TLC, GC-MS, HPLC Analyses and Testing the Antibacterial Effect of *Tragopogon pratensis* and *Vaccinium myrtillus*

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Plant extracts with antimicrobial potential are an important research directive in the current medical world, aiming to isolate active components in order to develop new chemotherapeutic agents that can be used in the treatment of various infectious disease. In the present study we determined the content of polyphenol carboxylic acids and flavonoids for two vegetal extracts, by means of TLC, GC-MS and HPLC techniques. Antimicrobial activity was determined on five bacterial species by performing agar diffusion method, using discs impregnated with standard antimicrobial substances and tested plant extracts. Our results showed that the alcoholic extracts Tragoponis pratensis folium and Myrtilli fructus contain polyphenolic compounds and had antimicrobial effect on a wide range of microorganisms (gram positive and negative).

Keywords: plant extracts, TLC, GC-MS, HPLC, antibiotic, synergism

Antibiotic resistance is considered to be one of the most pressing public health problems worldwide, because of the fact that the vast majority of bacteria become less sensitive to antibiotic treatment. The genetic capacity of bacteria to acquire multiple resistance to the used chemotherapeutic agents leads to an unfavorable progression of infectious diseases and, consequently, to therapeutic failure. Development of bacterial resistance to conventional antibiotics entailed developing research in the field of herbal extracts in order to find out new effective antibacterial agents.

Vegetable products have been used since ancient times as remedies for diseases, also playing a very important role in diet and nutrition. They contain essential nutrients such as carbohydrates, fats, essential proteins, vitamins, minerals, essential amino acids and fiber, and are also rich sources of carotene, ascorbic acid, riboflavin, folic acid and minerals (calcium, iron and phosphorus) [1-3].The bioactive substances within herbals have a wide range of therapeutic properties; one of the most valuable properties is the antimicrobial which is useful in the prevention or curative treatment of divers infections [4]. Today, according to the World Health Organization, 80% of the world's population use natural therapies [5]. In vitro studies demonstrated that plants have antibacterial effectiveness against many bacterial species, uncovering the importance of natural resources, less investigated in this regard, in fighting bacterial resistance and bacterial destruction [6]. Antimicrobial activity of plant extracts and natural active principles can be determined using antibacterial screening protocols [7].

The plant extract obtained from *Vaccinium myrtillus*, due to the contained anthocyanins and polyphenolic acids, exhibits antibacterial potential on *Escherichia coli* and *Proteus vulgaris*, thus being frequently used in the treatment of urinary tract infections. It also exerts antibacterial effect on *Staphylococcus aureus* and *Salmonella enterica* [8].

Tragopogon pratensis L. subsp pratensis, known as *goat's beard (*Asteraceae family*)*, is a biennial plant, found in Europe and North America, that grows in the plain and on the roadside. It is commonly found in England, the southeastern part of Scotland and central Ireland. The plant has a height of 30-100 cm, it blooms from June to October, and the flowers have a diameter of 3.5 cm. The root and stem contain a milky latex. The chemical composition of the plant has been elucidated by chemical and spectral techniques: it contains nine triterpenic saponosides, known as tragoponoids A-I, found throughout the plant structure, along with five other triterpenic glycosides [9].

The fresh roots of the plant are traditionally used for the preparation of dietary salads, recommended for obese or diabetic persons. Another use of the plant is obtaining chewing gum by collecting and drying the plant latex in glass pots [10].

Tragopogon pratensis is used to treat liver and gall bladder diseases. Furthermore, the *Tragopogon pratensis* extract possesses detoxifying properties, it stimulates appetite and digestion, and it also can be consumed by

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diabetes mellitus patients due to its high inulin content. The root is astringent, diuretic, depurative, expectorant, and stomachic. Recent studies have shown that the methanolic extract exhibits antiproliferative and tumor growth inhibiting properties [11].

The present study aims to investigate the antimicrobial potential of some rarely studied plant extracts: *Tragoponis pratensis folium* (*TPF*) and *Myrtilli fructus* (*M-fr*), taking as a reference the acknowledged antibacterial effect of the antibiotics of choice (control +) on reference strains derived from: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 700603). As a negative control, we used ethyl alcohol, which was also utilized for the preparation of plant extracts.

