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A Comprehensive Analysis to Determine the Phytochemical Characteristics of *Fraxinus americana* and *Fraxinus excelsior* Leaves Extracts: Influence of the Extraction Method

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Abstract

A comprehensive investigation was carried out to determine the phytochemical properties of *Fraxinus americana* and *Fraxinus excelsior* leaves extracts. In addition, infusion extraction method using only water as the extraction solvent and ultrasound-assisted extraction technique were followed comparatively to extract the phenolic compounds from the leaves to estimate the efficacy of the traditional hot water infusion method. Initially, total antioxidant capacities, total phenolic contents, and radical scavenging activities of the extracts were determined. Afterwards, 34 potential phenolic compounds were analyzed by LC-ESI-MS/MS technique. The paper gives a comprehensive insight to the literature about the phytochemical properties of *Fraxinus americana* and *Fraxinus excelsior* leaves extracts.

Keywords: *Oleaceae*; Genus *Fraxinus*; Traditional preparation; Hot water infusion technique; LC-ESI-MS/MS

1. Introduction

Genus Fraxinus (family Oleaceae) contain significant amounts of phytochemicals aimed at scavenging the reactive oxygen species (ROS) causing important damage to cellular structures. This valuable content has led to intensive research in phenolic compounds in the leaves of Oleaceae family.

Natural products from plants have proven their worth as main sources of chemical compounds having medicinal properties. To this end, several studies were carried out to determine the phytochemical profiles of different plant materials [1,2].

People traditionally prefer the hot water infusion, which is the easiest way to extract the pharmaceutical properties of plants. On the other hand, more complicated extraction methods, such as microwave-assisted extraction (MAE) [3,4], ultrasound-assisted extraction (UAE) [5–7], supercritical fluid extraction (SFE) [8,9], accelerated solvent extraction (ASE) [10,11], soxhlet [12,13] and heat reflux extraction (HRE) [13] were all followed to extract the bioactive compounds (especially phenolic compounds) with a higher yield before analytical determination of the phenolic contents of plants. However, these extraction techniques were usually carried out using a mixture comprising methanol which is not suitable for human consumption to enhance the extraction efficiency.

In this study, infusion extraction (IE) and UAE techniques were followed to determine the antioxidant capacities, radical scavenging activities and phenolic profiles of leaves extracts of two different species of fraxinus (*Fraxinus excelsior* and *Fraxinus americana*). UAE was carried out using methanol/water mixture (70/30, v/v) which is an efficient aid to extract the bioactive compounds from different plant parts. Then the phytochemical contents of the leaves were determined by using the extracts obtained by UAE. Otherwise, IE was performed using only water. Therefore, the efficacy of the IE method was compared to the efficient UAE technique. For this purpose, leaves extracts of both species obtained by UAE and IE methods were firstly screened for their total antioxidant capacities (TACs), total phenolic contents (TPCs) and radical scavenging activities (RSAs) via CUPRAC, Folin-Ciocalteu and DPPH methods, respectively. Afterwards, liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) technique was used for scanning 34 individual phenolics in the extracts.

Present work is quite original in its nature since it provides information on the efficacy of the simple hot

water infusion method that is traditionally applied at homes compared to UAE method, which has proven to be effective extracting phenolic compounds from plant matrices. On the other hand, this is the first comprehensive report in terms of number of individual phenolics determined in two species of genus Fraxinus. It gives a comprehensive insight to the literature about the phytochemical properties of *Fraxinus excelsior* and *Fraxinus americana* leaves extracts.

2. Materials and Methods

2.1. Apparatus

A Jasco V-530 UV/Vis Spectrophotometer was used for the spectrophotometric measurements. Agilent 1260 LC system hyphenated to an Agilent 6420 Triple Quadrupole MS system was used to determine the individual phenolics.

2.2. Reagents

All commercial phenolic standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fluka (St. Louis, MO, USA) and HWI Analytik (Ruelzheim, Germany). Trolox, neocuproine, methanol and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate, copper (II) chloride, Folin-Ciocalteu reagent and formic acid were purchased from Merck (Darmstadt, Germany).

