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EFFECT OF SAMPLE-PREPARATION METHODS ON THE HPLC QUANTITATION OF SOME PHENOLIC ACIDS IN PLANT MATERIALS

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SUMMARY

The extraction yield of phenolic acids from *Sambucus nigra* L. inflorescence and *Polygonum aviculare* foliage has been determined for different methods of liquid–solid extraction – Soxhlet extraction, ultrasonicfication (USAE), microwave-assisted extraction (MASE), and accelerated solvent extraction (ASE). Methanol was used as extractant. The crude extracts were evaporated to dryness and prepared for fractionation of the phenolic acids by liquid–liquid extraction (LLE) or solid-phase extraction (SPE). Samples containing the phenolic acid fraction were analysed by RP-HPLC. Quantitative analysis was performed by the external standard method and use of a calibration plot for each standard. It was found that yield depends primarily on the plant material from which the phenolic acid fraction was extracted. For *Sambucus nigra* L. inflorescence the most effective was exhaustive extraction in a Soxhlet apparatus. For *Polygonum aviculare* foliage the most effective methods were MASE and USAE. Both LLE and SPE gave similar recoveries and repeatability.

INTRODUCTION

In the process of quantitative analysis of plant secondary metabolites, preliminary treatment of the plant material is one of the most time-consuming steps. The first problem is extraction of the compounds from the plant material – usually performed by liquid–solid extraction (LSE).

The goal of every extraction process is rapid and effective isolation of compounds from a matrix by use of a minimum amount of solvent. Traditional methods, for example percolation, exhaustive Soxhlet extraction, or direct extraction with boiling solvent under reflux are most often used. Traditional techniques are starting points in the development of new, more effective, methods of extraction which apply solvents at higher temperatures and pressures. These methods require shorter extraction times, use small amounts of solvents, enable simultaneous parallel processing of several samples, and are automatic. Modern extraction methods, which include microwave-assisted solvent extraction (MASE), pressurised solvent extraction (PLE; called also accelerated solvent extraction, ASE), and supercritical fluid extraction (SFE) are usually more expensive [1].

In MASE there are two different modes – closed and open. In both types, however, electromagnetic irradiation induces rapid movement of solvent molecules which generates thermal energy and results in rapid extraction of the analyte at either high pressure (closed system) or normal pressure (open system) [1,2]. In PLE, extraction proceeds at high pressure in a closed vessel heated in a thermostatted electric oven [3,4]. The extract is then rapidly removed from the extracting vessel with a fresh portion of solvent. Between conventional and modern techniques lies ultrasound-assisted extraction (USAE). Ultrasound of approximately 16 kHz frequency loosens matrix structures and makes contact with solvent molecules much easier [5]. The method also has disadvantages, for example inability to renew the solvent and the need for filtration after the process [1,5].

The next step in sample preparation is purification of the crude extract. Plant extracts contain much ballast material, both non-polar (chlorophylls, waxes, lipids) and polar (tannins, sugars). Most often liquid–liquid extraction (LLE) is used, which takes advantage of solubility differences of hydrophobic substances, which have affinity for non-polar solvents, and hydrophilic substances, which have affinity for aqueous solutions. Although the analytes can be easily obtained by evaporation of the solvent, the method has many disadvantages – for example emulsions can be formed and the process is time-consuming. Purification can also be achieved by solid-phase extraction (SPE). This method uses a variety of adsorbents and ion-exchangers and is widely used for a variety of purposes [3,6–8].

The objective of the work discussed in this paper was comparison of different extraction techniques for isolation of phenolic acids from two common plant materials used in therapy – the flowers of *Sambucus nigra* L. (family *Caprifoliaceae*) and the foliage of *Polygonum aviculare* L. (fa-

mily *Polygonaceae*). Phenolic acids, derived from shikimic acid, belong to three groups: benzoic acid derivatives (with analgesic, antipyretic, anti-inflammatory, and antiseptic properties), cinnamic acid derivatives (with cholagogic, choleritic, and antibiotic properties), and depsides (with hypotensic, sedative, antihistamine, and cholagogic properties) [9–11]. Determination of phenolic acid concentrations is an important task in investigations of the quality of plant material and in the standardisation of plant drugs, so elaboration of adequate procedures for sample preparation and analysis of this compound group is a genuine problem.

