Quantitative Analysis and Antibacterial Activity of Some Coumarins Extracts

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Extracts of melilot, cinnamon and ash were analyzed with TLC and molecular absorption spectrometry. With TLC method coumarin and coumarins derivatives (fraxetin, psoralen and scopoletin) contained in the studied plants were identified. The coumarin concentrations in melilot (637.84 mg/100g d.w) and cinnamon (58.594 mg/100g d.w.) were the highest. Melilot and cinnamon extracts have been tested against two reference bacterial strains and against two clinical bacterial strains in order to preliminary evaluate their antimicrobial profile. Our results showed a significant response of bacterial growth on culture media when exposed to coumarin derivatives found in melilot and cinnamon extracts. Overall, the inhibitory activity of extracts was higher on Gram positive bacteria on one hand and on clinical strains on the other hand suggesting an important practical aspect.

Keywords: coumarin, melilot, cinnamon, ash, antibacterial activity, TLC, molecular absorption spectrometry

Coumarins and coumarins-derivatives have showed considerable interest due to their potential in human health. They are widely used in different therapies, in perfume industry and, due to their pleasant aromatic odor, in the food industry as flavoring ingredients [1]. Coumarins and coumarins-derivatives are naturally found in some plant species [2]. Coumarin-derivatives might represent an important sources of bio-active compounds [3, 4].

Coumarins and coumarin derivatives demonstrate significant biological activities like anti-inflammatory [5] and anti-diabetic [6]. They also prove to be enzyme inhibitors [7-10]. Coumarins and coumarin-derivatives were recognized as valuable antibacterial [11, 12], antifungal [13] and antiviral agents [14].

As a disadvantage, use of high amount of coumarins and coumarins-derivatives is toxic to human body and can effectively determine carcinogenic effects [15]. The European Food Safety Authority has established a tolerable daily dose (TDI) of 0.1 mg / kg / day which should not be exceeded [16]. A high content of coumarins and coumarins-derivatives was found in cinnamon, ash (*Fraxinus excelsior*) and melilot (*Melilotus officinalis*) [17].

In melilot (*Melilotus officinalis* L.) the following coumarin derivatives have been identified: melilotozida (2-*O*-coumaric acid glycoside); melilotina (3,4-dihidro-cumarina); meliloti acid (acid dihidrocumaric); melilotincumarinic acid (acid ester meliloti and cumaric) [17].

In ash (*Fraxini folium*) the following coumarin derivatives have been found: fraxin, fraxoside, (fraxetol 8-glucoside) fraxetol and izofraxetol, esculetin, isoesculetin [18].

In cinnamon (*Cinnamomum aromaticum*) were identified coumarin compounds and derivatives of cinnamic aldehyde, namely: coumarin, cinnamaldehyde, 2-methoxycinnamaldehyde, 2-hydroxycinnamaldehyde, coniferaldehyde, cinnamic acid, 2-hydroxycinnamic acid and cinnamic alcohol [4, 19-20]. Extraction of coumarin and coumarin-derivatives from vegetable products with polar solvents (water, ethanol, methanol) was found to be most effective [3,4, 21-22].

The aim of the study was to determine the amount of coumarin derivatives present in three vegetable products: melilot (*Melilotus officinalis*), cinnamon (*Cinnamomum aromaticum*) and ash (*Fraxini folium*), products known for their therapeutic action and applications in the food industry. The study was followed by the antibacterial activity testing of the two most rich in coumarin extracts.

Experimental part

Plant materials

Aerial part of melilot (*Melilotus officinalis* L.) was collected in May 2015 in natural populations at flowering stage from Constanta County, Romania. The freshly cut plants were dried in the drying room at ambient temperature (about 20–22ºC) until constant weight was achieved.

Ash leaves (*Fraxini folium*) were harvested in June 2015 from Dobrogea County. The leaves were dried at ambient temperature until constant weight was achieved.

Cinnamon (*Cinnamomum aromaticum*) was purchased from local market (Constanta City, Romania).

Chemicals

All used reagents were analytical grade reagent.

Standards of **2*H*-chromen-2-one** (coumarin), fraxetin, psoralen and scopoletin e"99% (HPLC) were obtained from Sigma-Aldrich.

