

# GC-MS, HPLC, TLC of *Dorycnium herbaceum* Tincture Species and Synergistic / Antagonist Effect Testing in Combination with Antibiotics

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*The study objectives were to determine the chemical composition and the synergistic / antagonistic effect of the association between hydroalcoholic extract from the Dorycnii pentaphylli herba (DPH) and the antibiotics of choice, on five reference strains. The tincture contains flavonoids and polyphenol carboxylic acids in low concentrations. DPH has an antagonistic effect on three of the drugs tested (amoxicillin + clavulanic acid, levofloxacin, amikacin), the therapeutic effect being completely canceled and has no significant effect on two of them (ceftazidime, cefotaxime).*

**Keywords:** GC-MS, polyphenols, plant extracts, antagonism

Nowadays bacterial infections are still a major cause of morbidity and mortality, both among people and among animals. The antibiotic resistance phenomenon, frequently encountered lately, shows an increase in the number of bacterial species able to develop resistance mechanisms to antimicrobial classics. The development of different resistance mechanisms to antibiotics within the same strain resulted in the so-called multi-drug resistant bacteria [1]. In cases of multidrug-resistant pathogens, resistance to second and even third-line antibiotics is thus sequentially acquired an illustrative example being represented by nosocomial infections with *Staphylococcus aureus*. Some pathogens such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* have also a high level of intrinsic resistance [2]. To limit this undesirable phenomenon we must adopt a proper therapeutic conduct of antibiotics administration. The appearance of antibiotic resistance and inadequate antibacterial treatment are important public health problems worldwide. Appropriate antibiotic administration, the correct combination with chemotherapy drugs together or with the appropriate dietary supplements and the respect of the dosage are essential for proper management of infectious conditions.

Nowadays herbal therapies are commonly used to fight bacterial infections alone or as adjuvant allopathic therapy [3].

Current research has demonstrated that DPH has important hypoglycemic effects, restoring glucose homeostasis after several weeks of use, activity observed by testing in mice with streptozotocin diabetes [4]. The therapeutic properties found in literature refer to strong antibacterial effect on *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus mirabilis*, a more reduced effect on *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and lack of effect on *Escherichia coli* [5].

In this study we determined the chemical composition of the plant extract *Dorycnii pentaphylla Herba* (DPH) originated from *Dorycnium herbaceum* species (*Fabaceae*) by various methods. We also determined the effects of synergistic / antagonistic effects arisen between the plant extract to be tested and the antibiotic of choice used, with known antibacterial effect on the bacteria

chosen. For testing I have used reference strains derived from *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*. In making and interpreting qualitative antibiograms we seek the respect with US standards, developed by CLSI (Clinical Laboratory Standards Institute).

## Experimental part

### Plant material

*Dorycnium herbaceum* were harvested from Botanical Garden of Craiova City, Dolj County, Romania, between may and june 2012. Herba was air-dried in the shade, at room 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 in the form of a tincture, 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 collection of the Pharmacognosy Laboratory of the Faculty of Pharmacy of Craiova [6].

### Thin Layer Chromatography

Flavonoid heterosides, their corresponding aglycones and polyphenol carboxylic acids were identified using aluminum backed silica gel GF<sub>254</sub> Merck TLC plates (20×20cm, activated for 60 min. at 105°C) as the stationary phase, ethyl acetate-formic acid-water (80:8:12, v/v/v) as mobile phase A [7] and toluene-dioxane-glacial acetic acid (80:25:4, v/v/v), mobile phase B [8]. Analyzed samples were 20% ethanol solutions (for mobile phase A) and hydrolyzed ethanol solutions extracted with apolar solvent (for mobile phase B). To obtain the hydrolyzed solutions, 3 mL of each extract were refluxed with 3 mL of 10% HCl solution for 30 min in an electric bath. After cooling they were extracted twice with 7 mL of diethyl ether. Apolar layers were combined, filtered through anhydrous sodium sulphate and brought to the residue which was solubilized in 3 mL of absolute methanol. 0.1 mg/mL methanol solutions of rutin, hyperoside, isoquercitrin, kaempferol, luteolin, chlorogenic acid (Roth, Germany), quercetol,

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ferulic acid (Sigma-Aldrich, Germany) and caffeic acid (Merck, Germany) were used as reference solutions. 10  $\mu$ L of the test samples/reference solutions were applied to the starting line, the tapes having 1 cm width with 1.5 cm between them. The plates were developed over a path of 8 cm (mobile phase A) and 16 cm (mobile phase B). Revelation was done by sputtering 10% ethanol solution of diphenylboriloxyethylendiamine (DFBOA, Sigma-Aldrich, Germany). The plates were examined in UV light ( $\lambda$ 365 nm) and photographed before and after the revelation, using an Epson Photo PC 3000 Z Mod G 790A and Camag Reprostar 3 System [9].

