



# Extraction of phenolic antioxidants from *Pyrus elaeagrifolia* Pallas: process optimization, investigation of the bioactivity and $\beta$ -glucuronidase inhibitory potential

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## Abstract

This study aimed to develop a mathematical model for the extraction of *Pyrus elaeagrifolia* Pallas leaves using the Box–Behnken design, to investigate bioactivity and bacterial  $\beta$ -glucuronidase (GUS) inhibitory potential of the extracts obtained under optimum conditions. Results showed that the mathematical models using extraction temperature, time and ethanol concentration as effective parameters fit the real data well. The optimum extraction conditions to maximize phenolic content and antioxidant activity were found 79.7 and 78.4% ethanol, 74.9 °C, and 45.0 and 35.9 min, respectively. The chlorogenic acid was detected as the major phenolic compound in the chromatographic analysis. The highest antibacterial activity achieved against *Staphylococcus aureus*. The extracts (150  $\mu\text{g}/\text{mL}$ ) showed 67.9%, and 75.2% cytotoxic effect on the MCF-7 and A549 cells, respectively, and displayed inhibitory potential against GUS. The antioxidant property together bioactivity makes *Pyrus elaeagrifolia* Pallas leaves potent source for functional food/food ingredient applications and nutraceutical development.

**Keywords** *Pyrus elaeagrifolia* · Optimization ·  $\beta$ -Glucuronidase · Box–Behnken · Bioactivity

## Introduction

*Pyrus elaeagrifolia* Pallas, of family Rosaceae and genus *Pyrus* L., is a species of pear native to Anatolia, Ukraine and southeast Europe [1]. *Pyrus* species are good source of vitamin C and dietary fiber. Their fruits and leaves are consumed either fresh or dried, and they have also been traditionally used for medical purposes due to their antitussive, anti-inflammatory, dissipating phlegm and diuretic anticonstipative activities [2, 3]. In literature, it was reported that the *Pyrus* leaves have much higher and more varied phenolic contents than the flesh of the fruit [4]. *Pyrus* leaves are rich in phenolics like arbutin, isoquercitrin, sorbitol, and tannin [5]. They are also used for the treatment of inflammation of the

bladder, bacteriuria, high blood pressure and urinary stones; as well as, they have diuretic, hypoglycemic, hypolipemic property and cytotoxic activity [6, 7].

In recent years, plant extracts are gaining a wide interest in the food industry as they are potential antimicrobials and antioxidants [8, 9]. They are also Generally Recognized as Safe (GRAS) to be used in foodstuffs or to be incorporated into the packaging material thus they promote the inhibition of the undesirable quality losses and growth of undesirable microorganisms [10–12]. In this context, bioactive compounds derived from plants have been shown to possess antimicrobial activities against various bacteria including foodborne pathogens [13–15]. Several plant extracts inhibited the growth of, or killed *Salmonella* spp., *E. coli* O157:H7 and *L. monocytogenes* [16]. Therefore they can be important tools for prevention of the bacterial food born diseases such as Salmonellosis, bloody diarrhea called hemorrhagic colitis [17], and Listeriosis. Plant extracts having bioactivity are also used for the inhibition of enzymes related to health and diseases.  $\beta$ -Glucuronidase (GUS) is an enzyme found in some microorganisms, mammalian and plant tissues. This enzyme is responsible for drug metabolite detoxification and to produce reactive metabolites which are related to some diseases and cancer development [18, 19]. The phenolic

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GUS inhibitors are also suggested as potential hepatoprotective agents and various natural dietetic compounds were investigated for the inhibition of GUS activity [20–22].

The response surface methodology (RSM) approach is a collection of statistical and mathematical methods for modeling and analysis [23–26]. It is used to optimize processes related to food systems, including the extraction of phenolic compounds [27, 28] where their bioactivity is highly affected by the extraction conditions [29, 30] and it is very efficient in terms of time, material and cost [31]. Therefore, choosing the appropriate extraction conditions such as; solvent, temperature, time, stirring speed, and ratio of solid and liquid phases is of great importance for achieving the desired outcomes of extraction and to preserve the bioactivity of the *Pyrus* species. In literature, there are limited numbers of studies about *P. elaeagnifolia*. To the best of the authors' knowledge, there is no research about the extraction optimization for the *P. elaeagnifolia* leaves. Since the health demands of the consumers have boosted the market of plant-based nutraceuticals and food supplements, evaluation of the phenolic composition and antioxidant activity, as well as assessment the importance of this plant species as a potential source of bioactive compounds by presenting the bioactivity studies in terms of antioxidant, antimicrobial, cytotoxic property and GUS inhibitory potential, are considerably important. Therefore, with this study, cost saving ways of providing bioactive compounds of *P. elaeagnifolia* leaves with relevant experimental set-up may provide opportunities to produce high value-added products. In this context, the aim of this study was to optimize the extraction conditions of *P. elaeagnifolia* leaves by applying the Box–Behnken design and to investigate the bioactivity and GUS inhibitory potential of the extracts obtained under optimum conditions.