Apparatus

Spectrometric measurements were carried out using a Jasco V-630 Spectrophotometer (double-beam spectro-photometer with single monochromator, silicon photodiode detectors, range 190 to 1100 nm, fixed bandpass of 1.5 nm and high-speed scanning up to 8,000 nm/min).

The UVA - 14 T lamp (Ultra- Lum. Inc. Carson - USA) working in the range l = 245 to 365 nm, was used to identify coumarins and coumarin-derivatives with TLC.

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Sample	20µL (0.005 g extract in 0.5 mL methanol)
Standard	10µL (0.05 g standard in 5 mL methanol)
Stationary phase	silica gel F254 silica gel 60 (previously activated 1 h at 105°C)
Mobile phase	Toluene - Et_2O (1 : 1, v/v, saturated with 10% acetic acid, upper phase)

Table 1
TLC WORKING CHARACTERISTICS

TLC (Thin Layer Chromatography) method

Coumarine derivative from selected plants were identified by Thin Layer Chromatography method (table 1) [23-26].

TLC was performed on 20x20 cm plastic plate, silica gel coated with fluorescent indicator F254 and on 20x20 cm aluminium plate, silica gel 60 both purchased from Merck, Germany.

Sample preparation

0.5 g of powdered herbal material from each plant was extracted with 10mL of 50% methanol at 50°C for 40 min, 2 times successively. The extractive solution was evaporated and the resulting residue was collected with 3 mL methanol. 20µL sample of resulting solution was applied as spots width band.

Standard preparation

0.05 g of each standard (coumarin, fraxetin, psoralen and scopoletin) was dissolved in 5 mL methanol.10µL sample of standard solutions was applied as spots width band.

TLC was performed as per characteristics presented in table 1.

The spots of coumarins were detected under UV 365 nm before and after spraying the plates with 10% methanolic KOH.

The relationship between the distance travelled by the solvent front and the substance was expressed as the Rf value. [27]

Spectrophotometric method

Quantitative analysis is based on the colour reaction of hydroxy aromatic acid (resulted from lactone hydrolysis) with diazonium salts. Absorbance was measured with Jasco V-630 Spectrophotometer at 520 nm.

0.5 g of powdered dry plant was triturated with 1 g sand and 1 mL distilled water. After heating 1 h at 40°C the mixture was triturated with 5 mL ethanol and 10 mL ethylic ether added drop-by-drop. The solution was filtered on Whatman No. 1 filter paper. The described procedure was applied twice until the sample has been broken down. The reunited extractive solutions volume was let 24h at ambient temperature for complete evaporation of ether. The solution was then transferred into a 100 mL volumetric flask, brought to the mark with sulfuric acid solution of 0.05 N and homogenized 30 min at 70°C resulting solution (A). Solution (A) was obtained after cooling and filtration.

5 mL solution (A) were transferred into a 50 mL volumetric flask, treated with 5 mL of 7% sodium carbonate solution and heated 5 min at 85°C. After cooling at ambient temperature, 5 mL of 0.05 N sulfuric acid solution and 5 mL *p*-nitrobenzenediazonium chloride (1,5 mmols/L) were added [28].The mixture was strongly homogenized until a red color azo compound was obtained. The mixture was brought to the mark with distilled water and right away measured at 520 nm [18, 23, 29].

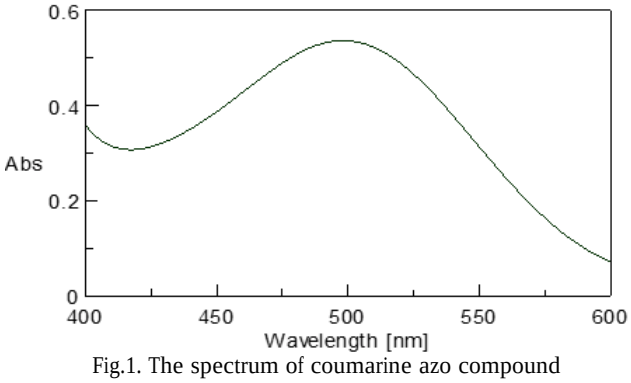

Fig.1. The spectrum of coumarine azo compound

The maximum absorption value of coumarine azo compound is presented in figure 1.