Experimental part

Material and methods

Samples preparation

The vegetal products were used as tinctures, obtained by simple percolation, in 1:5 ratio of vegetal product / solvent (ethanol 70°) (the preparation method complies with the Romanian Pharmacopoeia, 10th Edition - F.R. X [12]). The control sample of each tested tincture is stored in the Collection of the Laboratory of Pharmacognosy, Faculty of Pharmacy from Craiova [13].

Thin Layer Chromatography

The silica gel aluminum plates of 20×20 cm activated 60 min at 105°C (GF254 Merck plates) were used as stationary phase for the flavonoidic heterosis, their corresponding aglycones and polyphenol carboxylic acids determination; the mobile phases (A) and (B) were ethyl acetate-formic acid -water (80:8:12, v/v/v) [14], and toluene-dioxane-glacial acetic acid (80:25:4, v/v/v), respectively. The analyzed samples were: 20% ethanol solution, obtained from ethanol 70° (carried out using mobile phase A) and hydrolyzed ethanol solutions extracted with non-polar solvent (carried out sing mobile phase B). The hydrolyzed solutions were obtained as follows: 3 mL each of the tincture was refluxed with 3 mL of 10% HCl for 30 min, on thermostatically controlled water bath. After cooling they were extracted twice with 7 mL of diethyl ether. Nonpolar layers were combined, filtered through anhydrous sodium sulfate and brought to the residue, which was solubilized in 3 mL of absolute methanol. We used as reference solutions methanolic solutions of 0.1 mg/mL of rutin, ferulic acid (Fluka), hyperoside, isoquercitrin, kaempferol, luteolin, chlorogenic acid (Roth), quercetol (Sigma), caffeic acid (Merck). We applied applied at a distance of 1 cm from the starting 10µL of the test sample/reference solutions, with 1.5 cm between them, the migration distance of 8 cm (for mobile phase A), and 16 cm (for mobile phase B), respectively. The spots visualization was performed by sputtering 10% ethanolic solution of diphenylboryloxyethylendiaamine (DFBOA). The UV examination was performed at λ 365 nm before and after the spots visualization, using a Camag Reprostar 3 lamp with Epson Phota Z incorporated camera [15].

HPLC analysis of flavonoids and polyphenol carboxylic acids within tinctures

Equipment and working conditions for HPLC analysis were as follows: HPLC Jasco MD-2015, two-pump, thermostat, UV-DAD detection system, degassing system; eluent A (acetonitrile); eluent B (0.1% phosphoric acid); working gradient: *pre-run* →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 [16].

Gas chromatography coupled with mass spectrometry (GC-MS) tinctures analysis

Tinctures were analyzed by gas chromatography coupled with mass spectrometry (GC-MS) using a Shimadzu GCMS-QP2010 apparatus. The separation of the volatiles compounds was performed on a capillary column with weakly polar stationary phase, 5% phenyl, 95% polymethoxysiloxane, Alltec 15894, Ate 5 of 30 m length, internal diameter 0.32 mm, and 0.25 µm thickness of stationary phase. The working parameters for GC-MS: injector temperature of 150 °C, injection volume of 0.5 µL; 30 kPa pressure; total flow 101.8 mL/min; column flow 1.62 mL/min; linear velocity 46.9 cm/s; split ratio 60; purge flow 3 mL/min; carrier gas helium; column temperature (temperature ramp) 80°C (1 min), 170°C (3 min), 200°C (3 min); MS detector: ion source temperature 220°C; the interface temperature 250°C; scan speed 1666 u/s. Identification of the volatiles compounds was performed by comparing the obtained mass spectra with the available data provided by the software from libraries (NIST 05) [17,18].