EXPERIMENTAL

Plant Material and Standards

Inflorescences of *Sambucus nigra* L. and foliage of *Ploygonum aviculare* L. were purchased from the herbal industrial plant Herbapol (Lublin, Poland). Dry plant material was milled and sieved. All phenolic acid standards were purchased from Sigma–Aldrich (Steinheim, Germany).

Extraction Procedures

Exhaustive extraction was performed in Soxhlet apparatus. Plant material (2 g) in a filter paper was placed in the thimble holder of the apparatus and extracted with methanol for 15 h.

Ultrasound-assisted extraction was performed in an Intersonic IS-4 ultrasonic bath equipped with a thermostat. Plant material (2 g) was soaked with 50 mL methanol in an Erlenmeyer flask and the flask was placed in the bath for 30 min. The extract was then filtered and the same plant material was soaked with a fresh portion of methanol and again extracted in ultrasonic bath. This was repeated three times and the methanol extracts were combined. Extraction was performed at both ambient temperature (20 ± 1°C) and 60°C.

MASE was performed with 80% methanol in water using a Plasmotronika UniClever BMZ (Wroclaw, Poland) bath. Dried and ground plant material (2 g) was placed in a laboratory flask with 50 mL extractant in the microwave bath and extracted using two-step extraction – 40% generator power for 1 min then 60% generator power (600 W) for 30 min – in both open and closed systems.

ASE was performed with a Dionex (Sunnyvale, CA, USA) ASE 200 instrument with solvent controller. Plant material (1 g) was mixed with

neutral glass and placed in a stainless-steel extraction cell. Flowers of *Sambucus nigra* L were soaked with 50 mL 80% methanol in water and foliage of *Ploygonum aviculare* L was soaked with 50 mL pure methanol. The extractions were performed at 100°C at a pressure of 60 bar for 10 min.

All extraction procedures were repeated three times. The extracts obtained were combined and purified by LLE and SPE.

Purification Procedures

LLE

All methanolic extracts (obtained by Soxhlet, USAE, MASE, and ASE) were evaporated to dryness under reduced pressure. The dry residues were soaked with 50 mL boiling water then cooled by refrigeration (4°C). After 12 h extracts were filtered and extracted with diethyl ether (8 × 50 mL). The diethyl ether extracts were evaporated to dryness and the dry residues were dissolved in methanol in 5-mL volumetric flasks and analysed quantitatively for phenolic acids by RP-HPLC.

SPE

Extraction was performed in an SPE chamber (Baker, Germany). C_{18} cartridges (Bakerbond) were conditioned with 10 mL methanol then 10 mL distilled water. The extract dissolved in 30% aqueous methanol (5 mL) was applied to the column and eluted with 2 mL distilled water into the same receptacles. Sodium bicarbonate solution (2.5%, 1.2 mL) was added to these eluates and the solutions were diluted to 25 mL with distilled water in volumetric flasks. In this way the phenolic acids were converted to the anionic form, which is readily adsorbed by anion exchangers.

Cartridges packed with quaternary amine (QA) chemically bonded to silica were used in the experiments. The cartridges were conditioned with 10 mL distilled water then 29 mL dilute (0.0625%) sodium bicarbonate. The extracts containing the phenolic acids in the anionic form were applied to cartridges prepared as described above and the phenolic acids were eluted from the adsorbent bed with 20 mL 1 M phosphoric acid–water–methanol 1:4:5 (*v*/*v*). The eluates obtained were analysed by RP-HPLC.

HPLC Analysis

Analysis was performed with a Knauer liquid chromatograph equipped with a 200 mm × 4.6 mm, d_p 5 μm, Hypersil ODS column, a UV–visible detector, and a Rheodyne injector with 20-μL loop. Isocratic elution

was performed with methanol–0.001 M phosphoric acid 22:78 (*v*/*v*) for extracts of *Sambucus nigra* and 25% aqueous methanol containing 1% acetic acid for extracts of *Polygonum aviculare*. The phenolic acid content of the extracts was determined by use of calibration plots constructed for every standard. Identification and measurement of the peaks was performed at 520 nm. Calibration plots were linear in range 0.0625–1 mg mL^{-1} ($R^2 > 0.9980$) for all the phenolic acids.