Calibration curve

In a series of 50 mL volumetric flasks were introduced volumes of 0.1, 0.2, 0.5, 0.8, 1.0 mL of 0.02% coumarin standard solution and also 5 mL of 7% sodium carbonate solution each. The solutions were heated 5 min at 85°C. After cooling at ambient temperature 5 mL of 0.05 N sulfuric acid solution and 5 mL *p*-nitrobenzenediazonium chloride (1.5 mmols/L) [28] were added resulting a red azo compound. The resulting solutions were brought to the mark with distilled water and right away measured at 520 nm (fig. 2).

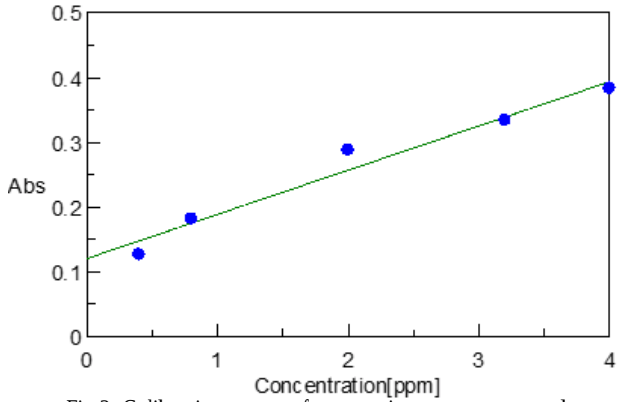

Fig.2. Calibration curve of coumarine azo compound

The characteristics of the obtained calibration curve are: Y = A * X + B; A = 0.0682698; B = 0.120774; Correlation Coefficient = 0.982603; Standard Error = 0.334711;

Coumarin concentration of standards and samples were expressed as coumarin azo compound and calculated as follow (eq. 1) and (eq. 2):

$$A_{520\ nm} = 0.0682698 \times C_{coumarin} + 0.120774 \quad (1)$$

$R^2 = 0.982603$ mg/mL (ppm)

$$C_{coumarin} = \frac{A_{520nm} - 0.120774}{0.0682698} = \\ = 14.6477 \times A_{520nm} - 1.76906 \quad (2)$$

The content of coumarin expressed as mg/100 g dry weight was calculated using the formula (eq. 3):

$$C = \left[\frac{V_{s,flask}(mL) \times 10^{-3}}{m_{sample}(g)} \times F \times C_{det.} \left(\frac{mg}{L} \right) \right] \times 100 \ \frac{\text{mg coumarin}}{100\text{g d.w.}} \quad (3)$$

Antibacterial activity

Sample extraction of coumarins and their derivatives

50 g of powdered dry plant (melilot and cinnamon) were extracted with 250 mL methanol by heating 40 minutes at 50°C. The resulted extracted solutions were filtrated and 130 mL melilot extractive solution and 132 mL cinnamon extractive solution were obtained.

Disfusimetric well method

Effect of coumarin as well as melilot and cinnamon extracts has been assayed by disfusimetric well method. Tested bacteria were as follows: *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, and two clinical strains, *Klebsiella sp*, and *Proteus sp*. Bacterial strains were grown overnight on Tryptone Soy Broth (TSB – Oxoid) [composition (g/L): enzymatic digest of soy bean, 3.0; pancreatic digest of casein, 1.0; sodium chloride, 5.0; di-potassium hydrogen phosphate, 2.5; glucose, 2.5, *p*H = 7.3] for 16-24 h. The bacterial strains were inoculated by spreading on Muller Hinton agar (MHA – Oxoid) [composition (g/L): beef extract, 3.0; casein hydrolysate, 17.5; starch, 1.5; agar-agar, 17.0, pH = 7.3]. Subsequently, wells (d = 9 mm) were performed on MHA by using a sterile test tube. Coumarin was dissolved in methanol (1%, 3% and 10% w/v). 50µL of coumarin solutions with different concentration were pipetted into wells. Inoculated plates were incubated at 37°C and inhibition zones were measured after 48 h of incubation.

Results and discussions

TLC method

By TLC method there were identified coumarins derivatives, other than simple coumarin, observing their blue fluorescence under $UV_{365\ nm}$ (table 2).