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

Equipment and working conditions for HPLC analysis were as follows: HPLC Jasco MD-2015 equipped with degassing system, binary gradient, thermostat and UV-DAD detection system; eluent A (acetonitrile); eluent B (0.1% phosphoric acid); working gradient: prerun  $\rightarrow$  10% A, 90% B; 13.1 min.  $\rightarrow$  22% A, 78% B; 14.1 min.  $\rightarrow$  40% A, 60% B; 20.1 min.  $\rightarrow$  40% A, 60% B; 50 mPA pressure; detection: 330 nm; retention times [min.] for flavonoids, 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 [10].

#### *Tinctures analysis by gas chromatography coupled with mass spectrometry (GC-MS).*

Tincture was analyzed by gas chromatography coupled with mass spectrometry (GC-MS) to an apparatus Shimadzu GCMS-QP2010. The separation of the volatiles compounds was performed on a capillary column with weakly polar stationary phase, 5% phenyl, 95% metoxipoli siloxane, Alltec 15894, Ate 5 of 30 m length, internal diameter 0.32 mm and stationary phase thickness of 0.25  $\mu$ m layer. The working parameters for GC-MS: injector temperature of 150°C, injection volume: 0.5  $\mu$ L; 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 mass spectra obtained with the data of the software from libraries (NIST 05).

#### *Testing anti-bacterial potential*

There was poured nutrient agar (Mueller-Hinton) in Petri plates, with a diameter of 100 mm, in a uniform layer of 4 mm. Inoculum preparation was performed by 2-3 standard colony suspending in physiological saline, turbidity of the suspension is nephelometrically controlled. The culture medium must have a pH of 7.2-7.4 and a suitable composition suitable for development of the bacterial species tested. 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 submission of the samples. The microorganisms to be tested coming from standard reference strain, purchased from the Institute Cantacuzzino, are classified as sensitive to the antibiotics of choice. To test the antibacterial effect we used the diffusion method of the nutrient agar (*Kirby-Bauer*) according to FR X. Sterile filter paper tablet ( $\varnothing = 6$  mm), previously sterilized, were impregnated with a volume of 25  $\mu$ L of plant extract to be tested and then were maintained in oven 24 h to evaporate the alcohol. Sample discs are prepared in the same conditions. The disks impregnated with antibiotic (control +) were chosen based on the sensitivity of bacterial species. Deposition of the slices impregnated 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. Deposition of the test discs was performed 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 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 [11, 12].

#### **Results and discussions**

In the specialist literature was noted that plants containing active antibacterial principles (polyphenols,

In the specialist literature was noted that plants containing active antibacterial principles (polyphenols, flavonoids, essential oils, vitamins) can be used in the

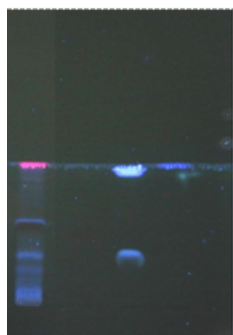


Fig. 1. Chromatogram obtained before revelation (mobile phase A) 1.DPH; 2.rutin, hyperoside, isoquercitrin (bottom-up); 3.chlorogenic acid, caffeic acid (bottom-up); 4.ferulic acid

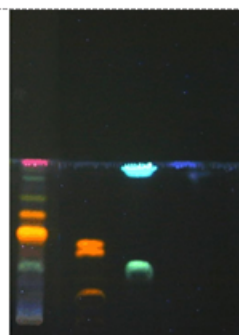


Fig. 2. Chromatogram obtained after revelation (mobile phase A) 1.DPH; 2.rutin, hyperoside, isoquercitrin (bottom-up); 3.chlorogenic acid, caffeic acid (bottom-up); 4.ferulic acid



