Screening of Polyphenol Content and *In vitro* Studies of Antioxidant, Antibacterial and Cytotoxic Activities of *Capsicum Annuum Extracts*

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In this study the polyphenolic profile of Capsicum annuum extracts was established by a RP-HPLC method. 11 acids derived from hydroxycinnamic acid and five flavonoids were quantified. The ellagic acid was present in high concentrations in all tested samples. The C. annuum extracts were also investigated for their antioxidant, antimicrobial and cytotoxic properties. In conclusion, the results of this study reveal that Capsicum annuum alcoholic extracts exhibit significant antioxidant properties, the highest antioxidant activity being obtained for the the green pepper extract (723.795 mg/L GAE, 3.315 mg/L trolox equivalents and 327.394 mg/L QE). Moreover, the antibacterial activity of the tested extracts was significantly higher as compared to the capsaicin standard used as positive control towards Enterococcus faecalis, E. coli and Bacillus subtilis strains and similar to capsaicin for Staphylococcus aureus strain. The results of the in vitro cytotoxicity assay revealed that the tested extracts revealed a good biocompatibility and did not alter the morphology of HACAT cells. Taken together, these biological properties indicate the potential of the obtained extracts to be used in different biomedical applications, as anti-rheumatic, anti-inflammatory and antimicrobial agents.

Keywords: polyphenolic profile, RP-HPLC, ultrasonication extraction, Capsicum annuum L., antioxidant, antimicrobial, cytotoxicity

Plants contain a variety of substances that neutralize the excess of reactive oxygen species, i.e.: polyphenols, flavonoids, vitamins, terpenoids. These are called natural antioxidants and their effects in the prevention and treatment of diseases caused by the reactive oxygen species are intensely studied [1].

Polyphenols exhibit these properties due to the reducing character of the phenolic group. Consequently researchers have turned to natural antioxidants and numerous phytoextracts were analyzed to establish their antioxidant activity [2].

Coupled high performance liquid chromatography is a separation technique suitable for complex matrixes such as plant extracts [3,4] applied successfully for the quantitative determination of various classes of compounds.

The aim of this research was to establish the pharmacological value of *Capsicum annuum* extracts base on its polyphenol content and to study its *in vitro* antioxidant, antibacterial and cytotoxic activities.

Experimental part

Materials and methods

Plant material

Selected pulps of *Capsicum annuum* were collected from the cultivated flora in Craiova City, Dolj County, Romania and then were dried at room temperature for 20 days at constant temperature and humidity.

Extract preparation

An amount of 1 g of fine powdered dried material (weighed on the analytical balance with accuracy of 0.0001 g) was subject to ultrasonication extraction for 1 hour at room temperature using a Bandelin Sonorex bath. Ethanol was used as extraction solvents in the ratio 1:20 (m/v).

HPLC Analysis

A Thermo_Finnigan liquid chromatograph with Surveyor Plus HPLC quaternary pump LCPMPP with built-degasser, Surveyor Plus HPLC autosampler ASP, thermostat Peltier; and a HPLC "Diode Array" PDA5P detector with a 5 cm flow cell were used for quantitative determination of the polyphenols.

The used method was published in the literature [5] and the operational parameters were kept unchanged in order to allow dosing of a large number of polyphenols, i.e.: a Hypersil Gold C18 column 5 μm (250 mm x 4.6 mm) was used with working temperature of 20°C; mobile phase: 0.5% acetic acid in water (A), acetonitrile (B) in gradient elution (starting from 90% A -10 % B for 20 min , 60% A - 40% B for the next 40 min. The A percentage was reduced and maintained to 55% for the next 10 min and then increased gradually to 90% and maintained at that value for the final five minutes of the analysis in order to rebalance the column; flow rate: 1 mL/min and the injection volume: 5 μL; UV-VIS detection, λ=278 nm;

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Antioxidant activity

Total content of polyphenols

Total polyphenol content was determined using Folin-Ciocalteu method. The calibration curve (the equation of the calibration: y=0.010x, R_c = 0.998) was plotted using a stock solution of gallic acid (1000 mg/L in water) and the results were expressed in mg/L gallic acid equivalent (GAE) of prepared extracts [6].

