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Cytotoxic, genotoxic and antimicrobial activity of caffeic and rosmarinic acids and their lithium, sodium and potassium salts as potential anticancer compounds



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ABSTRACT

Purpose: The aim of this study was to examine the cytotoxic, genotoxic, antioxidant and antimicrobial activity of caffeic and rosmarinic acids and their salts with Li, Na and K with use of *Escherichia coli* K-12 *recA:gfp* strain as a model organism.

Methods: Cytotoxic potency of tested chemicals were calculated on the basis on the dose that confers inhibition percentage such as 20% for each concentrations of analysed chemicals. Genotoxic properties were calculated on the basis of the fold increase (FI) of SFI values normalized with control. Antioxidant potencies were established on the base of DPPH assay. Antimicrobial activity of chemicals were established on the value of minimal inhibitory concentration (MIC).

Results: Obtained results indicated that lower concentrations of tested compounds exhibited stronger GFP fluorescence response after rosmarinic acids and their salts treatment. Genotoxic effects seemed to be independent of the salt ions. The caffeic acid salts with Li, Na and K showed reduced genotoxic effect in comparison to the caffeic acid while increased cytotoxic effect than that of caffeic acid. Moreover, caffeinate salts exhibited better antimicrobial activity against *E. coli* (MIC = 250 µg/mL) than K caffeinate salt (MIC > 500 µg/mL). The MIC values of Li, Na and K rosmarinate salts were above 500 µg/mL against all tested microorganisms.

Conclusion: The results of the experiment show that there is no clear positive correlation between the antioxidant potency of caffeic and rosmarinic acids and their Li, Na and K salts and their cytotoxic effect. Used salts ions Li, Na and K do not significantly affect the antioxidant effect of natural phenolic compounds and they do not have a significant impact on the biological parameters such as cyto- and genotoxicity. Perhaps it is connected with the reaction environment including polarity of the solvent (water).

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1. Introduction

Natural polyphenols have potential health benefits in a number of oxidative stress-associated diseases (such as cancer). Due to their antioxidant properties, in *in vitro* and *in vivo* experiments, these natural compounds have ability to tumor cell death induction, interfere with carcinogenesis, tumor growth and dissemination. Some preclinical experiments showed, that polyphenols in combination with conventional chemoradiotherapy or

with other polyphenols through the induction of apoptosis, the inhibition of coming out and spreading of cancer. In many experiments antioxidants, antithrombosis, antihypertensive, anti-fibrosis, antiviral, and anti-tumor properties of caffeic acid (3,4-dihydroxycinnamic acid) (CA) (Fig. 1) were proofed [1–4].

The previous studies revealed, that caffeic acid exerts protective effects against UVB-induced skin damage, by suppressing interleukin-10 and mitogen-activated protein kinase (MAPK) activation in mouse skin [1].

Koraneekit et al. [4], presented the synergistic effect of cisplatin and caffeic acid on cervical cancer cell lines. The simultaneous combination of caffeic acid-cisplatin allowed to decrease the dosage of cisplatin dose used, which could lead to a reduction of

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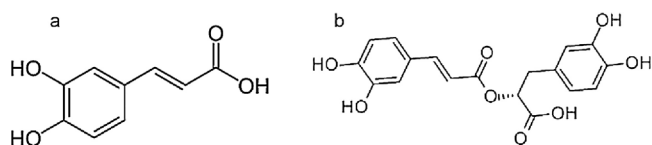


Fig. 1. Chemical structure of caffeic acid (3,4-dihydroxycinnamic acid) (a.) and rosmarinic acid (b).

side-effect of this drug. The addition of caffeic acid to the cell lines, treated with cisplatin, increased the efficiency of cisplatin anticancer activity. Caffeic acid has been predicted to act as a chemopreventive agent and emerging adjuvant for cancer chemotherapy [1–4].