RESULTS AND DISCUSSION

The benzoic acid derivatives gallic, protocatechuic, *p*-hydroxybenzoic, and vanillic acids, the cinnamic acid derivatives *p*-coumaric, caffeic, and ferulic acids, and the depside chlorogenic acid were identified in extracts from *Sambucus nigra* L. inflorescence. The qualitative and quantitative composition of extracts obtained by a variety of extraction methods were different, however. For most of the acids (protocatechuic, *p*-hydroxybenzoic, vanillic, and ferulic) the highest yields were obtained by extraction in a Soxhlet apparatus. For the other extraction methods the extraction yields

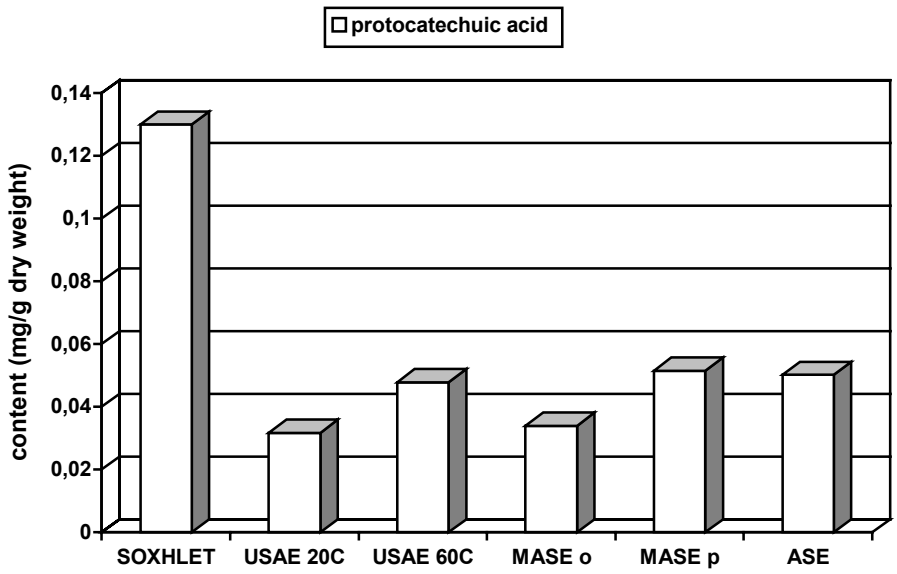

Fig. 1

Dependence on the method of extraction of the yield of protocatechuic acid from *Sambuccus nigra* L. inflorescence

for these acids were similar. For chlorogenic and *p*-coumaric acids the highest extraction yields were obtained by use of microwave-assisted extraction in an open system. Figures 1 and 2 show histograms of extraction yield for protocatechuic and *p*-hydroxybenzoic acids.

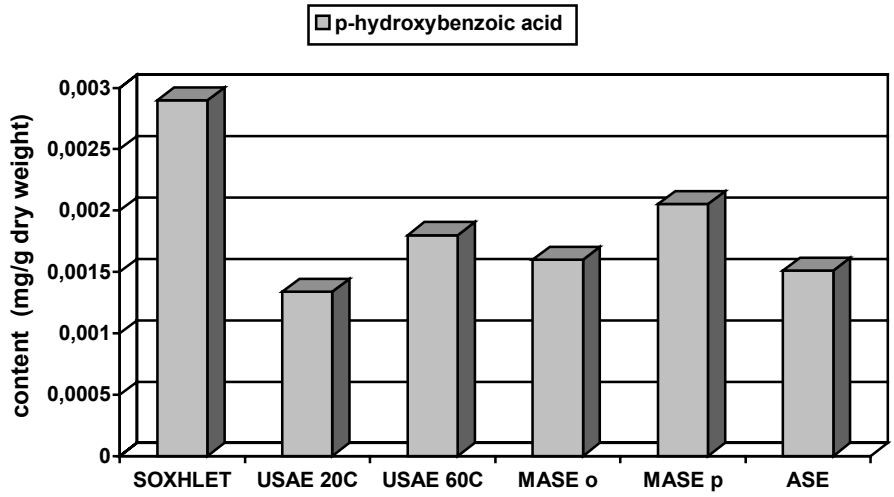

Fig. 2

Dependence on the method of extraction of the yield of *p*-hydroxybenzoic acid from *Sambuccus nigra* L. inflorescence