Concentration range of the standards was between 0-250 mg/L gallic acid. The results were read spectrophotometrically at 765 nm.

From the obtained extracts, 200 µL were taken over which were added 2.5 mLof Folin - Ciocalteu reagent diluted 1:10. After 4 min, 2 mL of sodium carbonate (75 g/L) were added in each sample and the mixtures were then left for 2 h at room temperature (23°C).

The colour intensity of the blue complex formed with the antioxidant compound is directly proportional to the concentrations of polyphenolic compounds.

Total flavonoid content

Total flavonoid content was assessed with $AlCl_3$ 10 % solution [7], by evaluating the absorption maximum of the obtained colored solutions, based on a calibration curve. From each ethanolic extract 0.5 mL were taken and were mixed with 1.5 mL ethanol, 0.1 mL potassium acetate, 0.1 mL 10% $AlCl_3$, and 2.8 mL bidistilled water. The mixtures thus obtained were kept at room temperature for 30 min then the absorbance was measured at 415 nm. Calibration curve (R_c = 0.998) was made using quercetin as standard (concentrations between 0-100 mg/L quercitin). Total flavonoid content was expressed in mg/quercitin equivalent in prepared extracts.

Total antioxidant activity

Total antioxidant activity was measured using DPPHr adical (2,2-diphenyl-1- picryl hydrazyl). Color change from blue to yellow was measured at 517 nm according to a procedure given by Oliveira et al., 2008 [8]. Thus, 0.05 mL sample was mixed with 2.9 mL 0.004% DPPH methanolic solution [9]. Mixes were left for 30 min at room temperature. Absorbance was read compared to the control at 517 nm.

The calibration curve was built with trolox in range 0-2.5 mM/L trolox (y = -0.434x + 1.244, R_c = 0.997). Antioxidant activity was expressed in Trolox equivalent, TEAC.

Antimicrobial activity

The antimicrobial activity of the obtained compounds was assayed on Gram-negative (*Escherichia coli* ATCC 8730, *Pseudomonas aeruginosa* ATCC 27853), and Gram-positive (*Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633) bacterial reference strains. Microbial suspensions of 1.5 x 10^8 CFU·mL^{-1} obtained from 15 to 18 h bacterial cultures grown on solid media were used. The antimicrobial activity was tested on Mueller–Hinton Agar (MHA) medium. The qualitative screening was performed by an adapted diffusion method as previously reported [10]. The quantitative assay of the antimicrobial activity was performed by liquid medium (tryptic soy broth) microdilution method in 96 multi-well plates. Two-fold serial dilutions of the extracts alcoholic solutions (1:20) were performed in a 200 µL volume of broth, and each well was seeded with 50 µL of microbial inoculum. Culture positive controls (wells containing culture medium seeded with the microbial inoculum) were used. The plates were incubated for 24h at 37°C, and the minimal inhibitory concentration (MIC) values were considered as the lowest concentration of the tested compound that inhibited the growth of the microbial overnight cultures, as compared to the positive control, revealed by a decreased value of absorbance at 600 nm (Apollo LB 911 ELISA reader) [11].

Cytotoxic activity

In this purpose, monolayers of HACAT (a spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin) cells were seeded in a 24 well plate and treated for 24 h with 10 µL of the alcoholic extracts (1:20). The morphology of the HACAT cells was examined and photographed at the inverted microscope.