## Materials and methods

### Materials

*Pyrus* leaves used in this study were harvested in the second half of August, 2015 from natural habitats (Afyonkarahisar, Turkey). The leaves samples of *Pyrus elaeagnifolia* Pallas ssp. *elaeagnifolia* grown in Afyonkarahisar - Sultandağ region were identified by Prof. Dr. Mustafa Kargıoğlu and Prof. Dr. Ahmet Serteser from Afyon Kocatepe University, Faculty of Art and Science, Botany Department where a voucher sample was deposited in the herbarium of the laboratory. p-nitro phenyl β-D-glucuronide, *Escherichia coli* β-glucuronidase (EC.3.2.1.31), 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), Folin–Ciocalteu reagent, and NaHCO<sub>3</sub> were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Na<sub>2</sub>CO<sub>3</sub>, and nutrient agar was obtained from Merck (Germany).

Standard sterile discs (8 mm) obtained from Oxoid (Basinstoke, UK). HPLC Standards were purchased from Sigma (St. Louis, MO, USA). All solvents and reagents were analytical or HPLC grade.

### Box–Behnken design

Box–Behnken design using Response surface methodology (RSM) approach was applied to determine the effect of extraction parameters (independent variables) on the responses (R). Data for the design matrix in Box–Behnken design can be found in supplementary material (ESM1). Experiments were performed using shade-dried, ground and sieved leaves by batch solvent extraction which was carried out in a water bath (Memmert, Germany) using conical flask covered with aluminum foil. The total volume for the extraction media was 150 mL to analyze the effect of the three selected extraction parameters. The parameters as independent variables used for Box–Behnken design were: solvent concentration ( $X_1$ ), extraction temperature ( $X_2$ ), and extraction time ( $X_3$ ). The other parameters; particle size, stirring speed and liquid-to-solid ratio were fixed at 106–150 μm, 100 rpm and 10:1 v/w, respectively. The responses in RSM design were total phenolic content (TP), and antioxidant activity (AA%), and they were represented as R1 and R2, respectively. The experimental data fitted the quadratic polynomial model:

$$R = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \sum \beta_{ij} X_i X_j + \epsilon \quad (1)$$

where R is the process response (Eq. 1).  $X_i$  and  $X_j$  are the independent variables.  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$ , and  $\beta_{ij}$  are the constant coefficients of intercept, linear, quadratic, and interaction terms, respectively [20].  $\epsilon$  is a random error which allows describing uncertainties between predicted and experimental values. Analysis was performed using Design-Expert® software ver. 7 (Stat Ease, USA).

### Determination of total phenolic content (TP)

The TP analysis was performed using the Folin–Ciocalteu method [32]. The results of TP were expressed as gallic acid equivalents (GAE) in mg/g of the sample. The samples obtained from each separate trial (n = 3), were analyzed in triplicate.

### Antioxidant activity assay

The antioxidant activity of the samples were measured using DPPH free radical scavenging activity assay as given previously in details [30]. The percent of radical scavenging activity (AA%) of the extracts was calculated using the following equation:

$$AA(\%) = \frac{A_c - A_s}{A_c} \times 100 \quad (2)$$

where  $A_c$  is the absorbance of the control sample and  $A_s$  is the absorbance of reagents and plant extract mixture. The samples obtained from each separate trial ( $n=3$ ), were analyzed in triplicate.

### HPLC analysis

Phenolic composition of the leaf samples were analyzed by HPLC system (Shimadzu Corp., Kyoto, Japan). Analysis was performed according to previously described method [33] with some modifications. DAD detector ( $\lambda_{\text{max}}$ ; 280 nm) was used for the analysis. 250 × 4.6 mm i.d., 5  $\mu\text{m}$  column used was filled with Agilent Eclipse XDB-C18 (Wallborn, Germany). The mobile phase used was A:3% acetic acid, B: Methanol. The flow rate of the system was 0.8 ml  $\text{min}^{-1}$  with an injection volume of 20  $\mu\text{l}$ . The temperature was set at 30 °C. The data were analyzed using the Shimadzu Class-VP Chromatography Laboratory Automated Software system. The standards were acquired from Sigma (St. Louis, MO, USA).

