5). Correlating the antioxidant activity with the phytochemical characterization, we can note that the pure compounds emodin and kaempferol are more active than the complex mixture which may explain the antagonistic effect observed in the binary combination of F3/F6 and the ternary one. This result highlighted how much important is attached to the combined effect in increasing antioxidant activity and eliminating the other non active fractions which leads to a favorable orientation of the antioxidant assessment.

Similar to our purpose, the comparison of the scavenging activity of plant crude extracts and fractions has been widely reported (Li et al., 2009) have demonstrated higher antioxidant activity in fractions when compared to the total crude extract of *Lysimachia foenum gracum*, which has been explained by the accordance between the total phenols and flavonoids amounts and the antioxidant potency. Similar to this study (Anagnostopoulou et al., 2006) have also evaluated the antioxidant effect of crude extract and different fractions of *Citrus sinensis*, the fractions have stronger antioxidant activity than the total extract but there was no well correlation between total phenolic content and the antioxidant capacity regarded that the both analysis do not follow the same mechanisms. The different magnitude of the observed antioxidant activity in fractions as compared to the total extract may due to the interferences between many compounds present in the crude extracts, taking into consideration that the crude extract is a complex mixture of molecules.

Many factors could explain the findings of this work because the interactions occurring between different compounds certainly affect the antioxidant efficiency either positively or negatively. (Peyrat-Maillard et al., 2003) have evaluated the synergetic and antagonistic effects between phenolic antioxidants in a binary mixture and the confirmation of structure–activity relationships; they have demonstrated that the efficiency increases with the number of OH (Hydroxy groups) or the presence of a methoxy group and also depending on the chemical structure of molecules. For instance, it was reported that *R. alaternus* contains 3.4% of a-Tocopherol in hexane extract and 442 ppm (parts-per-million) in dry leaves (Corporation and View, 1993) and according to another study, this compound has shown different mixture effects: antagonism with cinnamic acids and synergism with ferulic acid (Peyrat-Maillard et al., 2003). Moreover, in the same study the ferrulic acid has no mixture effect with a group a flavonoids, and for the flavonoids themselves the glycosylation of quercetin in rutin has decreased its efficacy (Peyrat-Maillard et al., 2003).

3.4. The mixture optimization

In order to generate the optimal zone of the fractions combination to meet the expectations of all the responses (Fig. 4) an optimizer of response has been used following the formula:

$$\mathbf{Y} = 0.0462\mathbf{X}_1 + 0.1575\mathbf{X}_2 + 0.0376\mathbf{X}_3 - 0.1941\mathbf{X}_1\mathbf{X}_2 - 0.1419\mathbf{X}_2\mathbf{X}_3$$

The most important antioxidant activity with an IC_{50} of 37.00 µg/mL was given by a mixture containing 651 µg of F3, 207.00 µg of F5 and 142.00 µg of F6 towards the total crude extract of *R. alaternus* with a

