

# Isolation and seasonal variation of fruticin in fruits of false indigo-bush (*Amorpha fruticosa* L. Fabaceae) from Serbia

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Received: April 3, 2020

Accepted: May 19, 2020

Published on-line: September 14, 2020

Published: December 25, 2020

Fruticin (amorphin) is a constituent of the fruit of the false indigo-bush (*Amorpha fruticosa* L.), which belongs to the class of rotenoid glycosides, and shows several interesting pharmacological activities. The aim of this study was to isolate and chemically characterize this natural product, as well as to determine the optimal period of the year for *A. fruticosa* fruits collection. Fruticin was obtained by re-crystallization of the precipitate that formed after partial evaporation of the extract, prepared by 3-fold extraction of powdered plant material by chloroform - ethanol (1:1, v/v). The structure of the final product was determined by various techniques of instrumental analysis (NMR, UV and MS), and confirmed by comparing the obtained spectra with corresponding data in available literature. The content of fruticin in *A. fruticosa* fruit was determined by LC-DAD-MS, using the external standard method based on the constructed calibration curve. Limits of detection (LOD) and quantification (LOQ) were also determined. A substantial increase in fruticin content was observed during the ripening period (>50 %). It has also been established that the optimal time for fruit collection is mid-December. Obtained results indicate that the content of fruticin in the *A. fruticosa* fruit is highly dependable on the time of collection. Since the biosynthesis of secondary plant metabolites is influenced not only by the time of collection, but by numerous other factors as well, additional studies are needed to define, with greater certainty, the conditions that are necessary for design of prospective efficient and sustainable production process.

**Key words:** *Amorpha fruticosa* L., fruticin, isolation, chemical characterization, seasonal variation

<http://dx.doi.org/10.5937/leksir2040052S>

## 1. INTRODUCTION

*Amorpha fruticosa* L. (Fabaceae), also known as false indigo-bush or desert false indigo, is a shrub autochthonous to North America. It was first introduced in Europe by the beginning of the 18th century as a horticultural plant esteemed for its ornamental qualities, as well as a melliferous species. Due to its strong and well-developed root system, *A. fruticosa* has been planted as a protection against erosion and along the railroads to firm up the railway embankments (Krpan et al., 2011). *A. fruticosa* blooms in late spring till early summer. In early autumn, *A. fruticosa* bears fruit regularly and abundantly. Its fruits are small (up to 1 cm long) warty pods, containing one seed each. In addition to generative reproduction, it also propagates by vegetative multiplication through rootstock shoots. It is best suited to neutral, slightly acidic or alkaline, moist soils and grows near water, along the river banks, where from it spreads by water (seed pods are buoyant), especially

during floods (Krpan et al., 2011).

Thanks to specific biological properties and ability to easily adapt and invade disturbed ecosystems of lowland floodplain forests, pastures, meadows or vacant soils, *A. fruticosa* escaped cultivation and expanded around the globe (Hulina, 2010). Today, it is recognized as one of the most invasive species for Europe, with serious deleterious effects on local autochthonous lowland flora (Krpan et al., 2011). As a heliophilic plant, it competes for the light and forms dense thickets that overshadow and suffocate the native flora, changing successional patterns and reducing biodiversity (Kozuharova et al., 2017; Krpan et al., 2011; Szigetvári, 2002). It is also widespread in Serbia, especially in Vojvodina, Mačva, and wider Belgrade area (Lazarević et al., 2012; Radulovic et al., 2008). Bearing in mind the damage that this plant causes not only to floristic diversity and forestry, but also to water management, drainage systems, roads etc., its presence should be reduced, or con-

trolled by any cost. So far, the methods of biological (Gagić et al., 2008), chemical (Blagojević et al., 2015) and mechanical (Szigetvári, 2002) control of the distribution of this plant have been proposed, but with certain limitations.