### Antibacterial tests

Extracts obtained under optimum extraction conditions were analyzed for their antimicrobial activity using the Kirby-Bauer disc diffusion assay [34]. *Bacillus cereus* (ATCC7064), *Staphylococcus aureus* (ATCC29213), *Listeria monocytogenes* (ATCC 7644), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC25922) *Pseudomonas aeruginosa* (ATCC27853) were tested for their susceptibility this study. Bacterial strains grown on nutrient agar at 37 °C for 18 h were suspended in 5 mL saline solution (0.85% NaCl) and adjusted to a turbidity of 0.5 McFarland standard. Experiments were performed with daily prepared subcultures which are standardized for inoculation on agar surface corresponding to certain numbers of cfu/ ml. Log phases of growth curves were taken into account to reach approximate inoculation numbers also the standardized inoculums were confirmed by measuring OD values. 100  $\mu\text{l}$  of bacteria culture during the lag phase inoculated onto agar surface. Nutrient agar plates were allowed to air-dry for approximately 10 min before 8 mm sterile discs were impregnated with 20  $\mu\text{L}$  of the plant extract (syringe filtered using a 45  $\mu\text{m}$  membrane) placed on petri dishes. Dishes were incubated at 37 °C for 24 h as previously described [30]. After incubation at 37 °C for 24 h, the antibacterial activity was evaluated by measuring the inhibition zone diameter in mm. Blank discs impregnated with ampicillin (10  $\mu\text{g}/\text{disc}$ ) and gentamicin (15  $\mu\text{g}/\text{disc}$ ) were used as positive controls. Results were

expressed as the inhibition zone diameter in mm. The culture samples obtained from each separate trial ( $n=3$ ), were analyzed in triplicate.

### $\beta$ -Glucuronidase inhibition assay

$\beta$ -Glucuronidase inhibition assay was carried out as previously described [35] with slight modifications. A total volume of 2.5 ml reaction mixture was used which contained 10 mM phosphate buffer (pH 7.0), 0.4 mM p-nitro phenyl  $\beta$ -D-glucuronide, and 2.0 Fishman unit/ml *Escherichia coli*  $\beta$ -glucuronidase. One Fishman unit liberates 1.0  $\mu\text{g}$  of phenolphthalein from phenolphthalein glucuronide for per hour at 37 °C and at pH 6.8. Reaction was carried out for 7 min. The reaction was terminated by adding 0.25  $\text{Na}_2\text{CO}_3$  (1.5 ml), and the absorbance was measured at 405 nm. Controls were devoid of test samples. The percent inhibition was calculated as follows:

$$\text{Inhibition}(\%) = \frac{A_c - A_s}{A_c} \times 100 \quad (3)$$

where  $A_c$  is the absorbance of the control sample and  $A_s$  is the absorbance of reagents and plant extract mixture. The samples obtained from each separate trial ( $n=3$ ), were analyzed in triplicate.

### Cell culture studies and cytotoxicity tests

Extracts obtained under optimum extraction conditions were analyzed for their cytotoxic activity against A549 (lung adenocarcinoma cell line) and MCF-7 (breast adenocarcinoma cell line) in the Plant, Drug and Scientific Research Center (Eskisehir, Turkey). RPMI supplemented with 10% FBS and 1–2% penicillin–streptomycin was used as culture media. The incubations were performed using  $\text{CO}_2$  incubator (CB 150 E3, BINDER GmbH, Germany) by maintaining cultures at 37 °C in a 95% humidified atmosphere with 5%  $\text{CO}_2$ . Both cell lines were treated with various concentrations (5–150  $\mu\text{g}/\text{mL}$ ) of the plant extract and cell proliferation inhibition was determined by MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay using a cell counter (CEDEX-XS Analyzer, Roche GmbH, Germany) after 48 h incubation. The culture samples obtained from each separate trial ( $n=3$ ), were analyzed in triplicate.

## Results and discussion

### Fitting the models

Phytochemicals in many plants have received much attention in recent years due to their health benefits. But the

stability of some bioactive compounds in the plant material can be affected during preparation or extraction processes. In this context, the RSM can identify the important experimental parameters, and optimizes simultaneously their levels in order to achieve the optimal response with the minimum necessary number of experiments. Before the RSM experiment, single factor approach was first applied to determine the required levels in RSM design [29]. Consequently, the appropriate range of solvent concentration, extraction temperature, and time were predetermined to be used in RSM design. The two other important parameters in aqueous mass transfer systems; particle size and liquid-to-solid ratio [36], were fixed during extraction at 106–150  $\mu\text{m}$  and 30:1 v/w, respectively. According to the design matrix extractions were performed. The results of antioxidant activity and total phenolic content of the extracts were revealed in design matrix (ESM1). Data in ESM1 showed that increase in TP resulted an increase in AA% indicating a correlation between them as previously suggested by other workers [37, 38]. In the 1990s, the phenolic compounds were classified as general antioxidants [39], and this was considered as a panacea to explain their global mechanism of action. In this study, 66% of antioxidant activity was achieved for the *Pyrus* extract in run#4. Such antioxidant actions suggested that *Pyrus* extract may be important in the prevention of carcinogenesis where antioxidant activity may enhance cellular defense and help to prevent oxidation damage to cellular components.

