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Effect of DIC Technology on Phenolic Content and Antioxidant Activity of Rheum ribes L. rhizome Extracts

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***Abstract-Rheum ribes* L. (rhubarb) is one of the most important medicinal Mediterranean plants. Its rhizomes' aqueous decoctions have become common among patients suffering from various ailments. Instant Controlled Pressure-Drop (DIC) is a high temperature-short time treatment (HTST) followed by an abrupt pressure-drop (ΔP/Δt>500 kPa.s-1) towards a vacuum (about 5 kPa). It increases the expansion and reduces the tortuosity to ensure better diffusivity and higher availability of the active molecules. In this work, we assessed the impact of DIC treatment of *R. ribes* rhizomes on the phytochemical content and antioxidant activity of their aqueous extract. Rheum ribes rhizomes were collected, dried, ground, and sieved to be then treated with DIC technology. DIC treatment significantly impacted the total phenolic (TPC) and flavonoid (TFC) contents and scavenging capacity at low saturated steam pressures. Indeed, the highest TPC and TFC values and the greatest antioxidant activity were recorded at 260 kPa. However, there was no significant influence of the DIC treatment time on TPC and TFC. Future work will focus on optimizing the DIC processing parameters for the highest yield of phytochemicals in the aqueous extracts of *R. ribes* rhizomes.**

Keywords–DPPH·, DIC Technology, Instant Controlled Pressure-Drop DIC,Low Saturated Steam Pressures, High Temperature-Short Time Treatment (HTST)

Abbreviations–Détente Instantanée Contrôlée (DIC), Gallic acid equivalent (GAE), Quercetin equivalent (QE), Raw material (RM), db (dry basis).

INTRODUCTION

Over the past years, medicinal plants have been gaining increasing interest for their activity in the treatment of various human ailments[1]. This goes to natural polyphenols that are secondary metabolites exclusively synthesized by plants [2]. Studies have confirmed that these molecules are powerful antioxidant, antitumoral[3], and anti-inflammatory agents [4], which explains their role in the treatment of several chronic diseases associated with oxidative stress, such as cardiovascular diseases, cancers, and type II diabetes [3]. *Rheum* genus comprises a group of medicinally important species that are rich in phenolic compounds, mainly concentrated in their subterranean parts [5]. Indeed, much research has been conducted in the past decades on the antioxidant, antibacterial, cytotoxic, and many other biological properties of *Rheumribes* L. rhizomes. Several types of polyphenols were isolated from *Rheumribes* rhizomes extracts, such as flavonoids (quercetin, naringenin, and chrysin) [6], and anthraquinone derivatives (chrysophanol, physcion, rhein, and aloe-emodin) [7], [8]. Abdulla et al. (2014) have compared the phenolic profile of Iraqi *Rheumribes* L. rhizomes extracted in two different solvents. Results have shown that the phenolic content was higher in ethanol extract compared with aqueous extract. Consequently, the antioxidant activity of ethanol extract was higher than that of aqueous extract [9]. Another comparative study of the phenolic content in different parts of Turkish *Rheum ribes* plant, extracted in different solvent (water, ethanol, and ether), have confirmed that ethanol extract of rhizomes is the richest in phenolic compounds among almost all the other parts of the plant [10]. These studies

justify people's beliefs for ages in the therapeutic properties of *Rheum ribes* L. rhizomes decoctions. For many plants, the compact structure tends to resist solvent diffusion during extraction. Changes in the cellular structure are then necessary to improve the extraction process. In general, solid-liquid extraction is divided into two phases: a rapid extraction of free accessible solutes existing in open superficial cells followed by slow diffusion of solutes enclosed in the unruptured cells [11]. Instant Controlled Pressure-Drop (DIC) is a high temperature-short time treatment (HTST) followed by an abrupt pressure-drop ($\Delta P/\Delta t$>500 kPa.s-1) towards a vacuum (about 5 kPa). Studies have shown that DIC technology is capable of intensifying the last slow step by reducing mass transfer resistance and making tissues more porous [12]. In particular, numerous studies in the agro-industrial field have proven the effectiveness of DIC technology to increase active-molecule availability and the extractability of flavonoids [13], essential oils [14], [15] and polyphenols [16]. When applied to plants, it was notable that DIC treatment increases the availability of phenolic compounds in powdered grapes [17], pomegranate peels[11], green coffee beans[18] and many other plant matrices. Hence, the importance of studying the effect of DIC treatment on the availability of phenolic compounds extracted from *Rheum ribes* L. rhizomes, in the hope of being able to increase the yield of bioactive contents in aqueous decoctions as prepared traditionally. Therefore, the objective of this study was to investigate the effect of DIC treatment on the extractability of phenolic compounds from a Lebanese species of *Rheum ribes* L. rhizomes. The antioxidant activity of treated and control samples was also studied using DPPH assay.

