Determination of Polyphenol and Flavonoid Profiles and Testing the Antibacterial Effect of *Acanthus longifolius* Comparative with *Vaccinium myrtillus*

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From our research we noticed that Acanthi balcanici herba (ABH) contains luteolin and quercetol and Myrtillus fructus (M-fr) contains chlorogenic acid, apigenol and rutoside. ABH has no antibacterial effect on any species tested, completely cancel the therapeutic effect of three drugs (amoxicillin + clavulanic acid, levofloxacin, amikacin) and have no significant effect on the other two antibiotics (cefotaxime and ceftazidime) when they are associated. All bacterial species were sensitive to M-fr and the extract has synergistic effect in combination with levoflaxacin, amikacin and cefotaxime.

Keywords: HPLC, TLC, poliphenols, plant extracts, antibacterial effect

Infectious diseases represents a significant cause of morbidity and mortality worldwide, accounting for approximately 50% of all deaths in tropical countries and 20% of deaths in the Americas [1]. The most frequent type of infections are located at the level of: urinary tract, respiratory tract, tissue and mucous membranes, bloodstream, digestive tract (including diarrhoea due to *Clostridium difficile*) and others [2]. Rapid appearance of resistant bacteria worldwide, endanger the effectiveness of antibiotics, which have revolutionized medicine and saved millions of lives. The resistance to antibiotics was attributed to overuse and abuse of these drugs and to the lack of development of new antimicrobial agents by the pharmaceutical industry [3]. Nowadays plant extracts with antimicrobial potential represents an important directive in the medical world, aiming to isolate active components or to develop new chemotherapeutic agents with applicability in treatment or as adjuvant therapy in infectious disease [4]. In vitro studies have shown that plants have antibacterial efficiency against many bacterial specie [5]. According to World Health Organization, natural therapies are used by 80% of world population [6]. The antimicrobial activity of plant extracts and active ingredients of basic plant can be determined using antibacterial screening protocols [7]. Plant extract from species *Vaccinium myrtillus*, thanks to the content of anthocyanins and phenolic acids, has antibacterial potential on *Escherichia coli* and *Proteus vulgaris*, being commonly used to treat urinary infections [8]. It also has an antibacterial effect on *Staphylococcus aureus* and *Salmonella enterica*[9].

Acanthus balcanicus is a species with broad leaves and white-pink flowers that grows in southwestern Romania, in Oltenia and Banat, in sunny places. The shape of leaves has been a model in ancient sculpture, for decorating the chapiter of Corinthian columns. The plant was used in ancient practices for the preventive role against plague [10]. In the literature there is no information about the chemical composition and pharmacological action of the species *Acanthus balcanicus*.

In this study we determined the chemical composition of the plant extracts *Acanthi balcanici herba (ABH)* and *Myrtillus fructus (M-fr)* using TLC, HPLC and GC-MS methods. We also determined the antibacterial potential of tincture *ABH*, not studied till now, comparative with *M-fr*. We tested the synergistic / antagonistic effects arisen between the plant extract to be tested and the antibiotic of choice used. For testing we have used reference strains derived from *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneumoniae*.

Experimental part

Plant material

Fresh herba of *A. balcanicus*. were collected in june 2013, in the city of Craiova, Romania. *V. myrtillus* was bought as fresh fruits from a supermarket in July, 2013. Both plant samples were air dried in the shade, at ambient temperature. Herbarium voucher samples are deposited in the Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, Romania.

Preparing the sample

Vegetable product was used as tinctures, manufactured by simple leaching, in a ratio of vegetable / solvent (ethanol 70°) of 1:5 (F.R. X). The control sample of the tested tincture can be found in the Pharmacognosy Laboratory of the Faculty of Pharmacy of Craiova [11].