In RSM, a three-factor, three-level Box–Behnken design model was used. The results of modeling were expressed by the following quadratic polynomial equations (Eqs. 4, 5). To represent the exact numeric values, equations are given in terms of actual factors.

$$\begin{aligned}
 R1 = & 0.7580 + 0.3531(X_1) + 0.2366(X_2) + 0.2694(X_3) \\
 & + 0.0008(X_1)(X_2) - 0.0010(X_1)(X_3) \\
 & - 0.0006(X_2)(X_3) - 0.0022(X_1^2) - 0.0010(X_2^2) \\
 & - 0.0006(X_3^2) \quad (4)
 \end{aligned}$$

$$\begin{aligned}
 R2 = & -9.1397 + 0.8558(X_1) + 0.5929(X_2) + 0.5537(X_3) \\
 & - 0.0003(X_1)(X_2) - 0.0031(X_1)(X_3) \\
 & - 0.0015(X_2)(X_3) - 0.0041(X_1^2) - 0.0022(X_2^2) \\
 & - 0.0026(X_3^2) \quad (5)
 \end{aligned}$$

Results showed that the most significant effect ( $p < 0.05$ ) on both responses were the temperature variable ( $X_2$ ) with the linear coefficients of 0.3531 and 0.8558 for R1 and R2, respectively. This can be explained by the effect of temperature on the diffusion coefficient of molecules. Higher temperatures result in higher diffusion rates and increased solubility [36, 40]. The importance of temperature in extraction was also reported by other researchers [23, 25, 30].

Statistical tests were undertaken by ANOVA for the two fitted quadratic polynomial models (Table 1). The significance of the models and each coefficient was determined using an F-test and p values [23], and a lack-of-fit test was used to verify the adequacy of the fit. The insignificant lack-of-fit for the two models indicated that both models adequately fit the experimental data. Results showed that the interaction terms  $\beta_{12}$ ,  $\beta_{23}$ , and the time factor were not significant in both models ( $p > 0.05$ ). The F values (60.91 and 24.52 for R1 and R2, respectively) and p values (less than 0.05) were significant for both models. The determination coefficients ( $R^2$ ) were calculated as 0.987 for R1, and 0.969 for R2. The  $R^2$  values being close to 1 confirm that both experimental models fit the real data well. The 3D response surface plots for the effects of the independent variables are given in Fig. 1. Results showed that higher extraction temperature and solvent concentration resulted in higher R1 and R2. A similar case was observed in previous researches [28, 41]. This result was also in good agreement with the previous finding where the factor time ( $X_1$ ) was found insignificant for the two responses.

### The optimum extraction conditions results

The optimum extraction conditions were calculated for both responses. Further extraction processes were applied under optimized conditions to verify the suitability of the equation model, and results are given in Table 2. The lower error (%) values between the experimental and predicted results confirmed that the optimum extraction conditions had been achieved and consistent results were obtained.

The results of optimum solvent concentrations revealed that the ethanol–water mixture was more efficient than a mono-component solvent. It has been reported that polyphenolic compounds with several hydroxyl groups are hydrophilic and generally have higher solubility in hydroalcoholic mixtures than in a pure alcoholic solvent [42]. The increase in the solubility of phenolic compounds in the ethanol–water mixture has also been suggested by other researchers [23, 30, 42]. Furthermore, the optimum solvent concentration results were in good agreement with the previous reports on the extraction of phenolics from different plant species. It was reported that ethanol concentrations of 80% for mango-steen peel [29], 75.3% for *Flos Chrysanthemi* [23], and 75% for black bamboo leaves [43] were optimum for the extraction of phenolics. The optimum extraction temperature for the maximum response was calculated as 74.9 °C for both responses and the optimum extraction times were 45.0 and 35.9 min for R1 and R2, respectively. Determination of optimum extraction temperature is especially important for the process economy and to prevent decomposition of thermo-sensitive compounds. Additionally, it has been reported that phenolic compounds might be re-adsorbed on the plant