MATERIALS AND METHODS

I. *Materials*

A) Plant material

Rheum ribes L. rhizomes were collected from the Chouf area, Mount Lebanon, Lebanon, growing at an altitude of 1140 meters. The rhizomes were thoroughly dry cleaned to remove soil debris that may carry contaminants then they were kept in a ventilated dark place until usage.

B) Chemicals

Folin Ciocalteu reagent, aluminum chloride, sodium carbonate, L-ascorbic acid, Gallic acid, and quercetin standards were all obtained from Sigma-Aldrich Co. (Schnelldorf, Germany). Analytical grade methanol and absolute ethanol were obtained from Carlo Erba Reagents (Val-de-Reuil, France). DPPH free radicals were obtained from Merck Group (Fontenay sous Bois, France).

II. Methods

A) DIC process

a) Experimental setup

Allaf et al. in 1992 have fully studied the fundamental analyses of instant thermodynamics, entirely defined the experimental setup of a DIC process, and largely described the adequate machines [19]. The latter is mainly composed of many elements as presented in figure 1: a processing vessel (1), where the ground rhizomes were installed to be treated, a vacuum system, which consists of a large volume vacuum tank (3) doubled by a cooling jacket, and a vacuum pump (4) to maintain a low pressure (5 kPa) in the vacuum tank, and a great section instant opening pneumatic valve (2) separating the processing vessel to the vacuum system. It is, indeed, the key element for the creation of an abrupt pressure-drop within the processing vessel in less than 0.2 sec.

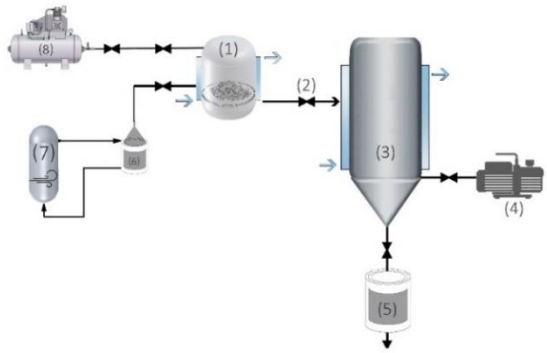

FIGURE **1**
SCHEMATIC DIAGRAM OF THE DIC REACTOR : (1) TREATMENT VESSEL; (2) PNEUMATIC VALVE; (3) VACUUM TANK; (4) VACUUM PUMP; (5) AIRLOCK; (6) CONDENSATE COLLECTION TRAP; (7) STEAM GENERATOR; (8) AIR COMPRESSOR

The different steps occurring in the processing vessel during a typical DIC treatment are presented in figure. 2 [20]. Firstly, dried rhizomes(about 35 g) are placed in the processing vessel (stage A).The initial pressure drop (stage B) is used to create the vacuum, therefore, to take out the air from the processing vessel, which acts as an insulator between the saturated steam and rhizomes to be treated. Phase C is characterized by the injection of saturated steam at high pressure. This step involves vapor condensation on the cold surface of raw material. At this level, the treated particles become subject to mass (condensed water) and heat transfer. Saturated steam injection is maintained for a period (Stage D) to ensure a relatively uniform level of both temperature and humidity within the treated matrix. The high-pressure/high-temperature plateau is then followed by an abrupt pressure-drop towards vacuum (5 kPa) (stage E), which triggers the auto-vaporization of water. Consequently, treated rhizomes are rapidly cooled which protects the quality of thermosensitive active molecules. Also, rhizomes gain a new porous structure that increases their specific surface area, thus improving solvent extraction.

