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ESSENTIAL OILS FROM *AMORPHA FRUTICOSA* L. FRUITS – CHEMICAL CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY

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Abstract: *Amorpha fruticosa* L. is mainly known as an ornamental plant, but also as the source of the medicinal substance amorphine. Indigo bush is an invasive shrub in the *Fabaceae* family, native to eastern part of North America. The fruits of this species contain the rotenoid compound amorphine which was used for its cardio-sedative properties in nervous complaints, vegetative neurosis and paroxysmal tachycardia. The present study aims to assess the antimicrobial activity of the essential oil from *Amorpha fruticosa* fruits in correlation with its chemical composition. The volatile oil was extracted by hydrodistillation (1.3 - 1.8% dry basis) from the fruits of indigo bush, harvested from three locations near Iasi. Gas-chromatography coupled with mass spectrometry was applied in order to analyse the composition of volatile oil, revealing the following main compounds: γ-muurolene, α-zingiberene, δ-cadinene and α-eudesmol. The antimicrobial activity was tested against four Gram-positive bacteria (*Staphylococcus aureus, Sarcina lutea, Bacillus cereus, B. subtilis*), two Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) and against three fungi (*Candida albicans, C. glabrata, C. sake*). The volatile oil manifested moderate antibacterial activity against Gram-positive bacteria and no antifungal activity that can be explained by the absence of phenolic compounds and the low content of oxygenated monoterpenes.
Key words: *Amorpha fruticosa* L. fruits, essential oil, GC-MS, antimicrobial activity.

Introduction

Since ancient times, medicinal plants were the basis of remedies used in traditional medicine. They are the source of bioactive compounds that have contributed to the discovery of new therapeutic agents. Increased incidence of infectious diseases and microbial resistance to antibiotics prompted the search and identification of new antimicrobial agents, either natural or synthetic.

Volatile oils have been used since ancient times for their bactericidal, fungicidal, anti-parasitic properties in medicine, food, cosmetics and agriculture. Today, their use in therapy shows an increased interest in terms of safety in administration, lack of side effects, easy acceptance by patients and the health benefits. In addition to uses in drug industry, essential oils are also used as functional ingredients for the foods, beverages and cosmetics. The antimicrobial effects of essential oils vary depending on the species, chemotype, chemical composition and pedoclimatic conditions (Bakkali et al., 2008).

Amorpha fruticosa L. (family *Fabaceae*, indigo bush) is widely distributed in North America, southern Canada and northern Mexico. Populations of *Amorpha fruticosa* in Europe were introduced as ornamental plants. It presents as a shrub with a stem of 1-3 m,

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with imparipinnate compound leaves with stipules. The purple flowers are clustered in racemes. The fruit is an indehiscent pod of 8-9 mm with 1(2) seeds. (Alexan et al., 1991). Indigo bush has been used as a Chinese folk medicine for hypertension, hematomas and contusions. Various organs of the species (seeds, root, leaves) contain rotenoid compounds, known for their insecticide activity, but also with antimicrobial and anticancer properties (Fang and Casida, 1998; Gao et al., 2003; Sangthong et al., 2011). Flavanones and rotenoids from roots of indigo bush also manifest antibacterial activity by inhibition of bacterial neuraminidase (Kim et al., 2011).

Only few data are available regarding the volatile oil of *Amorpha fruticosa* fruits (Lis and Gora, 2001; Popescu et al., 1973) and none referring to its antimicrobial activity. Therefore, the aim of present study was to investigate the in vitro antibacterial and antifungal activities of the essential oil isolated from the fruits of *Amorpha fruticosa*, collected from Romania, in relation to its chemical compositions.

Materials and methods

The fruits of *Amorpha fruticosa* were harvested from three different locations in and near the city of Iasi: CUG – S1 collected on 10.11.2011, Nicolina – S2 collected on 21.10.2011, Ciric – S3 collected on 01.10.2014. The plant material was authenticated and the voucher specimens were deposited in the Herbarium of Department of Plant and Animal Biology, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania. The plant material was dried at room temperature, under shade.

Air-dried fruits (50 g) were subjected to hydrodistillation in a Clevenger apparatus for 4h. The collected oil was dried over anhydrous sodium sulphate and stored at 4°C until analysis. Yields of the essential oils (v/w%) were calculated on a dry weight basis and were expressed as average values of the results from three extractions (Farmacopeea Română, 1993).