Thin Layer Chromatography

For determining the polyphenols profile we used the following conditions:

- stationary phase: silica gel G F_{254} Merck, aluminum plates of 20×20 cm activated 60 min. At 105°C;
- mobile phase (A) ethyl acetate-formic acid -water (80:8:12, v/v/v) [12];
- mobile phase (B) toluene-dioxane-glacial acetic acid (80:25:4, v /v/v) [13].
- samples: ethanol solution (70°) at 20% concentration (mobile phase A) and hydrolyzed ethanol solutions extracted with apolar solvent (mobile phase B).
- reference solutions: methanol solution of 0.1 mg/mL of rutin, ferulic acid (Fluka), hyperoside, isoquercitrin, kaempferol, luteolin, chlorogenic acid (Roth), quercetol (Sigma), caffeic acid (Merck).

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- method: 10 μL of the test sample/reference solutions was applied to the starting line, tapes applied have a width of 1 cm from it and 1.5 cm between them. The migration distance was of 8 cm (mobile phase A) and 16 cm (mobile phase B). Revelation was made using DFBOA reactive sputtering 10% ethanol solution; UV examination (λ 365 nm) before and after the revelation, at a lamp Camag Reprostar 3 with Epson Phota Z incorporated camera [14].

Analysis by HPLC chromatography technique of flavonoids and polyphenol carboxylic acids of tinctures

HPLC analysis was performed using the following equipment and working conditions: HPLC Jasco MD-2015, two-pump, thermostat, UV-DAD detection system, degassing system; eluent A (acetonitrile); eluent B (0.1% phosphoric acid); working gradient: *prerun* →10% A, 90% B; 13.1 min. → 22% A, 78% B; 14.1 min. → 40% A, 60% B; 20.1 min. → 40% A, 60% B; 50 mPA pressure; detection: 330 nm; retention times [min.] for flavonosids, flavonoid aglycones and polyphenol carboxylic acids: chlorogenic acid - 7.12, caffeic acid – 7.964, ferulic acid - 13.147, rutoside - 15.19, isoquercitrin - 15.68, rosmarinic acid - 17.58, apigenin-7-glucoside - 17.65, quercetol - 18.71, kaempferol – 20.25 [15].

Tinctures analysis by gas chromatography coupled with mass spectrometry (GC–MS)

Analysis of the volatile compounds from the tincture was carried out in a gas chromatograph coupled to a mass spectrometer (Shimadzu GCMS-QP2010), equipped with an capillary column with weakly polar stationary phase, 5% phenyl, 95% metoxipoli siloxane, Alltec 15894, Ate 5 (30 m × 0.320 mm × 0.25μm). It was used the following conditions: injector temperature of 150°C, injection volume 0.5 μL at a ratio of 1:60 (split mode), column temperature (temperature ramp) 80°C (1 min), 170°C (3 min), 200°C (3 min);. The carrier gas (helium) flow was set at 3 mL/min. The temperatures of the transfer line, ion source, and quadrupole were 250, 220 and 150°C. Mass spectra were obtained with a scan range of 40 to 500 m/z and a solvent delay time of 3 min, and identification of the volatiles compounds was performed by comparing the mass spectra obtained with the data of the software from libraries (NIST 05) [16].

Testing anti-bacterial potential

To test the antibacterial effect we used the diffusion method of the nutrient agar (*Kirby-Bauer*) according to FR X. Agar Petri plates (diameter of 100 mm, in a uniform layer of 4 mm) are inoculated with a standardized inoculum of the test microorganism. The microorganisms to be tested coming from standard reference strain, purchased from the Institute Cantacuzzino, are classified as sensitive to the antibiotics of choice. The disks impregnated with antibiotic (control +) were chosen based on the sensitivity of bacterial species. Sterile filter paper tablet (Ø = 6 mm), previously sterilized, impregnated with a volume of 25 μL of plant extract are placed on the agar surface. After this, incubation of the plates was carried out for 18 hours at 37^{0}C, in the inverted position. Generally, antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones are measured.

The reading of the results was conducted by eye, using a graduated ruler, measuring the average diameter of the inhibition zone (DZI) in millimeters, induced by the test samples. Results were expressed as average values obtained by calculating the arithmetic mean of diameters for the three tests. Very small colonies were not considered, neither subsequent invasion of the inhibition zone or discrete increments within the zone of inhibition [17].