Antibacterial potential test

There was poured nutrient agar (Mueller-Hinton) in Petri plates with a diameter of 100 mm, in a 4 mm uniform layer. Inoculum preparation was performed by 2-3 standard colony suspending in physiological saline, turbidity of the suspension is controlled by nephelometry. The culture medium must have a *p*H of 7.2-7.4 and a suitable composition that is proper for development of the tested bacterial species. Seeding was carried out by flooding the nutrient medium with the bacterial suspension, followed by removal of the excess. Drying is achieved by keeping the inoculated plates for 10 min at room temperature (22 °C) prior to the samples insertion in the oven. The microorganisms to be tested coming from standard reference strain were purchased from the Cantacuzino Institute, being sensitive to the antibiotics of choice. In order to test the antibacterial effect of our vegetal extracts we used the diffusion method of the nutrient agar (*Kirby-Bauer*) according to F.R. X [11]. Sterile filter paper tablet (Ø = 6 mm), were impregnated with a volume of 25 µL of plant extract to be tested, and then were maintained in oven for 24 h, at 20°C, so that alcohol evaporates. Sample discs are prepared in the same conditions. The disks were impregnated with the proper antibiotic (control +), according to the sensitivity of bacterial species. Deposition of the impregnated slices with test samples was carried out after drying of the paper, about 15 min after sowing, using an ophthalmic forceps, applying each sample to be analyzed on the surface of the culture medium. Test discs were placed at 1.5 cm distance from the edge of the Petri dish and 3 cm distance from each other. Incubation was carried out for 18 h at 37°C, in the inverted position of the Petri plate. The results were read by visual inspection, using a graduated ruler, and the average diameter of the inhibition zone (DZI), in millimeters, induced by the test samples was recorded. Results were expressed as average values obtained by calculating the arithmetic mean of diameters

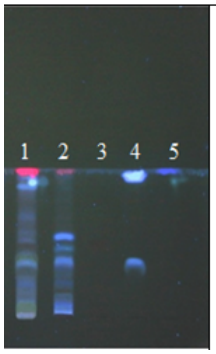

Fig. 1 Chromatogram obtained before spots visualization (mobile phase A) 1. TPF; 2. M-fr; 3. rutin, hyperoside, isoquercitrin (bottom-up); 4. chlorogenic acid, caffeic acid (bottom-up); 5. ferulic acid.

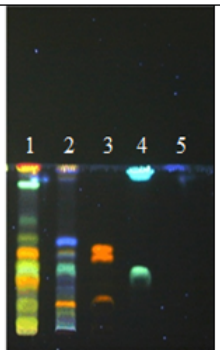

Fig. 2 Chromatogram obtained after spots visualization (mobile phase A)1. TPF; 2. M-fr; 3. rutin, hyperoside, isoquercitrin (bottom-up); 4. chlorogenic acid, caffeic acid (bottom-up); 5. ferulic acid

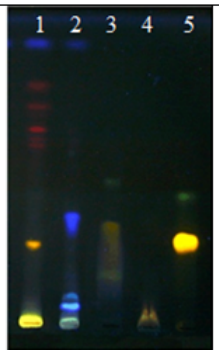

Fig. 3 Chromatogram obtained before spots visualization (mobile phase B) 1. TPF; 2. M-fr; 3. quercetol, kaempferol (bottom-up); 4. myricetin; 5. luteol

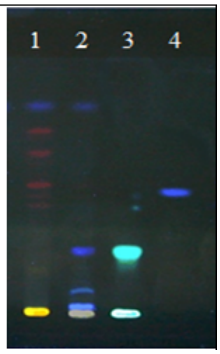

Fig. 4 Chromatogram obtained after spots visualization with DFBOA, (mobile phase B)1. TPF; 2. M-fr; 3.chlorogenic acid; =4.ferulic acid.

