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ORIGINAL PAPER

Sorbus umbellata (Desf.) Fritsch var. *umbellata* Leaves: Optimization of Extraction Conditions and Investigation Antimicrobial, Cytotoxic, and β-Glucuronidase Inhibitory Potential

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Abstract

This study aimed to optimize the extraction conditions for *Sorbus umbellata* (Desf.) Fritsch var. *umbellata* leaves to maximize the phenolic content and their antioxidant activity and to investigate β-glucuronidase (GUS) enzyme inhibitory, antimicrobial and cytotoxic potentials of the extracts obtained under optimum conditions. The optimum extraction conditions were found to be 78.2 and 79.7% solvent, 73.1 and 71.5 °C, and 89.9 and 88.8 min to maximize phenolic content and antioxidant activity, respectively. Low values of coefficient of variations indicate the high reliability and reproducibility of the conducted extraction experiments. Bioactivity results showed that extracts had cytotoxic effect on the MCF-7 and A549 cells where the highest cell proliferation inhibition was observed for the A549 cell line (71.8% at 150 μg/mL). *Staphylococcus aureus* showed highest zone of inhibition (19.3 mm) in all bacteria followed by *Escherichia coli*. Additionally, extracts displayed potential GUS inhibitory activity. In conclusion, *Sorbus umbellata* leaf extract can be obtained by optimized cost-saving extraction and has a potential bioactivity to be utilized as a food ingredient for high value-added products and/or nutraceuticals development where it can combat oxidative stress and GUS mediated reactive metabolite formation.

Keywords Extraction · Optimization · *Sorbus umbellata* · Antioxidant · Antibacterial · Cytotoxicity

Introduction

Sorbus is a plant genus consisting of 100–200 species of trees and shrubs in the subfamily *Maloideae* of the rose family *Rosaceae* [1]. *Sorbus umbellata* (Desf.) Fritsch var. *umbellata* is one of the 12 species (17 taxa) growing in Turkey, and the most common varieties are *Sorbus domestica* (L.) Crantz, *Sorbus aucuparia* (L.), *Sorbus umbellata* (Desf) Fritsch, and *Sorbus torminalis* (L.) Crantz [2]. Leaves of sorbus species have been used in various traditional medicine systems. The leaves of *Sorbus umbellata* (Desf.) Fritsch var. *umbellate* were traditionally used as tea against diabetes and blood coagulation [3]. The leaves of other sorbus species such as *Sorbus aucuparia* L. were used for prostatitis, cancer, diarrhea; the leaves of *Sorbus domestica* L. were used for prostatitis, diabetes, nephritis, gallbladder ailments, diuretic, diarrhea, kidney stones, cholesterol lowering, the leaves of *Sorbus torminalis* (L.) Crantz var. *Torminalis* were used for diabetes and stomach ache [4].

Extraction techniques used to obtain natural products from plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for finding new sources for natural antioxidants, functional foods and nutraceuticals, food ingredients, and dietary supplements. An economical tool that is commonly used in the experimental design step of extraction is response surface methodology (RSM), and it is very efficient in terms of time, material and cost [5–7]. RSM successfully optimizes processes related to food/natural product systems, including the extraction of phenolic compounds [8–11] where bioactivities of those systems are highly affected by extraction conditions. The main aim of this study was to optimize the extraction conditions of *Sorbus* leaves and to

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investigate the potential bioactivity of the extracts obtained under optimum conditions. Antioxidant, cytotoxic and antimicrobial activity of the extracts were investigated and potential of the Sorbus as a new inhibitor for the microbial β-glucuronidase enzyme (EC.3.2.1.31) was analyzed to reflect if there is a more realistic health promoting role by mediating reactive metabolite formation related to health and diseases in human intestinal system. To the best of the authors' knowledge, there is no research in the literature about health promoting potential of *Sorbus umbellata* (Desf.) Fritsch var. *umbellata* leaves. This is also the first detailed report on the optimization of extraction conditions for maximal phenolic and antioxidant activity.

Materials and Methods

Materials

Sorbus leaves were harvested in the second half of August from natural habitats (Afyonkarahisar Sultandağı region, Turkey; 2016). For enzymatic assays, *p*-nitro phenyl β-D-glucuronide, β-glucuronidase from *Escherichia coli* (EC.3.2.1.31), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), Folin–Ciocalteu reagent, $NaHCO_3$ were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Nutrient agar was obtained from Merck (Germany). 8 mm diameter standard sterile discs obtained from Oxoid (Basinstoke, UK). HPLC standards of gallic acid, protocatechuic acid, catechin, *p*-hydroxy benzoic acid, chlorogenic acid, caffeic acid, epicatechin, syringic acid, vanilin, *p*-coumaric acid, ferulic acid, sinapic acid, benzoic acid, *o*-coumaric acid, rutin, rosmarinic acid, eriodictiol, cinnamic acid, quercetin, luteolin, kamferol, apigenin were purchased from Sigma (St. Louis, MO, USA). All solvents and reagents were analytical or HPLC grade.