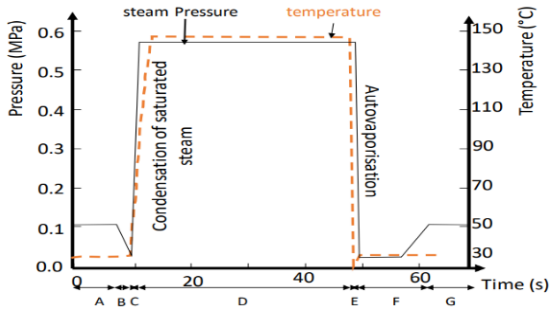

FIGURE 2
DIFFERENT THERMAL AND DYNAMIC PHASES OF A DIC TREATMENT

b) Experimental design

The methodology of n-variable 5-level central composite rotatable design of experiment (DoE) allowed studying the effect of DIC operating parameters (saturated steam pressure P and processing time t) and identifying their impacts on the different measured dependent variables. The design of numerous experiments in the field of process engineering was based on response surface methodology (RSM) [18]. As a preliminary trial, a pressure ranging from 300 to 700 kPa for a processing time varying from 20 to 80 seconds was applied to rhizomes cut into 5 mm thick slices. This first DoE that was applied to sliced rhizomes didn't show statistically significant results, and this goes to the heterogeneity in plant material that has dim the variability in response variables resulting from the DIC treatment. Despite all, this trial still helped to choose correctly the new independent variables intervals. In the new design of experiments DoE detailed in this article, the saturated steam pressure used ranged from 200 to 600 kPa for a processing time of 10 to 40 seconds (table 1) applied to ground rhizomes. Since the treatment temperatures are related to saturated steam pressures according to Antoine's equation for pure water (1)[21], we can determine at what temperatures we have treated our rhizomes in the function of the saturated steam pressure injected. Saturated steam pressures ranging from 200 to 600 kPa correspond to temperatures varying from 120 to 160 °C.

$$P_v = 10^{10.09938 - \frac{1681}{(-43.037+T)}} \qquad (1)$$

where P_v is the saturated steam pressure in Pa and T is the temperature expressed in K.

As listed in table 2, there were four factorial-points (trials n° 5, 6, 8, and 9), four star-points (trials n° 2, 3, 11, and 12), and 5 replicates of the central-point treated at the same experimental conditions (trials n° 1, 4, 7, 10, and 13).

TABLE I
LEVELS OF INDEPENDENT VARIABLES ORGANIZED IN A 2-PARAMETER, 5-LEVEL EXPERIMENTAL DESIGN

Coded level	- α	-1	0	+1	+ α
Saturated steam pressure (kPa)	200	260	400	540	600
Processing time (s)	10	14	25	36	40

α (axial distance) = $\sqrt[4]{2^K}$, in our case k = 2 and α = 1.414

B) Extract preparation

Rhizomes were first ground by Grindomix, a Retch mechanical grinder (Haan, Germany), and sieved to retain and subsequently process particles between 0.4 and 2 mm in size. Approximately 10 g of DIC-treated and control samples (raw material, RM) underwent a 24-hour maceration in 100 ml of distilled water with constant magnetic stirring at room temperature, a vacuum filtration through Whatman No. 1 filter papers, and freezing at -18°C for 48 hours. Finally, freeze-drying took place at -55 °C under 7.9 Pa using a Martin Christ- Alpha 1-2 LD plus freeze drier (Harz, Germany). The freeze-dried extracts were stored in dark glass containers at room temperature until usage.

C) Total phenolic content determination

Measurement of the total phenolic content of the DIC-treated aqueous extracts and the control involved the use of Folin Ciocalteu reagent as described by Wong and his colleagueswith slight modifications [22]. This included preparing a mixture of 100 µl of extract solution (2 mg/ml) and 500 µl of Folin Ciocalteu reagent (10%), vortexing and then incubating in the dark at room temperature for 5 min before adding a volume of 2 ml Na2CO3 (7%), vortexing a second time, and then incubating the new mixture for 30 min in the dark. The absorbance of the mixture was then measured using a Thermo Scientific-Helios Omega UV-VIS spectrophotometer (Massachusetts, USA) at 750 nm against a blank. The concentration of phenolic compounds calculated in milligrams of gallic acid equivalent (GAE) per gram of extract uses a standard gallic acid curve with a calibration (range of gallic acid concentrations 3.125-200 µg/ml; Curve equation: Absorbance = 0.0037 ± 0.0024 (µg) [gallic acid]; R^2= 0.9993). All determinations were performed in triplicate.