Sample	Rf Value
Scopoletin Standard	0.25-0.30
Fraxetin Standard	0.25-0.40
Coumarin Standard	0.65-0.67
Psoralen Standard	0.50
Melilot extract	0.65
Ash extract	0.25
Cinnamon extract	0.67

Table 2
Rf VALUES OF COUMARIN AND COUMARINS DERIVATIVES

The presence of coumarin derivatives by TLC method was noticed by the fluorescent spots appeared on the plates after examination under UV 365 nm.

Comparing with to standards, the presence of coumarin in cinnamon (Rf = 0.67) and coumarin derivatives in ash (Rf = 0.25) was detected.

The coumarin derivatives in heterosides form, known to be in melilot, remained in band [24].

Spectrophotometric method

Table 3 presents coumarin concentrations in all studied plant extracts measured at 520 nm.

The results show a noticeable high coumarin concentration in melilot comparing to coumarin content in cinnamon and ash.

Considering the quantitative analysis data, the melilot and cinnamon extracts with the highest coumarin content were selected for antibacterial activity testing.

The amount of coumarin in the extractive solutions of melilot and cinnamon was determined to be 0.245% and 0.02%, respectively.

Antibacterial activity

Coumarin had a variable effect depending on its concentration and bacterial species. Thus, the lowest effect was observed against *Proteus sp.* when only the solution of 10% coumarin was used, in which case it was recorded an inhibition zone of 6 mm (fig. 3). A variable response was also observed by Basile et al (2009) in case of some

No.	Sample	$A_{520\ nm}$	Conc (ppm)	Conc. mg/100 g dry weight
1.	Melilot (*Melilotus officinalis* L.)	0.3385	3.1892	637.84
2.	Cinnamon (*Cinnamomum aromaticum*)	0.1408	0.2929	58.594
3.	Ash (*Fraxini folium*)	0.1298	0.1322	26.440

Table 3
COUMARIN CONTENT IN INVESTIGATED PLANT EXTRACTS

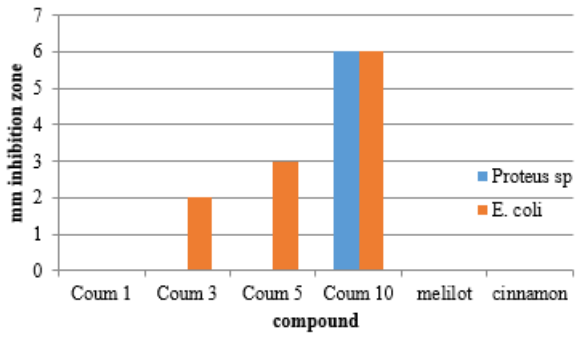

Fig.3. Inhibitory activity of coumarin solutions (coum 1 = 1%; coum 3 = 3% coumarin; coum 5 = coumarin 5%; coum 10 = coumarin 10%), melilot and cinnamon extracts on *E. coli* ATCC and *Proteus sp.*

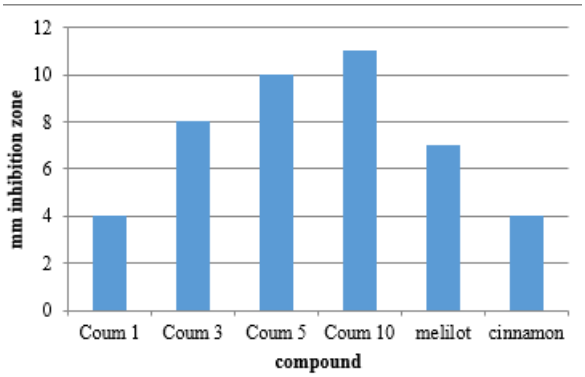

Fig. 4. Inhibitory activity of coumarin solutions (coum 1 = 1%; coum 3 = 3% coumarin; coum 5 = coumarin 5%; coum 10 = coumarin 10%), melilot and cinnamon extracts on *Klebsiella sp*