Experimental Design and Extraction

The Box–Behnken design was applied to determine the best combination of extraction variables. Analysis was performed using Design-Expert® software ver. 7 (Stat Ease, USA). A more detailed description can be found in supplementary material (ESM 1).

Determination of Total Phenolic Content (TP)

The TP analysis was performed using the Folin-Ciocalteu method as previously described [12]. The results of TP were expressed as gallic acid equivalents (GAE) in mg/g of the sample. All the tests were carried out in triplicate.

Antioxidant Activity (AA) Assay

An antioxidant activity test was performed on the Sorbus extracts based on DPPH free radical scavenging activity as previously described [13]. All the tests were carried out in triplicate.

HPLC Analysis

Phenolic characterization analyses were carried out by the HPLC system (Shimadzu Corp., Kyoto, Japan) equipped with a diode array detector (DAD) at λ_{max} of 280 nm as previously tested and validated for plant extracts [13].

β-Glucuronidase Activity Measurements

β-Glucuronidase inhibition assay was carried out as previously described with slight modifications [14]. A more detailed description can be found in supplementary material (ESM 1).

Antibacterial Tests

Antibacterial tests were performed using The Kirby-Bauer disc diffusion assay for bacterial strains according to the method previously described in detail [13]. A more detailed description can be found in supplementary material (ESM 1).

Cell Culture Studies and Cytotoxicity Tests

Cell survival was determined by an MTT (methylthiazolyldiphenyl-tetrazolium bromide) test using a cell counter (CEDEX-XS Analyzer, Roche GmbH, Germany). A more detailed description can be found in supplementary material (ESM 1).

Results and Discussion

Extraction Process Modeling and Optimized Extraction Conditions

In RSM, a three-factor, three-level Box–Behnken design was used to determine the responses R1 (TP) and R2 (AA %) for the independent variables; X_1, X_2, and X_3. To analyze the effect of only the three selected parameters, particle size and liquid-to-solid ratio were fixed at 106–150 μm and 30:1 (*v*/*w*), respectively. The empirical relationships between the two responses and the independent variables were expressed by the following quadratic polynomial equations (Eq. 1 and Eq. 2) in terms of actual factors.

$$R1 = -21.6127 + 0.3004(X_1) + 0.6265(X_2) + 0.2744(X_3) + 0.0022(X_1)(X_2) + 0.0025(X_2)(X_3) - 0.0030(X_1^2) - 0.0066(X_2^2) - 0.0022(X_3^2) \quad (1)$$

$$R2 = 16.03674 - 0.2450(X_1) + 0.3742(X_2) + 0.2310(X_3) + 0.0073(X_1)(X_2) + 0.0061(X_2)(X_3) - 0.0079(X_2^2) - 0.0028(X_3^2) \quad (2)$$

According to these model equations, the temperature variable (X_2) had the most significant ($p < 0.05$) effect on both responses (linear coefficients of 0.6265 and 0.3742 for R1 and R2, respectively). This was followed by the concentration term. However, the interaction term β_{13} was not significant for concentration X_1 and time X_3 in both models, and the quadratic term (β_{11}) was not significant for concentration in R2 ($p > 0.05$) with p values of 0.3201 and 0.0528, respectively.

Effects of factors on the responses were determined statistically by ANOVA. Table 1S (online resource) presents the results of ANOVA for the two fitted quadratic polynomial models. The significance of the models and each coefficient was determined using an F-test and p values [15], and a lack-of-fit test was used to verify the adequacy of the fit. The F values (72.73 and 37.98 for R1 and R2, respectively) and p values (less than 0.05) were significant for both models. However, the results of the lack-of–fit test were not significant for the two models indicating that both models adequately fit the experimental data. Determination coefficients (R^2) were 0.986 for R1 and 0.967 for R2. The R^2 values being close to 1 confirm that both experimental models fit the real data well [16, 17]. The coefficient of variations (C.V) for R1 and R2 were calculated as 3.1 and 4.39%, respectively. These low values of C.V indicate the high reliability and reproducibility of the conducted experiments [18, 19]. To the best of the authors' knowledge, there is no study in the literature concerning the antioxidant activity of the leaf extracts of *Sorbus umbellata* (Desf.) Fritsch var. *umbellata*. The results of RSM experiments revealed that extraction process can successfully expressed by a mathematical model and the analyzed leaf extracts had potential antioxidant activities. These results are in good agreement with those reported by previous studies demonstrating the antioxidant potential of other *Sorbus* species, such as *S. aucuparia*, *S. domestica*, *S. aria*, and *S. torminalis* [20–22].