The GC-MS analysis was performed on an Agilent 7890 gas chromatograph equipped with an Agilent 5975C mass spectrometer with electron impact ionization. Separation of the constituents was performed on a DB-5 MS capillary column (30 m x 0.25 mm i. d., film thickness 0.25 μm). A volume of 0.3 μL was injected in the split mode (split ration 1:50). The carrier gas was helium (1 mL/min). The oven temperature was initially 40°C, then was programmed to raise at 250°C at a rate of 4°C/min and to 280°C at a rate of 10°C/min. The final temperature was held for 9.5 min. The individual components were identified by comparing their mass spectral data with those of Wiley 275 mass spectral database.

The antimicrobial activity was studied using Gram positive bacteria (*Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341, *Bacillus cereus* ATCC 14579, *B. subtilis*), Gram negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) and pathogenic yeasts (*Candida albicans* ATCC 10231, *C. sake*, *C. glabrata* ATCC MYA 2950). All these strains were obtained from the Culture Collection of the Department of Microbiology, Faculty of Medicine, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania.

Antimicrobial activity was evaluated by agar disc diffusion method according to described protocols (CLSI, 2012). Sterile stainless steel cylinders (5 mm internal diameter; 10 mm height) were applied on the agar surface in Petri plates. 100 μL of each volatile oil

sample were added to each cylinder. Commercial available discs containing ampicillin (25 µg/disc), chloramphenicol (30 µg/disc) and nystatin (100 µg/disc) were used. The plates were incubated at 37°C for 24 h (bacteria) and at 24°C for 48 h (yeasts). After incubation the diameters of inhibition zones were measured.

The antimicrobial activity of volatile oil samples was also quantitative determined by the broth microdilution method, with an inoculum of 10^6 CFU/ml. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of tested oils were measured by broth microdilution method, with an inoculum of 10^6 CFU/mL (CLSI, 2012). Double dilutions of each oil sample were tested. The microorganisms used in this assay were *Staphylococcus aureus* ATCC 25923 and *Sarcina lutea* ATCC 9341. MIC was the lowest concentration of oil where complete inhibition of visible growth was observed after 24 h incubation at 37°C. The minimum bactericidal concentration was determined by transferring 10 µL from each dilution of oil showing no growth on the MIC on the surface of agar plate. The subcultures are incubated 20 hours and the MBC read as the least concentration, which produced ≥ 99.9% killing of the bacteria.

Mueller Hinton broth and agar were obtained from Merck (Darmstadt, Germany), while Sabouraud 4% glucose agar was from Fluka Biochemika (Buchs, Switzerland). The antibiotics discs were purchased from Himedia (Mumbai, India).

Results and discussions

The dried fruits of *Amorpha fruticosa* were subjected to steam distillation and pale yellow oils were obtained in yields of 1.3% (S1), 1.5% (S2) and 1.8% (S3), respectively. The volatile oils were analyzed by GC-MS and the results are presented in Table 1.

Table 1. Chemical composition (%) of volatile oils from *Amorpha fruticosa* fruits

Retention time (min.)	Compound	Aria (%)		
		S1	S2	S3
8.012	tricyclene	-	0.02	-
8.400	α-pinene	3.82	10.86	0.91
8.916	camphene	0.07	0.64	0.02
9.052	verbenene	-	0.05	-
9.859	β-pinene	0.52	1.33	0.04
10.102	6-methyl-5-hepten-2-one	0.02	0.04	0.05
10.287	myrcene	4.62	1.38	0.66
10.870	α-phellandrene	0.05	0.26	-
11.240	α-terpinene	0.06	0.45	0.03
11.522	p-cymene	0.21	1.04	0.04
11.687	limonene	0.78	2.42	0.12
11.813	1,8-cineole	-	0.83	0.05
11.959	(Z)-β-ocimene	1.84	0.89	2.76
12.309	(E)-β-ocimene	0.38	0.39	0.93
12.737	γ-terpinene	0.22	0.90	0.04
13.709	α-terpinolene	0.07	0.33	0.04
13.884	dehydro-p-cymene	-	0.06	-
14.254	linaalol L	0.27	3.23	0.46
14.973	fenchol	-	0.04	-
15.255	allo-ocimene	0.09	0.08	0.13
16.014	(-)-isopulegol	0.13	0.37	0.08