D) Total flavonoid content determination

Measurement of flavonoids content of DIC-treated and control aqueous extracts was based on the aluminum chloride colorimetric method as described by Chandra et al., (2014), using quercetin to plot the standard calibration curve [23]. A volume of 0.6 ml of diluted extracts (0.4 mg/ml) was mixed with 0.6 ml of methanolic aluminum chloride (2%), then the mixture was incubated for 60 min in the dark at room temperature. Finally, the absorbance of the mixture was measured using a UV-Vis spectrophotometer at 420 nm against a blank. The assessment of the concentration of flavonoids calculated in milligrams of quercetin equivalent (QE) per gram of extract implied a plotted standard curve of quercetin (range of quercetin concentrations 1-40 µg/ml; curve equation: Absorbance = 0.0194 + 0.0238 (µg) [quercetin]; R^2 = 0.9978). All determinations were carried out in triplicate.

E) Free radicals scavenging activity

Assessment of the antioxidant activity of the aqueous DIC-treated and control extracts was performed using DDPH as described by Rammal et al. (2013). Equal volumes of a DPPH solution dissolved in absolute ethanol (0.15 mM) and extract dilutions from 1000 to 100 µg/ml were mixed. The mixture was shaken vigorously and left to stand in the dark at room temperature. After 30 min, the absorbance of the solution was measured at 517 nm, and antioxidant activity was calculated using the following equation:

$$\%\, Scavenging\ capacity = \left(\frac{Abs_{control} - Abs_{extract}}{Abs_{control}}\right) * 100$$

Absolute ethanol and distilled water were used as blanks, while a mixture of DPPH solution and distilled water (1:1) v/vwas used as a negative control. To evaluate the antioxidant capacity of the extracted rhizomes, the concentration providing 50% of the total inhibition percentage for each extract was calculated (IC50). Ascorbic acid was used as a standard.

F) Statistical analysis

Response variables were total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity (IC50). Their values were statistically treated through Statgraphics Centurion XV (version 15.2.06). Consequently, a second-order polynomial mathematical model of the independent variables can express the response variable as defined in (2).

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{ii} x_i^2 + \beta_{ij} x_i x_j + \varepsilon \qquad (2)$$

Yrepresents the response variable, β_i, β_{ii}andβ_{ij} are the regression coefficients,$x_i$andx_j are the independent variables,ε represents the random error, i and j are the factors indices.

RESULTS AND DISCUSSIONS

I. Total phenolic content

Table 2 lists the results of the Folin-Ciocalteu method for phenolic determination in DIC-treated and control (RM) extracts. The highest TPC values are recorded for samples 8 (91.6±1.7 mg GAE/ g extract), 11 (92.1±0.3 mg GAE/g extract), and 9 (94.0±0.9 mg GAE/g extract). These samples were treated at 260, 200, and 260 kPa for 14, 25, and 36 sec, respectively. Meanwhile, the lowest TPC values are attributed to samples 5 (69.4±0.2 mg GAE/g extract), 2 (72.1±1.9 mg GAE/g extract), 3 (77.7±0.9 mg GAE/g extract), and 6 (79.6±0.8 mg GAE/g extract) treated at 540, 600, 400, and 540 kPa for 36, 25, 40, and 14 seconds, respectively. These results reveal that the higher the temperature, which is correlated to the saturated steam pressure, the lower the phenolic compounds.

Hence, it is worth noting that only the samples 8, 11, and 9 of DIC treated rhizomes had a TPC content higher than the control sample (89.3±1.5 mg GAE/g). Two mechanisms may explain the loss of phenolic compounds especially for samples treated at high saturated steam pressures. Either the autovaporization contributes to expulse the polyphenols into the vacuum tank, or they underwent a thermal degradation due to their exposure to high saturated steam pressure. To test the first hypothesis, the condensed water resulting from the DIC treatment was collected from the vacuum tank to determine its phenolic content. The average TPC value was about 0.06 mg GAE/g db (dry basis), which is negligible compared with the phenolic content of raw-material and DIC-treated extracts. Therefore, the most probable hypothesis is that the high DIC pressure causes a thermal degradation of the phenolic compounds. A similar conclusion was reached by [25] who have also reported a decrease in TPC value in "Henna" leaves samples treated at high temperatures probably because of thermal degradation. Previous studies have shown that polyphenols in red onions are severely damaged at high temperatures [26]. Moreover, [27] adopted a DoE to optimize the most adequate condition for the extraction of polyphenols in red cabbage. Their results confirmed that temperatures above 120°C have been severely destructive to polyphenols. According to these findings, in addition to Antoine's equation for pure water (1), we can determine the reason why *Rheum ribes* L. rhizomes phenolic contents were conserved at low saturated steam pressures (i.e., low temperatures) and degraded at higher saturated steam pressures (i.e., high temperatures).