The extracts obtained were purified by LLE and SPE. Both procedures have been used for isolation of the phenolic acids fraction from crude methanolic extracts, as has been described elsewhere [12,13]. In our experiments similar extraction yields were obtained by both methods of purification but extracts obtained by LLE contained less unwanted ballast material. Liquid–liquid extraction also seems to be the most reproducible method giving high recoveries. Recoveries of phenolic acids were measured by fortifying extracts with standards; the recoveries obtained (± *RSD*) were: protocatechuic acid, 91.45 ± 1.5%; *p*-hydroxybenzoic acid, 89.84 ± 1.3%; and gallic acid, 90.21 ± 3.93%. The extraction yields of SPE and LLE for protocatechuic and *p*-hydroxybenzoic acids are compared in Figs 3 and 4. In subsequent experiments LLE was used for sample preparation for all extracts before HPLC analysis.

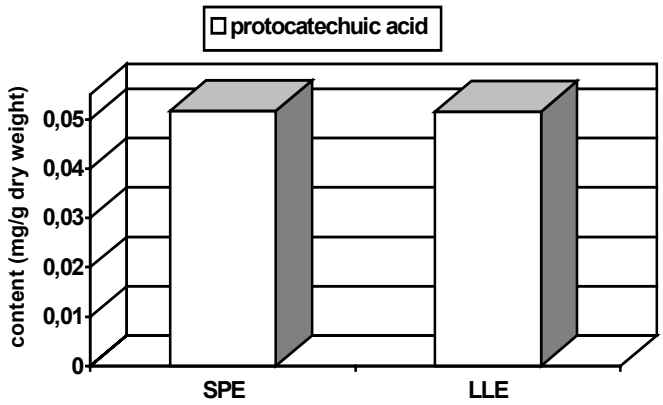

Fig. 3

Dependence on the method used (LLE or SPE) of the purification yield of protocatechuic acid from extracts of *Sambuccus nigra* L. inflorescence (obtained by MASE)

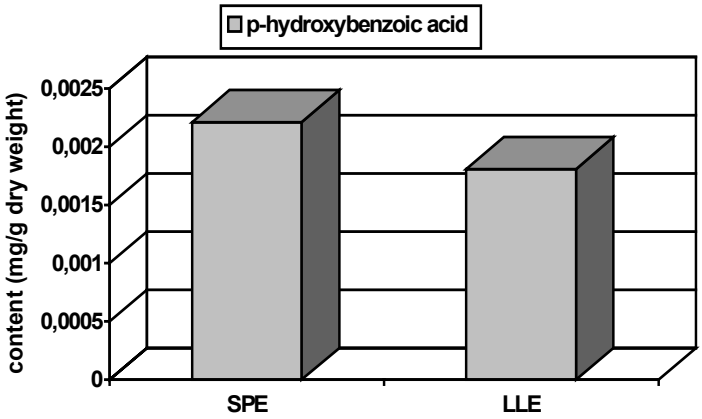

Fig. 4

Dependence on the method used (LLE or SPE) of the purification yield of *p*-hydroxybenzoic acid from extracts of *Sambuccus nigra* L. inflorescence (obtained by USAE at 60°C)

The benzoic acid derivatives gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, and syringic acids and the cinnamic acid derivatives caffeic and ferulic acids were identified in extracts of *Polygonum aviculare* plant. Although, again, the extracts obtained were quantitatively and qualitatively different, the results obtained by extraction using the different methods

were quite different from those obtained for *Sambucus nigra* inflorescence. For this plant material exhaustive extraction in Soxhlet apparatus resulted in poor yield whereas high extraction yield was achieved by use microwave-assisted techniques, especially MASE in a closed system. It should be borne in mind that this technique is unsuitable for extraction of non-polar furanocoumarins, because of possible isomerisation or decomposition of the hydrophobic compounds imperatorin and phellopterin and simultaneous high yield of the more polar xanthotoxin and bergaptene from *Archangelica officinalis* [14] and *Pastinaca sativa* fruit [15]. For the polar benzoic acid derivatives gallic, protocatechuic, and *p*-hydroxybenzoic acids it was a very effective method of extraction (Figs 5 and 6). The very low extraction yield of accelerated solvent extraction is apparent from these histograms whereas for *Sambucus nigra* inflorescence the yield of ASE was similar to that of the other modern extraction techniques. Figure 7 shows a typical chromatogram obtained from the phenolic acids fraction from *Polygonum aviculare*.