Polyphenols	***Concentration, mg/L***			
	Capsicum annuum (red variety)	*Capsicum annuum* (red variety, without seeds and ribs)	*Capsicum annuum* (green variety)	*Capsicum annuum* (green variety, without seeds and ribs)
Gallic acid	7.093	1.118	20.240	3.794
Vanilic acid	1.591	1.109	0.612	0.000
Chlorogenic acid	0.000	14.073	0.000	0.874
Cafeic acid	2.099	2.269	2.305	0.235
Syringic acid	0.230	0.223	0.310	2.017
Epicatechin	1.474	2.812	3.381	0.376
Coumaric acid	0.428	0.756	0.236	0.332
Sinapic acid	0.154	1.280	0.059	0.399
Ferulic acid	0.353	4.322	0.511	2.789
Salicylic acid	6.320	9.452	9.346	1.183
Rutin	3.641	10.045	5.839	5.552
Elagic acid	27.668	204.305	20.050	0.897
Myricitn	7.740	6.344	3.466	0.591
Transcinamic acid	0.899	9.805	0.890	0.422

Table 1
THE AMOUNT OF POLYPHENOLS DETERMINED IN THE *CAPSICUM ANNUUM* ALCOHOLIC EXTRACTS BY RP-HPLC

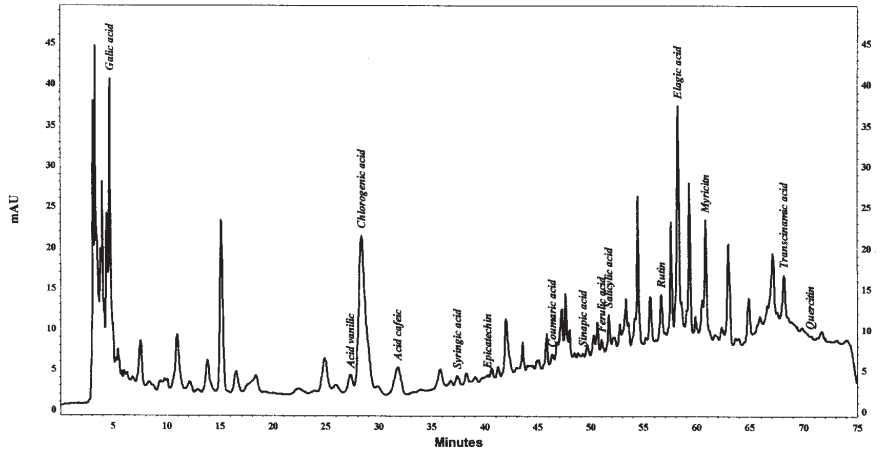

Fig. 1. Chromatogram of *Capsicum annuum* (red variety)

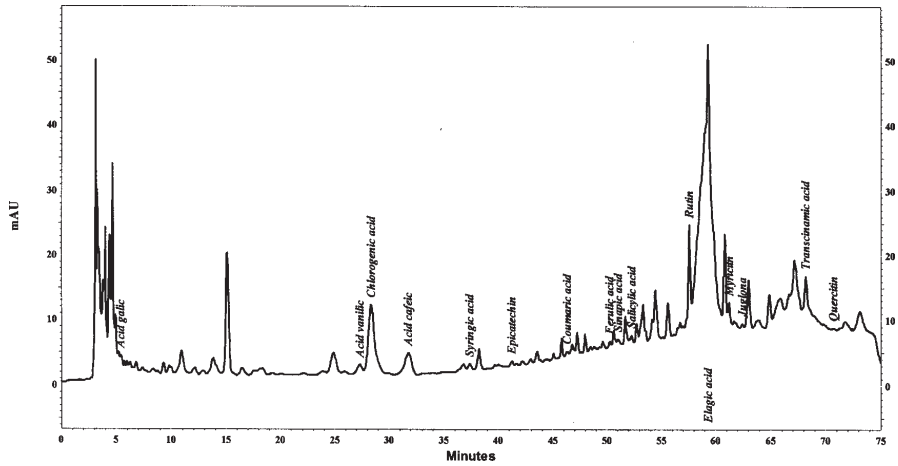

Fig.2. Chromatogram of *Capsicum annuum* (red variety, without seeds and ribs)