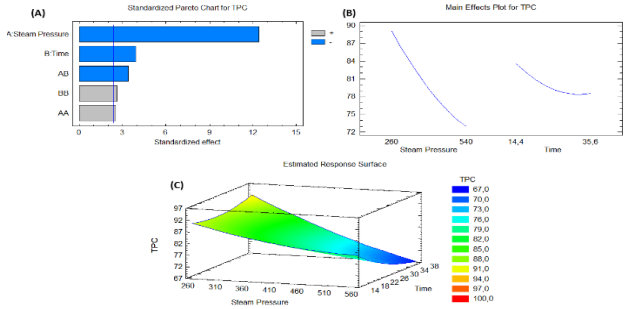

FIGURE 3
PARETO CHART (A), MAIN EFFECT PLOT (B), AND RESPONSE SURFACE (C) FOR TOTAL PHENOLIC CONTENT TPC

Results of TPC (table 2) allowed using the response surface methodology (RSM) and expressing the results through Pareto chart, main trends, and surface values depending on the operating parameters of DIC as independent variables (figure 3). Pareto chart expresses ANOVA for DIC operating parameters P and t and their combinations on TPC values of aqueous extracts and shows them from the most to the least significant effects. Bars that exceed the vertical line that indicates a defined level of the p-value (here equal to 0.05) are considered statistically significant. Pareto chart (figure 3.A) shows that the variation of the two DIC parameters P and t has a significant effect on TPC values. However, the variation of saturated steam pressure was the most significant, while the main effect plot (figure 3.B) reveals negative correlations

between the phenolic contents and both saturated steam pressure and processing time.

The polynomial equation (3) estimated from RSM indicates with R^2= 96.49% the high goodness-of-fit of the model of TPC. The overall results revealed that DIC treatment of *R. ribes* L. rhizomes at low saturated steam pressure allows an increase in phenolic contents' extractability.

$$TPC = 112.054 - 0.0773P - 0.227t + 0.000089P^2 - 0.002Pt + 0.0162t^2 \quad (3)$$

Equation (3)also confirmed that DIC conditions of saturated steam pressure of P=200 kPa and thermal treatment time of t=40 s, relate to the optimum value of TPC at 100.5 mg GAE/g db extract, which is about 12.63% higher than the raw material.

II. Total Flavonoid content

Results of aluminum chloride colorimetric method for the determination of flavonoids in DIC-treated and control aqueous extracts are listed in table 2. The three highest TFC values were recorded for the same samples that showed the highest TPC values i.e., samples 8 (92.0±0.1 mg QE/ g extract), 11 (99.4±1.2 mg QE/g extract), and 9 (101.1±0.7 mg QE/g extract). These samples were treated at 260, 200, and 260 kPa for 14, 25, and 36 sec, respectively. Meanwhile, the lowest TFC value is attributed to Sample 2 (63.6±1.5 mg QE/g extract) treated at 600 kPa for 25 sec, which also matches low TPC value. TFC value of the raw material (80.7±2.4 mg QE/g) was lower than all the other TFC values of DIC treated rhizomes except for sample 2 which was treated with the highest saturated steam pressure.