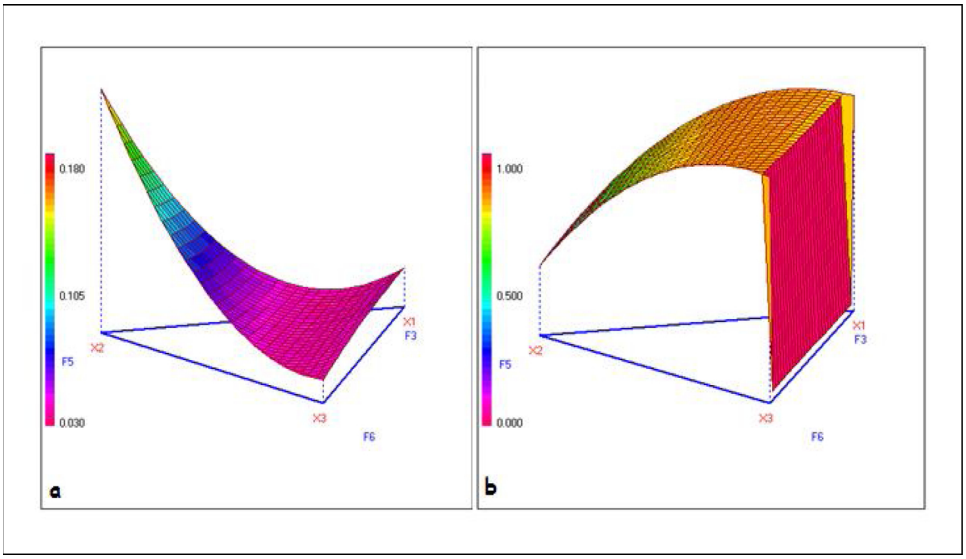

Fig. 4. 3D surface plots for (a) the IC_{50} variations of different combinations of the studied fractions (b) Optimal design regions of IC_{50} of the mixture of fractions.

percentage of 82.54% to predict the highest antioxidant activity of the three fractions. F5 has noticed an antioxidant activity lower than the crude extract, however when it was added to F3 then F6 the combination has noticed a very interesting antioxidant potency which may be explained by the dose-dependent remembering that F5 is a complex mixture and the main compound of F5 was emodin. The binary combination of emodin and kaempferol has shown antagonistic effect but when adding F5, the emodin concentration becomes more important leading to more interesting antioxidant potent. Previous studies reported that higher ration of phytochemical compounds can obtain higher antioxidant potent and that the hydroxyl radical scavenging activity can be concentration dependent (Wang et al., 2009).

4. Conclusion

To the best of our knowledge, the present survey is the first to investigate the intra-combined antioxidant effect between fractions from *Rhamnus alaternus* leaves ethanolic extract. In general, the obtained results indicated that the separate fractions are more active than the crude extract and the binary mixture of the most active fractions has shown higher significant antioxidant activity than crude extract, fractions separately and ternary mixture. Moreover, the response optimizer allowed us to optimize the mixture presenting the highest antioxidant activity at 82.54%. Data in this work may orient scientific assessment in both food and health applications and worth further *in vivo* studies and evaluation of the structure- activity relationship of emodin and kaempferol as pure active compounds.

Author contributions

Ikrame Zeouk carried out the experiment, wrote the manuscript with support from Ignacio A. JIMÉNEZ, Jacob L. MORALES and Isabel L. BAZZOCCHI to identify the active compounds.

Wessal Ouedrhiri performed and developed the mixture design analysis and the mixture matrice.

Khadija Bekhti designed and revised the study.

Declaration of Competing Interest

None.

Acknowledgment

We are grateful to Professor IBN TATTOU Mohammed and Professor EL OUALIDI Jalal director of the herbarium of the Scientific Institute in Rabat-Morocco (Mohammed V University-Rabat) for their valuable assistance and collaboration to identify the reported plant in this study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.112054.

References

Abou-chaar, C.I., Kabbara, R.A., Shamlian, S.N., 1982. A Chromatographic Study of the Anthraquinones of Rhamnus alatevnus L .111. Extraction, Isolation and Chroma tographic Characterization of the Anthraglycosides of the Stem Bark. Int J Crude Res 20, 13–18.

Ben Ammar, R., Miyamoto, T., Chekir-ghedira, L., 2018. Isolation and identification of new anthraquinones from Rhamnus alaternus L and evaluation of their free radical scavenging activity. Nat. Prod. Res. 6419, 1–7. https://doi.org/10.1080/14786419.2018.1446135.

Anagnostopoulou, M.A., Kefalas, P., Papageorgiou, V.P., Assimopoulou, A.N., Boskou, D., 2006. Radical scavenging activity of various extracts and fractions of sweet orange peel (Citrus sinensis). Food Chem. Food Chem. 94, 19–25. https://doi.org/10.1016/j.foodchem.2004.09.047.

Bag, A., Chattopadhyay, R.R., 2015. Evaluation of synergistic antibacterial and antioxidant efficacy of essential oils of spices and herbs in combination. PLoS One 10, 1–17. https://doi.org/10.1371/journal.pone.0131321.

