UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
DOCTORAL SCHOOL

PHYSICO - CHEMICAL RESEARCH ON *Sambucus ebulus* L. SPECIES

PhD THESIS ABSTRACT

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CONTENTS

Keywords: *Sambucus ebulus* L., GC, HPLC, MS.

CHAPTER 1. GENERAL CONSIDERATIONS REGARDING *Sambucus ebulus* L. SPECIES

1.1. Systematic classification

Originally, the genus *Sambucus ebulus* belonged to the *Caprifoliaceae*[1] botanical family, but after long taxonomic debates, both *Sambucus* genus and several other genera (*Viburnum*) were moved by Vernon in 1987[2] to *Adoxaceae* family. This botanical family belongs to the order *Dipsacales*, Magnoliopsida *class, Asteridae* subclass. Sambucus genus includes about 30 species, of which the best known are: *Sambucus nigra*, *Sambucus racemosa*, *Sambucus palmensis*, *Sambucus canadensis* and *Sambucus ebulus* L.

1.2. *Sambucus ebulus* L. distribution

Sambucus ebulus similar to *Sambucus nigra* species is a species widespread in both Central and South America and in Asia, North Africa and the United States.

1.4. Phytoterapeutic potential

In the literature, references appear on the use of leaves, flowers and fruits as an expectorant, diuretic and purgative in various inflammatory diseases such as rheumatoid arthritis, fever, pulmonary edema, burns, and various lacerations having bacteriostatic and diuretic action.

[1] Iaroşenko P D (1962). *Geobotanica*, Ed. Academiei R.P.R., Bucureşti.
[2] Vernon H (1987). Flowering plants of the World. Andromeda Oxford LTD, Heywood.

CHAPTER 2. EXTRACTION AND ANALYSIS METHODS OF CHEMICAL CONTENT OF SAMBUCUS EBULUS L. SPECIES

Among the most popular extraction methods are: Soxhlet method, microwave extraction, ultrasonic extraction, maceration, hydrodistillation, turbo extraction, vibro extraction etc.

Among the identification and dosage techniques of the compounds contained in the obtained extracts are: gas-chromatography coupled with mass spectrometry (GC-MS), thin-layer chromatography (TLC), high performance liquid chromatography coupled with mass spectrometry (HPLC-MS), atomic absorption spectrometry (AAS), Fourier transform infrared spectroscopy (FT-IR).

ORIGINAL CONTRIBUTIONS

CHAPTER 3. PRELIMINARY IDENTIFICATION AND CHARACTERIZATION

3.1. Macroscopic characteristics

In this first research stage were described morphological and organoleptic characteristics of plant organs, the aspect (inside, outside) and characteristic features: dimensions, color, odor.

3.2. Microscopic characteristics

Microscopic characterization intended to emphasize the structural differentions between vegetative organs from *Sambucus ebulus* L. species. We worked on a Bel Photonics microscope BIO3 model with videocamera MSE-100 and Bel MicroImage Analzyer soft.

3.4. Determination of moisture, dry matter and ignition residue for Sambucus ebulus L. species

Table 1. Moisture and raw ash for *Sambucus ebulus* L. species

No.	*Sambucus ebulus* L. sample	Weight (g)	Dried weight (g)	Humidity (%)	Ignition residue	Ash (%)
1.	**Roots**	1,4216	1,2987	8,645	0,0989	6,956
2.	**Stems**	2,1261	1,9463	8,456	0,0860	4,044
3.	**Flowers**	1,4758	1,3555	8.8749	0,1240	8,402
4.	**Fruits**	2,9378	2,5349	13,7143	0,0931	3,169
5.	**Leaves**	3.3317	3,0672	8.6235	0,2536	7,612

Preliminary phytochemical reactions

For the identification of the main classes of active ingredients contained in such species we used the extracts obtained by Soxhlet method. In order to identify this classes of compounds we used the methods prescribed by Romanian Pharmacopoeia ed. X.