**Table 1** ANOVA for response surface quadratic model

Response	Source	Sum of squares	df	Mean square	F value	p value
R1 <sup>a</sup>	Model	121.51	9	13.5	60.91	<0.0001
	X <sub>1</sub> -concentration	46.62	1	46.62	210.34	<0.0001
	X <sub>2</sub> -temperature	53.26	1	53.26	240.28	<0.0001
	X <sub>3</sub> -time	0.18	1	0.18	0.8	0.3994
	X <sub>1</sub> X <sub>2</sub>	0.6	1	0.60	2.71	0.1437
	X <sub>1</sub> X <sub>3</sub>	2.4	1	2.40	10.82	0.0133
	X <sub>2</sub> X <sub>3</sub>	0.53		0.53	2.38	0.1670
	X <sub>1</sub> <sup>2</sup>	7.65	1	7.65	34.51	0.0006
	X <sub>2</sub> <sup>2</sup>	0.72	1	0.72	3.26	0.1141
	X <sub>3</sub> <sup>2</sup>	8.05	1	8.05	36.3	0.0005
	Residual	1.55	7	0.22		
	Lack of fit	1.06	3	0.35	2.91	0.1647
	Pure error	0.49	4	0.12		
	Cor total	123.06	16			
R2 <sup>b</sup>	Model	487.34	9	54.15	24.52	<0.0001
	X <sub>1</sub> -concentration	202.31	1	202.31	91.61	<0.0001
	X <sub>2</sub> -temperature	199.3	1	199.3	90.25	<0.0001
	X <sub>3</sub> -time	1.25	1	1.25	0.57	0.4767
	X <sub>1</sub> X <sub>2</sub>	0.08	1	0.08	0.04	0.8559
	X <sub>1</sub> X <sub>3</sub>	21.21	1	21.21	9.60	0.0174
	X <sub>2</sub> X <sub>3</sub>	3.33	1	3.33	1.51	0.2591
	X <sub>1</sub> <sup>2</sup>	28.15	1	28.15	12.75	0.0091
	X <sub>2</sub> <sup>2</sup>	3.12	1	3.12	1.41	0.2735
	X <sub>3</sub> <sup>2</sup>	23.41	1	23.41	10.60	0.0139
	Residual	31.67	7	2.21		
	Lack of fit	28.03	3	3.64	3.21	0.1445
	Pure error	3.64	4	1.13		
	Cor total	967.04	16			

<sup>a</sup>The coefficient of determination ( $R^2$ ) of the model was 0.987

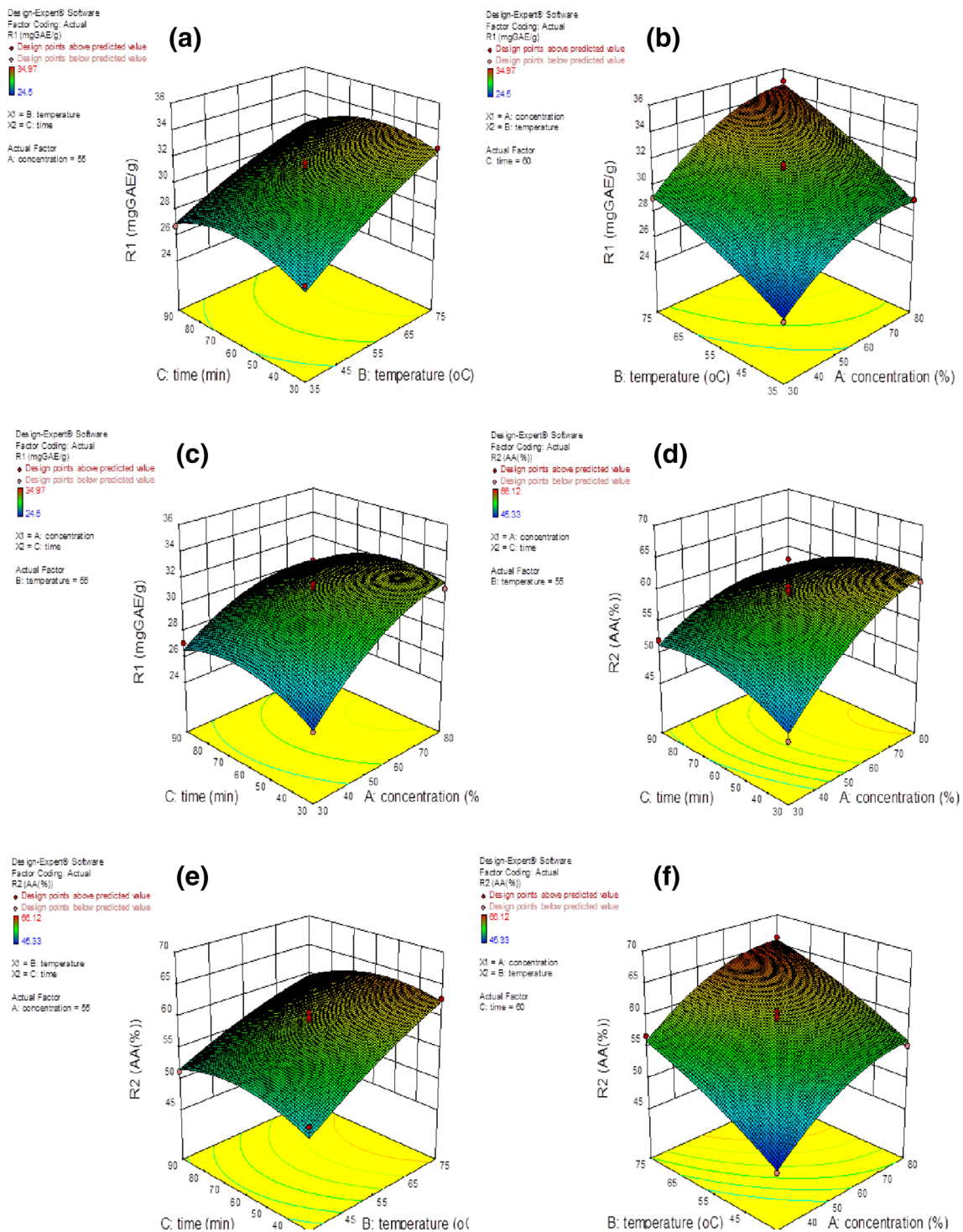
<sup>b</sup>The coefficient of determination ( $R^2$ ) of the model was 0.969