Baj, T., Baryluk, A., Sieniawska, E., 2018. Industrial Crops & Products Application of mixture design for optimum antioxidant activity of mixtures of essential oils from Ocimum basilicum L., Origanum majorana L. and Rosmarinus o ffi cinalis L. Ind. Crop. Prod. 115, 52–61. https://doi.org/10.1016/j.indcrop.2018.02.006.

Ben Ammar, R., Bhouri, W., Ben, M., Boubaker, J., Skandrani, I., Neffati, A., Bouhlel, I., Kilani, S., Mariotte, A., Chekir-ghedira, L., Dijoux-franca, M., Ghedira, K., 2009. Antioxidant and free radical-scavenging properties of three flavonoids isolated from the leaves of Rhamnus alaternus L. (Rhamnaceae) : A structure-activity relationship study. Food Chem. 116, 258–264. https://doi.org/10.1016/j.foodchem.2009.02.043.

Ben Ammar, R., Neffati, A., Skandrani, I., Ben Sghaier, M., Bhouri, W., Ghedira, K., Chekir-Ghedira, L., 2011. Anti-lipid peroxidation and induction of apoptosis in the erythroleukaemic cell line K562 by extracts from (Tunisian) Rhamnus alaternus L. (Rhamnaceae). Nat. Prod. Res. 25, 1047–1058. https://doi.org/10.1080/14786419.2010.490783.

Bhouri, W., Boubaker, J., Kilani, S., 2012. Flavonoids from Rhamnus alaternus L. (Rhamnaceae): Kaempferol 3-O-β -isorhamninoside and rhamnocitrin 3-O-β isorhamninoside protect against DNA damage in human lymphoblastoid cell and enhance antioxidant activity. S. Afr. J. Bot. 80, 57–62. https://doi.org/10.1016/j.sajb.2012.02.005.

Bhouri, W., Sghaier, M.Ben, Kilani, S., Bouhlel, I., Dijoux-Franca, M.G., Ghedira, K., Ghedira, L.C., 2011. Evaluation of antioxidant and antigenotoxic activity of two flavonoids from Rhamnus alaternus L. (Rhamnaceae): kaempferol 3-O-β-isorhamninoside and rhamnocitrin 3-O-β-isorhamninoside. Food Chem. Toxicol. 49, 1167–1173. https://doi.org/10.1016/j.fct.2011.02.011.

Blois, M.S., 1958. Antioxidant determinations by the use of a stable free radical. Nature 181, 1199–1200. https://doi.org/10.2307/3280933.

Boussahel, S., Dahamna, S., Ruberto, G., Siracusa, L., Harzallah, D., 2013. Phytochemical study and antioxidant activities of leaves extracts from *Rhamnus alaternus* L. Pharmacogn. Commun. 3, 46–53. https://doi.org/10.5530/pc.2013.1.10.

Cavalcante Alencar, P.O., Lima, G.C., Barros, F.C.N., Costa, L.E.C., Ribeiro, C.V.P.E., Sousa, W.M., Sombra, V.G., Abreu, C.M.W.S., Abreu, E.S., Pontes, E.O.B., Oliveira, A.C., de Paula, R.C.M., Freitas, A.L.P., 2019. A novel antioxidant sulfated polysaccharide from the algae Gracilaria caudata: *in vitro* and in vivo activities. Food Hydrocoll. 90, 28–34. https://doi.org/10.1016/J.FOODHYD.2018.12.007.

Çelik, E.E., Rubio, J.M.A., Andersen, M.L., Gökmen, V., 2018. Interactions of coffee and bread crust melanoidins with hydroxycinnamic and hydroxybenzoic acids in aqueous radical environment. Food Res. Int. 108, 286–294. https://doi.org/10.1016/j.foodres.2018.03.057.

Chou, T., 2007. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. Pharmacol. Rev. 58, 621–681. https://doi.org/10.1124/pr.58.3.10.

Corporation, T., View, M., 1993. Antioxidant activity of Mediterranean plant leaves: occurrence and antioxidative importance of a-Tocopherol. JAOCS 70, 807–809.