Table 2. Phytochemical analysis of the ether extracts of *Sambucus ebulus* L. species

Identification reactions	Active principles	Roots	Leaves	Fruits	Stems
Carr-Price reaction	carotenoids	+	+	+	-
NeoClevenger method	Volatile oils	+	+	+	-
Liebermann-Burchard reaction	sterols	+	+	+	+
Hidrolysis	Fatty acids	+	+	+	+
Cyanidol reaction	flavonoid aglycone	+	+	+	-
Mayer, Bertrand, Ehrlich reagents	alkaloid bases	+	+	+	-
Borntranger reaction	emodols	+	+	+	-
+ present active principles;	- absent active principles;				

Table 3. Phytochemical analysis of alcoholic extracts of *Sambucus ebulus* L. species

Identification reactions	Active principles	Roots	Leaves	Fruits	Stems
The reaction with $FeCl_3$	taninuri catehice	+	+	+	-
Fehling reaction	polifenoli	+	-	-	-
Liebermann-Burchard reaction	saponozide	+	+	+	-
ninhydrin	aminoacids	+	-	+	-
Coloraţie în mediu acid	antocianozide	+	+	+	-
Shibata reaction	flavonosides	+	-	-	-
+ present active principles;	- absent active principles;				

3.6. Analysis by FTIR spectroscopy

To record the spectra of ethanol and ether residues a NICOLET FTIR apparatus was used in the 4000-400 cm^{-1} domain, with potassium bromide. Device control and data acquisition was performed using Spectra Omnic software. Spectra were plotted by milling dry residue with potassium bromide of spectral purity.

Table 4. The main IR frequencies (cm-1) obtained for ethanol and ether residues

Sample/ characteristic frequencies in IR (cm^{-1})					Attribution
Roots	Leaves	Fruits	Stems	Aerial parts	
3.386	3.435	-	-	3.385	Stretching vibration O-H şi N-H (amino acids, amines, amides)
-	-	3.008	-	3.008	$\nu_{C\text{-}H(asim)}$ (fats, fatty acids)[3]
2.924	2.925	2.926	2.929	2.926	Asymmetric stretching vibration $-CH_3$, $-CH_2-$ (Carboxylic acids)
2.853	2.854	2.854	2.854	2.857	$\nu_{(sim)}$ $-CH_3$ groups
1.733	1.735	1.745	1.735	1.733	Stretching vibration -C=O (esters)
1.462	1.462	1.460	-	1.457	$\nu_{C\text{-}H(bending)}$ ($-CH_2$ group from proteins)
1.378	1.377	1.377	-	1.376	Deformation vibration (C-H)
1.260	1.247	1.241	1.247	1.249	$\nu_{C\text{-}O}$, $\nu_{O\text{-}H\ def}$ (poliphenols)
1.172	1.171	1.166	1.171	1.170	$\nu_{C\text{-}O\text{-}C}$
1.093	1.094	1.098	1.094	1.072	$\nu_{C\text{-}H(deformation)}$, $\nu_{C\text{-}O}$, $\nu_{C\text{-}C(bending)}$ – carbohydrates
1.023	-	1.032	-	1.045	$\nu_{C\text{-}O}$ ($_{bending}$) polysaccharides
801	802	723	-	801	$\nu_{C\text{-}H}$ (bending out of plane)
-			-	552	Vibration of phosphate group

[3] Schulz H., Baranska M. Identification and quantification of valuable plant substances by IR and Raman spectroscopy, *Vibrational Spectroscopy*, 43: 13-25, 2007.

3.7. Analysis of metal cations from Sambucus ebulus L. species by atomic absorption spectrometry

Analyses were performed by flame and graphite furnace AAS on a Varian DUO device AA240FS and graphite furnace AAS on an atomic absorption spectrometer Nova 400G - Analytik Jena with autosampler and software MPE60 WinAAS 3.17.0.