For extraction of ferulic acid from *Polygonum aviculare* foliage the most effective technique was ultrasonification (Fig. 6). It should also be mentioned that for the benzoic acid derivatives ultrasound-assisted solvent extraction (USAE) resulted in much higher yields of protocatechuic

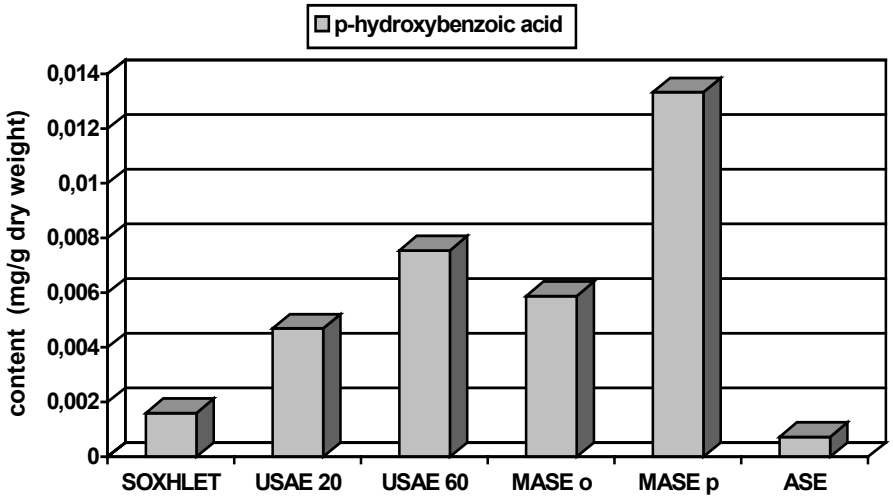

Fig. 5

Dependence on the method of extraction of the yield of *p*-hydroxybenzoic acid from *Polygonum aviculare* L. foliage

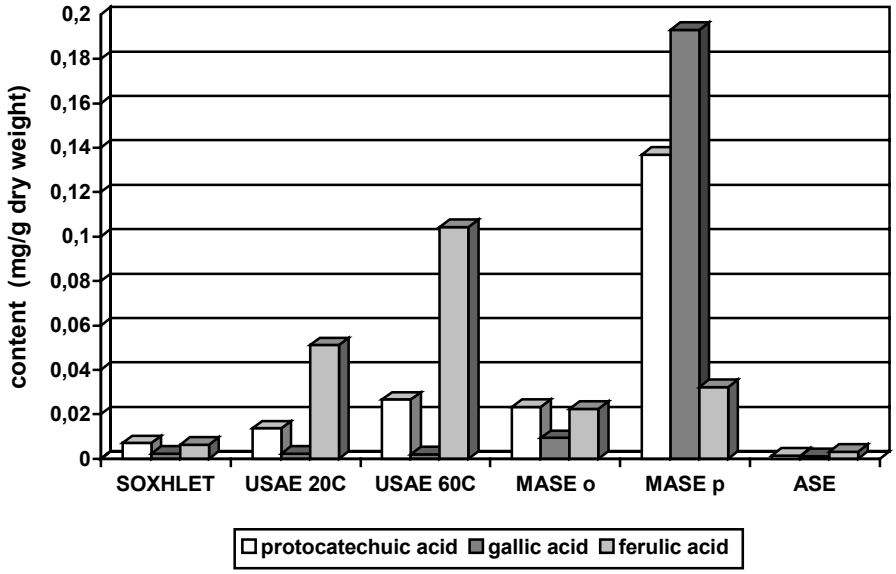

Fig. 6

Dependence on the method of extraction of yields of protocatechuic, gallic, and ferulic acids from *Polygonum aviculare* L. foliage

and *p*-hydroxybenzoic acids and similar yields of gallic acid as exhaustive extraction in Soxhlet apparatus.