Fig. 3. Chromatogram obtained before revelation (mobile phase B) 1.DPH 2.quercetol, kaempferol (bottom-up); 3.myricetin; 4.luteol

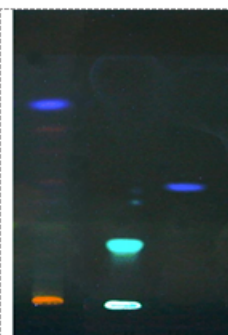


Fig. 4. Chromatogram obtained after DFBOA revelation, (mobile phase B) 1.DPH 2.chlorogenic acid; 3.ferulic acid

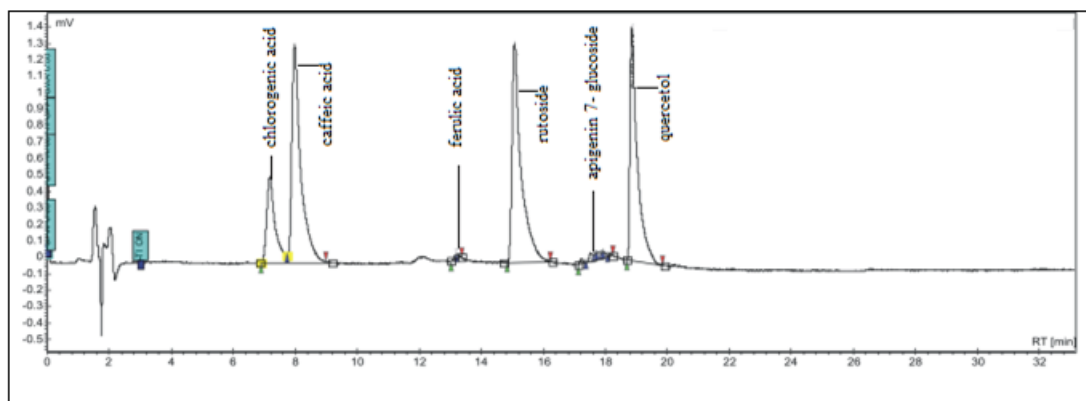


Fig. 5. HPLC chromatogram for some flavonoids standards and flavonoid aglycone and polyphenol carboxylic acids

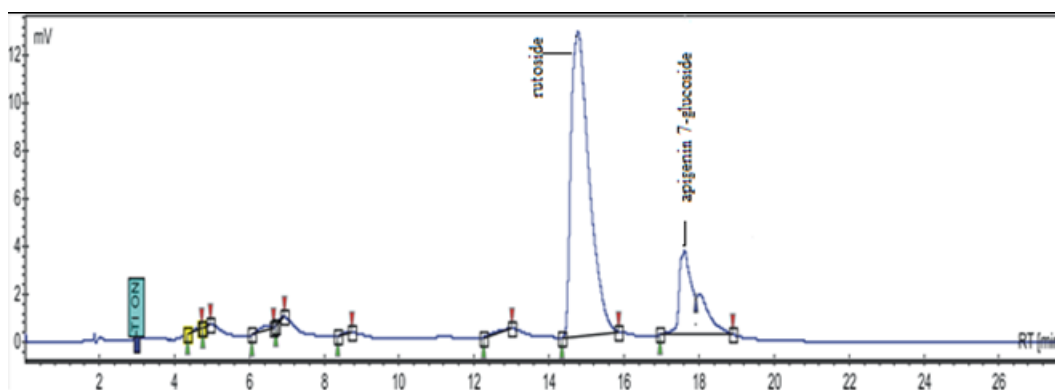


Fig. 6. HPLC chromatogram of the *DPH* tincture

prevention or therapeutic treatment of infections [13, 14, 15]. Polyphenols and flavonoids also have notable antiviral effects (comparable to acyclovir and oseltamivir) [16] or antifungal medicines [17].

When analyzing polyphenol carboxylic acids were identified chlorogenic acid, and ferulic acid was absent. Many components of polyphenol carboxylic acid type were highlighted in the form of characteristic bands, but due to lack of standards they could not be accurately identified. In addition to the flavonoid aglycone, TLC analysis also revealed the presence of chlorophylls.

Retention times for the main flavonoid components, polyphenol carboxylic acids and flavonoid aglycone are shown in figure 5. From the analysis of experimental results, there was observed that *DPH* tincture contains in its traces apigenin-7-glucoside flavonoid and rutin.