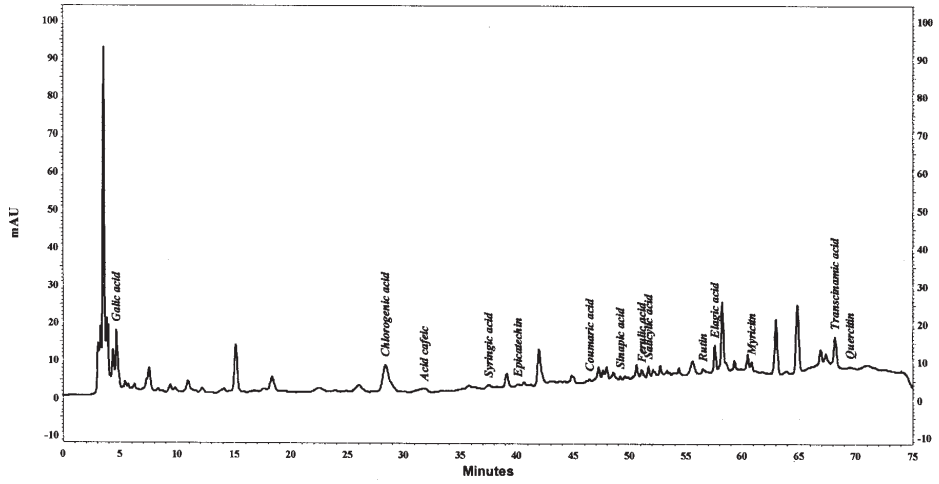

Fig. 3. Chromatogram of *Capsicum annuum* (green variety)

Chemicals and reagents

All chemicals and reagents were of purchased from Sigma-Aldrich, Merck or Fluka and were of analytical grade.

Results and discussions

HPLC analysis

Eleven acids derived from hydroxycinnamic acid and five flavonoids were identified and assayed in the extracts of red and green peppers (tables 1-4).

The largest amount was found for ellagic acid (27 mg/L in red peppers, 204 mg/L in pulp of red pepper without seeded and ribs and 20 mg/L in dried green pepper). Gallic acid was found in larger quantities in dried green pepper, compared to the red one.

It can be noticed by analyzing the obtained chromatograms of the four extracts (figs. 1-4) that the highest content of polyphenols was found in red peppers, while significantly decreasing amounts of polyphenols were obtained by removing seeds and ribs.

Antioxidant activity

Total phenolic and total flavonoid content and antioxidant capacity for the four extracts were determined and are shown in table 2. The total content of polyphenols and flavonoids is extremely high, even compared to other species of medicinal plants recognized for their antioxidant capacity [12, 13]. The amount of phenolic compounds in this species varies with the colour, our findings showing a greater accumulation in dried green pepper.

DPPH test is a simple and rapid method, widespread as a good method to estimate the total antioxidant capacity of complex mixtures. In this category enter plant extracts[14]. 1,1-diphenyl-2 hydrazyl picryl is a stable purple radical that after its reduction becomes yellow. The change in coloration is measured spectrophotometrically.

In terms of reducing properties, the studied *Capsicum annuum* products can be ordered as follows: dried green pepper> green pepper pulp> whole red peppers> red pepper pulp.

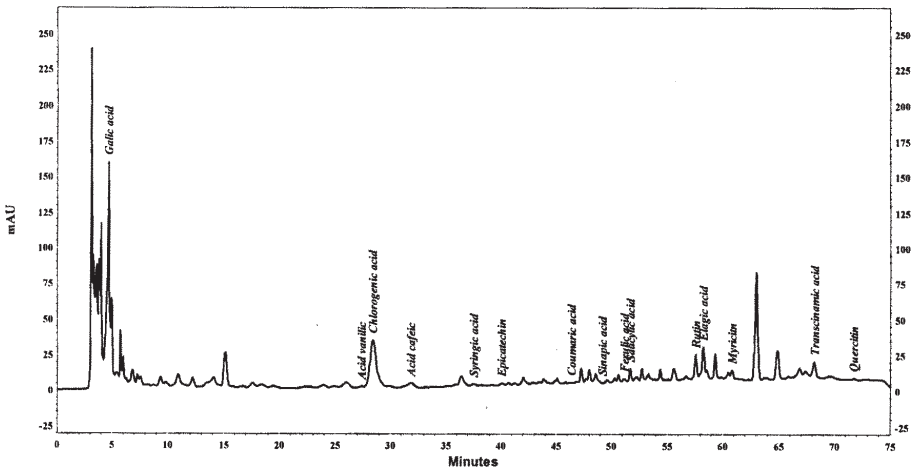

Fig. 4. Chromatogram of *Capsicum annuum* (green variety, without seeds and ribs)

