Journal of Herbal Medicine 29 (2021) 100485

Contents lists available at ScienceDirect

Journal of Herbal Medicine

journal homepage: www.elsevier.com/locate/hermed

Research paper

Characterization of phenolic compounds isolated from the *Fraxinus angustifolia* plant and several associated bioactivities

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ARTICLE INFO

Keywords:
F. angustifolia
Flavonoids
Antioxidant
Cytotoxic
Colon cancer
Pancreatic lipase

ABSTRACT

In Algeria, *Fraxinus angustifolia* Vahl (Oleaceae) is a prominent ingredient for the treatment of inflammatory diseases caused by oxidative stress. This study aimed to make a comparison between the phenolic compound compositions of the ethanolic and aqueous extracts (decoction and infusion) of this plant and to test the antioxidant, cytotoxic and anti-pancreatic lipase activities of the ethanolic extracts from the leaf and bark. The identification and quantification of phenolic compounds was carried out by HPLC-DAD-MS and the antioxidant activity was assessed according to three methods: Ferric Reducing Antioxidant Power (FRAP), 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging activity and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity. The cytotoxic effect was investigated using the human colon adenocarcinoma cell line HCT-116; while pancreatic lipase inhibitory activity was examined using 4-nitrophenyl butyrate (NPB) as a substrate. A large variety of phenolic compounds were detected and the content of the leaf was much higher than that of the bark. The ethanolic extracts from the leaf had a higher antioxidant capacity than those of the bark, however, the latter showed greater antilipase activity and cytotoxicity than the former. These results suggest that this plant may be an important source of active compounds with a variety of biological activities which justify its use in traditional medicine.

1. Introduction

In recent years the role of certain plant secondary metabolites as protective dietary constituents has become an increasingly important area of research for human nutrition (Martins et al., 2015). In association with the well-known health benefits related to the consumption of fruit and vegetable-rich diets, research on the protective effects of plant-derived phenolic compounds (polyphenols) has been developed (Cesar, 2010). There is no doubt that among the large number of phenolic compounds found in plants, flavonoids play a central role (Grotewold, 2006). The benefits of these compounds have been proven through several studies focused on antioxidant, anti-cancer (Atmani et al., 2009), antiinflammatory, antiatherosclerotic, antithrombogenic, antiviral (Nijveldt et al., 2001), antihepatotoxic, antiallergic, and anti-ulcerogenic effects (Ghedira, 2005). With the increasing demand for herbal medicinal products and natural products for health care universally, medicinal plant extract manufacturers have started using the most appropriate extraction technologies in order to produce extracts of defined quality (Handa et al., 2008). Extraction by solvents using alcohol or water (infusion or decoction) are efficient extraction methods to recover flavonoids from medicinal plants but the amount of these compounds in each extract varies according to the plant (Martins et al., 2015).

The genus *Fraxinus* belongs to the family Oleaceae, which comprises of around 600 species in 25 genera (Wallander and Albert, 2000; Perez et al., 2005). The olive (Oleaeuropaea L.), cultivated for its fruit and oil, belongs to this family and has been studied extensively with regard to its chemical constituents. The compound classes that have been reported to be commonly associated with olives include iridoid glucosides, phenylethanoids, coumarins, and lignan glucosides (Bianco et al., 2001;

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https://doi.org/10.1016/j.hermed.2021.100485
Received 15 April 2019; Received in revised form 28 November 2020; Accepted 6 July 2021
Available online 15 July 2021

Jensen et al., 2002; Ryan et al., 2002; Cardoso et al., 2005). Some Fraxinus species are very typical of Sicily where they are cultivated to commercialize the sweet gum that the tree exudes. Sicilian gum, known as manna, is very rich in elenolic acid, tyrosol and hydroxytyrosol, demonstrating high antioxidant and antiinflamatory activities (Attanzio et al., 2019). Just as many species from *Fraxinus* plants have medicinal virtues, *Fraxinus angustifolia* Vahl is used in Algerian folk medicine. In particular, different plant parts have been used to treat many inflammatory diseases like rheumatism, arthritis and gout (Beloued, 1998). Despite the fact that the leaves and bark of *F. angustifolia* were reported to be used in decoctions and infusions as anti-rheumatism treatments and against hemorrhoids and fever (Baba-Aissa, 1999), there is no report about their bioactive compound composition.

To the best of the authors knowledge, this is the first report on the identification and quantification of different groups of phenolic compounds, including phenolic acids, flavonoid glycosides, secoiridoids, hydroxycoumarin and phenylethanoid in the ethanolic, infusion and decoction extracts from the leaf and bark of *F. angustifolia*. Studies carried out on *F. angustifolia* and other *Fraxinus* species such as *Fraxinus ornus* and *Fraxinus pennsylvanica*, focused on investigating the content of total polyphenols, flavonoids, tannins, and proanthocyanidins, using colorimetric methods with leaf and stem extracts (ethanol, methanol and water) (Touhami et al., 2017; Tahirović et al., 2017; Atmani et al., 2011). Ayouni et al. (2016) identified verbascoside, calceolariosides (A and B), oleuropein, kaempférol-(3-O-rutinoside), Quercetin 3-O-glucoside and rutin as the main antioxidant components in the leaf and bark of *F. angustifolia*. However, for the quantification, they also used colorimetric methods. The focus of this work was concentrated on extracting phenolic compounds from the *F. angustifolia* leaf and stem bark using ethanol and water, quantifying these compounds and then testing the antioxidant, cytotoxic and anti-pancreatic lipase activities of the ethanolic extract.

2. Material and methods

2.1. Chemicals and reagents

Authentic standards of rutin (quercetin-3-O-rutinoside), p-hydroxybenzoic acid, caffeic acid, ferulic acid, esculin, fraxetin, protocatechuic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH free radical), ferric chloride, 2,2′-dipyridyl (99 % minimum purity), Trolox (97 % purity), Porcine pancreatic lipase, p-nitrophenyl butyrate, and trichloroacetic, methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Quimica (Madrid, Spain) and, kaempferol-3-O-rutinoside (nicotiflorin), kaempferol-3-O-glucoside, hydroxytyrosol, fraxin, fraxidin, oleuropein, and verbascoside were purchased from Extrasynthese (Genay, France). All cell culture reagents were purchased from Gibco (Madrid, Spain). All solvents were of HPLC grade purity (Romil and Teknokroma, Barcelona, Spain). Pure deionized water was obtained from a Milli-Q50 system (Millipore Corporation, Bedford, MA).

2.2. Plant material

Leaves and bark of *F. angustifolia* were harvested in July 2015 from the province of Amizour, Northeastern Algeria. The genus and species of the tested plant were confirmed by a botanist affiliated with the laboratory of Botany and voucher specimens [PLM 120] have been deposited at the Herbarium of the Department of Biology, University of Bejaia, Algeria. Samples were air-dried and then ground to powder (< 63 μm) by an electric mill (KIKA Labortechnic, Staufen, Germany).

2.3. Preparation of F. angustifolia extracts

For infusion, a sample (2.5 g) was added to 100 mL of boiling distilled water, left to stand at room temperature for 20 min, and then the mixture was filtered under reduced pressure.

The decoction was prepared by adding 100 mL of distilled water to 2.5 g of the sample. The mixture was heated (heating plate, VELP scientific), boiled at 100 °C for 20 min and then filtered under reduced pressure.

For the ethanolic extract, plant material (4 g) was mixed with 100 mL of ethanol:water (96:4, v/v) in an Ultraturrax (Ultra- Turrax T25, Janke & Kunkel/IKA Labortechnik) for 1 min (at maximum speed). The hydroalcoholic extract was filtered through filter paper (Whatman nº 1) and the residue was extracted again under the same conditions.

For the same plant, two parts were used: leaves and bark. All plant extracts were made in duplicate and stored at −20 °C until analysis by HPLC.