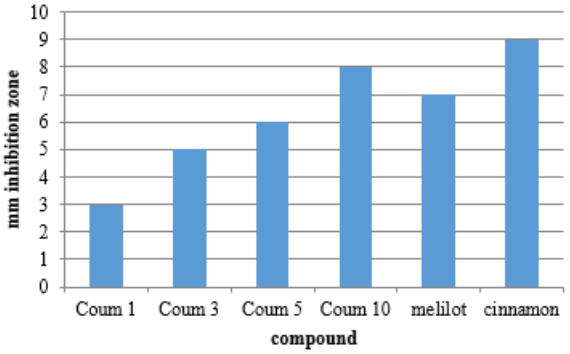

Fig. 5. Inhibitory activity on *B. cereus* ATCC of coumarin solutions (coum 1 = 1%; coum 3 = 3% coumarin; coum 5 = coumarin 5%; coum 10 = coumarin 10%), melilot and cinnamon extracts

plant-extracted coumarin derivatives, some inhibiting *Proteus* while others being ineffective on this species [30]. For *Escherichia coli*, the inhibition zone increased progressively from 3 mm (3% coumarin) to 6 mm (10% coumarin) (fig. 3). In contrast, *Klebsiella sp* was highly sensitive even to 1% coumarin solution when it was recorded an inhibition zone of 4 mm (fig. 4). Inhibitory effect has increased as the concentration of coumarin increased reaching 11 mm inhibition zone for 10% coumarin solution (fig. 4). In regard of *Bacillus cereus,* the inhibitory activity increased in similarly as concentration of coumarin increased, reaching 10 mm zone of inhibition for 10% coumarin solution (fig. 5). Kayser et Kolodziej (1999) showed that simple coumarins demonstrated a broad variability regarding the level of inhibitory activity, similarly to our experiment [31]. Melilot extract has not had any effect on the *Proteus sp* and *E. coli* (fig. 3), but proved an important inhibitory activity against *B. cereus* and *Klebsiella sp* (fig. 4, 5). Unlike this, the cinnamon extract inhibited markedly *B. cereus* and to a less extent *Klebsiella sp* (fig. 4, 5). On average, based on the effect against all bacteria tested, the inhibitory activity of coumarin solution increased gradually from 1.75 mm inhibition zone for 1% solution to 7.75 mm in case of 10% solution of coumarin. (fig.6). Inhibitory effects of melilot and cinnamon extracts were moderate with mean zones of inhibition ranging from 3.25 to 3.50 mm. In comparison with coumarin solutions, these two extracts were almost equal to 3% coumarin solution (fig. 6).

As shown before, concentration of coumarin derivatives found in melilot and cinnamon extract ranged from 0.58 mg/mL (cinnamon) to 6.37 mg/mL (melilot) and their average activity was close to 3% (w/v) coumarin solution. Therefore, inhibitory activity of plant-extract coumarin

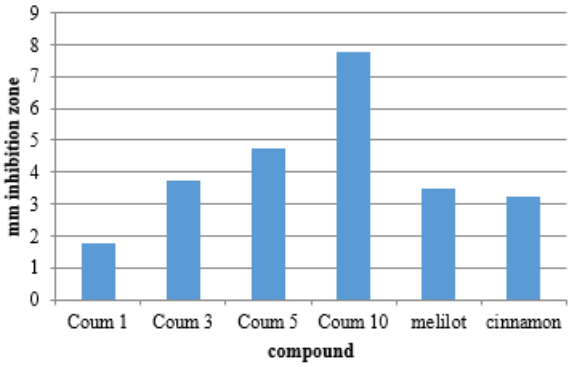

Fig. 6. Average inhibitory activity of coumarin solutions (coum 1 = 1%; coum 3 = 3% coumarin; coum 5 = coumarin 5%; coum 10 = coumarin 10%), melilot and cinnamon extracts on four bacterial strains tested

derivatives was higher and more efficient than coumarin itself. This is due, probably, to specificity of molecular structure of derivatives found in plant extracts that allowed a better interaction of coumarin moiety with bacterial biological macromolecules [31].

In another paper were studied antioxidant activity and total phenols content in selected spices [32].

Conclusions

All studied plants present rich content of coumarin and coumarin derivatives. In the case of melilot (637.84 mg/100g d.w) and cinnamon (58.594 mg/100g d.w.), the fact which justifies their applications is the highest concentration in coumarin and coumarin derivatives.

Melilot and cinnamon extracts proved significant antibacterial activity, especially on Gram positive bacteria. In comparison with coumarin solution alone, derivatives found in plant extracts were more efficicient in inhibiting the bacterial growth. Among the two extracts, cinnamon extract was very active on *Bacillus cereus* ATCC while melilot extract was a little more efficient on Gram negative clinical strains like *Klebsiella sp.*

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