2.3. Plants

Fraxinus excelsior and Fraxinus americana leaves (Figure 1) were washed with distilled water, and dried in the dark. Dried plant leaves were kept at +4 °C. Dried plant material was powdered and sieved before the extraction.

Figure 1. The leaves (a) *Fraxinus excelsior*, (b) *Fraxinus Americana*

2.4. Extraction procedures

For UAE, sample powder (0.2 g) was extracted initially with 20 mL of methanol/water mixture (70/30, v/v) in ultrasonic bath for 60 min. Then, the procedure was repeated twice in a row using 20 and 10 mL of the same mixture for 45 and 15 min respectively to enhance the extraction efficiency. For IE, 50 mL of ultrapure water was added on 0.2 g sample powder and brewed for 1 hour at 95 °C.

2.5. LC-ESI-MS/MS technique

Two different chromatographic techniques were followed to determine 34 phenolic compounds in the leaves extracts. 31 of these compounds were scanned following the procedure described in our previous work [14]. On the other hand, oleuropein (a secoiridoid) and phenyl ethyl alcohols (hydroxytyrosol and tyrosol), which are specific groups of Oleaceae family, was determined by a separate method given below. The chromatographic column was Poroshell 120 EC-C18 (100 mm x 4.6 mm I.D., 2.7 µm). The mobile phase was made up from solvent A (5mM ammonium acetate solution) and solvent B (methanol). The gradient profile was set as follows: 0.00 min 5% solvent B, 2.00 min 25% solvent B, 4.00 min 50% solvent B, 6.00 min 95% solvent B, 8.00 min 95% solvent B, and 9.00 min 5% solvent B. The injection volume was 5.0 µL. The column temperature was 25 °C and the flow rate was 0.4 mL/min.

All the method details and LC-ESI-MS/MS chromatograms for the 31 phenolic compounds were provided in the previous work [14]. MS/MS parameters and retention times of each compounds determined by the second method proposed were provided in Table 1 and a representative LC-ESI-MS/MS chromatogram of the phenolics were represented in Figure 2.

2.6. Total phenolic content, cupric reducing antioxidant capacity, and DPPH radical scavenging activity assays

The Folin–Ciocalteu method [15] was followed to determine the TPCs of the extracts. Total antioxidant capacities (TACs) of the extracts were determined following the method, which is CUPRAC of Apak et al. [16]. The scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was used to determine the RSAs of the leaves extracts [17].

Table 1. MS/MS parameters for target compounds and retention times.

Compounds	R_t (min)	Precursor ion	MRM1 (CE, V)	MRM2 (CE, V)	Linear range (µg/L)	R^2
Hydroxytyrosol	5.667	153.0 $[M-H]^-$	123.0 (10)	94.9 (18)	25-500	0.9986
Tyrosol	6.263	137.0 $[M-H]^-$	119.1 (12)	105.8 (12)	25-500	0.9965
Oleuropein	6.941	539.2 $[M-H]^-$	377.1 (10)	275.1 (16)	25-500	0.9975

R_t, retention time; MRM, multiple reaction monitoring; CE, collision energy.