2.4. Analysis and quantification of phenolic compounds by HPLC/DAD/MS

An HPLC Waters Alliance (Manchester, U.K.) system fitted to a Mediterranea Sea18 reverse-phase analytical column (25 cm length ×4.6 mm i.d., 5 μm particle size; Teknokroma, Barcelona) was used. An elution gradient was used with solvents A (water with 1 % formic acid) and B (acetonitrile with 1 % formic acid). The elution program was as follows (percentages refer to proportion of eluant B): 5–25 % (0–30 min); 25–50 % (30–45 min); 50–100 % (45–47 min); 100–25 % (47–50 min); 25–5% (50–52 min); 5 % (52–55 min). The flow was maintained at 1 mL min^{-1} and the column end was connected directly to a DAD (diode array detector) (Waters 996, Millipore, Manchester, U.K.), subsequently, a part of the flow (0,4 mL/min) was directed to an on-line quadrupole mass analyzer (ZMD, Micromass, Waters, Inc., Manchester, U.K.). Electron spray ionization (ESI) mass spectra were obtained at ionization energy of 70 eV, capillary voltage was 3 kV, desolvation temperature 120 °C, source temperature 80 °C and extractor voltage 12 V.

Stock standard solutions of each compound (chlorogenic acid, p-hydroxybenzoic acid, cafeic acid, protocatechuic acid, hydroxytyrosol, ferulic acid, esculin, fraxin, fraxetin, fraxidin, verbascoside, isoverbascoside, oleuropein) were prepared by dissolving 10 mg of analytical standard in 10 mL of 80 % ethanol. All solutions were stored at −20 °C. An intermediate solution containing all standard compounds (60 μg /mL) was prepared in 80 % ethanol, and dilutions from this solution were taken at different levels for calibration curves and validation experiments (matrix effects, precision and accuracy). Triplicate injections were made for each standard and sample. Analytes were identified by comparing retention time, UV and *m/z* values obtained by mass spectrometry (MS) with those of standards obtained under the same conditions. The calibration curves were used for quantitation. Peak areas were compared with calibration curves generated by three repeated injections of known standards at seven concentrations (20−60 μg/mL). Linearity ranges for calibration curves were determined.

2.5. Evaluation of bioactivity

2.5.1. Antioxidant and free radical scavenging bioassays

In order to establish a relationship between the phenolic compound composition and the antioxidant power of *F. angustifolia*, the aqueous and ethanolic extracts were evaluated for their ferric reducing antioxidant power (FRAP), soluble antiradical activity (DPPH) and ABTS scavenging activity.

2.5.1.1. Ferric reducing antioxidant power assay (FRAP). FRAP assay was performed using the method of Psarra et al. (2002), with slight modifications. In a 96-well microplate, 10 μL of each extract, standard and their dilution were mixed with 10 μL of 6 mM $FeCl_3$ (in 6 mM citric acid), then the microplate was set at 50 °C for 20 min. After this time, 180 μL of dipyridyl solution (5 g/l in 1.2 % trichloroacetic acid) was added and the mixture was incubated for 30 min at 37 °C, in the dark. A blank well without $FeCl_3$ was included and each assay was carried out in

quadruplicate. Absorbance was measured at 490 nm and the results were expressed as millimoles of Trolox equivalent (TE) per kilogram of sample.

2.5.1.2. DPPH free radical scavenging activity assay. A modification of the method described by Rodriguez et al. (2005), was used to evaluate the free radical scavenging activities of the extracts using a 96-well microplate. A solution of DPPH was prepared by mixing 3.8 mg of DPPH$^{\bullet}$ and 50 mL of methanol. Then, 5 µL of the extracts, the standard and their dilution were placed in wells with the addition of 195 µL of DPPH$^{\bullet}$ solution, after which the microplate was incubated at 37 °C for 30 min, in the dark. Each sample was repeated at least three times and a blank with only methanol was prepared in parallel. To calculate the efficient concentration (EC_{50}), which represents the amount of antioxidant necessary to decrease the initial absorbance by 50 %, a calibration curve with a linear regression for each antioxidant solution was used. The activity was expressed as millimoles of Trolox equivalent antioxidant capacity (TEAC) per kilogram of sample.

2.5.1.3. $ABTS^{\bullet+}$ scavenging activity assay. A radical cation $ABTS^{+}$ was prepared by mixing 7 mM ABTS stock solution with 2.45 mM of $K_2S_2O_8$ (1/1, V/V) and the solution was kept in the dark for 14–16 h. To obtain 0.700 ± 0.02 absorbance of $ABTS^{+}$ measured at 734 nm, the ABTS stock solution was diluted with 80 % ethanol. The reaction mixture consisted of 13 µL sample solution and 187 µL free radical solution. This mixture was then incubated for 6 min and the reading of the absorbance was registered at 750 nm (Re et al., 1999). Relative $ABTS^{+}$ scavenging percentages were calculated by the following equation:

% of inhibition = 100 × (1-A100 % /A_S). A 100 % :Absorbance of ABTS, A_S : Absorbance of sample.

2.6. Cell viability studies (MTT essay)

To study the cytotoxic activity of *F. angustifolia* ethanolic extracts, human colon adenocarcinoma cell line HCT-116 (ATCC, #CCL 247 Bethesda, MD, USA) were grown at 37 °C and in a humidified atmosphere of 5% CO_2 in air. McCoy's 5A medium supplemented with 10 % heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 g/mL streptomycin, was used as the growth medium. The cells were seeded in 96-well plates at a density of 10^4 cells/well, in order to have 70–80 % confluence. Cell viability was studied by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) colorimetric assay method described by Jaramillo et al. (2016) and all essays were carried out in three separate experiments. Microplates were incubated with plant extracts at indicated concentrations or with the vehicle (Dimethyl sulfoxide (DMSO) < 0.1 %) for 12 h, 24 h and 48 h. Then 200 µL of MTT solution was added to these microplates. After 3 h, the MTT was eliminated and replaced with 100 µL of DMSO < 0.1 % and the experiment was stopped after 1 h. The concentration was spectrophotometrically quantified at 570 nm with a microplate reader and the results were shown as percent of cell viability (100 % viability).

2.7. Pancreatic lipase inhibition

The anti-pancreatic lipase activity of *F. angustifolia* ethanolic extract was evaluated using 4-nitrophenyl butyrate (NPB) as substrate according to Jaradat et al. (2017), with some modifications. A stock solution of NPB (10 mM in dimethyl formamide) and six sample dilutions of 0, 2.5, 5, 7.5, 10, and 12.5 mg/mL (in Tris−HCl buffer 100 mM) were prepared. A stock solution of pancreatic lipase enzyme (20 mg/mL) was made immediately before being used. The experiment was performed using a 96-well microplate that contains 20 µL of lipase in each well, a volume of sample (0, 10, 20, 30, 40, 50 mL) corresponding to the previous concentration and finally, the volume was completed with Tris−HCl to 160 mL. The mixture was incubated at 37 °C for 30 min. Then, 20 µL of NPB was added and the microplate was again incubated for 30 min at 37 °C. A kinetic reading by a microplate reader at 415 nm for 15 min was carried out and the results were expressed as a percentage of inhibition as a function of the concentration. All assays were performed in triplicate and the calculated inhibition percentages were the mean averages of 3 observations.