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (Fig. 1) and is known as (R)- α -[[3-(3,4-dihydroxyphenyl)-1-oxo-2E-propenyl]oxy]-3,4-dihydroxybenzoic acid, with a molecular formula of $C_{18}H_{16}O_8$. RA is a phenolic compound found in *Rosmarinus officinalis*, *Thymus mastichina*, *Forsythia koreana*, *Ocimum sanctum*, *Hyptis pectinata* and some plants belonging to the *Agastache* genus of the *Lamiaceae* family. The antiviral, antibacterial, anticancer, antiinflammatory and antioxidant activities of rosmarinic acid have been reported [5–10].

In spite of most microorganisms have similar biochemical pathways as higher organisms, there is no complete compatibility in genotoxicity tests using bacteria and human assays. *Salmonella* Ames assay is most widely used bacterial assay for mutagenic and carcinogenic screening of compounds. There is a correlation between mutagenicity as measured by the Ames assay and carcinogenicity in mammals [11,12]. Kirkland et al., [13] demonstrated that this correlation is not perfect because mutations are only one of many stages in tumor development. Moreover, it could be possible to meet with specific mutagenic response to the bacteria or the test protocol, e.g., bacterial specific metabolism, exceeding a detoxification threshold, or the induction of oxidative damage to which bacteria may be more sensitive than mammalian cells *in vitro* or tissues *in vivo*, or an *in vitro* metabolic activation preparation that does not mimic the *in vivo* situation [13]. Additionally, the Ames assay is simple to perform but requires high replication, a wide range of controls, extensive culturing and time-consuming enumeration. The Ames test actually measures mutagenicity. On the other hand, activation of SOS repair system following DNA damage has been used to measure the mutagenic and genotoxic effects of various chemicals and physical treatments. These microbial bioassays – also called as microbial biosensors – used for cyto- and genotoxic assessment offer some advantages; they are inexpensive, have short life cycles, simple to apply, sensitive and easy to measure. In addition, the most of microorganisms have similar biochemical pathways as the higher organisms and respond rapidly to environmental changes [13,14].

The aim of this study was the assessment of cytotoxic, genotoxic effects, antioxidant potency and antimicrobial activity of caffeic and rosmarinic acid and their salts with Li, Na and K toward *Escherichia coli* K-12 *recA:gfp* as microbial biosensor strain.

The alkali metals constitute the logical series, where in the order: Li, Na, K atomic radius is steadily increasing and the degree of oxidation is constant. To select the alkali metals to this study following criteria (which are important from the point of view of further possible application) were taken into account: (1) as small as possible harmfulness of the human body and the environment; (2) the possibility of the practical application because of the good solubility of the alkali metal compounds in water and polar solvents; (3) the availability, ease of preparation and stability.

Escherichia coli K-12 contained plasmid transcriptional fusion between *recA* promoter which belongs to DNA-damage genotoxin-inducible group of bacteria promoters, involved in the SOS regulon response was selected as a testing model. In this genetic construct fast folding variant of *gfp* gene – *gfpmut2* was used [13]. Bacterial biosensors, detect the genotoxic mode of action of some chemicals and drugs, are based on fluorescence measurements. This leads to an increase concentration of GFP protein and as a consequence – its fluorescence [14–16]. *RecA* promoter-operator, as it was previously shown, is useful to detect genotoxins, environmental chemicals, anticancer drugs and some new candida the drugs [12,14–21]. So far, many authors have presented the cytotoxicity analysis of caffeic and rosmarinic acids. There is no literature presented some correlation among cyto-, genotoxic properties of tested chemicals and their antioxidant potency using the *recA:gfp* genetic system in *E. coli*. Do salts ions (Li, Na and K) affect the antioxidant effect of natural phenolic compounds?

Cytotoxic potency of tested chemicals were calculated on the basis on the dose that confers at certain inhibition percentage such as 20% for each concentrations of analysed chemicals. Genotoxic properties were calculated on the basis of the fold increase (FI) of SFI values normalized with control. Antioxidant potencies were established on the base of DPPH assay. Antimicrobial activity of chemicals was established on the value of minimal inhibitory concentration (MIC).