particles under extensively extended extraction time [44], therefore extraction should be completed in particular time.

## HPLC results

Plant phenolic compounds can play an important role in the shaping of the biological activities of the plants. To analyze the phenolic composition of the leaves, HPLC analysis was performed and the chromatogram of the extract is shown in Fig. 2. Nine phenolic compounds were identified by a comparison with the reference standards mix. It was found that catechin hydrate (1.1 mg/g), chlorogenic acid (2.2 mg/g), caffeic acid (0.08 mg/g), epicatechin (0.19 mg/g), p-coumaric acid (0.05 mg/g), ferulic acid (0.18 mg/g), rutin (0.4 mg/g) rosmarinic acid (0.22 mg/g) and apigenin (0.08 mg/g) were present in the leaf extract. The possible arbutin peak at about min. 4 with a low retention time is also reported in previous

studies for the pear fruits [45]. However, this phenolic compound which is specific for the pear species can't be quantified due to lack of the reference compound. The phenolic compounds in *Pyrus* species have been reported to contribute their bioactivity such as antioxidant, antimicrobial, and cytotoxic properties [7]. The results of the current research showed that chlorogenic acid, arbutin (with respect to its peak intensity), and catechin hydrate were the three main phenolic compounds in *Pyrus* leaves. Those results are consistent with the previous reports about *Pyrus communis L.* leaf extracts [46]. It was reported that the chlorogenic acid was found beneficial against hyperglycemia, and regulated lipid metabolism which was most probably through its antioxidant mediated activity [47, 48]. The arbutin and its derivatives, like other phenols, exhibit antioxidant activity [49], and it was also reported that arbutin was a comparable or even superior antioxidant to hydroquinone against the free radicals



**Fig. 1** Response surface for the effects of independent variables on total phenolic content TP (R1) and radical scavenging activity AA% (R2). **a** R1 according to extraction temperature and time **b** R1 according to solvent concentration and extraction temperature **c** R1 accord-

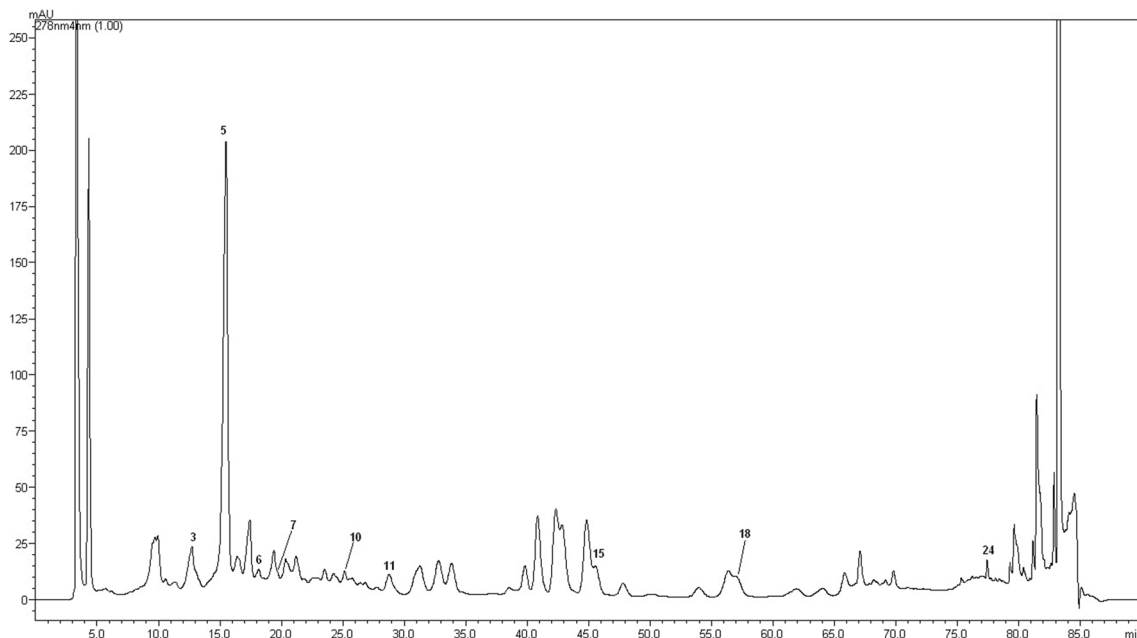
ing to solvent concentration and time **d** R2 according to extraction temperature and time **e** R2 according to time and extraction temperature **f** R2 according to solvent concentration and extraction temperature