2.8. Statistical analysis

All data values were expressed as mean ± standard deviation. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by Dunnett's test, using GraphpadInStat, version 5.03. The results were considered statistically significant at *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

3. Results and discussion

3.1. Characterization of phenolic compounds in leaf and bark extracts of *F. angustifolia*

The chromatograms of the ethanol, infusion and decoction extracts from the leaf and bark are shown in Figs. 1 and 2. Five phenolic compound groups corresponding to phenolic acids, flavonoids, coumarins, secoiridoids and phenylethanoid compounds were identified. These compounds were previously reported in the ash of *F. mandshurica*, *F. americana* and *F. pennsylvanica* (Eyles et al., 2007; Sanz et al., 2012), *F. excelsior* (Sanz et al., 2012) and *F. oxycarpa* (Jiménez-López et al., 2017). Their structures were proposed by comparison of the retention time, UV spectrum and product ion spectra in negative mode ($[M-H]^-$) with those of commercial standards. When standards were not available, tentative characterization was carried out by comparison of the experimental mass spectra with data from the scientific literature. The compounds were numbered in both chromatograms by their order of elution. The MS data are given in Table 1.

3.1.1. Coumarins

Four compounds were identified as hydroxycoumarins (7, 12, 14 and 17). Compound 7 exhibited an $[M-H]^-$ ion at *m/z* 339. After the neutral loss of 162 Da (hexoside), it presented a base peak at *m/z* 177, which corresponds to esculetin (main fragment ion at *m/z* 133), a fragmentation pattern is characteristic of esculin. This compound, which had been previously described in the phloem of Manchurian ash, *Fraxinus mandshurica* (Eyles et al., 2007), was confirmed by the comparison of the Rt, UV and mass spectra (of $[M-H]^-$) with those of the standard.

Compound 12, with a deprotonated molecular ion at *m/z* 369, was identified as fraxin, due to the fragment ions at *m/z* 207 [M−H-162] and 191 [M−H-CH3−162] (Godecke et al., 2005). This compound was also confirmed by comparison with commercial standards.

Compounds 14 and 17 were tentatively identified as fraxetin and fraxidin, respectively (Table 1). Fraxetin exhibited an $[M-H]^-$ ion at *m/z* 207, while fraxidin exhibited a $[M-H]^-$ ion at *m/z* 221. It was possible to identify each compound by comparing their UV spectra to those previously reported (Liu et al., 2005) and those of commercial standards.

3.1.2. Secoiridoids

Mass spectrum and fragmentation pattern, together with literature data, allowed for the identification of compounds 9, 24 and 25 as oleoside-type secoiridoids. Oleuropein (C compound 24) was identified by comparison with a standard, and this compound showed an $[M-H]^-$ ion at *m/z* 539 plus other ions at *m/z* 377, 307, and 275. Compound 9, with $[M-H]^-$ at *m/z* 389 and a base peak at *m/z* 345, was previously reported as oleoside (Cardoso et al., 2005; Jiménez-López et al., 2017). Compound 25 exhibited $[M-H]^-$ at *m/z* 523, and other fragment ions at *m/z* 361 (base peak) and 291, consistent with ligustroside.

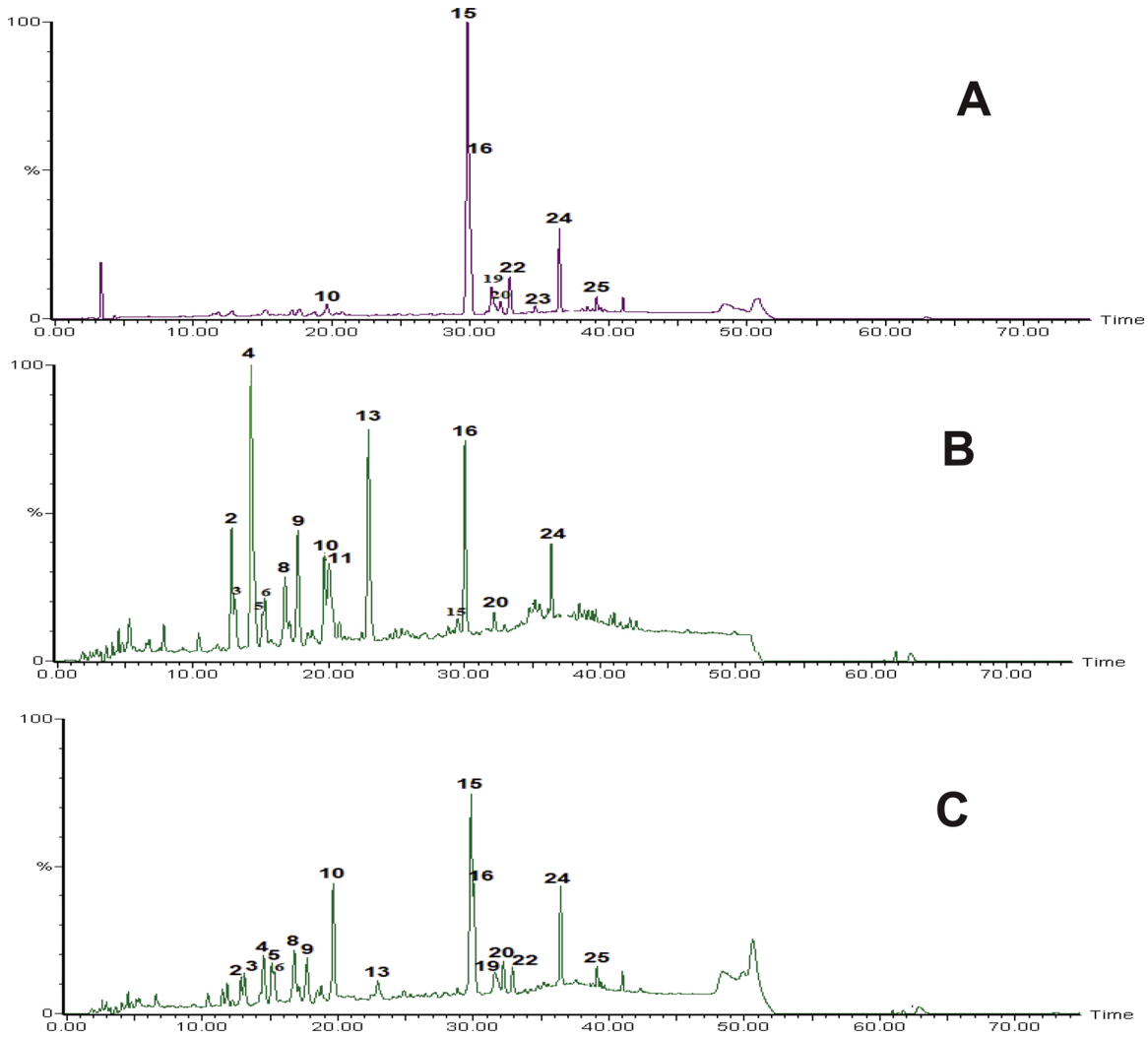

Fig. 1. Chromatographic profiles obtained from the HPLC-DAD of the leaf ethanolic extract (A), infusion (B), and decoction (C) extracts of *Fraxinus angustifolia*.

3.1.3. Phenylethanoids

Four compounds (16, 18, 20 and 21) were identified as phenylethanoid glycosides. Compound 16 and 20 were identified as verbascoside and isoverbascoside, respectively, by comparison with authentic standards. These two compounds were characterized by the $[M-H]^-$ ion at *m/z* 623 and showed two major ions at *m/z* 461 and 161.

Compounds 18 and 21 exhibited an $[M-H]^-$ ion at *m/z* 477 with almost identical UV and mass spectra, suggesting them to be isomers of the same compound. In both cases, their MS/MS spectra showed an $[M-H\text{-}162]^-$ ion at *m/z* 315, indicating the loss of a hexose moiety, and additional fragments at *m/z* 179 and 161 were found. This fragmentation pattern may be compatible with two calceolariosides. These phenylethanoids with $[M\text{-}H^-$, *m/z* 477] had been previously described in the ash of *Fraxinus excelsior* L. and *Fraxinus americana* L. (Sanz et al., 2012). In addition, the work of Nicoletti et al. (1988) and Ersöz et al. (2002) showed the 1H NMR and 13C NMR data to be identical to those reported for calceolariosides A and B, respectively.