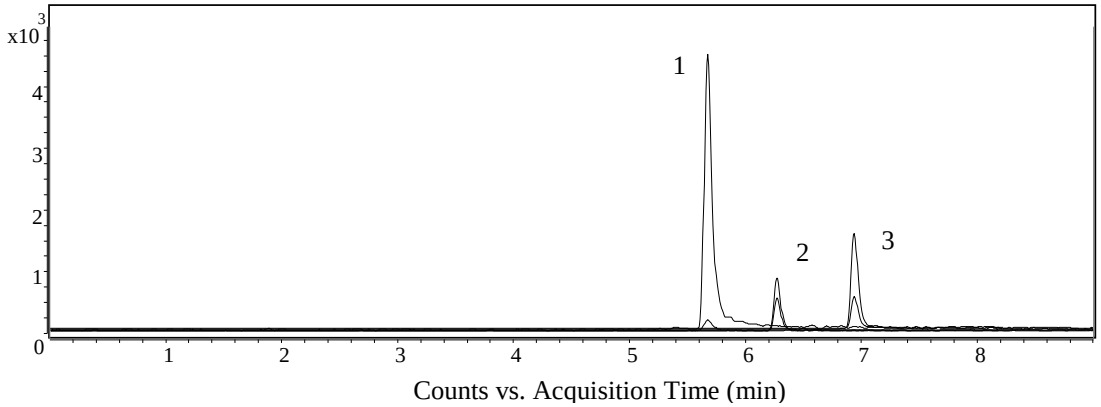

Figure 2. LC-ESI-MS/MS MRM chromatogram of the standard phenolic compounds. 1-3 represent the chromatograms of hydroxytyrosol, tyrosol and oleuropein, respectively.

3. Results and Discussion

TPCs, TACs and RSAs of the leaves extracts of both species were provided in Table 2. In all cases, the leaves extracts obtained by UAE showed higher antioxidant capacity and were found to be richer in terms of the phenolic compounds when compared to extracts obtained by IE. In addition, especially the results of the leaves extracts obtained by UAE technique which is more effective to extract phytochemicals from plant matrices showed that *Fraxinus americana* contains higher amount of phenolic compounds and has higher antioxidant capacity. In contrast, there was no significant difference in the RSAs of the leaves extracts of the species obtained by both extraction techniques.
LC-ESI-MS/MS method was used to determine the some potential phenolic compounds in both leaves extracts of the species. The phenolic compounds contents of the extracts were provided in Table 3. Among phenolics, 22 compounds were quantitatively determined with oleuropein as the dominant one. It is well known that secoiridoids are the most common compounds in Oleaceae [18]. However, their concentrations and those of their derivatives are also dependent on the season [19].

While the TPCs and TACs of the ultrasound-assisted extracts of the leaves of *Fraxinus excelsior* was lower (see Table 2), it was clear from Table 3 that the amount of oleuropein was determined higher in both leaves extracts of *Fraxinus excelsior*. This result reveals that *Fraxinus americana* leaves are richer in some other phenolic compounds (except for the 34 phenolic compounds involved in the study) showing antioxidant properties compared to *Fraxinus excelsior* leaves. The other dominant compounds in the extracts were verbascoside and hesperidin.

In concluding, oleuropein, tyrosol, hydroxytyrosol, protocatechuic acid, chlorogenic acid, 2,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, verbascoside, *p*-coumaric acid, ferulic acid, luteolin 7-glucoside, hesperidin, hyperoside, apigenin 7-glucoside and pinoresinol were determined in both leaves extracts of the species. In some cases, the IE technique provided a more efficient phenolic compound extraction from the leaves of the species compared to UAE. Especially, hydroxytyrosol and tyrosol (the phenyl ethyl alcohols) concentrations were higher in the both leaves extracts obtained via IE technique using only water as extractant. The results indicated that the hot water infusion technique is more efficient in extracting phenyl ethyl alcohols, which are more polar and relatively small phenolic compounds. Also, gallic acid and 3,4–dihydroxyphenylacetic acid were determined only in aqueous extract obtained by using IE technique. In contrast, quercetin was found only in methanolic extract obtained via UAE method. Finally, (+)-catechin, pyrocatechol, (−)-epicatechin, syringic acid, vanillin, taxifolin, rosmarinic acid, 3-hydroxybenzoic acid, 2-hydroxycinnamic acid, sinapic acid, eriodictyol and kaempferol were not detected in both extracts.

Table 2. TPCs, TACs and RSAs of *Fraxinus americana and Fraxinus excelsior* leaves extracts (n=3).

Species	Extraction technique	TPCs (mg GAE/g dry sample)	TACs (mg TE/g dry sample)	RSAs (mg TE/g dry sample)
Fraxinus americana	UAE	110.92±4.75	238.38±6.74	2.21±0.01
	IE	45.06±5.16	76.74±3.31	2.08±0.07
Fraxinus excelsior	UAE	70.21±3.64	138.45±5.07	2.19±0.04
	IE	46.45±4.49	91.42±2.66	2.08±0.07

Table 3. Quantitative results of phenolic compounds in *Fraxinus americana* and *Fraxinus excelsior* leaves extracts obtained by ultrasound-assisted extraction and infusion extraction techniques (n=3).