Table 5. Determination of metal cations of the *Sambucus ebulus* L. species by method 1

Sample	Ca (µg/g)	Fe (µg/g)	Mn (µg/g)	Mg (µg/g)	Zn (µg/g)	Pb (µg/g)	Cr (µg/g)	Ni (µg/g)
fruits	3196,9	97,1	16,4	1698,6	20,7	1,4	0,35	2,5
stems	2921,8	9,9	5,5	2127,14	6,2	0,78	0,15	1,32
leaves	9539,6	148	49,93	5469	23,12	0,76	1,37	0,99
roots	2805,1	200,25	37,1	1841,22	19,46	0,7	4,15	7,146

Table 6. Determination of metal cations of the *Sambucus ebulus* L. species by method 2

	Cu (µg/g)	Pb (µg/g)	Zn (µg/g)
green fruits	6,895	1,021	33,1
black fruits	4,962	0,728	27,9
leaves	1,923	0,876	47,5
roots	6,310	1,952	26,5
stems	7,374	2,980	35,6

Tabl 7. Determination of metal cations of the *Sambucus ebulus* L. species by method 3

No.	Product	Concentration (µg/g)					
		Cd	Cu	Cr	Zn	Pb	Fe
1.	Fruits	*	2.34	9.85	45.92	9.21	113.40
2.	Leaves	0.32	5.16	10.27	36.58	12.63	99.29
3.	Stems	*	2.16	*	16.58	*	*

CHAPTER 4. PHYTOCHEMICAL INVESTIGATIONS ON *Sambucus ebulus* L. SPECIES BY GC-MS AND HPLC / HPLC -MS

4.1. Analysis by gas chromatography coupled with mass spectrometry of the compounds contained in *Sambucus ebulus* L. extracts

We worked on a Hewlett Packard 6890 gas chromatograph with 5973 mass spectrometer detector, column: 30 mx 0.25 mm DB1 x 1 micrometres $T_{injector}$.: 290^{o}C, carrier gas flow (He) = 0.8 mL / min, split ration = 188: 1, Temp. MS source = 230^{o}C, quadrupole MS = 150^{o}C, MS interface = 300^{o}C. The assignment of the mass spectra was performed using the database Nist05 Library. Of all the compounds found in the leaves, were identified a number of 19 compounds in the ether extract (9 with high probability) and 51 compounds in alcoholic extract (29 with high probability), representing 87% of all constituents. Most of the compounds have been identified in fruit species, in the ether extract 51 compounds and 32 compounds in the alcoholic extract.

4.2. Analysis of some secondary metabolites classes by HPLC/ HPLC-MS

4.2.1. Brassicasterol, campesterol and stigmasterol analysis

Determination of phytosterols from Sambucus ebulus was achieved by:
- **HPLC** (Dionex Ultimate 3000 detector UVD-3000, Acclaim 120 C18 DIONEX length 250 mm, diameter 4 mm, LPG-3400 pump). The mobile phase was methanol: acetonitrile: isopropanol: water acidified (0.05% trifluoroacetic acid) = 75:5:10:10. The column temperature 21^{o}C, flow 0.5 mL / min, wavelength 230 nm.
- **Agilent LC-MS chromatography** (1200 Series MS detector and autosampler Qudrupole 6120). Chromatographic conditions: Column temperature detection 40 ° C, flow 0.6 mL / min, injection volume 5 µl for 35 minutes. The mobile phase was acetonitrile: isopropanol = 80:20 (%). Detection was carried out in the MSD scan.
Was preffered simultaneous determination of the three phytosterols, given that they have a particularly cumulative effect, but they don't have the same properties separately.