3.1.4. Phenolic acids

Eleven phenolic acids were identified. Compounds 2, 4, 10, 11 and 13 were identified as hydroxytyrosol, protocatechic acid, chlorogenic acid, p-hydroxybenzoic acid and caffeic acid, respectively, by comparing the Rt, UV spectra and MS in negative mode to their authentic standards. Compound 1, with the $[M-H]^-$ ion at *m/z* 315, suffered the neutral loss of 162 Da, yielding *m/z* 153, corresponding to hydroxytyrosol. This fragmentation pattern is consistent with hydroxytyrosol-O-hexoside. In addition, compound 6, with the $[M-H]^-$ ion at *m/z* 353, yielded four peaks at *m/z* 191, 179, 173, and 135. The UV spectra and fragmentation pattern of these two compounds were compatible with chlorogenic acid but they had different retention times. This result indicated that compound 6 was a chlorogenic acid isomer. In addition, the negative ion mode spectrum of compounds (3, 5 and 8) presented deprotonated molecular ions at *m/z* 371. The ion at *m/z* 371 produced an ion at *m/z* 209 [M−H-162] resulting from the loss of hexosyl moiety. The loss of hexosyl moiety generated an ion at *m/z* 191 [hydroxyferulate-H2O]. These three corresponded to hydroxyferulic acidhexosides. Compounds 3, 5 and 8 were tentatively identified as hydroxyferulic acid hexoside 1, hydroxyferulic acid hexoside 2 and hydroxyferulic acid hexoside 3, respectively.

3.1.5. Flavonoids

Four flavonol-O-glucoside were identified in the leaf extracts of *F. angustifolia* by comparing the Rt, UV and MS (*m/z*) to those of commercial standards. Compounds 15, 19, 22, 23 were identified as rutin, quercitin-3-O-glucoside, nicotiflorin and kaempherol-O-glucoside, respectively.

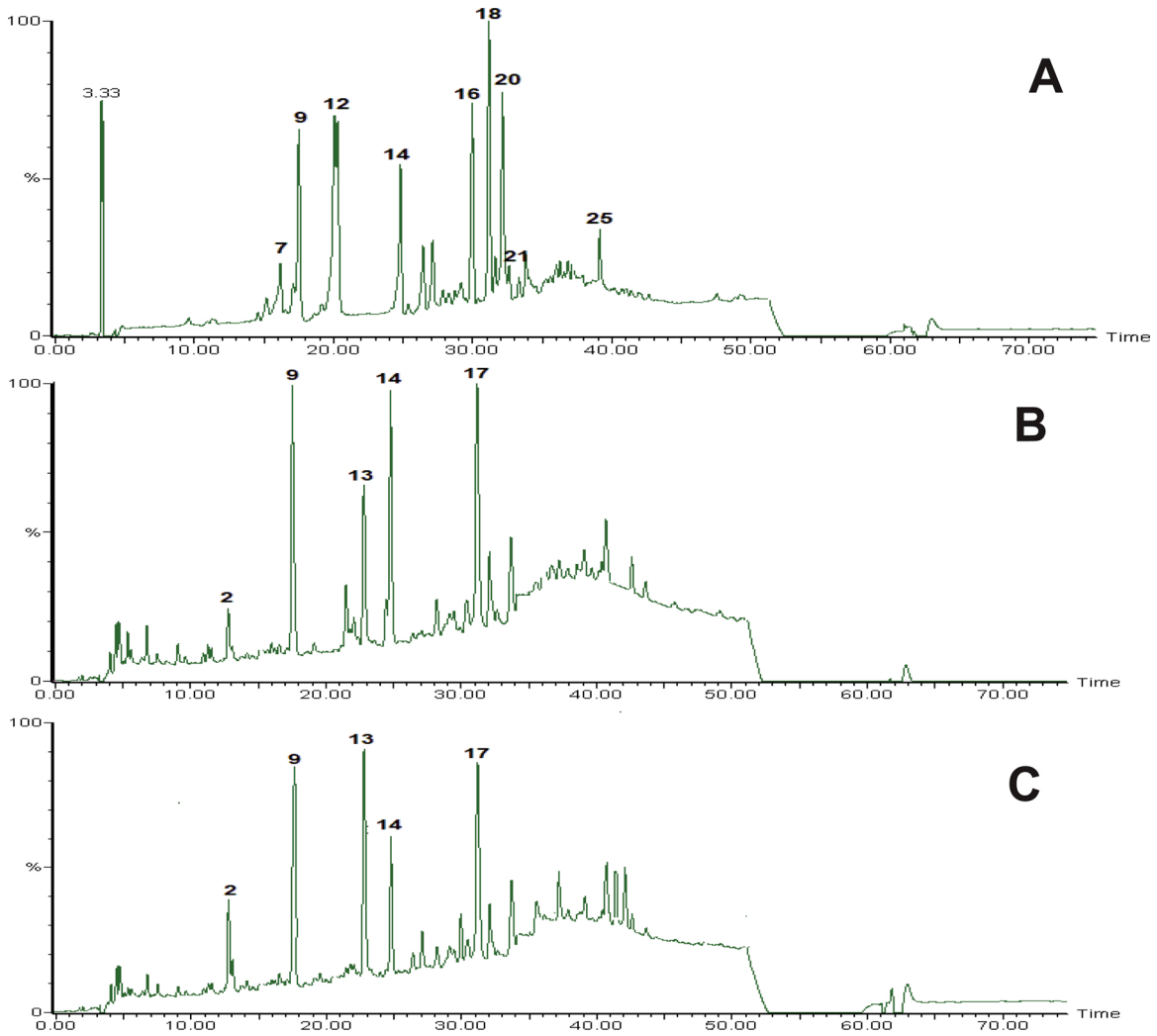

Fig. 2. Chromatographic profiles obtained from the HPLC-DAD of the stem bark ethanolic extract (A), Infusion (B), and decoction (C) extracts of *Fraxinus angustifolia.*

3.2. Comparison of phytochemical compounds in the leaf and bark extracts of F. angustifolia

Both qualitative and quantitative differences were observed among the two organs and the different extraction methods. Comparisons of 25 individual phenolic compounds revealed a number of differences in composition as listed in Tables 2 and 3. The contents of total phenolic compounds of the ethanolic extracts of leaf and stem bark were higher (24 and 17 g/kg DW, respectively) than the leaf and stem bark infusions (16 and 8 g/kg DW) and decoction extracts (19 and 8 g/kg DW), respectively. In fact, extraction yields depended on solvents used, vegetable matrix, duration and method of extraction with different physical and chemical parameters (Cowan, 1999; Akowuah et al., 2005; Naczk and Shahidi, 2004; Falleh et al., 2008).

Regardless of the extraction methods, the results clearly showed that the leaf was the organ which showed the highest level of phenolic compounds compared to the bark. In particular, the leaf was distinguished from the bark by the presence of flavonoids, mainly rutin (15), quercitin-3-O-glucoside (19), nicotiflorin (22), and kaempherol-O-glucoside (23), which were absent from the bark. Previous studies suggested the presence of a greater number of flavonoids in the leaf extracts of *F. angustofolia* than in the stem bark (Medjahed et al., 2016; Ayouni et al., 2016). Ten phenolic acids were present in the leaf, but only cafeic acid (13) and hydroxytyrosol (2) were found in the bark. Some simple phenolic compounds were reported to occur in *Fraxinus oxycarpa* (6-O-caffeoyl-β-D-glucopyranoside) (Hosny, 1998) and in the leaves of *F. angustifo*lia (caffeoylquinic acid and chlorogenic acid) (Falé et al., 2013). Fraxin (12), fraxetin (14), fraxidin (17), esculin (7), and calceolarioside A (18) were the only ones detected in the bark. The absence of coumarin in the leaf extracts was previously reported for the leaves of other *Fraxinus* species such as *Fraxinus ornus* and *Fraxinus pennsylvanica (*Tahirović et al., 2017*), Fraxinus excelsior* (Carnat et al., 1990) *and F. angustifolia (*Ayouni et al., 2016*)*. Finally, oleuropein (24) was only detected in the leaf extracts but not in the bark. In both organs, verbascoside (17), isoverbascoside (22) and ligstroside (26) were identified in high amounts.