[50]. Additionally, antioxidant activity of catechins is well established in vitro [51]. Thus, the potential value of those detected phenolic compounds in *Pyrus* leaf is

considered for the biologically active phytochemicals since they can promote health, prevent diseases and, in part, via antioxidant activity.

**Table 2** Optimal experimental conditions, experimental and predicted values of responses obtained at optimized conditions

Maximized response	X <sub>1</sub> (%)	X <sub>2</sub> (°C)	X <sub>3</sub> (min.)	Experimental values	Predicted values	Error (%)
R1 (TP)	79.7	74.9	45.0	35.01	34.06	-2.7
R2 (AA %)	78.4	74.9	35.9	66.99	64.41	-3.8

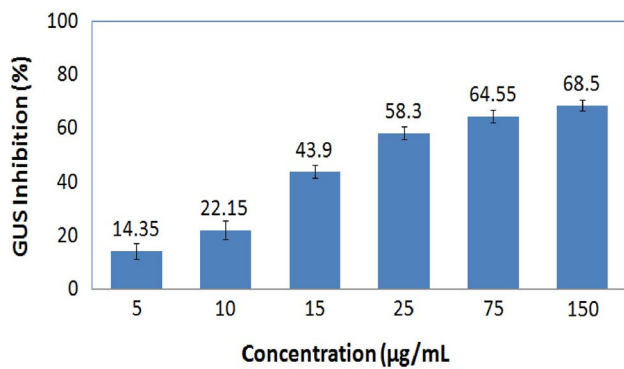
**Fig. 2** HPLC chromatogram of Pyrus leaf extract (3:catechin hydrate, 5:chlorogenic acid, 6:caffeic acid, 7:epicatechin, 10:p-coumaric acid, 11:ferulic acid, 15: rutin, 18: rosmarinic acid, 24:apigenin)**Table 3** Antibacterial activity of Pyrus leaf extract on test microorganisms

Test microorganism	Zone diameter (mm)
<i>Staphylococcus aureus</i>	18.0 ± 1.6
<i>Bacillus cereus</i>	10.7 ± 1.2
<i>Bacillus subtilis</i>	11.3 ± 1.7
<i>Pseudomonas aeruginosa</i>	10.3 ± 0.9
<i>Listeria monocytogenes</i>	8.7 ± 0.5
<i>Escherichia coli</i>	15.0 ± 0.1

### Antibacterial tests results

The stability of some bioactive compounds in the plant material is affected during some processing or preparation of plant. Therefore, the bioactivity tests were performed using extracts obtained under optimum extraction conditions. The results of antibacterial activity tests are given in Table 3. Results showed that *Pyrus elaeagrifolia* leaf extracts have moderate antibacterial activity. The extracts showed the

lowest antimicrobial effect against *Listeria monocytogenes*, and the highest antimicrobial effect on *Staphylococcus aureus* with a zone diameter of 18.0 mm. In literature, there are limited numbers of studies about antimicrobial activity of Pyrus species. It was reported that aqueous extracts of the young shoots of the pear Pyrus spp. exhibited strong antibacterial activity against the bacterium *Erwinia amylovora* [52]. In another study, it was indicated that *Pyrus pashia* showed significant activity against *Klebsiella pneumonia*, *Shigella flexneri* and *Escherichia coli* [53]. The sensitivity of bacteria to phenolic compounds depends on the bacterial species and the structure of the phenolics [54, 55]. There are differences in cell structure of gram positive and negative bacteria since the gram negative bacteria has a lipopolysaccharide outer membrane, transfer of molecules is achieved through the cell membrane with porins which significantly decreases the ability of antimicrobial to penetrate the outer plasma membrane [20]. However in this study, higher inhibitions were achieved for *S. aureus* (gram positive), as well as *E.coli* (gram negative). The phenolics (mainly chlorogenic acid, arbutin and catechin detected by HPLC in Pyrus extract)