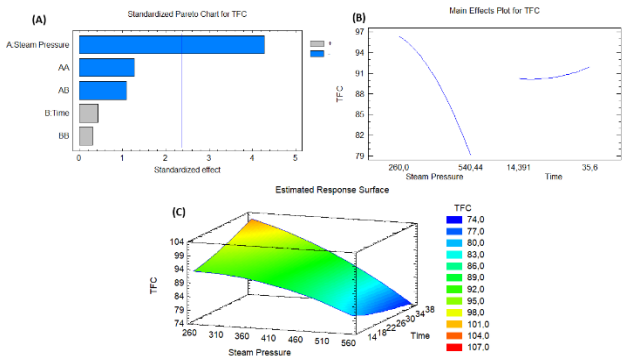

FIGURE 3
PARETO CHART (A), MAIN EFFECT PLOT (B), AND RESPONSE SURFACE (C) FOR TOTAL FLAVONOID CONTENT TFC

Pareto chart (figure 4.A) shows that the saturated steam pressure variation is the only operative DIC parameter to have a significant effect on TFC values, while the main effect plot (figure 4.B) reveals that there is a negative correlation. The response surface presentation (figure 4.C) reveals the polynomial equation (4) estimated from RSM with R^2= 75.45%, which indicates the goodness-of-fit of TFC model versus P and t.

$$TFC = 74.321 + 0.0992P + 0.60441t - 0.00013P^2 - 0.002Pt + 0.00574t^2 \quad (4)$$

Moreover, the equation above allows the optimization of DIC treatment conditions. At saturated steam pressure of P=200 kPa and thermal treatment time of t=40 s, the optimum value of TFC was 105.8 mg QE/g extract, which is about 31% higher than the raw material. The overall results revealed that DIC treatment of *R. ribes* rhizomes at low saturated steam pressure allows an increase in the flavonoid extractability.

III. Antioxidant activity

The values of antioxidant activity against DPPH free radicals of DIC treated and control rhizome samples are shown in table 2. The highest antioxidant activities are those of samples 9 (54.6±0.2 µg/ml), 11 (57.3±0.1 µg/ml), and 8 (64.6±0.4 µg/ml) that previously recorded the highest TPC and TFC values. The highest IC50 of all extracts was recorded for sample 2 (90.0±0.9 µg/ml) that was treated with the highest saturated steam pressure (600 kPa).

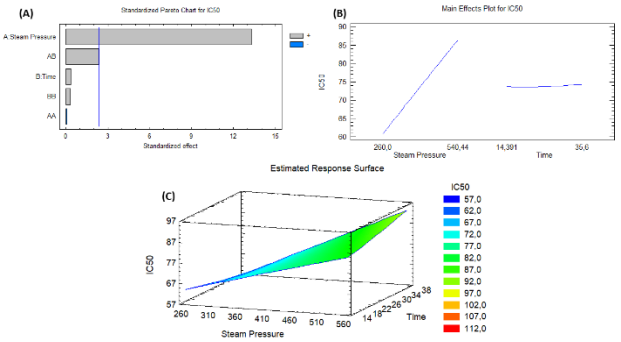

FIGURE 3
PARETO CHART (A), MAIN EFFECT PLOT (B), AND RESPONSE SURFACE (C) FOR IC50

Pareto chart (figure 5.A) shows that the saturated steam pressure variation is the only one to have a significant effect on IC50 values. In addition, the main effect plot reveals that the IC50 of treated rhizomes is positively correlated with the saturated steam pressure. The polynomial equation estimated from RSM is shown in (5) with R^2= 96.3% which indicates that the fitted model explains well the variability of IC50.

$$IC50 = 58.64 + 0.04215P - 0.96328t - 0.000005P^2 + 0.00213Pt + 0.002895t^2 \quad (5)$$

The response surface methodology (figure 5.C) results confirmed that DIC conditions of saturated steam pressure of P=200 kPa, and thermal treatment time of t=40 s, relate to the optimum value of IC50 at 49.9 µg/ml, which is about 29.9% lower than the IC50 of the raw material. The overall results (figure 5.B) revealed that DIC treatment of *R. ribes* rhizomes at low saturated steam pressure increases the antioxidant activity of aqueous extracts, without any

significant effect of the treatment time.This result ties well with the previous findings where sample 2 recorded the least concentration in TPC and TFC knowing that the antioxidant activity in *R. ribes* rhizomes is due to the presence of various secondary metabolites such as polyphenols [9]. A simple regression of TPC vs IC50 (Figure 6. A) gives a correlation coefficient of -0.919, which implies a relatively strong and negative relationship between the two dependent variables. Authors have already shown that a positive correlation exists between total phenolic content and antioxidant capacity [5]. Another simple regression of TFC vs IC50 (Figure 6 B) reveals a correlation coefficient of -0.778 which implies a moderately strong and negative relationship between the two variables. From these results, it is worth concluding that TPC is the main factor for the determination of the antioxidant activity of an extract, while TFC is a secondary factor which implies that the major antioxidant polyphenols are not from the flavonoid type.