Demmak, R.G., Bordage, S., Bensegueni, A., Boutaghane, N., Hennebelle, T., Mokrani, E.H., Sahpaz, S., 2019. Chemical constituents from Solenostemma argel and their cholinesterase inhibitory activity. Nat. Prod. Sci. 25, 115. https://doi.org/10.20307/nps.2019.25.2.115.

Embuscado, M.E., 2015. Spices and herbs: Natural sources of antioxidants – a mini review. J. Funct. Foods. https://doi.org/10.1016/j.jff.2015.03.005.

Fumio Nanjo, K., GOTO, I., SETO, R., SUZUKI, M., SAKAI, M., HARA, Y., 1996. Effects of tea catechins and their derivatives. Free Radic. Biol. Med. 21, 895–902.

Guo, S., Mao, W., Han, Y., Zhang, X., Yang, C., 2010. Bioresource Technology Structural characteristics and antioxidant activities of the extracellular polysaccharides produced by marine bacterium Edwardsiella tarda. Bioresour. Technol. 101, 4729–4732. https://doi.org/10.1016/j.biortech.2010.01.125.

Huang, S.-W., Frankel, E.N., 1997. Antioxidant activity of tea catechins in different lipid systems. J. Agric. Food Chem. 45, 3033–3038. https://doi.org/10.1021/jf9609744.

Kelly, F.J., 2010. Oxidative Stress : Its Role in Air Pollution and Adverse Health Effects POLLUTION STRESS : AND ITS ROLE ADVERSE IN AIR HEALTH EFFECTS. Occup. Environ. Med. 60, 612–616.

Kim, Y.M., Lee, C.H., Kim, H.G., Lee, H.S., 2004. Anthraquinones isolated from Cassia tora (Leguminosae) seed show an antifungal property against phytopathogenic fungi. J. Agric. Food Chem. 52, 6096–6100. https://doi.org/10.1021/jf049379p.

Larson, A.R., 1988. The antioxidants of higher plants. PhytoLhemwy 27, 2375–2395.

Li, H., Hao, Z., Wang, X., Huang, L., Li, J., 2009. Bioresource technology antioxidant activities of extracts and fractions from Lysimachia foenum-graecum Hance. Bioresour. Technol. 100, 970–974. https://doi.org/10.1016/j.biortech.2008.07.021.

Maia, E.C.R., Borsato, D., Moreira, I., Spacino, K.R., Rodrigues, P.R.P., Gallina, A.L., 2011. Study of the biodiesel B100 oxidative stability in mixture with antioxidants. Fuel Process. Technol. 92, 1750–1755. https://doi.org/10.1016/j.fuproc.2011.04.028.

Mari, S.H., Varras, P.C., Atia-Tul-, Wahab, Choudhary, I.M., Siskos, M.G., Gerothanassis, I.P., 2019. Solvent-dependent structures of natural products based on the combined use of DFT calculations and 1H-NMR chemical shifts. Molecules 24. https://doi.org/10.3390/molecules24122290.

Mishra, S.K., Tiwari, S., Shrivastava, A., Srivastava, S., Boudh, G.K., Chourasia, S.K., Chaturvedi, U., Mir, S.S., Saxena, A.K., Bhatia, G., Lakshmi, V., 2014. Antidyslipidemic effect and antioxidant activity of anthraquinone derivatives from Rheum emodi rhizomes in dyslipidemic rats. J. Nat. Med. 68, 363–371. https://doi.org/10.1007/s11418-013-0810-z.

Montoro, P., Braca, A., Pizza, C., Tommasi, N.De, 2005. Structure – antioxidant activity relationships of flavonoids isolated from different plant species. Food Chem. 92, 349–355. https://doi.org/10.1016/j.foodchem.2004.07.028.

Moussi, K., Nayak, B., Perkins, L.B., Dahmoune, F., 2015. HPLC-DAD profile of phenolic compounds and antioxidant activity of leaves extract of Rhamnus alaternus L. Ind. Crop. Prod. 74, 858–866. https://doi.org/10.1016/j.indcrop.2015.06.015.