For that reason, some alternative approaches to the problem of *A. fruticosa* control have recently emerged, with focus on the possibilities of the employment of this plant, rather than its eradication (Ciuvăţ et al., 2016; Kozuharova et al., 2017). *A. fruticosa* has a great bio-pharmaceutical relevance, since it is recognized as a rich source of bioactive compounds with a high diversity of pharmacological activities. Its constituents are secondary metabolites of different chemical nature, such as stilbenoids (amorfrutins), flavonoids and their derivatives, including the subgroups of flavanones, isoflavones, rotenoids, as well as terpenoids (Kozuharova et al., 2017; López Díaz, 2016; Muharini, 2016). These constituents exhibit different activities and mechanisms of action, including PPAR $\alpha$ / $\gamma$  agonist effect, cytotoxicity, inhibition of TNF- $\alpha$  and NF- $\kappa$ B, antimicrobial (antiviral, antibacterial and antifungal) activity, etc. Wide spectrum of pharmacological activities make this plant stand out due to its potential application in the treatment of different pathologies, such as diabetes mellitus, some types of cancer, as well as various infections (Kozuharova et al., 2017; López Díaz, 2016; Muharini, 2016).

In other terms, if recognized as an inexpensive source of pharmacologically active compounds for pharmaceutical and related industries, this harmful weed could be eventually brought under the sustainable control. In this context, the aim of this paper is to present the results of investigations on isolation and chemical characterization of fruticin (amorphin), a rotenoid glycoside known as the most abundant constituent of *A. fruticosa* fruits, in an attempt to better understand its pharmacological values.

## 2. MATERIALS AND METHODS

### 2.1. Plant material

The plant material (fruits, in different stages of maturity) was collected in the Autumn 2019, from a meadow at Kumodraž (southern suburbs of Belgrade). Sample 1 (500 g) was collected in September 2019 and used for the extraction of fruticin. Samples 2-7 (approximately 100 g per sample) were collected in 14-days intervals starting from the beginning of September 2019 and used for the quantification of fruticin. Each sample has been dried at the room temperature, for 7 days. Identification of the plant was performed in the Department of Botany, Faculty of Pharmacy, Belgrade where a voucher specimen was deposited (No. 4206HFF).

### 2.2. Isolation of fruticin

Ground and sifted pods of *A. fruticosa* (100,2 g) were extracted as described by Khodzhaev et al. (1982) by 3-fold digestion with the mixture of chloroform-ethanol (1:1, v/v) for 5,5 h, 4 h and 2,5 h. Combined extracts were reduced by vacuum evaporation to approximately 1/10 of the starting volume and left aside overnight. Resulting precipitate was separated off using a suction filter, suspended three times in chloroform (the solvent being removed by suction each time) and the product was well dried in vacuo. Finally, it was re-crystallized, first from boiling water, then from ethanol, dried and accurately weighed.

### 2.3. Chemical characterization

NMR spectra ( $^1\text{H}$ , 400 MHz;  $^{13}\text{C}$ , 101 MHz) were recorded on Bruker AscendTM 400 instrument. Prior to analysis, the sample of fruticin was dissolved in deuterated dimethyl sulfoxide (DMSO- $d_6$ ). Chemical shifts ( $\sigma$ ) were expressed as ppm, and coupling constant (J) in Hz. As internal standard,

tetramethylsilane was used ( $\sigma = 0$  ppm). UV and mass spectra of fruticin were obtained using Agilent 1260 Infinity liquid chromatograph, equipped with a diode array and a single quadrupole mass detectors, after a chromatographic separation as described in the section 2.4.3.