Our team is involved in examination of chemical reaction and electron structure of natural ligands for many years. In our previous papers we have studied the influence of metals on the molecular structure and electronic charge distribution of biologically important ligands such as nicotinic, picolinic, salicylic, benzoic acids [22–27]. We have showed that toxic metals such as Hg(I), Hg(II), Ag(I), Pb(II), Cd(II) and alkali metals disturb uniform electronic system of examined ligands, while 3-d transition metals, lanthanides and aluminum stabilized electronic systems by bonds delocalization effect. We have showed that a degree of perturbation of the uniform distribution of electronic system of ligands and its aromatic properties depended on an ionic potential of metals. This studies have been realised using FT-IR, FT-Raman, NMR, X-ray methods and theoretical calculations. In the next works we showed also in several cases that the molecular structure and electronic charge distribution determine the biological activity of ligand and complexes [25–27].

2. Materials and methods

2.1. Chemicals preparation

Caffeic and rosmarinic acids were commercially obtained (Sigma Aldrich, UK). Chemicals were dissolved in Milli-Q[®] water and were transferred to phosphate buffered saline – PBS buffer (1.44 g Na_2HPO_4 , 0.24 g KH_2PO_4 , 0.2 g KCl, 8 g NaCl in 1000 mL distilled water, pH = 7.0) with bacteria strains.

Lithium, sodium and potassium, caffeinates or rosmarinates were prepared by dissolving the powder of acids in a water solution of the appropriate alkali metal hydroxide in a stoichiometric ratio (1:1). All reagents were Aldrich analytical chemicals, with the exception of LiOH (Sigma). The solutions were than heated in a shaker to ca 70 °C for to completely dissolve the acid (somewhere 1 h). The pH of the aqueous solutions of the acids was about 4, while the pH of the saline solutions was about 6. The products precipitated by slow evaporation. Then the salts were dissolved and crystallized from demineralised water. After drying, the IR spectra were recorded. The correctness of the synthesis was determined by the disappearance of characteristic bands in the acid spectrum.

2.2. Bacteria growth condition

Escherichia coli K-12 MG1655 strains were cultured overnight in LB agar medium (Merck, Germany) at 30 °C supplemented with 100 µg/mL of kanamycin (Sigma-Aldrich, Germany). Colonies were carried to LB broth medium (10 g NaCl, 10 g tryptone and 5 g yeast extract per 1000 mL of distilled water) with 100 µg/mL of kanamycin and incubated overnight at 30 °C. Bacteria cells were refreshed in LB broth with 100 µg/mL of kanamycin and incubated 2 h at 30 °C. Afterwards, the cells were washed with PBS buffer.

2.3. Monitoring of bacteria growth and concentration

At the beginning of the experiment, the initial bacteria cells density was standardized to OD (Optical Density) value by using spectrophotometer (Glomax[®], Multi Detection System, Promega) at the wavelength of 600 nm. The concentration of bacteria cells per mL of PBS was assessed by series dilutions system and expressed as CFU (colony forming units)/mL values.

Dynamic growth of bacteria strains treated with chemicals was monitored by the use of standard spectrophotometer analysis of Optical Density values, at the wavelength of 600 nm.

The values of bacteria growth inhibition (GI) during the treatment (after 24 h) with compounds used at the start of bacteria incubation was calculated according to the formula:

$GI (\%) = OD_{CS} (\%) - D_{ODTS} (\%)$, where: $OD_{CS} (\%)$ – Optical Density of control sample, =100%, $D_{ODTS} (\%)$ – the decrease in the value of Optical Density of bacteria samples treated with chemicals in relation to OD value of control sample.

2.4. Genotoxicity testing of caffeic and rosmarinic acids and their complexes with lithium, sodium and potassium

1 mL of bacteria cells (4×10^7 CFU/mL) were suspended in 4 mL of PBS buffer and the both acids and their salts with Li, Na and K were used for genotoxicity testing at concentrations of 10^{-3} , 10^{-4} and 10^{-5} mg/mL.

The concentration range of the analysed chemicals was selected experimentally from the minimum level of *recA:gfp* construct sensitivity and according to the reviewed references recommendation [18–21]. The time of bacteria incubation with chemicals (24 h) was estimated for monitoring the sensitivity of *recA:gfp* genetic construct. The control sample in PBS buffer was not treated with chemicals. For verification the correct activity of *recA* promoter, *Escherichia coli* K-12 strain containing pUA66 plasmid without the promoter was used as the control. Additionally, for assessment of genotoxic sensitivity of *recA:gfp* construct, 4% acetone was used as the negative control and 50 µM methyl-N-nitrosoguanidine (MNNG, known genotoxin) as the positive control.