3.2.1. Phenolic composition in leaf extracts

The percentage of each phenolic compound group in the different leaf extracts is illustrated in Fig. 3. In the leaf ethanolic extracts, flavonoids represented 61 % of the total contents of phenolic compounds, followed by phenylethanoids (21 %) and secoiridoids (15 %), while phenolic acid represented only 3%. Remarkably, in the infusion and decoction extracts of the leaf, phenolic acids were the predominant compounds representing 57 % and 33 % of the total phenolic compounds, respectively. The second major group of compounds was secoiridoids, which represented 25 % and 23 %, followed by phenylethanoid at 14 % and 20 % in the infusion and decoction extracts, respectively. Flavonoids represented the lowest percentage of the total phenolic compounds in the infusion (4%) and a higher amount in decoction, at around 24 %. Our results showed that among the different phenolic compounds, flavonoids and phenolic acid were highly affected

Table 1
Characterization of phenolic compounds from the leaf and stem bark extracts of *F. angustifolia.*

Peak	Compound	R_t (min)	$[M-H]^-$	λ_{max} (nm)	[M-H] Fragment m/z
1	Hydroxytyrosol-O- hexoside	11.84	315	255, 280	315, 153, 123
2	Hydroxytyrosol	12.86	153	256, 280	153, 123
3	Hydroxyferulicacidhexoside	13.13	371	289, 326	371, 209,191
4	Protocatechicacid	14.31	153	260, 293	153,109
5	Hydroxyferulicacidhexoside	14.54	371	289, 326	371, 209,191
6	Chlorogenicacid isomer1	15.13	353	294, 324	353, 191, 179
7	Esculin	16.19	339	292, 333	339, 391, 177, 147, 137
8	Hydroxyferulicacidhexoside	16.81	371	289, 326	371, 209,191
9	Oleoside	17.74	389	221,264	389, 345, 209
10	Chlorogenicacid	19.68	353	293, 326	353, 191, 179
11	P-Hydroxybenzoicacid	20.03	137	208, 255	137
12	Fraxin	20.15	369	292, 342	369, 207, 191, 354
13	Cafeicacid	22.96	179	288, 323	179, 147
14	Fraxetin	24.78	207	208, 333	207, 193, 109
15	Rutin	29.84	609	255, 354	609, 463, 303
16	Verbascoside	30.04	623	291, 328	623, 461, 161
17	Fraxidin	31.17	221	296, 341	221, 191, 163, 135
18	Calcelarioside A	31.18	477	289, 327	477, 315, 179, 161
19	Q-3-O-glucoside	31.59	463	253,351	463, 301
20	isoverbascoside	32.23	623	291, 328	623, 461, 161
21	Calcelarioside B	32.61	477	289,327	477, 315, 161, 135
22	Nicotiflorin	32.89	593	265, 348	593, 447, 285
23	K-O-glucoside	34.70	477	264, 348	447, 285
24	Oloropeine	36.44	539	236, 280	539, 377,307, 275, 223, 149
25	Ligstroside	39.13	523	240, 278	523, 361, 291, 223, 139

by the extraction methods and the solvent used. The decrease in the content of flavonoids detected in water extraction (infusion and decoction) may be partially due to the oxidation of this compound in the presence of water at high temperatures. Flavonoids are known to degrade in water at temperatures of 100 °C and above (Palma et al., 2001; Srinivas et al., 2011). The number and type of substituents as well as the position of the hydroxyl group affect the thermal stability of flavonoids, as compounds possessing a smaller number of substituents are less stable at high temperatures (Biesaga, 2011).

Table 2 shows the quantitative and qualitative differences in the composition of phenolic compounds among the different extraction methods from the leaf. Rutin (15) was the main compound in the ethanolic and decoction extracts. It represented 48 % and 18 % of the total contents of phenolic compounds, respectively. The amount of this

Table 2
Content of selected individual and total phenolic compounds in the ethanolic, infusion and decoction extracts of the leaf of *F. angustifolia.*

	Leaf		
	Ethanolic	Infusion	Decoction
Hydroxytyrosol hexoside	–	–	0.56 ± 0.04 3 %
Hydroxytyrosol	–	0.77 ± 0.01 5 %	0.63 ± 0.06 3 %
Hydroxy ferulic acid hexoside	–	0.39 ± 0.04 2 %	0.36 ± 0.03 2 %
Protocatechic acid	–	2.03 ± 0.04 12 %	0.77 ± 0.06 4 %
Hydroxy ferulic acid hexoside	–	0.59 ± 003 4 %	0.61 ± 0.02 3 %
Chlorogenicacid isomer1	–	0.41 ± 0.01 3 %	0.69 ± 0.01 4 %
Hydroxy ferulic acid hexoside	–	0.71 ± 0.04 4 %	0.65 ± 0.01 3 %
Oleoside	–	2.26 ± 0.02 14 %	1.36 ± 0.11 7 %
Chlorogenic acid	0.70 ± 0.08 3 %	1.78 ± 0.04 11 %	1.65 ± 0.03 9 %
P-Hydroxybenzoic acid	–	1.24 ± 0.17 8 %	–
Cafeic acid	–	1.44 ± 0.15 9 %	0.23 ± 0.01 1 %
Rutin	11.19 ± 0.34 48 %	0.67 ± 0.03 4 %	3.37 ± 0.13 18 %
Verbascoside	2.85 ± 0.15 12 %	1.53 ± 0.14 9 %	1.72 ± 0.20 9 %
Q-3-O-glucoside	0.94 ± 0.04 4 %	–	0.53 ± 0.06 3 %
isoverbascoside	2.05 ± 0.07 9 %	0.74 ± 0.05 5 %	2.06 ± 0.27 11 %
Nicotiflorin	2.04 ± 0.13 9 %	–	0.65 ± 0.04 3 %
K-O-glucoside	0.24 ± 0.02 1 %	–	–
Oleuropein	3.04 ± 0.18 13 %	1.91 ± 0.12 12 %	2.50 ± 0.04 13 %
Ligstroside	0.50 ± 0.01 2 %	–	0.54 ± 0.07 3 %
Total	23.55 ± 0.58a	16.47 ± 0.02c	18.89 ± 0.25b

Diff ;erent letters within the same row mean that there are significant diff ; erences ($P < 0.05$). nd, not detected. [a]Results correspond to the mean ± standard deviation of three replicates.

compound decreased significantly in the infusion extracts, where it represented only 4% of the total content of phenolic compounds. Makris and Rossiter (2001) and Fuentes- Alventosa et al. (2009), working with green asparagus, quantified a 43.9 % decrease in total flavonols when the asparagus was boiled for 60 min. Therefore, rutin (the major compound in Asparagus) must be oxidatively cleaved rather than hydrolyzed. Although hydrolysis might have occurred to some extent, the quercetin was oxidized as soon as it was liberated from the sugar and, thus, did not accumulate in detectable amounts. In addition, nicotiflorin and Q-3-O-glucoside represented 9% and 4% of the total phenolic compounds in the ethanolic extract and 3% in the decoction extracts, while they were not detected in the infusion extracts. K-O-glucoside was only identified in the ethanolic extract at a very low amount of around 1% of the total content of phenolic compounds.