## 2.4. LC-DAD-MS analysis

### 2.4.1. Construction of calibration curve and determination of the limits of detection and quantification

A quantity of 5.1 mg of fruticin was accurately weighed and dissolved in LC-MS grade methanol with the aid of ultrasonication. The resulting solution was transferred to a volumetric flask (5 mL) and made up to the marking. The prepared stock solution (1.02 mg/mL) was used to make a series of dilutions whose concentrations ranged between 10.2 and 680  $\mu\text{g/mL}$ . The limit of detection (LOD) and limit of quantification (LOQ) were determined. LOD is the lowest concentration of analyte in a sample that can be detected, while LOQ could be defined as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. LOD and LOQ were determined by calculating the standard response deviation (Sd) and the slope of the calibration curve (a) using following expressions (Malenović and Stojanović, 2010):

$$\text{LOD} = 3.3 \times \frac{Sd}{a}$$

$$\text{LOQ} = 10 \times \frac{Sd}{a}$$

### 2.4.2. Preparation of extracts

The extracts for fruticin assay were prepared essentially as described by Genkina et al. (1971). In brief, approximately 1 g of powdered herbal substance was weighed accurately, macerated 24 h with methanol (25 mL) and further shaken for 4 h at the room temperature. Prior to LC-DAD-MS analysis, the extract was filtered and quantitatively transferred into a volumetric flask. Each extract was prepared in triplicate.

### 2.4.3. Fruticin assay

Chromatographic separation was performed on reverse-phase analytical column Zorbax SB-Aq (3.0 $\times$ 150 mm, stationary phase particle size 3.5  $\mu\text{m}$ ), at 25  $^\circ\text{C}$  and mobile phase flow rate of 0.35 mL/min ((injection volume: 3  $\mu\text{L}$ ; phase A - 0.1 % solution of formic acid in water; phase B - acetonitrile). The mobile phase gradient was programmed as follows: 25 % B (0 min), 90 % B (30 min), 90 % B (36 min), 25 % B (38 min), 25 % B (40 min). The chromatograms were recorded at 296 nm. Mass spectra were recorded at a mass range of 100 - 1000  $m/z$ . Electrospray ionization was performed in negative mode under atmospheric pressure ( $\text{N}_2$  flow 10 L/min, nebulizer pressure 40 psig,  $\text{N}_2$  temperature 350  $^\circ\text{C}$ , capillary voltage 3500 V; the molecular ion was obtained at a fragmentation voltage of 100 V). Identification of fruticin in analyzed extracts was performed by comparison of retention times (Rt) and UV spectra of present constituents with Rt and UV spectrum of previously isolated fruticin, which were obtained under the same chromatographic conditions (Claisse et al., 1964). Fruticin was quantified by the external standard method at 296 nm.

## 3. RESULTS AND DISCUSSION

The structure of fruticin was determined on the basis of good fit of the obtained  $^1\text{H}$  and  $^{13}\text{C}$  NMR, UV and MS spectral data with data from the literature and calculated molecular weight (Crombie et al., 1975; Li et al., 1993; Liang et al., 2015; Sariaslani and Rosazza, 1983; Wu et al., 2015). The yield of the isolated compound was 0.14 g.

### 3.1. Spectral data

**<sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>):**  $\sigma$  7.71 (<sup>1</sup>H, d,  $J$  = 7.2 Hz, H-5), 6.68 – 6.54 (2H, m, H-4, H-7), 6.51 (<sup>1</sup>H, s, H-10), 5.47 (<sup>1</sup>H, t,  $J$  = 7.9 Hz, H-2), 5.32 (<sup>1</sup>H, s, H-1'), 5.21 (<sup>1</sup>H, s, H-1'), 5.13 (<sup>1</sup>H, m, H-12a), 4.52 (2H, m, H-12), 4.32 (<sup>1</sup>H, d,  $J$  = 13.2 Hz, H-3'), 4.27 – 4.06 (4H, m, H-12, H-3', H-1'', H-1'''), 3.98 – 3.85 (2H, m, H-6a, H-6''), 3.77 – 3.45 (9H, m, -OCH<sub>3</sub>, -OCH<sub>3</sub>, H-6'', H-4''', H-5'''), 3.19 – 2.90 (4H, m, H-1, H-2'', H-3'', H-4'').