Table 16. Chromatographic characteristics of brassicasterol, campesterol and stigmasterol standards

Standard	Molecular formula	Retention time (min)	Ion $[M-H_2O+H]^+$
Brassicasterol	$C_{28}H_{46}O$	3.73	470
Campesterol	$C_{28}H_{48}O$	4.28	383
Stigmasterol	$C_{29}H_{48}O$	4.49	395

Table 17. Resulted chromatographic characteristics in the plant extracts

Sample	Phitosterol	t_R (min)	Area (mAu·min)	Height (mAu)	Concentration (mg/100 g)
S. ebulus flowers	Brassicasterol	3,59	174,09	886,88	29,222
	Campesterol	4,18	965,29	2691,67	136,310
S. ebulus leaves	Brassicasterol	3,63	73,98	394,13	12,418
	Stigmasterol	4,46	163,05	952,64	132,748
S. ebulus stems	Campesterol	4,21	755,37	2787,99	109,411
S. ebulus roots	Campesterol	4,20	674,08	2771,32	95,118
S. ebulus fruits	Brassicasterol	3,31	221,80	478,27	37,230
	Campesterol	4,19	568,67	2699,82	80,244

The presence of relatively high amounts of brassicasterol and campesterol in all vegetative organs of the species explains its inflammatory and antirheumatic activities. Campesterol is in a quantity greater than brassicasterol, an actually rare fact in the plant world[4]. The flowers are rich in campesterol (136 mg %) followed by strains (109.411 mg/100 g)[5].

4.2.2. Anthocyanins separation

For the three reference compounds the following retention times were obtained: delphinidin - 6.28 min., pentunidin - 6.89 min. and cyanidin 3,5-diglycozide - 7.35 min.

[4] Gabay O, Sanchez C, Salvat C, Chevy F, Breton M, Nourissat G, Wolf C, Jacques C, Berenbaum F (2010). Osteoarthritis Cartilage, 18:106.
[5] Bubulică M.V., **Chirigiu L.**, Popescu M., Simionescu A., Anoaica G., Popescu A (2012). *Analysis of sterol compounds from Sambucus ebulus,* Chemistry of natural compounds. 48(3):520-521.

Table 18. Chromatographic characteristics resulted at the determination of anthocyanins

Sample	Antocyanins	t_R (min)	Area (mAu·min)	Heigh (mAu)
S. ebulus flowers	Delphinidin + pentoze	5,89	196,43	609,99
	Petunidin + pentoze	6,67	749,98	2596,46
	Cyanidin 3,5-diglycoside	7,22	213,17	1025,88
S. ebulus leaves	Delphinidin + pentose	5,89	133,14	434,31
	Petunidin + pentose	6,76	727,31	2387,9
	Cyanidin 3,5-diglycoside	7,29	814,56	2354,67
S. ebulus stems	Petunidin + pentose	6,69	928,48	2578,0
S. ebulus roots	Petunidin + pentose	6,61	1003,86	2832,31
S. ebulus fruits	Delphinidin + pentoze	5,72	111,12	230,88
	Petunidin + pentose	6,67	678,53	2688,87

Anthocyanins presented in this species are readily soluble, are potent inhibitors of free radicals having thus extremely high antioxidant activity and anti-inflammatory properties and thus explaining the immunostimulant activity of *Sambucus ebulus* L.

Surprisingly, the highest amount of anthocyanins were found in the roots, but numerically, most of the compounds found in the leaves and fruits, taking into account that in high-performance liquid chromatography the separate characteristic peak area is directly proportional to the concentration of this compound found in the extract, it can be said that in these two vegetative organs (fruit and leaves) are high amounts of cyanidin and petunidin. Stems contain only a high percentage of petunidin + pentose.

4.2.3. α and β-amyrin separation

The chromatographic characteristics obtained for the 5 samples are shown in Table 19.

Table 19. Chromatographic characteristics obtained for α and β amyrin

Sample	Compound	t_R (min)	Area (mAu·min)	High (mAu)
Sambucus ebulus flowers	α - amyrin	3,25	228,21	1409,07
	β - amyrin	4,23	51,03	229,99
Sambucus ebulus leaves	α - amyrin	3,33	77,15	330,73
	β - amyrin	4,4	181,23	956,08
Sambucus ebulus stems	β - amyrin	4,00	630,59	2827,83
Sambucus ebulus roots	β - amyrin	4,07	624,99	2840,44
Sambucus ebulus fruits	α - amyrin	3,42	52,69	248,86
	β - amyrin	4,07	531,22	2786,11

It should be noted that the racemic mixture of the two isomers gives the properties in question. As shown in Table 19, flowers, leaves and fruits have the two isomers, whereas in the roots and stems only the β-isomer is present.