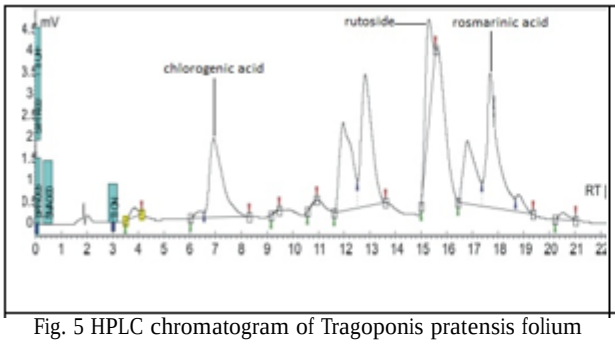

Fig. 5 HPLC chromatogram of Tragoponis pratensis folium (TPF) extract

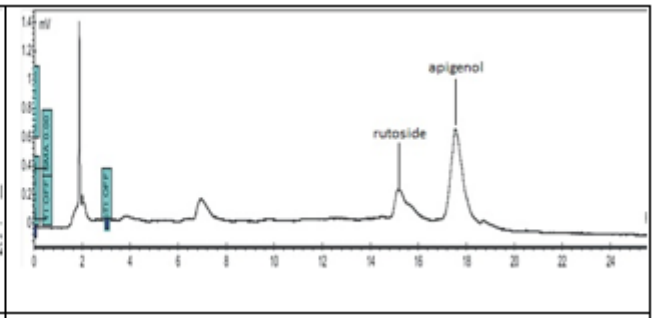

Fig. 6 HPLC chromatogram for Myrtilli fructus (M-fr) extract

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 [16-18].

Results and discussions

Thin Layer Chromatography analysis (TLC)

TLC is a less expensive and fast separation method, which allows investigation of a large number of vegetal compounds and drug molecules [19-20]. Hyperoside, luteol and chlorogenic acid were identified in the *TPF* extract via TLC analysis, and rutoside, chlorogenic acid and caffeic acid were identified in the *M-fr* extract using the same analytical method, as presented in figures 1-4. The presence of chlorophylls was also revealed in both extracts. For some phytocompunds (*i.e.* quercetol, kaempferol, myricetin, and luteol) TLC separation with mobile phase B the reagent-based visualization was no longer required (fig. 3). At the same time, in the case of mobile phase B separation, ferulic acid was identified only after spraying the TLC plate with the spot visualization reagent (DFBOA) (fig. 4).

HPLC analysis

HPLC is an important analytical method that enables both identification and quantitative determination of the tested components [21]. Figures 5 and 6 present the HPLC chromatograms for our *TPF* and *M-fr* analyzed tinctures.

The HPLC results presented in table 1 indicate specify that traces of apigenol and rutoside were experimentally identified in both vegetal extracts. In the *TPF* extract, two polyphenolcarboxylic acids were identified in high concentration: 258.25 µg/mL rosmarinic acid and 270 µg/mL chlorogenic acid, respectively.

GC-MS analysis

Table 1

HPLC RESULTS (CHEMICAL IDENTITY AND CONCENTRATION,µg/mL) OF T*RAGOPONIS PRATENSIS FOLIUM* (TPF) AND *MYRTILLI FRUCTUS* (M-FR) EXTRACTS

Tinctures	Chlorogenic acid [µg/ml]	Rosmarinic acid [µg/ml]	Kaempferol [µg/ml]	Querceto l [µg/ml]	Apigenin-7-gluco-side [µg/ml]	Apigenol [µg/ml	Isoquercitrin [µg/ml]	Rutoside
TPF	270	258.25	–	–	–	+	–	+
M-fr	–	–	–	–	–	+	–	+

Compound	Chemical formula	RT (min)	TPF %	M-fr %
ethyl methyl carbonate	$C_4H_8O_3$	1.069	–	60.22
ethoxyacetic acid	$C_4H_8O_3$	1.064	48.85	–
ethanimidic acid, ethyl ester	C_4H_9NO	1.123	13.42	–
2-propen-1-ol -acetate	$C_5H_7O_2$	1.299	2.57	–
2,5-dimethyl-hexanediol	$C_8H_{18}O_2$	1.372	2.62	–
1,4,9-decatriene	$C_{10}H_{16}$	12.342	32.53	–

Table 2
GC-MS RESULTS OF T*RAGOPONIS PRATENSIS FOLIUM* (TPF) AND *MYRTILLI FRUCTUS* (M-FR) EXTRACTS

Test Product		*Staphylococcus aureus*	*Escherichia coli*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
	TPF	22,1***	18,2***	21,1***	19,6***	21,8**
	M-fr	25,4***	20,4***	17,4***	18,9***	23,3***
DZI (mm)	amoxicillin + clavulanic acid (control +)	32,6***	nt	nt	nt	nt
	levofloxacin (control +)	nt	34,2***	nt	nt	nt
	amikacin (control +)	nt	nt	33,6***	nt	nt
	ceftazidime (control +)	nt	nt	nt	28,6***	nt
	cefotaxime (control +)	nt	nt	nt	nt	35,8***
	ethyl alcohol 70°	15*	7,3*	0*	0*	0*