Results and discussions

The analysis of flavonosids and corresponding aglycones (mobile phase A) comparative with the reference substances, helped us to identify only some active components as rutoside and chlorogenic acid in *M-fr.* In *ABH* we observed the presence of numerous polyphenolic compounds and flavones but due to lack of standards they could not be accurately identified (table 1). Besides the flavonoid aglycone, TLC analysis revealed the presence of chlorophylls. Polyphenol acids analysis (mobile phase B)

Sample	Value of Rs	Color of spot after revelation	Observation
ABH	0.13	light blue	phenolic compound
	0.26	blue	phenolic compound
	0.35	light green	polyphenol acid
	0.42	blue-green	polyphenol acid
	0.58	blue	phenolic compound
	0.64	blue	phenolic compound
	0.72	blue	phenolic compound
M-fr.	0.15	light green	polyphenol acid
	0.20	yellow-orange	**rutoside**
	0.38	blue-green	**chlorogenic acid**
	0.46	blue	phenolic compound
	0.53	blue	phenolic compound
	0.92	violet	caffeic acid
Rutoside	0.20	yellow-orange	r.s
Hyperoside	0.43	yellow-orange	r.s
Isoquercitrin	0.50	yellow-orange	r.s
Chlorogenic acid	0.38	blue-green	r.s
Caffeic acid	0.92	blue-green	r.s
Ferulic acid	0.95	blue-green	r.s

Table 1
Rf VALUE OBTAINED AFTER REVELATION (MOBILE PHASE A) R.S -REFERENCE SUBSTANCE

Sample	Value of Rf	Color of spot after revelation	Observation
ABH	0.27	intensive yellow	luteol
	0.33	yellow-orange	quercetol
	0.40	orange	flavonic aglycone
	0.43	green	flavonic aglycone
	0.60	red	chlorophyll
	0.75	red	chlorophyll
	0.86	red	chlorophyll
	0.90	blue	phenolic compound
M-fr.	0.05	blue	phenolic compound
	0.10	blue	phenolic compound
	0.23	blue violet	chlorogenic acid
	0.90	blue	phenolic compound
Myricetin	0.03	yellow	r.s. (not migrated)
Luteol	0.27	yellow	r.s.
Quercetol	0.33	yellow	r.s.
Kaempferol	0.48	green	r.s.
Chlorogenic acid	0.23	Blue-green	r.s.
Ferulic acid	0.44	blue	r.s.

Table 2
Rf VALUE OBTAINED AFTER REVELATION (MOBILE PHASE B)

Table 3
HPLC RESULTS OF PLANT EXTRACTS

Tinctures	Chlorogenic acid [µg/ml]	Rosmarinic acid [µg/ml]	Kaempferol [µg/ml]	Quercetol [µg/ml]	Apigenin-7-glucoside [µg/ml]	Apigenol [µg/ml]	Isoquercitrin [µg/ml]
ABH	-	-	-	+	-		-
M-fr.	+	-	-	-	-	+	-

		ABH	*M-fr*
Compound	Retention time	%	%
Etil-metil-carbonat ($C_4H_8O_3$)	1.069	68.19	60.22

Table 4
GC- MS RESULTS OF VOLATILE COMPOUND FROM PLANT EXTRACTS

Table 5
DZI BACTERIAL GROWTH AVERAGE, AFTER TESTING THE TINCTURE *RESISTANT **INTERMEDIARY ***SENSIBLE*; nt-NOT TESTED*

Test products	*Staphylococcus aureus*	*Escherichia coli*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
ABH	0*	0*	0*	0*	0*
M-fr	25,4***	20,4***	17,4***	18,9***	23,3***
amoxicillin + clavulanic acid	32,6***	nt	nt	nt	nt
levofloxacin	nt	34,2***	nt	nt	nt
amikacin	nt	nt	33,6***	nt	nt
ceftazidime	nt	nt	nt	28,6***	nt
cefotaxime	nt	nt	nt	nt	35,8***

were identified in *M-fr* chlorogenic acid and in *ABH* luteola and quercetol (table 2).