GC-MS analysis carried out after the tincture *DPH* library of spectra allowed the identification of a few of volatile compounds. In addition, another part of the substances identified in the mass spectra was not considered because

it represents artifacts arising directly from the chromatography column or by reaction of the active components in natural compounds spinal and tinctures. The results of GC-MS analysis of the volatile compounds 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 the probe *DPH* were identified six volatile compounds, such as: acid ethyl ester ethanimidic, 2-propen-1-ol acetate, 1,1 diethoxy ethane.

The antimicrobial activity of the *DPH* extract was determined using antibacterial screening [18]. From our research we observed that the plant extract combination with standard antibiotic makes the antibacterial effect disappear (amoxicillin + clavulanic acid, levofloxacin, amikacin) or insignificant decreased it (ceftazidime, cefotaxime).

The antimicrobial activity of polyphenols has been intensively studied and hundreds of publications reporting the antimicrobial activity have been published. In an era of

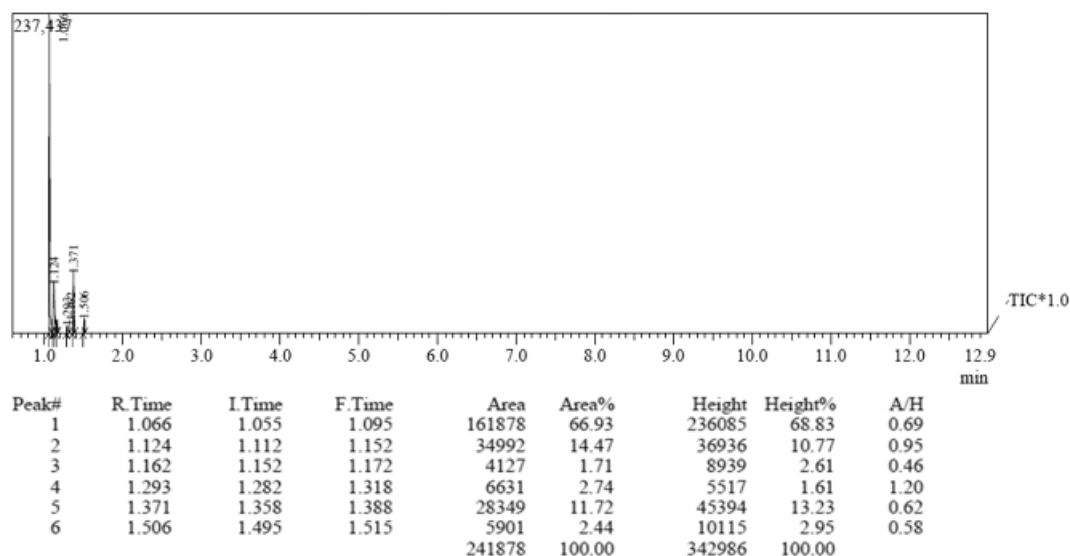


Fig. 7. Gas chromatography and identified by mass spectrometry paintings for volatile compounds in *DPH*



**Table 1**  
DZI BACTERIAL GROWTH AVERAGE, AFTER TESTING THE TINCTURE ASSOCIATED WITH THE ANTIBIOTIC OF CHOICE

Tincture for testing	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Proteus vulgaris</i>	<i>Pseudomona s aeruginosa</i>	<i>Klebsiella pneumoniae</i>
(M+) + DPH	0*	0*	0*	28,1***	31,3***
Amoxicillin + 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		31,8***

\*resistant ;\*\*intermediary; \*\*\*sensible M+= (SAMPLE+) nt-not tested

increasing antibiotic resistance, the development of new strategies to fight infections is welcome. Further studies are needed to evaluate the therapeutic potential of polyphenols alone or in combination with currently available antibiotics [19].

### C]onclusions

The tested plant product contains flavonoids and polyphenol carboxylic acids in small quantities. *DPH*specie tincture canceled (amoxicillin + clavulanic acid, levofloxacin, amikacin) standard antibiotics antibacterial effect in association with the above. *DPH*has no significant effect on ceftazidime and cefotaxime, the bacterias remained sensitive to the antibiotics. There are hereby imposed restrictions on the consumption of *DPH* tincture if the patient is treated with some antibiotics because they can completely cancel the effect.

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