16.373	p-menth-8-en-3-ol	0.07	0.23	0.05
16.869	endo-borneol	-	0.10	-
17.171	4-terpineol	0.08	0.48	-
17.696	α-terpineol	0.09	0.69	-
18.785	citronellol	0.32	0.60	0.11
20.788	l-bornyl acetate	0.04	0.16	0.01
22.888	α-cubebene	0.27	0.15	0.07
23.024	eugenol	-	-	0.04
23.637	α-ylangene	1.15	1.42	2.67
23.880	α-copaene	2.72	3.05	4.98
24.619	methyl eugenol	-	-	0.43
24.862	α-gurjunene	0.59	0.61	1.00
25.066	α-bergamotene	-	-	0.12
25.290	β-cariofilen	4.79	3.20	2.66
25.873	aromadendrene	1.12	1.12	1.91
26.310	(E)-β-farnesene 1458	-	0.60	-
26.087	β-selinene	0.22	-	0.31
26.398	α-humulene	2.60	1.00	1.66
26.651	γ-curcumene	0.19	0.22	0.45
27.088	γ-muurolene	7.17	7.30	9.85
27.263	ar-curcumene	3.41	3.30	2.99
27.740	α-zingiberene	7.63	5.79	6.88
28.031	α-farnesene	-	-	0.42
28.274	γ-cadinene	5.34	3.19	7.21
28.459	δ-cadinene	5.72	5.82	7.77
28.576	β-sesquiphellandrene	2.51	2.83	2.91
28.819	cadina-1,4-diene	1.16	1.14	2.18
28.926	α-cadinene	0.76	0.64	1.04
29.227	elemol	0.14	-	-
29.577	nerolidol B	-	0.28	0.42
30.073	spathulenol	-	-	0.37
30.219	caryophyllene oxide	1.15	0.54	-
30.588	viridiflorol	0.51	0.30	1.33
31.969	α-cadinol	3.07	-	2.64
31.687	γ-eudesmol	3.00	2.35	-
32.436	α-eudesmol	7.89	5.65	6.51
33.185	α-bisabolol	2.82	2.09	2.14
Monoterpene hydrocarbons		12.73	21.1	5.72
Oxygenated monoterpenes		0.96	6.57	1.22
Sesquiterpene hydrocarbons		47.35	41.38	57.08
Oxygenated sesquiterpenes		18.58	11.21	13.41
Others		0.06	0.2	0.06
Total identified		79.68	80.46	77.49

A number of 45 compounds were identified in S1 sample which accounted for 79.68% of the total oil composition, 50 compounds were identified in S2 sample which accounted for 80.46 % and 46 compounds were identified in S3 sample representing 77.49% of the total oil chemical composition.

The terpene composition from *Amorpha fruticosa* fruits was dominated by sesquiterpene hydrocarbons (47.35%, 41.38% and 57.08%, respectively). β-cariofilen, γ-muurolene, α-zingiberene, γ-cadinene and δ-cadinene were always the major constituents in sesquiterpene fractions of all the samples. Monoterpene hydrocarbons (12.73%, 21.1% and 5.72%, respectively) and oxygenated sesquiterpenes (18.58%, 11.21% and 13.41%,

respectively) are found in smaller amounts compared to sesquiterpene hydrocarbons. Although in S2 sample sesquiterpene hydrocarbons prevailed, α-pinene (10.86%) is present in high percentage in the oil. However, in the other two samples, the amounts of α-pinene were much smaller (3.82% in S1 and 0.91% in S3). Oxygenated monoterpenes (0.96%, 6.57% and 1.22%, respectively) were almost lacking in the volatile oil of *Amorpha fruticosa* fruits.

The speciality literature contains few data regarding the chemical composition of *Amorpha fruticosa*. Lis and Gora (2011) examined the composition of dried and fresh ripe fruits of *Amorpha fruticosa* fruits from Poland, collected in October. They found that α-pinene (19.6%), myrcene (17.7%), δ-cadinene (6.9%), γ-muurolene + ar-curcumene (6%) were the most aboundant compounds in the volatile oil from dried fruits. In our study, the main components were: α-eudesmol (7.89%), α-zingiberene (7.63%) and γ-muurolene (7.17%) in sample S1; α-pinene (10.86%), γ-muurolene (7.30%) and δ-cadinene (5.82%) in sample S2; γ-muurolene (9.85%), δ-cadinene (7.77%) and γ-cadinene (7.21%) in sample S3. The quantitative and qualitative differences may be atributted to the phytogeographic origin, environmental conditions and time of harvest.