The optimum parameters were calculated using Design-Expert 7.0 (Table 2S, online resource). Those values were numerically higher than expected. Further extraction processes were applied under optimized conditions to verify the suitability of the equation model. 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 overall results of RSM showed that the TP content increased with the increasing ethanol concentration. The optimum solvent concentrations (78.2 and 79.7% for R1 and R2, respectively) were in good agreement with the previous reports on the extraction of phenolics: 75.3% for *Flos Chrysanthemi* [15], 75% for black bamboo leaves [23], 70% for black pepper [24], and 80% ethanol concentration for mangosteen peel [25]. Furthermore, these results also revealed that the ethanol-water mixture was more efficient than a mono-component solvent where using the combination of organic solvent and water facilitated the extraction of all compounds that were soluble in both water and organic solvents. It has been reported that polyphenols with several hydroxyl groups, such as glucosides, are hydrophilic and generally present higher solubility in hydroalcoholic mixtures than in a pure alcoholic solvent [26]. Increased solubility of phenolic compounds in the mixture of ethanol and water has also been suggested by other researchers [13, 15, 26].

The optimum extraction temperature was found to be 73.1 °C for R1 and 71.5 °C for R2. According to the 3D plots (Fig. 1S, online resource), increased temperature resulted in an accelerated increase in R1 and R2 up to a certain value, then showed a decelerating trend (the surface became planer rather than curved). This result was confirmed by the negative quadratic effects of temperature (X_2) in both response models [19]. The accelerating effect of temperature was also reported by other researchers [13, 15, 27]. 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 [19, 28, 29]. It was reported that phenolics were sensitive to heat where heat treatment using boiling water on green fruit extracts led to a 7.6% reduction in phenolic content even in short term (10 min.) heat process [30]. Therefore, an upper limit should be determined in experiments where bioactivity is of major importance to avoid degradation of thermosensitive phenolics.

The optimum extraction times for maximum response were 89.9 and 88.8 min for R1 and R2, respectively. The negative quadratic effects of time (X_1) in both response models (Eq. 1 and Eq. 2) revealed deceleration in the extraction responses. This can be explained by Fick's second law of diffusion, which states that a final equilibrium should be reached between solute concentrations in the solid matrix and bulk solution after a certain time [19]. Additionally, it has been reported that phenolic compounds might be re-adsorbed on the smashed plant particles under extensively extended extraction time conditions [31]. Therefore, extending the extraction time more than the optimum values would not lead to significant differences in the amount of phenolic compounds extracted.

The Phenolic Characterization Results

Various polyphenols have been found in different *Sorbus* species, and flavonoids have been reported as the main bioactive components in *S. aucuparia, S. aria*, and *S. intermedia* [32]. These studies referred to several quercetin and kaempferol glycosides in different parts of the plant, such as inflorescences, leaves, and fruits. However, to date, no study has been undertaken on the phenolic characterization of the leaves of

Sorbus umbellata (Desf.) Fritsch var. *umbellata*. The phenolic profiles of *Sorbus* leaf extracts were analyzed by HPLC. The HPLC chromatogram of the extract is shown in Fig. 2S (online resource). A total of seven compounds were identified by a comparison with the reference standards mix but some of the peaks in the chromatograms were not identified due to a lack of reference compounds. The results showed that catechin hydrate (0.38 mg/g), chlorogenic acid (0.89 mg/g), caffeic acid (0.07 mg/g), epicatechin (0.84 mg/g), *p*-coumaric acid (0.03 mg/g), ferulic acid (0.05 mg/g), rutin (0.77 mg/g) and quercetin (0.04 mg/g) were present in the leaf extract where chlorogenic acid and epicatechin were the two main phenolic compounds in *Sorbus* leaves. Comparing with the current literature about the retention times (t_R) data for the polyphenols in *S. domestica* leaf extracts (at UV λmax: 280 nm.), the two unidentified peaks in this study between 23 and 25 min might be procyanidin trimer B-type (t_R: 23.1 min.), procyanidin dimer hexoside (t_R: 25.1 min.), and the peak at t_R ~14.5 min might be procyanidin B2 (t_R: 14.8 min.) [33]. Those compounds detected in leaf extract in this study can contribute to its medicinal and antioxidant properties. In literature it was reported that chlorogenic acid had several health benefits, such as reduction in the relative risk of cardiovascular diseases, type 2 diabetes, and Alzheimer's disease [34]. It also has antibacterial and anti-inflammatory properties, and exhibits hepatoprotective effects against acetaminophen toxicity [34]. It was reported that (−)-epicatechin modulated the production of reactive oxygen species, and played a role for the improvement of parameters related to cardiovascular disease [35]. Additionally, it was shown that (−)-epicatechin and catechin improved insulin sensitivity [SR1]. The phenolic compounds; *p*-coumaric acid and quercetin reported to prevent TNFα-induced increase of inflammation and oxidative stress [35] and rutin showed cytoprotective effects including antioxidative, and neuroprotective action [SR2].