Compound	*Fraxinus americana*		*Fraxinus excelsior*	
	UAE (µg/g dry sample)	IE (µg/g dry sample)	UAE (µg/g dry sample)	IE (µg/g dry sample)
Oleuropein	27085±1896	17299±1211	55192±3863	29443±2061
Tyrosol	119±16	363±25	154±12	406±27
Hydroxtyrosol	161±19	496±27	118±15	392±26
Gallic acid	nd	1.0±0.1	nd	2.3±0.5
Protocatechuic acid	54.4±1.0	12.8±1.0	3.9±0.2	61.3±4.4
3,4 -Dihydroxyphenylacetic acid	nd	0.4±0.1	nd	1.6±0.1
(+)-Catechin	nd	nd	nd	nd
Pyrocatechol	nd	nd	nd	nd
Chlorogenic acid	265±16	963±52	1765±25	153±18
2,5-Dihydroxybenzoic acid	5.3±0.1	1.6±0.2	2.3±0.1	4.9±0.3
4-Hydroxybenzoic acid	1.7±0.1	3.5±0.1	3.0±0.1	2.3±0.1
(−)-Epicatechin	nd	nd	nd	nd
Vanillic acid	3.6±0.7	4.6±1.0	4.0±0.3	3.1±0.6
Caffeic acid	2.9±0.2	33.0±1.3	6.9±0.6	23.0±0.7
Syringic acid	nd	nd	nd	nd
3-Hydroxybenzoic acid	nd	nd	nd	nd
Vanillin	nd	nd	nd	nd
Verbascoside	18585±265	846±134	2809±79	6850±968
Taxifolin	nd	nd	nd	nd
Sinapic acid	nd	nd	nd	nd
p-Coumaric acid	5.3±0.3	10.9±0.3	4.9±0.3	9.5±0.6
Ferulic acid	2.9±0.1	11.4±0.8	9.0±0.4	4.5±0.3
Luteolin 7-glucoside	984±31	0.6±0.1	0.8±0.1	304±12
Hesperidin	14369±238	11086±1131	23691±1147	7092±306
Hyperoside	793±7.8	317±16	805±53	329±25
Rosmarinic acid	nd	nd	nd	nd
Apigenin 7-glucoside	372±51	0.8±0.1	1.0±0.1	156±18
2-Hydroxycinnamic acid	nd	nd	nd	nd
Pinoresinol	5.1±0.3	56.6±2.5	58.8±7.4	7.5±1.6
Eriodictyol	nd	nd	nd	nd
Quercetin	0.8±0.1	nd	0.9±0.1	nd
Luteolin	17.0±1.3	nd	nd	3.1±0.2
Kaempferol	nd	nd	nd	nd
Apigenin	7.8±0.5	nd	nd	1.6±0.2

4. Conclusion

Present work addressed two important issues. The first one was a detailed investigation in terms of number of

individual phenolic compounds in two species of genus Fraxinus. Twenty two compounds were identified and quantified in the extracts with oleuropein as the dominant one. The other important issue was the demonstration of the efficacy of the simple hot water infusion technique which can easily be applied at home. It was clear from the results that both extraction solvent and extraction technique play important roles in the extraction efficacy of the phenolics from different plant leaves. Although, higher amounts of oleuropein (approximately 2-fold) were extracted in all cases from the leaves of genus Fraxinus by using UAE method with methanol as the extractant, it was considered that the hot water infusion technique was also effective enough to extract phenolic compounds from the leaves. In addition, some relatively polar compounds (especially phenyl ethyl alcohols) were only determined in the extracts obtained by IE method using only water as the extraction solvent.

Author's Contributions

Buse Aydoğan: Performed the experiment and result analysis.

Mustafa Cittan: Drafted and wrote the manuscript, performed the experiment and result analysis.

Ali Çelik: Supervised the experiment's progress, result interpretation and helped in manuscript preparation.

Kenan Dost: Assisted in analytical analysis on the structure.

Ethics

There are no ethical issues after the publication of this manuscript.

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