CHAPTER 5. EVALUATION OF BIOLOGICAL ACTIVITY OF SOME EXTRACTS FROM A *SAMBUCUS EBULUS* L.

5.1.2.1. The total content of phenolic compounds

Of the obtained extracts 200 µl were taken and 2.5 mL of Folin - Ciocalteu reagent diluted 1: 10 were added. After 4 minutes 2 mL of sodium carbonate (75 g / L) were added in each sample, than were left for 2 hours at 23°C.

Absorbance was measured at 765 nm.

5.1.2.2. The total content of flavonoids

The determination was achieved with 10% aluminum chloride solution (method Roy et all., 2010), subsequently was assessed the obtained absorption maximum, based on the calibration curve. Total flavonoid content was expressed in mg/g (quercetin equivalents).

The highest polyphenolic content is in *Sambucus ebulus* fruits, followed by flowers and leaves. For the obtained fruit extract was performed also the evaluation of the juice of fresh fruits, especially considering their use in ethnomedicine in this form.

Table 20. The total content of phenolic compounds and flavonoids and the antioxidant activity of the tested extracts

No.	Sample	Total phenolics (mg GAE/g)		Total flavonoids (mg QE/g)	
		m.p.*	m.u.**	m.p.*	m.u.**
1.	*Sambucus ebulus* L. flowers	-	41,8 ± 0,98	3,75 ±2,31	19,06 ±1,06
2.	*Sambucus ebulus* L. roots	-	32,06± 0,56	-	20,07 ±1,23
3.	*Sambucus ebulus* L. fruits	9,25 ± 1,08	43,28± 0,71	3,96 ±1,84	22,71 ±0,61
4.	*Sambucus ebulus* L. leaves	-	35,73± 1,26	-	13,55 ±1,36
5.	*Sambucus ebulus* L. stems	-	30,21 ± 0,74	2,52 ± 1,20	13,54 ± 0,78
* fresh plant material ** Dried plant material average ± DS (n = 3).					

5.1.2.3. Permanganometric determination of the content in organic oxidizable substances of *Sambucus ebulus* L. species

Permanganometric titration was performed in 4N sulfuric acid medium. Volumes of permanganate solution used were determined. The results are shown in Table 21.

Table 21. Permanganometric determination of the content in organic oxidizable substances of *Sambucus ebulus* L. species

	$m_{probă}$ (g)	V_{H2SO4} (mL)	V_{H2O} (mL)	$V_{KMnO4\ 0,1\ N}$ (mL)
Stems				
Fruits	10,00	20,00	60,00	118,5
Leaves	10,00	20,00	60,00	89,5

5.1.2.4. Cyclic voltammetry studies

Cyclic voltammograms obtained for the determination of antioxidant activity of the extracts were performed using a potentiostat model PGZ-402 Universal Pulse voltammetry Dynamic EIS produced by Radiometer Copenhagen in 2007 equipped with soft Volta Master 4. Cyclic voltammetry studies were made in the range of - 200 ÷ 1000 mV at ambient temperature at a rate of 100 mV bias / s

Extracts tested at pH 7.1 had the best antioxidant activity, which is particularly important if we take into account the aim of obtaining pharmaceutical formulations which have high antioxidant activity.

5.1.2.5. Determination of chelating activity of the extracts

Determination of chelating ability of the extracts was performed by the method of Dinis et al. with some adjustments. The absorbance was measured at 562 nm using Na_2EDTA as a positive control. Plant extracts have the role of the complexing agent that disrupts the formation of the Fe-Ferosine complex, the coloration intensity decreases.

5.2. Phytotoxicity evaluation

Phytotoxicity of the extracts from various plants was then correlated with their cytotoxic properties, and even anticancer activity. The plant used to test phytotoxicity is the wheat (*Triticum vulgare,* Dropia variety, *Gramineae*). Table 22 presents the results obtained from *Triticum* test.