**<sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>):**  $\sigma$  189.25 (C-6), 166.74 (C-3a), 158.22 (C-13a), 149.84 (C-9), 148.06 (C-10a), 144.38 (C-2'), 143.67 (C-8), 129.67 (C-5), 113.73 (C-5a), 113.30 (C-13b), 113.03 (C-1'), 111.26 (C-7), 105.57 (C-6b), 104.91 (C-4), 103.94 (C-1''), 102.74 (C-1'''), 101.92 (C-10), 85.03 (C-2), 76.99 (C-3''), 76.20 (C-5''), 73.83 (C-2''), 73.02 (C-3'''), 72.20 (C-12a), 71.01 (C-2'''), 70.59 (C-4''), 68.51 (C-6''), 68.22 (C-3'), 67.79 (C-4'''), 66.19 (C-12), 65.36 (C-5'''), 56.63 (-OCH<sub>3</sub>), 56.03 (-OCH<sub>3</sub>), 44.03 (C-6a), 31.27 (C-1).

**UV:**  $\lambda_{\max 1}$  = 200 nm,  $\lambda_{\max 2}$  = 236 nm and  $\lambda_{\max 3}$  = 296 nm.

**ESI-MS m/z (negative mode, fragmentor voltage 100 V):** 703.2 [M-H]<sup>-</sup>, 749.1 [M+HCOOH-H]<sup>-</sup>.

### 3.2. Fruticin content

The basic attributes of the calibration curve are given in Table 1. The content of fruticin (%) in the plant material is shown in Table 2

**Table 1.** Basic attributes of the calibration curve

Attribute	Value
Data points [N]	9
Concentration range [ $\mu\text{g}/\text{mL}$ ]	10.2 - 680
Calibration equation	$y = 9777.2x - 4.4957$
Correlation coefficient [ $r^2$ ]	0.9999
LOD [ $\mu\text{g}/\text{mL}$ ]	6
LOQ [ $\mu\text{g}/\text{mL}$ ]	19

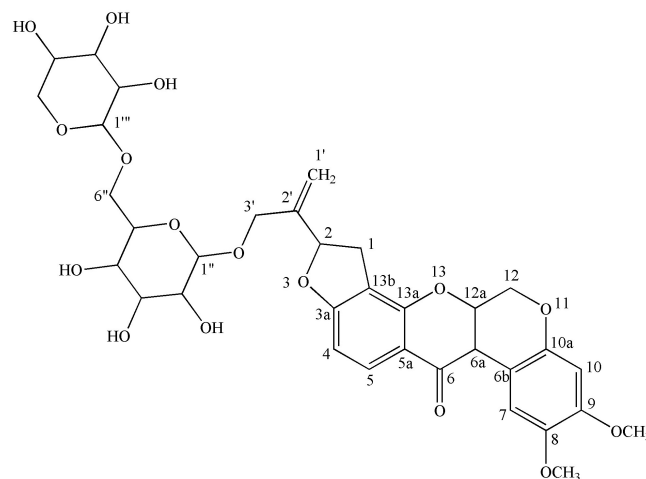
The highest content of fruticin was determined in the sample of *A. fruticosa* fruits collected in December 2019. The content of fruticin gradually increased over time, which leads to the conclusion that the fruit as a potential raw material for drugs should be collected at a later stage of ripening.

**Table 2.** Contents of fruticin in investigated plant material

Date of collection	Fruticin contents	Standard deviation
	[%]	[%]
9-Sep-2019	0.6557	0.0191
23-Sep-2019	0.6373	0.0395
10-Oct-2019	0.7491	0.0605
24-Oct-2019	0.7909	0.0318
13-Nov-2019	0.9308	0.0405
9-Dec-2019	0.9586	0.0236