GUS Inhibitory, Antibacterial and Cytotoxix Activity Results

The phenolic compounds in *Sorbus* species can make them a potent inhibitor for bacterial β-glucuronidase (EC 3.2.1.31) enzyme. This bacterial enzyme in the intestinal system is responsible for reactive metabolites and carcinogenic compound formation, and there are ongoing efforts to find natural dietetic inhibitors of this enzyme [14, SR3-SR6]. Results showed that plant extracts obtained under optimum conditions (Table 2S) have an inhibitory activity against enzyme in dose dependent manner (Fig. 3S, online resource). IC_{50}, which is the concentration of the *Sorbus* extract required to inhibit the enzyme's activity by 50% was calculated as 117.9 (μg/mL). In literature, it was reported that the IC_{50} values of *Nymphaea pubescens* Willd. plant flower and pedicel extracts against GUS were 270.27 and 868.46 μg/ ml, respectively [SR7]. In another study IC_{50} values of *Swertia chirayita* and *Swertia decussate* extracts against GUS were found as 210.97 and 269.7 μg/ml, respectively [SR5]. Compared to the recent literature, *Sorbus* extracts are active against GUS and this plant has a potential to be utilized as a novel GUS inhibitor.

Table 3S (online resource) presents the comparative analysis results on the antimicrobial activity of the selected test bacteria using an agar diffusion test. The inhibitory zones for the positive controls using ampicillin and gentamicin varied in the range between 19.8–25.6 mm, and 21.5–29.2 mm, respectively. When the results were compared with antibiotics, the *Sorbus* leaf extracts obtained under optimum conditions showed low antimicrobial activities against *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes*, where the clear zones had almost the same results. These extracts had the highest antimicrobial effect on *Staphylococcus aureus* with a zone diameter of 19.3 mm, followed by *Escherichia coli*. To the best of our knowledge, there is no published report on the antibacterial activity of *Sorbus umbellata* (Desf.) Fritsch var. *umbellata*. Results were in good agreement with previous reports where *Sorbus aucuparia* fruit extracts have been shown to exhibit antibacterial activity against *B. cereus, S. aureus,* and *P. aeruginosa* [SR8], and *Sorbus sibirica* fruit extract inhibited *E. coli*, *B. thuringiensis*, *S. aureus*, *B. subtilis*, *S. cerevisiae*, with the strongest inhibition against *S. aureus* [SR9].

The effect of the *Sorbus* leaf extract was also investigated on the metabolic activity of A549 and MCF-7 cells which was obtained under optimum extraction conditions. The results of the MTT assay are given in Fig. 4S (online resource). The *Sorbus* extract presented cytotoxic activity against both cell lines. The metabolic activity of both cells showed a decreasing trend with the increase in extract concentration, and the highest cell proliferation inhibition was observed for the A549 cell line (71.8% at 150 μg/mL). The current results are in agreement with those of the limited number of studies on other *Sorbus* species. For example, it has been reported that *Sorbus aucuparia* L. leaves have a cytotoxic effect on prostatitis and cancer [20] *Sorbus commixta* Hedl. (Rosaceae) fruit on human lung cancer cells [SR10], ethanol extract of *Sorbus rufopilosa* on human colon carcinoma HT29 cells [SR11]. The cytotoxic activity of *Sorbus umbellata* (Desf.) Fritsch var. *umbellata* extract suggests that it is a potential nutraceutical candidate for cancer remedy.

Conclusion

The RSM design used in this study showed that extraction conditions including extraction time, temperature and solvent concentration markedly influenced the total phenolics, and antioxidant activity of *Sorbus* leaves. The high correlation of the models exhibited that the quadratic polynomial model could

be successfully used for optimizing the extraction parameters. The optimum extraction conditions were found as 78.2 and 79.7% solvent, 73.1 and 71.5 °C extraction temperature and 89.9 and 88.8 min of extraction time to maximize phenolic content and antioxidant activity, respectively. Additionally, *Sorbus* extracts obtained under optimum extraction conditions showed valuable GUS inhibitory, antioxidant, antimicrobial and cytotoxic activities. The data presented in this study will provide a basis for a cost-saving extraction process and show potential of *Sorbus umbellata* (Desf.) Fritsch var. *umbellata* leaf extract as a food ingredient or compound to produce high value-added products and/or nutraceuticals.

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Compliance with Ethical Standards

Conflict of Interest Authors have no conflict of interest to declare.

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