Table 3
AVERAGE DIAMETERS OF BACTERIAL GROWTH INHIBITION FOLLOWING *TRAGOPONIS PRATENSIS FOLIUM* (TPF) AND *MYRTILLI FRUCTUS* (M-FR) TINCTURES, SELECTED ANTIBIOTICS (CONTROL +), AND ETHYL ALCOHOL 70° DISTINCT TESTING

** resistant, *** intermediate, *** sensitive, nt – not tested; DZI- Average diameter of inhibition zones (mm)*

Table 4
AVERAGE DIAMETERS OF BACTERIAL GROWTH INHIBITION AFTER TESTING *TRAGOPONIS PRATENSIS FOLIUM* (TPF) AND MYRTILLI FRUCTUS (M-FR) TINCTURES ASSOCIATED WITH THE ANTIBIOTIC OF CHOICE

Test Product		*Staphylococcus aureus*	*Escherichia coli*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
DZI (mm)	(*M+*) + *Tragoponis pratensis folium*	34.9***	36.4***	34.2***	29.8***	36.4***
	(*M+*) + *Myrtilli fructus*	32***	34.9***	34.9***	29.1***	34.9***
	amoxicillin + clavulanic acid (*M+*)	32.7***	–	–	–	–
	levofloxacin (*M+*)	–	33.4***	–	–	–
	amikacin (*M+*)	–	–	33.4***	–	–
	ceftazidime (*M+*)	–	–	–	29.1**	–
	cefotaxime (M +)	–	–	–	–	31.8***
	(*M+*) + ethyl alcohol	32***	32***	0*	28.4***	29.1***

** resistant, *** intermediate, *** sensitive, nt-untested; DZI- Average diameter of inhibition zones (mm)*

The results of the GC-MS analysis, presented in table 2, emphasize that the presence of ethyl methyl carbonate is characteristic in the mass spectra for *M-fr* tincture, and ethoxyacetic acid, 1,4,9-decatriene, ethanimidic acid (as ethyl ester), 2,5-dimethyl-hexanediol, and 2-propen-1-ol-acetate are distinguished in the *TPF* tincture.

Antibacterial potential test

The antibacterial effects of *TPF* and *M-fr* tinctures, as well as of selected antibiotics are presented in table 3.

Our results presented in table 3 reveal that *TPF* extract is more effective against *Proteus vulgaris* and *Pseudomonas aeruginosa* compared to *Myrtilli fructus*, due to the fact that it displays higher values of the inhibition diameter; at the same time, *TPF* extract presents fewer antibiotic alternatives. The plant extract from *Myrtilli fructus* is more effective on *Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumonia* strains.

In table 4 are presented the antibacterial effects, in terms of average diameters of bacterial growth inhibition that are exhibit by the vegetal extracts and selected antibiotics association, on five microorganism species.

DZI- Average diameter of inhibition zones (mm)

Tinctures and antibiotic of choice association leads to synergism or potentiation, as observed by the greater diameter of bacterial growth inhibition. Antimicrobial properties of plants are conferred by their ability to synthesize certain secondary metabolites with relatively complex structures.

Conclusions

Plants extracts are commonly used in diverse infectious processes, as valuable alternatives and adjuvants as well to allopathic antibiotic therapy, with the benefit of better tolerance.

We determined that tinctures obtained from *Tragoponis pratensis folium* and *Myrtilli fructus* contain polyphenolic compounds using the TLC, GC-MS and HPLC techniques. Antimicrobial activity as determined by the agar diffusion method reveals that both tinctures exhibit antimicrobial effect on a wide range of micro-organisms (gram positive and negative). An important conclusion of our research is that synergism occurs when tinctures are associated with the antibiotic of choice, based on the greater diameter of bacterial growth inhibition in comparison to the antibiotic alone, thus unfolding new potential strategies in infectious diseases treatment.

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