Table 22. Root elongation of germinated caryopses during the five days

No.	Extract	Concentration	Root elongation (cm)					
			Day 1	Day 2	Day 3	Day 4	Day 5	% inhibition
1.	***Sambucus ebulus* leaves (Se_1)** (aqueous extract)	2.0 %	0.75	0.80	0.80	0.80	0.93	92.38
2.		1.0 %	0.80	0.95	0.95	0.95	1.12	90.82
3.		0.5 %	0.74	1.13	1.52	1.74	2.13	82.54
4.		0.25 %	1.01	1.62	2.38	2.38	3.22	73.61
5.	**Sambucus ebulus fruits (Se_2)** (aqueous extract)	2.0 %	1.00	2.26	4.54	4.71	6.59	45.98
6.		1.0 %	0.98	2.20	3.90	4.32	5.00	59.02
7.		0.5 %	1.05	2.52	5.27	6.37	6.82	44.098
8.		0.25 %	0.82	2.66	5.92	6.11	8.80	27.87
9.	**Sambucus ebulus roots (Se_3)** (aqueous extract)	2.0 %	0.74	1.12	1.46	1.43	3.48	71.47
10.		1.0 %	0.73	1.47	3.81	3.78	3.77	69.09
11.		0.5 %	1.21	1.47	3.78	4.10	8.30	31.96
12.		0.25 %	1.24	1.88	4.02	4.72	11.23	7.95
13.	**Sambucus ebulus stems (Se_4)** (aqueous extract)	2.0 %	0.77	1.16	1.22	1.40	5.62	52.29
14.		1.0 %	1.00	1.40	3.30	3.46	7.70	36.88
15.		0.5 %	1.10	1.74	3.62	4.81	7.26	40.49
16.		0.25 %	0.97	2.19	5.23	6.42	9.84	19.34
17.	**Blank (M_1)**	-	1.23	2.03	3.47	5.72	12.2	-

There is a specific mitoinhibitor effect at higher concentrations. A cytotoxic effect was observed in the 2% extract of the leaves and roots of (nuclei with 1-2 nucleoli, hypertrophy). At low concentrations, 0.5-1%, of the same extracts revealed a moderate mito-depressant effect, while the lowest concentration found investigated the effect of stimulation of cell division.

CHAPTER 6. DETERMINATION OF CHLOROPHYLL PIGMENTS, CAROTENES, NITROGEN, PROTEIN, PHOSPHORUS, POTASSIUM AND DRY SUBSTANCES

Fresh substance from the plant was powdered very well, than alcohol was added. Next day the mixture was filter and dilute again with alcohol.

Table 23. The content of carotene and chlorophyll pigments of the *Sambucus ebulus* L. species

Collection place	chlorophyll pigments		
	Ca	Cb	K
Dăbuleni	10,614	4,721	3,961
Amărăşti	10,214	4,659	3,890
Mârşani	10,427	4,718	3,943
Teasc	10,601	4,719	3,954

6.5. Determination of crude protein. Total nitrogen

Total nitrogen content of the plant material was determined by wet digestion with sulfuric acid in a version of the classic Kjeldahl method. Release of nitrogen from plant material can be realized by dry combustion - Dumas method, a less used method because it requires special equipment.

6.6. Determination of phosphorus

Readings were recorded spectrophotometrically at a wavelength of 420 or 470 nm to the reference sample - standard 0, which has a slight yellowish color.

6.7. Determination of potassium

To determine the total potassium in plant, the vegetal material was mineralized by ignition or wet with a mixture of concentrated mineral acids. The resulting solution was assesed for potassium determination by flame emission photometry.

Table 24. The results of the analyzes

Collection place	Nitrogen (%)	Proteins (%)	P_2O_5 (%)	K_2O (%)	Dry substance (%)
Amărăşti	3,01	19,3	1,90	1,59	28,50
Mârşani	3,06	19,4	1,92	1,60	28,53
Teasc	3,10	19,01	1,94	1,63	28,51

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