**Fig. 3**  $\beta$ -Glucuronidase inhibitory effects of *Pyrus* leaf extract (5–150  $\mu\text{g/mL}$ )

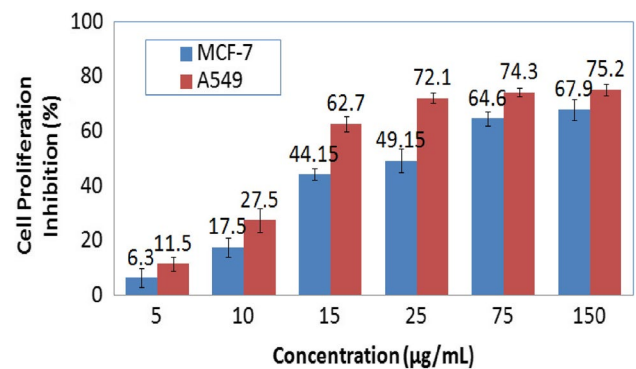
might have more effective role against antimicrobial action of extract. The known antimicrobial activities of catechins against bacteria were well established in literature [56]. Arbutin was also a potent antimicrobial agent and it was reported that bacterial species; *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* (MIC 10 mg/ml) were more sensitive to arbutin compared with *Listeria monocytogenes* (with a MIC of 0.30 mg/ml) [7].

### $\beta$ -Glucuronidase inhibition assay results

The GUS inhibitory potential of *Pyrus* leaf extract is investigated, and the results are presented in Fig. 3. The extracts inhibited the enzyme in a dose dependent manner where the highest inhibition was achieved at 150  $\mu\text{g/mL}$ . The  $\beta$ -glucuronidase inhibitors reduce the carcinogenic potential of toxic compounds, and are reported to be hepatoprotective [19, 23]. Since some carcinogens can be produced by bacterial GUS activity in the intestine, its activity is considered as cancer risk biomarker [57]. A diet composed of large amounts of GUS inhibitors of plant origin can be reason for the elimination of harmful effects of GUS such as; elimination of its promoting role for the intestinal tumors in large intestine [58]. On the other hand, it is important to note that among the intestinal flora *E.coli* has the highest activity of GUS [59]. In this study, *Pyrus* leaf extract showed also considerable antimicrobial action against *E.coli* within the tested bacteria. In this context, those results revealed that GUS inhibitory potential may have an important role in the bioactivity of the *Pyrus* leaf extracts.

### Cytotoxicity tests results

The results of cytotoxicity tests for the extracts obtained under optimum conditions against A549 and MCF-7 cells are given in Fig. 4. The current results indicated that *Pyrus* extracts have cytotoxic activity against both cell lines. The



**Fig. 4** Cytotoxicity results in terms of cell proliferation inhibition (%) of *Pyrus* leaf extract (5–150  $\mu\text{g/mL}$ ) against: MCF-7 breast adenocarcinoma cell line and A549 lung adenocarcinoma cell line

inhibition in the cell proliferation showed an increasing trend with the increase in extract concentration. Higher proliferation inhibition was observed for the A549 cell line. In literature, the cytotoxic effect of methanol extracts of *P. pyraster* (leaves and bark) were tested against melanoma cell line (Fem-x), and exhibited significant cytotoxic effects towards Fem-x cells [7]. Potential cytotoxicity of the *Pyrus* extract in this study might be dominated by the major detected phenolics in HPLC analysis. It has been reported that arbutin in *Pyrus* species has an important role in cytotoxicity, and its cytotoxicity was demonstrated for higher concentration (mM) in B16F10 melanoma cells [60–62]. The similar results for arbutin were obtained for two humane breast cancer cell lines, MCF-7/Adr and wild-type MCF-7/wt [63]. It was reported that chlorogenic acid has shown beneficial effects in colon cancer, brain cancer, breast tumors, lung cancer in phase I clinical studies [64, 65], as well as the inhibitory activities catechins against the growth of cancer cells have been demonstrated in different experimental systems [66, 67].

### Conclusion

Response surface methodology has proved to be effective in estimating the effect of three independent variables; extraction time, temperature and solvent concentration on the antioxidant activity and total phenolic content for predicting the optimal operational conditions. Modeling and analysis of optimum extraction conditions confirmed that these parameters maximized the phenolic content and antioxidant activity of the extracts. Optimal conditions for extraction process proposed by the Design-Expert software, were 45.0 and 35.9 min of extraction time 74.9 °C temperature, 79.7 and 78.4% solvent concentration to maximize the total phenolic content and the antioxidant activity, respectively. Bioactivity experiments revealed that the extracts obtained under



optimum extraction conditions had also GUS inhibitory, antimicrobial and cytotoxic activities. The current RSM design can be valuable in terms of cost saving extraction process, and *Pyrus elaeagrifolia* leaf can be a potent source to produce high value-added food products and/or nutraceuticals to combat oxidative stress and GUS mediated reactive metabolite formation.

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