Oleoside, oleuropein, protocatechic acid and chlorogenic acid were the predominant compounds in the leaf infusion extracts where they represented 14 %, 12 %, 12 %, and 11 % of the total phenolics respectively. These four compounds were also detected in the decoction extracts at 7%, 13 %, 4%, and 5%, respectively. However, among the four compounds, only oleuropein (13 %) and chlorogenic acid (3%) were detected in the ethanolic extracts. Verbascoside and isoverbascoside were identified in the different extraction methods, respectively; 12 % and 9% in the ethanolic extract; 9% and 5% in the infusion; and 9% and

Table 3
Content of selected individual and total phenolic compounds in the ethanolic, infusion and decoction extracts from the bark of *F. angustifolia.*

	Bark		
	Ethanolic	Infusion	Decoction
Hydroxytyrosol	–	0.59 ± 0.04 7 %	0.64 ± 0.11 8 %
Esculin	1.08 ± 0.01 6 %	–	–
Oleoside	2.27 ± 0.23 13 %	1.74 ± 0.07 21 %	1.61 ± 0.13 19 %
Fraxin	3.25 ± 0.1 19 %	–	–
Cafeicacid	–	1.51 ± 0.13 18 %	1.33 ± 0.11 16 %
Fraxetin	1.08 ± 0.12 6 %	1.69 ± 0.13 20 %	2.09 ± 0.05 25 %
Verbascoside	2.33 ± 0.10 14 %	–	–
Fraxidin	–	2.78 ± 0.30 33 %	2.80 ± 0.12 33 %
Calcelarioside A	2.71 ± 0.06 16 %	–	–
isoverbascoside	2.37 ± 0.04 14 %	–	–
Calcelarioside B	Trace	–	–
Ligstroside	1.90 ± 0.02 11 %	–	–
Total	16.99 ± 0.20a	8.31 ± 0.42b	8.47 ± 0.17b

Diff ;erent letters within the same row mean that there are significant diff ; erences ($P < 0.05$). nd, not detected. [a]Results correspond to the mean ± standard deviation of three replicates.

11 % in the decoction.

A very small amount of ligstroside was detected in the ethanolic and decoction extracts (2% and 3%) but not in the infusion extract. Caffeic acid, hydroxytyrosol, hydroxyferulic acid hexoside 1, hydroxyferulic acid hexoside 2, hydroxyferulic acid hexoside 3, and the isomer of chlorogenic acid were only detected in the infusion and decoction extracts with percentages between 3 % and 9 %. Hydroxytyrosol-O-hexoside was only detected in the decoction extract at a percentage of 3 %. In addition, p-hydroxybenzoic acid (8 %) was only found in the infusion extract.

3.2.2. *Phenolic composition in stem bark extracts*

In the bark extracts (Fig. 4), the composition of ethanolic extracts differed from those found in the infusion and decoction extracts. Phenylethanoids were the major compounds found in the ethanolic extract, which represented 44 % of the total phenolic compounds, followed by coumarins (32 %) and secoiridoids (24 %), while no phenolic acids were detected. By contrast, in the infusion and decoction extracts coumarins were the predominant compound (54 % and 58 %, respectively) followed by phenolic acid (25 % and 35 %, respectively) and secoiridoids (21 % and 23 %, respectively), yet no flavonoids were detected. Kostova and Iossifova (2007) showed that the main secondary metabolites isolated from the stem bark of *Fraxinus ornus* belonged to the groups of hydroxycoumarins, secoiridoids, phenylethanoids, and lignans.

The results presented in Table 3 show that the major compound in the ethanolic extract of the stem bark was fraxin, at about 19 % of the total content of phenolic compounds. In addition, calcelarioside A, verbascoside, isoverbascoside, oleoside, and ligstroside were also detected at 16 %, 14 %, 14 %, 13 %, and 11 %. Esculin and fraxitin were found in small amounts at around 6% for each compound. Esculin,

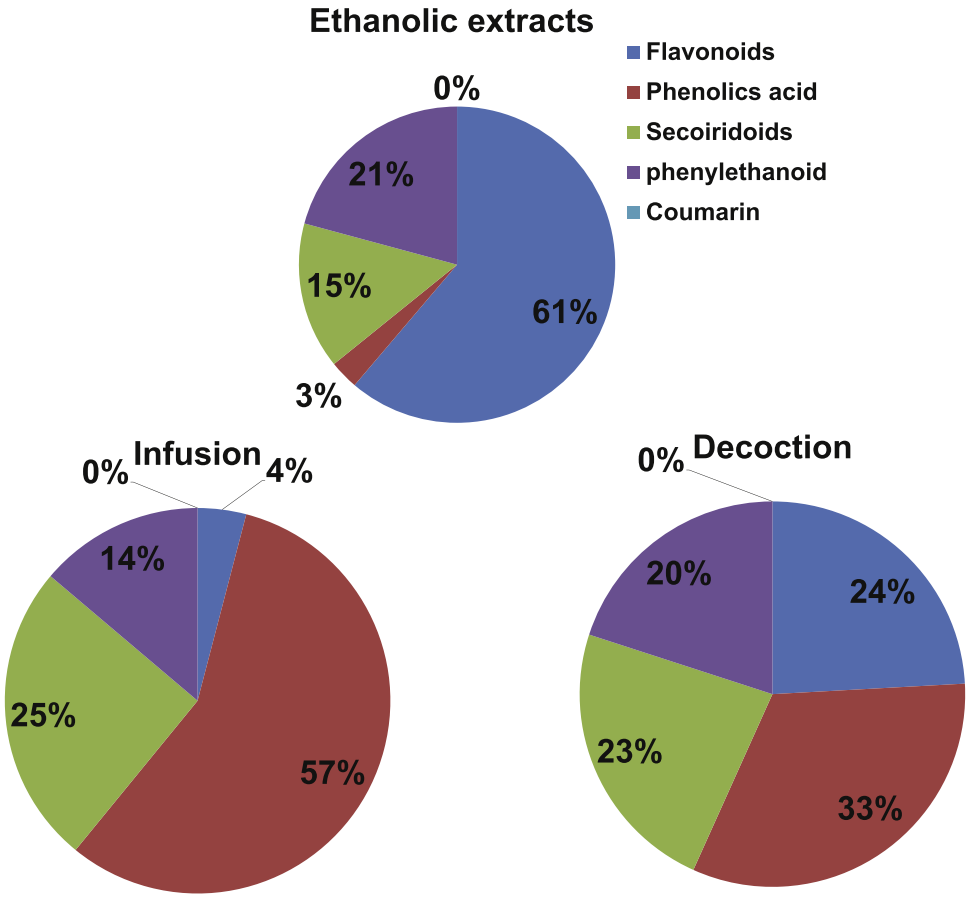

Fig. 3. Composition (%) of different groups of phenolic compounds detected in the ethanolic, infusion, and decoction extracts from the leaves of Fraxinus angustifolia.