The results of presented examination are of a preliminary character. Since the synthesis of secondary metabolites can be influenced by various external factors (e.g. soil type, altitude, atmospheric precipitation, temperature) and plant genetics,

it is necessary to conduct more extensive studies in order to be able to select the time suitable for *A. fruticosa* fruit collection with greater certainty. Rotenoids are considered as isoflavonoid derivatives that are biosynthesized by the formation of an additional oxygen ring between the B and C rings of the isoflavone skeleton. Fruticin (Figure 1), also known as amorphin, frutitsin, amorphigenin O-vicianoside or amorphigenin  $\alpha$ -L-arabinopyranosyl-1 $\rightarrow$ 6-O- $\beta$ -D-glucopyranoside, is one of the most abundant constituents of *A. fruticosa* fruit and one of the best known representatives of the whole rotenoids family (Buckingham, 1993; Kozuharova et al., 2017).



**Fig. 1.** Fruticin (amorphigenin  $\alpha$ -L-arabinopyranosyl-1 $\rightarrow$ 6-O- $\beta$ -D-glucopyranoside)

Based on this constituent, the drug Fruticin<sup>®</sup> (50 mg) was developed in the USSR and used in 1960s and 1970s as a sedative for neurotic conditions of various origins (cardiac neurosis, vegetative-vascular dystonia, paroxysmal neurogenic tachycardia). During the use of this drug, no contraindications and side effects were observed (Anonymous, 1968; 1974; Moiseev and Ustinova, 1967; Syropyatov and Dzeruzhinskaya, 2001). Eventually, due to the emergence of novel and, presumably, more effective synthetic drugs, Fruticin<sup>®</sup> was excluded from the USSR drug register in 1982 as obsolete (Anonymous, 1982). Although retracted from the drug register, pharmacological investigations on fruticin have been continued, as the results of experimental *in vitro* and *in vivo* studies indicated its significant medical potential. Fruticin showed an inhibitory effect on rat heart phosphodiesterase. Its activity was found to be greater than the inhibitory activity of potent flavonoids such as luteolin, kaempferol, quercetin (Petkov et al., 1983). Hypercoagulability in rabbits with experimentally induced atherosclerosis was inhibited by oral administration of fruticin (Aizikov et al., 1984). Administered at a dose of 5 mg/kg/day, it showed a hepatoprotective effect in rats when carbon tetrachloride/D-galactosamine was used as toxic agents (Kloutek et al., 1985). Fruticin prevented the development of experimental atherosclerosis in the presence of the combination of risk factors in rabbits. It reduced the level of cholesterol and lipoproteins that have atherogenic potential after administration of an oral dose of 10 mg/kg body weight in a model of hyperlipemic rats. In addition, it hindered the development of morphological manifestations of aortic atherosclerosis (Kurmuikov et al., 1986). The cytotoxic effect of fruticin was assessed *in vitro* on malignant breast (MCF-7) and colon (HCT-116) cancer cell lines using the MTT assay. The results showed that fruticin exhibited selective anti-proliferative activity against the MCF-7 cell line ( $\text{IC}_{50}$  = 0.95  $\mu\text{M}/\text{mL}$ ), even better than the positive control, cisplatin ( $\text{IC}_{50}$  = 7.07  $\mu\text{M}/\text{mL}$ ). These results indicate that fruticin could be

an important raw material for the production of anticancer drugs (Wu et al., 2015).

We believe that the medicinal potentials of fruticin have not been exhausted and that the use of this molecule should be re-evaluated by means of contemporary tests in the light of modern knowledge in physiological sciences.

## CONCLUSION

Our results suggest that *A. fruticosa* fruits, as potential raw material for industrial extraction of fruticin, should be collected during the dormant phase of the plant, in late autumn or in winter. The methodology of fruticin isolation shows promising potentials for scale-up and use in industrial or semi-industrial environment, with satisfactory purity of obtained product. LC-DAD-MS or HPLC-UV/DAD could be suitable as analytical techniques for quality control assurance. Further research on fruticin pharmacology is needed and already in progress.

## ACKNOWLEDGMENTS

Supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia; contract No. 451-03-68/2020-14/200161.

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