Myers, R.H., Khuri, A., Carter, W.H., 1989. Response surface methodology: 1966-l988. Technometrics 31, 137–157. https://doi.org/10.1080/00401706.1989.10488509.
Ouedrhiri, W., Balouiri, M., Bouhdid, S., Moja, S., Chahdi, F.O., Taleb, M., Greche, H., 2016. Mixture design of Origanum compactum, Origanum majorana and Thymus serpyllum essential oils: optimization of their antibacterial effect. Ind. Crops Prod. 89, 1–9. https://doi.org/10.1016/j.indcrop.2016.04.049.
Ouedrhiri, W., Bouhdid, S., Balouiri, M., El, A., Lalami, O., Moja, S., Chahdi, F.O., Greche, H., 2015. Chemical composition of Citrus aurantium L. Leaves and zest essential oils, their antioxidant, antibacterial single and combined effects. Available online. J. Chem. Pharm. Res. 7, 78–84. www.jocpr.com.
Peyrat-Maillard, M.N., Cuvelier, M.E., Berset, C., 2003. Antioxidant Activity of Phenolic Compounds in 2, 2 ′ -Azobis (2-amidinopropane) Dihydrochloride (AAPH) -Induced Oxidation : Synergistic and Antagonistic Effects. JAOCS 80, 1007–1012. https://doi.org/10.1007/s11746-003-0812-z.
Poprac, P., Jomova, K., Simunkova, M., Kollar, V., Rhodes, C.J., Valko, M., 2017. Targeting free radicals in oxidative stress-related human diseases. Trends Pharmacol. Sci. 38, 592–607. https://doi.org/10.1016/j.tips.2017.04.005.
Rocha, M., Alemán, A., Baccan, G.C., Elvira, M., Gómez-guillén, C., Montero, P., Prentice, C., 2018. Anti-inflammatory, antioxidant and antimicrobial effects of underutilized fish protein hydrolysate. J. Aquat. Food Prod. Technol. 27, 592–608. https://doi.org/10.1080/10498850.2018.1461160.
Shahidi, F., Zhong, Y., 2015. Measurement of antioxidant activity. J. Funct. Foods 1–25. https://doi.org/10.1016/j.jff.2015.01.047.
Sies, H., 1997. Physiological society symposium: impaired endothlial and smooth muscle cell function in oxidative stress - oxidative stress: oxidants and antioxidants. Exp. Physiol. 82, 291–295. https://doi.org/10.1113/expphysiol.1997.sp004024.
Spector, A., 2000. Review: Oxidative stress and disease. Clin. Interv. Aging 16, 193–201.
Tsahar, E., Friedman, J., Izhaki, I., 2002. Impact on fruit removal and seed predation of a secondary metabolite, emodin, in Rhamnus alaternus fruit pulp. OIKOS 2, 290–299.
Wang, T., Jónsdóttir, R., Ólafsdóttir, G., 2009. Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. Food Chem. 116, 240–248. https://doi.org/10.1016/j.foodchem.2009.02.041.
Xue, Y., Balmuri, S.R., Patel, A., Sant, V., Sant, S., 2018. Synthesis, physico-chemical characterization, and antioxidant effect of PEGylated cerium oxide nanoparticles. Drug Deliv. Transl. Res. 8, 357–367. https://doi.org/10.1007/s13346-017-0396-1.
Zeouk, I., Abdelhakim, El, Lalami, O., Bekhti, K., 2019. *In vitro* antibacterial activity of medicinal plants in the central north of Morocco : a possible source of alternative drugs against methicillin-resistant *Staphylococcus aureus*. Asian J. Pharm. Clin. Res. 12, 285–292.
Zheng, W., Wang, S.Y., 2001. Antioxidant activity and phenolic compounds in 32 selected herbs. J. Agric. Food Chem. 49, 5165–5170. https://doi.org/10.1016/j.foodchem.2007.04.038.
Zhuang, X., Dong, X., Ma, S., Zhang, T., 2008. Selective on-line extraction of trans-resveratrol and emodin from Polygonum cuspidatum using molecularly imprinted polymer. J. Chromatogr. Sci. 46, 739–742. https://doi.org/10.1093/chromsci/46.8.739.