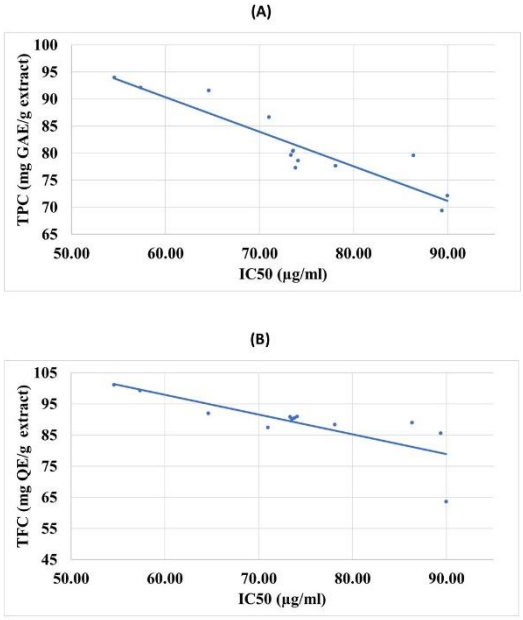

FIGURE 6
SIMPLE REGRESSIONS OF TPC VS IC50 (A) AND TFC VS IC50 (B)

Although the aqueous extraction of *Rheum ribes* L. rhizomes is not frequent in the literature, we sought in our study, to preserve the traditional concept of macerating the Lebanese species of *Rheum ribes* L. rhizomes in water. Others have already extracted rhizomes in many organic solvents such as ethanol [28], methanol [29], and chloroform [5]. Abdulla et al in 2014 have compared the antioxidant activity of aqueous and alcoholic extract of Iraqi *Rheum ribes* L. rhizomes. IC50 were respectively 25.62 and 4.73 µg /ml. When comparing our results to those obtained by Abdulla et al., (2014), it is interesting to point out that the Iraqi *Rheum ribes* L. has a higher antioxidant activity than the Lebanese one. This can be explained by the climatic difference between the two countries, as it was found that when plants are under hydric and thermal stresses, they increasingly produce secondary metabolites (i.e. phenols) [30]

CONCLUSION

In conclusion, results obtained from the present work have revealed that DIC technology has an impact on the extractability of active molecules from *Rheum ribes* L. rhizomes. Based on the present experimental design, it was possible to optimize the DIC parameters of saturated steam absolute pressure P= 200 kPa and thermal treatment time t= 40 sec for all the dependent variables. Indeed, among the three tested methods, the highest TPC, TFC, and antioxidant activities were attributed to samples treated at low values of pressure and temperature. This would be related to the thermal sensitivity of the bioactive molecules present in *Rheum ribes* L. rhizomes. A mild treatment in terms of saturated steam pressure seems, therefore, more suitable inthe present case. Therefore, these findings will be later tested by carrying out even lower saturated steam pressure values.

TABLE II
EXPERIMENTAL CONDITIONS OF DIFFERENT DOE TRIALS WITH THE TOTAL PHENOLIC CONTENTS (TPC), TOTAL FLAVONOID CONTENTS (TFC), AND IC50 OF DIC TREATED AND CONTROL (RM) EXTRACTS

Trial n°	Pressure (kPa)	Time (s)	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	IC50 (µg/ml)
Central points (1; 4; 7; 10; 13)	400	25	79.3±1.3	90.5±0.4	73.7±0.3
2	600	25	72.2±1.9	63.6±1.5	90.0±0.9
3	400	40	77.7±0.9	88.4±0.5	78.1±0.3
5	540	36	69.4±0.2	85.6±1.4	89.4±0.4
6	540	14	79.6±0.8	89.0±1.0	86.3±0.2
8	260	14	**91.6±1.7**	**92.0±0.1**	**64.6±0.4**
9	260	36	**94.0±0.9**	**101.1±0.7**	**54.6±0.2**
11	200	25	**92.1±0.3**	**99.4±1.2**	**57.3±0.1**
12	400	10	86.7±0.9	87.5±1.5	71.0±0.4
RM	-	-	89.3±1.5	80.7±2.4	71.3±0.5

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