One can attempt to explain the different yields of the methods used for extraction of phenolic acids from the different plant materials. The differences might be caused by the distinct cellular structures of flowers (for *Sambuccus nigra*) and foliage (stem and leaves, for *Polygonum aviculare*) and/or the different dimensions of the cells. Stems are largely composed of hard, mechanically resistant tissue, for example colenchyma, sclerenchyma, and elements of wood whereas leaves and flowers comprise mainly delicate parenchyma cells with large intercellular spaces. It is probable that destruction of the compact, hard structures of *Polygonum aviculare* stems and diffusion of solvent into this material requires more drastic extraction conditions than the delicate parenchyma cells of *Sambuccus nigra* flowers. Phenolic compounds can, moreover, form strong bonds with lignin, a component of the cell walls of stems and leaves. Such lignin complexes are difficult to break down, and then classic extraction methods are less efficient. In such circumstances techniques which destroy the cell structure – USAE and MASE – result in higher yields of the phenolic acids. For the delicate

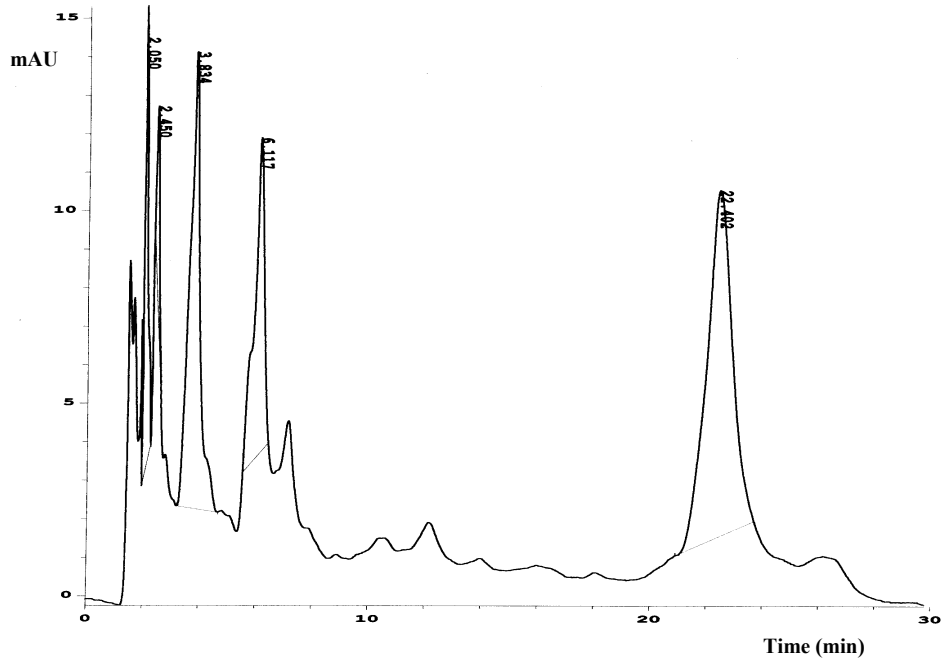

Fig. 7

HPLC chromatogram obtained from the phenolic acids fraction from *Polygonum aviculare* L. foliage extract. The phenolic acids in order of elution are gallic, protocatechuic, *p*-hydroxybenzoic, syringic, and ferulic acids (peaks with marked retention times)

parenchyma cells of *Sambuccus nigra* flowers the best yield is obtained by exhaustive Soxhlet extraction. The different silica content of plant tissues could also affect the extraction yield.

CONCLUSIONS

The qualitative and quantitative composition of extracts of *Sambucus nigra* inflorescence and *Polygonum aviculare* foliage, containing the phenolic acids fraction, depends on the method of extraction. The highest extraction yields of protocatechuic, *p*-hydroxybenzoic, vanillic, and ferulic acids from *Sambucus nigra* inflorescence were obtained by use of Soxhlet extraction. Ultrasonification, microwave-assisted solvent extraction, and accelerated solvent extraction result in similar extraction yield of phenolic acids from *Sambucus nigra* inflorescence. The highest extraction yields of protocatechuic, *p*-hydroxybenzoic, and gallic acids from *Polygonum avi-*

culare foliage were obtained by use of microwave-assisted extraction in a closed system. For extraction of ferulic acid from *Polygonum aviculare* foliage the highest yield was obtained by use of USAE. Ultrasonification resulted in higher yields of protocatechuic and *p*-hydroxybenzoic acids from *Polygonum aviculare* foliage than exhaustive extraction in Soxhlet apparatus; both techniques resulted in similar yields of gallic acid.

Similar yields were obtained when the purification methods LLE and SPE were investigated for these phenolic acids.

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