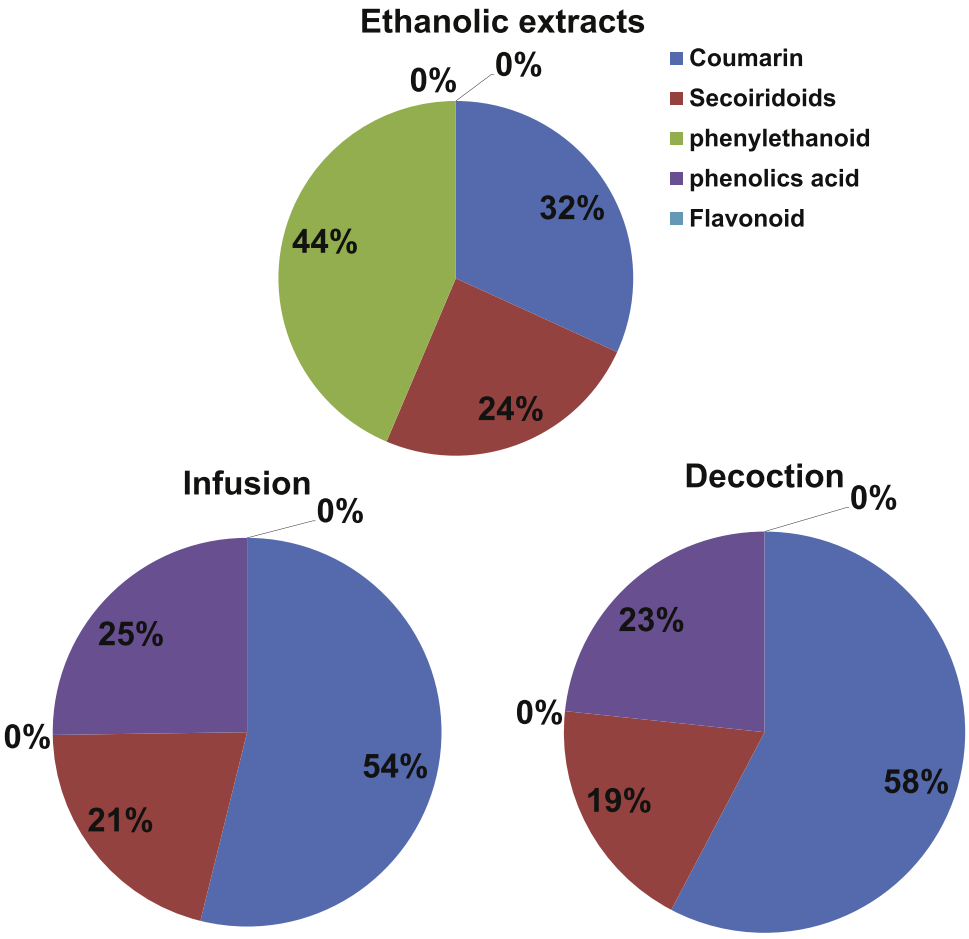

Fig. 4. Composition (%) of different groups of phenolic compounds detected in the ethanolic, infusion, and decoction extracts from the stem bark of *Fraxinus angustifolia*.

fraxin, verbascoside, isoverbascoside, and calcelarioside A were only detected in the ethanolic extracts but not in the infusion or decoction extracts.

In regards to the infusion and decoction extractions, 5 compounds were detected: cafeic acid, hydroxytyrosol, oleoside, fraxitin, and fraxidin. Among them, fraxidin was the main compound, representing 33 % of the total phenolic compounds. Fraxitin and oleoside were found in high amounts in the infusion (20 % and 21 %, respectively) and decoction extracts (25 % and 19 %, respectively); while caffeic acid and hydroxytyrosol showed the lowest percentages at 18 % and 7% in infusion extracts, respectively, and 16 % and 8% in decoction extracts, respectively.

3.3. Evaluation of radical-scavenging activities

The results in Table 4 show that the leaf ethanolic extract (100 mmolTrolox/Kg DW) exhibited significantly higher antioxidant activity than the ethanolic extract of the bark (75 mmol Trolox/Kg DW) by both DPPH and ABTS methods. However, they exhibited the same reducing power (61 and 65 mmol Trolox/Kg DW, respectively). The ferric reducing antioxidant power method predicts the presence of compounds with reducing capacity, which may indicate their antioxidant potential (Meir et al., 1995). In this study, the FRAP results indicated that *F. angustifolia* possessed compounds with reducing capacity, which may be potent antioxidants.

Table 4
Antioxidant activity of *F. angustifolia* leaf and bark ethanolic extracts.

	Leaf	Bark
DPPH (mmol Trolox/Kg dw)	99.92 ± 8.140	75.19 ± 0.280**
ABTS (mmol Trolox/Kg dw)	427.70 ± 1.930	358.54 ± 0.197***
FRAP (mmol Trolox/Kg dw)	60.82 ± 0.018	65.02 ± 0.022*

The antioxidant activities were determined by DPPH, ABTS, and FRAP assays. DPPH: DPPH radical scavenging activity; ABTS: ABTS+˙scavenging activity; FRAP: Ferric reducing antioxidant power. Results correspond to the mean ± SD. The values are significantly different *P < 0.05, **P < 0.01, and ***P < 0.001. The comparison is for different samples with the same method.

The high antioxidant activities of the leaf extracts against DPPH and ABTS may be due to the high content of flavonoid glycosides. Flavonoids have been proven to be excellent antioxidants, using a wide variety of *in vitro* and *in vivo* tests, and that their power is due to their ability to reduce free radical formation and to scavenge free radicals (Pietta, 2000; Atmani et al., 2009). In this study, the major flavonoid detected in the leaf ethanolic extract was rutin. The study by Ayouni et al. (2016) reported the strong involvement of rutin in the antioxidant activity of *F. angustifolia* leaf ethanolic extract. In addition, in accordance with the literature (Khatib et al., 2006; Dinda et al., 2011; Scognamiglio et al., 2014), two secoiridoids, oleuropein and ligstroside, were identified to be highly involved in the antioxidant activity of the leaves. However, in the bark extracts, the major compounds were phenylethanoides, which can be held responsible for the antioxidant activity of the bark. Ayouni et al. (2016) confirmed that phenylethanoids were identified as the major antioxidants in *F. angustifolia* stem bark extracts.

3.4. Pancreatic lipase inhibition

The pancreas synthesizes and secretes a lipolytic enzyme called pancreatic lipase (PL), which plays a key role in the efficient digestion of triglycerides. PL is responsible for the hydrolysis of 50–70 % of total dietary fats (Bustanji et al., 2011). PL inhibition is one of the most widely studied mechanisms for the determination of the potential efficacy of natural products as antiobesity agents (Jaradat et al., 2017).

The ethanolic extracts from the leaves and bark of *F. angustifolia* were tested for their porcine pancreatic lipase inhibition at different concentrations (0–12.5 mg/mL). The IC_{50} values for the plant extracts were calculated and the percentage of lipase inhibition was plotted, as shown in Fig. 5. The activity of lipase decreased by increasing the concentration of both extracts. The results shown in Fig. 5 indicate that at the highest concentration of 12.5 mg/mL, the bark extract inhibited 60 % of the lipase activity (IC_{50} value equal to 9.14 ± 0.98 mg/mL) while the leaf extract inhibited only 37 % (IC_{50} equal to 15.54 ± 3.06 mg/mL). These results suggested that the bark of *F. angustifolia* can be considered a particularly valuable source of effective anti-lipase substances. These results are consistent with those of Ahn et al. (2012), which showed that the stem bark of *F. rhynchophylla* might be beneficial in the treatment of obesity through the inhibition of pancreatic lipase, due to its active components coumarins, secoirioids and sesquilignans. Several phenolic compounds are known for their inhibitory effect against pancreatic lipase.

3.5. Cytotoxic activity

The ethanolic extracts from the leaves and bark of *F. angustifolia* were tested for their cytotoxic activities using the MTT assay. The HCT-116 cells were exposed to different doses of extracts (0–200 µg/mL) during intervals of 12, 24, and 48 h, and the results are summarized in Fig. 6.

The leaf ethanolic extract did not exhibit any significant cytotoxic effects, even after 12 and 24 h of incubation at low concentrations, whereas the same extract induced a significant decrease in the percentage of cell viability at 150 and 200 µg/mL from 84 % ($p < 0.001$) to 61 % ($p < 0.001$), respectively, after 48 h of incubation (Fig. 6A). As shown in Fig. 6B, the incubation of HCT-116 with different concentrations of bark ethanolic extracts exhibited a significant ($p < 0.001$) decrease in cell viability at 50 µg/mL and this activity started at 12 h and persisted until 24 h of incubation. The best cytotoxic activity was recorded at 12 h with the concentration of 200 µg/mL, which decreased the percentage of viability to 40 %. On the other hand, only the concentration of 200 µg/mL bark ethanolic extract was significantly ($p < 0.001$) cytotoxic against HCT-116 colon cancer cell lines with a percentage of viability of 56 % for a period of 48 h.