The antimicrobial activity of the volatile oils was tested against four Gram-positive bacteria (*Staphylococcus aureus, Sarcina lutea, Bacillus cereus, B. subtilis*), two Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*) and against three fungi (*Candida albicans, C. glabrata, C. sake*). The test was carried out by a disc diffusion method, using ampicillin, cloramphenicol and nistatin as references. The effects of three essential oils on the tested strains are shown in Table 2. The data obtained in the quantitative antimicrobial activity are presented in Table 3.

Table 2. Antimicrobial effects of volatile oils from *Amorpha fruticosa* fruits

Microorganism	**Diameter of inhibition zone (mm)**					
	S1	**S2**	**S3**	**Ampicillin (25 µg/disc)**	**Chloramphenicol (30 µg/disc)**	**Nystatin (100 µg/disc)**
Staphylococcus aureus ATCC 25923	10	0	10	26	24	nt
Sarcina lutea ATCC 9341	24	12	26	28	26	nt
Bacillus cereus ATCC 14579	0	0	0	0	21	nt
Bacillus subtilis	0	0	0	26	29	nt
Escherichia coli ATCC 25922	0	0	0	21	29	nt
Pseudomonas aeruginosa ATCC 27853	0	0	0	0	16	nt
Candida albicans ATCC 10231	0	0	0	nt	nt	18
Candida glabrata ATCC MYA 2950	0	0	0	nt	nt	19
Candida sake	0	0	0	nt	nt	20

nt – not tested

Table 3. MIC and MBC values of tested oils

Oil sample	***Staphylococcus aureus* ATCC 25923**		***Sarcina lutea* ATCC 9341**	
	MIC[a]	**MBC[a]**	**MIC[a]**	**MBC[a]**
S1	29.44	58.88	1.84	3.68
S2	nt	nt	3.68	13.68
S3	117.77	117.77	0.92	0.46

[a] values are expressed in mg/mL; nt-not tested

The volatile oils S1 and S3 exhibited good antibacterial activity against *Sarcina lutea* ATCC 9341 (24 mm and 26 mm, respectively) and moderate activity against *Staphylococcus aureus* ATCC 25923 (10 mm and 10 mm, respectively). The Gram-negative bacteria and fungi are not sensitive to the tested volatile oils. The oil samples were generally bactericidal at a concentration equal or up to twofold higher than the MIC value (Table 3).

Our results are similar to those obtained by other researchers. A study carried out on a 50% methanol extract from fruits with seeds of *Amorpha fruticosa* occuring in the Mississippi River Basin indicated antibacterial activity only on *Staphylococcus aureus* strain (d = 17 mm) and a lack of activity on *Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*. (Borchardt, 2008).

It is a known fact that essential oils of many medicinal plants have a broad spectrum of antimicrobial activities *in vitro*, attributed to the high content of phenolic derivatives like thymol, carvacrol, eugenol. Furthermore, oxygenated monoterpenes (α-terpineol, terpinen-4-ol, linalool) and sesquiterpenes (γ-muurolene, spathulenol, α-selinene, γ-cadinene) enhance antibacterial activity. These compounds are responsible for the high antimicrobial activity, and also for the broad spectrum of activity (Berger, 2007; Hong et al., 2004; Maciąg et al., 2004). Some researchers claim that α-pinene acts by affecting the integrity of the bacterial membrane (Park and Lee, 2011; Toroglu, 2007). Increased antifungal activity depends on the presence of compounds such as cinnamic aldehyde, eugenol and citral in the volatile oil (Balchin et al., 1998; Soković et al., 2008).

In case of the analyzed volatile oils, the lack of antibacterial activity on some of the tested strains can be explained by the absence of phenolic compounds and the high content of monoterpene hydrocarbons. Moreover, the oxygenated monoterpenes with superior antimicrobial activity compared to the monoterpene hydrocarbons, are low in the three samples.

Conclusions

The chemical composition of the volatile oil of *Amorpha fruticosa* fruits from Romania was studied in the present paper. Also, we tested for the first time the antimicrobial activity of this oil. Although in all tested samples sesquiterpene hydrocarbons predominate, there is a variation in the chemical composition depending on the time and area of harvest. Generally, the antimicrobial activity is weak, in accord with the chemical composition, except for a good activity against *Sarcina lutea* ATCC 9341 and a moderate activity against *Staphylococcus aureus* ATCC 25923.

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