No	Extract	Total polyphenol content mg/L gallic acid equivalents	Antioxidant capacity mg/L trolox equivalents	Total flavonoid content mg/L quercitin equivalents
1	*Capsicum annuum* (red variety, without seeds and ribs)	377.485	1.51	231.665
2	*Capsicum annuum* (red variety)	505.207	2.16	235.884
3	*Capsicum annuum* (green variety, without seeds and ribs)	330.37	2.295	64.545
4	*Capsicum annuum* (green variety)	723.795	3.315	327.394

Table 2
ANTIOXIDANT PROPERTIES OF *CAPSICUM ANNUUM EXTRACTS*

Antibacterial activity

Three of the four analyzed extracts , i.e. *Capsicum annuum* (red variety); *Capsicum annuum* (red variety, without seeds and ribs) and *Capsicum annuum* (green variety) were tested for their antimicrobial activity and cytotoxicity, using as positive control the capsaicin standard alcoholic solution.

The qualitative screening of the antimicrobial activity evidenced the occurrence of a bactericidal effect as intense as that observed for the positive control in case of four of the five tested bacterial strains, excepting *B.subtilis,* in which the decrease of the microbial culture density was observed, but the inhibitory effect was less intensive than that induced by the positive control (fig. 5).

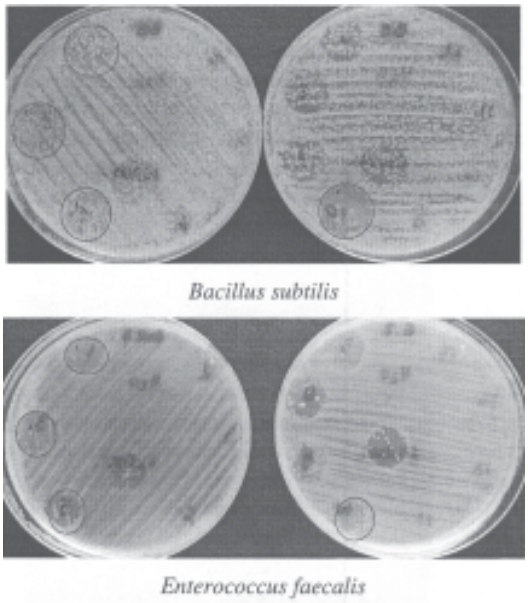

Fig. 5. The antimicrobial activity of the tested compounds evidenced in the qualitative assay revealing the occurrence of microbial growth inhibition zones 1- *Capsicum annuum* (red variety); 2- *Capsicum annuum* (red variety, without seeds and ribs); 3- *Capsicum annuum* (green variety); 4- Capsaicin standard

	E. faecalis	*P. aeruginosa*	*E. coli*	*S. aureus*	*B. subtilis*
1- *Capsicum annuum* (red variety)	0.025	0.025	0.05	>0.05	0.0009
2- *Capsicum annuum* (red variety, without seeds and ribs)	>0.05	>0.05	>0.05	>0.05	0.001
3- *Capsicum annuum* (green variety)	0.0125	0.025	0.025	>0.05	0.003
4-Capsaicin	>0.05	0.0003	>0.05	>0.05	0.003

Table 3
MIC (μg mL^{-1}) VALUES OF THE TESTED COMPOUNDS AGAINST THE TESTED MICROBIAL STRAINS (THE GRAY CELLS INDICATE THE CASES IN WHICH THE TESTED EXTRACTS EXHIBITED AN ANTIBACTERIAL ACTIVITY SUPERIOR TO THAT OBTAINED FOR THE POSITIVE CONTROL).