The concentration that inhibited 50 % cell growth (IC_{50} value) is a suitable parameter to compare the cytotoxic activity of the ethanolic extracts against the HCT-116 cells. These results indicated that the leaf crude extract was not cytotoxic for this line of cells at any of the concentrations tested (0–200 µg/mL) with IC_{50} values of 278 µg/mL, for the three time intervals studied. In comparison with the results of the leaf ethanolic extract, the IC_{50} value for the bark ethanolic extract was 85 µg/mL after 12 h and increased after 24 h and 48 h of incubation to 232 µg/mL. The latter result suggests that the bark ethanolic extract of *F. angustifolia* has a low concentration and short-term cytotoxic effect (12 h). Many studies have reported that plants belonging to genus *Fraxinus* have the ability to inhibit carcinogenesis by targeting various signaling network proteins associated with tumor cell multiplication (Sarfraz et al., 2017). It has been proven that two species from the *Fraxinus* genus have a cytotoxic effect, *in vitro*, against colorectal cancer cells: *Fraxinus sieboldiana* (HCT-8 cells) (Lin et al., 2007), and *Fraxinus excelsior* (SW742 cells) (Sardari et al., 2009).

4. Conclusion

In this study, several bioactivities of phenolic compounds, including antioxidant, cytotoxic and anti-pancreatic lipase, isolated from *F. angustifolia* extracts, were investigated. The results of the phytochemical screening showed a difference in composition between the two organs, bark and leaf, and the different methods of extraction. For both organs, ethanolic extracts exhibited the highest content of phenolic compounds. Five phenolic compound groups, flavonoids, phenolic acids, coumarins, secoiridoids and phenylethanoids were detected. The leaf ethanolic extract was rich in flavonoids with rutin being the major compound. However, phenolic acids were more easily extracted in the aqueous extract. Concerning the bark extract, no flavonoids were detected and the predominant compounds were phenylethanoids, such as isoverbascoside in the ethanolic extract and ligstroside in the infusion and decoction extracts. The ethanolic extracts of the bark and leaf of *F. angustifolia* were screened for their biological activities. The leaf extracts exhibited the highest antioxidant activity against DPPH and ABTS.

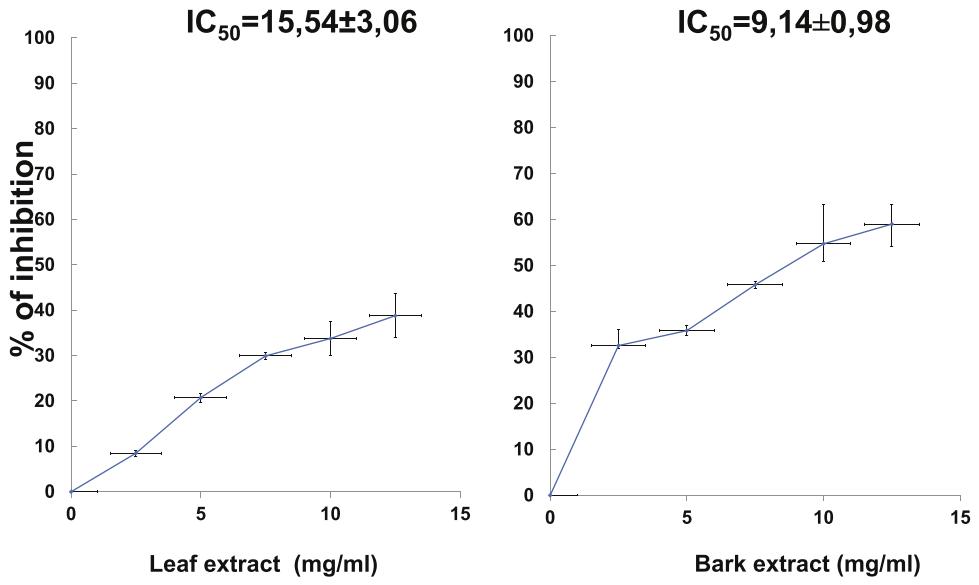

Fig. 5. The inhibitory effects of the leaf (A), and bark (B) ethanolic extracts of *Fraxinus angustifolia* on the activity of porcine pancreatic lipase. The activity was expressed as IC_{50} mg/mL of dry weight. Results correspond to the mean ± SD.

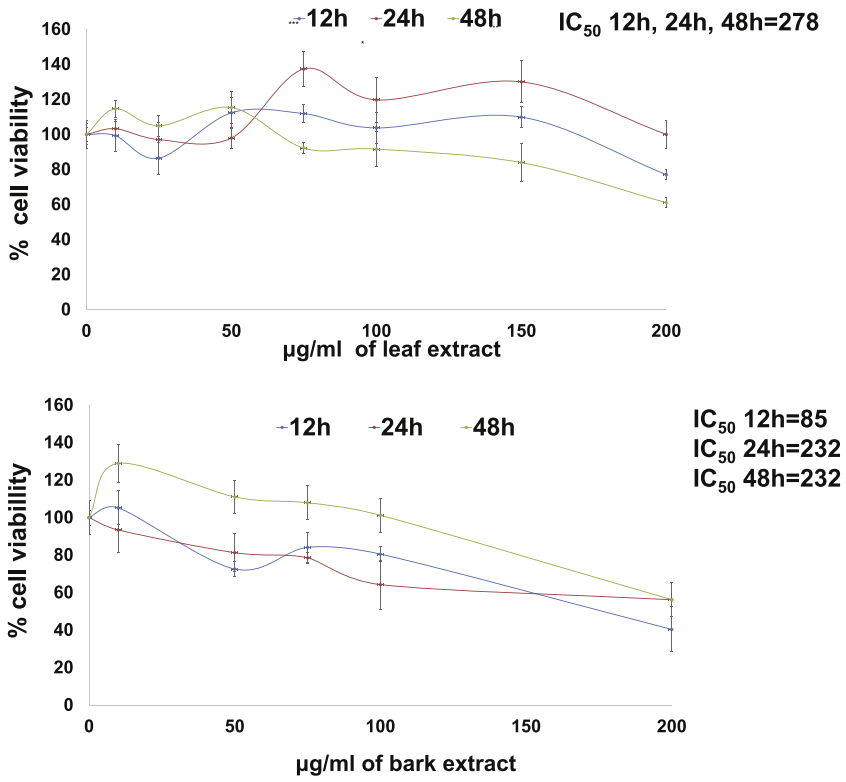

Fig. 6. Cell viability (%) of the HCT-116 cancer cell line after 12, 24, and 48 h treatment with various concentrations of leaf (A), and bark (B) ethanol extracts of *F. angustifolia*. The effects were measured by MTT cell viability assay and the cytotoxic activities are expressed as IC_{50} µg/mL of dry weight. Results correspond to the mean ± SD of four replicates.

Furthermore, it was found that the bark extract had better cytotoxic and anti-pancreatic lipase effects than the leaf extract. The results of this work suggest that using environmentally sustainable solvents it is possible to develop extracts from the *F. angustifolia* plant that can be applied in different commercial sectors such as food, cosmetics and pharmaceuticals. On the one hand, the extracts can be used as antioxidants of natural origin that replace synthetic antioxidants in many food and cosmetic applications. They could also be considered as a good alternative in the treatment and prevention of obesity. Finally, the *F. angustifolia* plant could be an important source of interesting molecules for the field of cancer prevention and treatment.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgment

This work was supported by the Spanish Ministry of Science and Innovation (AGL2017-82428-R).

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