HPLC analysis confirmed the preliminary results achieved by TLC, from the analysis of experimental results the following observations can be drawn: in *M-fr* sample was identified apigenol and chlorogenic acid and in *ABH* only quercetol (table 3).

The results of the GC-MS analysis of volatile compounds [18, 19] from the tincture obtained by relating the retention time of the gas chromatograms of the data provided by the mass spectrometer, led to the observation that volatile ethyl-methyl carbonate is present in both tinctures (table 4). In literature it is noted that vegetable extracts that contain anthocyanins and phenolic acids have potential antibacterial on the bacteria *Escherichia coli* and *Proteus vulgaris*, which causes frequently urinary infections and sepsis [20]. They also have antibacterial effect on *Staphylococcus aureus* and *Salmonella enterica* [21]. Studies confirm that plants rich in phenolic compounds have often antibacterial effect [22].

Analysis of antibacterial potential on *Staphylococcus aureus* showed no effect from *ABH* tincture and higher therapeutic potency of the tincture *M-fr,* but lower comparative with the antibiotic of choice (table 5). The combination between the reference standard antibiotic (amoxicillin + clavulanic acid) and *ABH* occurred

Table 6

DZI BACTERIAL GROWTH AVERAGE, AFTER TESTING THE TINCTURE ASSOCIATED WITH THE ANTIBIOTIC OF CHOICE *RESISTANT **INTERMEDIARY ***SENSIBLE *M+ = (SAMPLE +) nt-NOT TESTED*

Test products	*Staphylococcus aureus*	*Escherichia coli*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
(M+) + ABH	0*	0*	0*	25.4***	28.4***
(M+) + M-fr	32***	34.9***	34.9***	29.1***	34.9***
amoxiciliin + clavulanic acid (M +)	32.7***	nt	nt	nt	nt
levofloxacin (M +)	nt	33.4***	nt	nt	nt
amikacin (M +)	nt	nt	33.4***	nt	nt
Ceftazidime (M +)	nt	nt	nt	29.1**	nt
Cefotaxime (M +)	nt	nt	nt	nt	31.8***

antagonism action, the chemotherapeutic agent lost therapeutic properties, in association with *M-fr* no therapeutic effect changed (table 6).

Testing has revealed the natural resistance of *Escherichia coli* bacteria to *ABH* and susceptibility to *M-fr* (table 5). The association between levofloxacin and *ABH* canceled the antibacterial effect of antibiotic of choice while with *M-fr* appeared potentiation synergism (table 6).

Following the antibacterial sensitivity on the species *Proteus vulgaris* we observed no effect from *ABH* and antagonistic antibacterial effect to the association of amikacin with the plant extract. *M-fr* species is active on *Proteus vulgaris* and increased the antibacterial effect of amikacin when they were associated (table 5 and 6).

Tincture *M-fr* had a little effect on *Pseudomonas aeruginosa* and *ABH* no therapeutic efficacy (table 5). Combination of ceftazidime and *M-fr* presented addition synergism, and the association with *ABH* slightly decreased the effect (table 6).

No antibacterial effect was observed on species *Klebsiella pneumoniae* from *ABH,* and low effect from *M-fr* (Table 5). Potentiating synergism occured in the species *Klebsiella pneumoniae* with combination of cefotaxime and *M-fr*. The results showed that the antibacterial activity of cefotaxime against *Klebsiella pneumonia* is insignificant influenced by association with *ABH* tincture (table 6).

Conclusions

This work showed that both plant extracts contains phenolic compounds, *ABH* contains luteolin and quercetol and *M-fr* contains chlorogenic acid, apigenol and rutoside.

ABH tincture had no antibacterial activity on any microorganism tested and canceled the effects of standard antibiotics (amoxicillin + clavulanic acid, levofloxacin, amikacin), when associated with them. Our testing showed that tincture *M-fr* had an antimicrobial effect on a large range of microorganisms (gram positive and negative).

Experimental findings support the potential use of *M-fr* as a therapeutic agent for treating infectious diseases, supporting the use in complementary and alternative medicine.

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Manuscript received: 24.05.2017