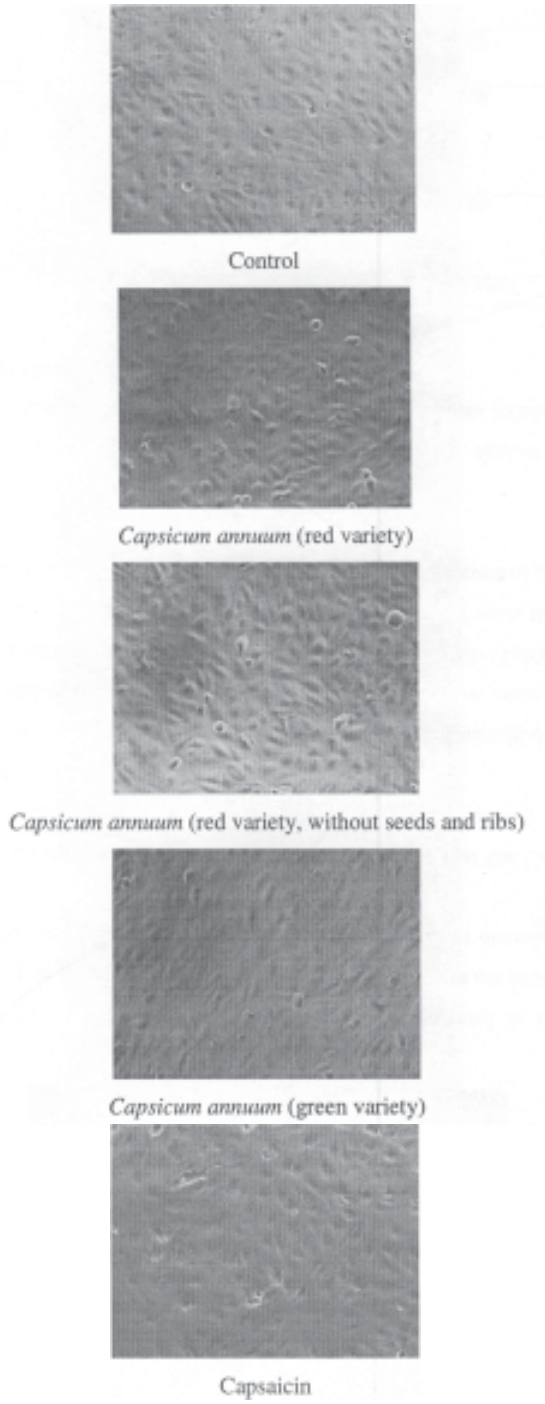

Fig. 6. Typical inverted microscopy images of HACAT cells treated for 24 h with 10 μl of the alcoholic extracts (1:20) (x200).

The quantitative assay of the antibacterial activity revealed that the *Capsicum annuum* red and green variety extracts exhibited a more intensive antibacterial activity as compared to the capsaicin positive control against *E. faecalis* (table 3).

Capsicum annuum (green variety) exhibited also a good antimicrobial activity, superior to that of the positive control against the *E. coli* strain, while *Capsicum annuum* (red variety) and *Capsicum annuum* (red variety, without seeds and ribs) against *B. subtilis* (table 3).

The tested extracts exhibited an antimicrobial activity similar with the capsaicin positive control against *S. aureus,* and less intensive than that of the capsaicin control against *P. aeruginosa* (table 3).

Cytotoxicity activity

All tested extracts presented good biocompatibility properties, as revealed by microscopy analysis, the HACAT cells being firmly attached, with a morphology similar to that seen in control (standard) growth conditions (fig. 6).

Conclusions

In conclusion, the results of this study reveal that *Capsicum annuum* alcoholic extracts exhibit significant antioxidant properties. Moreover, the antibacterial activity of the tested extracts was significantly higher as compared to the capsaicin standard used as positive control towards *Enterococcus faecalis, E. coli* and *Bacillus subtilis* strains and similar to capsaicin for *Staphylococcus aureus* strain. The results of the *in vitro* cytotoxicity assay revealed that the tested extracts revealed a good biocompatibility and did not alter the morphology of HACAT cells. Taken together, these biological properties indicate the potential of the obtained extracts to be used in different biomedical applications, as anti-rheumatic, anti-inflammatory and antimicrobial agents.

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