2.5. Analytical method for the intensity of *gfp* gene fluorescence (IF) analysis

After exposition of bacteria cultures to the tested chemicals, the strains were washed with PBS buffer and the intensity of fluorescence of *gfp* gene in the volume of 1 mL of bacteria cells suspension (4×10^5 CFU/mL) in PBS buffer was measured in the spectrofluorometer (Glomax[®], Multi Detection System, Promega). The measurements were done at excitation and emission wavelengths of 485 and 507 nm, respectively.

2.6. Assessment of SFI values

The specific fluorescence intensity (SFI) value measured with a spectrofluorometer is defined as culture fluorescence intensity (IF)

divided by optical density (OD) at 600 nm for cell culture. SFI value was calculated according to the below formula [18–20] for monitoring the dynamic of *gfp* expression after bacteria treatment with chemicals:

$SFI = \frac{IF}{OD}$; where: SFI – Specific Fluorescence Intensity, IF – The raw fluorescence intensity of the strains at excitation and emission wavelengths of 485 and 507 nm, OD – Optical Density at 600 nm of the strains.

2.7. The percentage stimulation of green fluorescence protein expression ($S_{gfpexp.}$) value

The percentage stimulation of *gfp* ($S_{gfpexp.}$) was calculated according to the formula:

$S_{gfpexp.} (\%) = I_{TS} (\%) - SFI_{CS} (\%)$; where: $I_{TS} (\%)$ – the increase of SFI values for tested compounds as a response to the level of *gfp* expression in comparison with the control sample, $SFI_{CS} (\%)$ – SFI for the control sample = 100%.

2.8. The cytotoxic effect calculation

To compare the potency of cytotoxic effect of tested chemicals the dose that confers inhibition percentage such as 20% for each concentrations of analysed chemicals was calculated (Fig. 3), according to the formula:

$D (mg/mL) = 20\% C (mg/mL) / GI (\%)$; where: –
 $D (mg/mL)$ – the values of tested chemicals doses which caused 20% of inhibition of GI values,
 $C (mg/mL)$ – concentrations of tested chemicals: 10^{-3} , 10^{-4} , 10^{-5} , $GI (\%)$ – growth inhibition.

2.9. The fold increase values calculations

The dose-response data was expressed as fold increase (FI) of SFI value normalized with control and calculated according to the formula: $FI = SFI_{TS} / SFI_{CS}$; where: FI – fold increase of SFI values; SFI_{TS} – SFI values of tested sample; SFI_{CS} – SFI values of control sample.

2.10. Statistical analysis

Data obtained from eight measurements ($n = 8$) are expressed as a mean $\pm$ standard deviation (SD). Data were analysed using standard statistical analyses, including one-way Student's test for multiple comparisons. *P*-values less than 0.05 were considered significant.

2.11. Antioxidant DPPH assay

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) is a stable free radical. In methanol solution DPPH shows a strong absorption band at 517 nm (purple). By reacting with a substance that donates an electron or hydrogen, it forms a reduced DPPH form. The color change from purple to yellow is observed. The decrease in absorbance is proportional to the amount of oxidized form that remains in solution. Parameter IC_{50} is an antioxidant concentration that causes the fall of initial radical concentration by 50%. (the more reactive antioxidant has a lower IC_{50} value). Absorbances at 517 nm were measured with UV-vis spectrophotometer (The Nanocolor[®] VIS, Macherey-Nagel).

Eight substances were tested: solutions of rosmarinic acid, caffeic acid and solutions of their salts which were made by dissolving acids in aqueous solutions of lithium, sodium and potassium hydroxides. Absorption was measured at 517 nm after

30 min incubation of a mixture of 2.5 mL of DPPH (methanol solution) and 50 μ L of tested aqueous solutions.

2.12. Antimicrobial activity analysis of caffeic and rosmarinic acids and their salts with lithium, sodium and potassium

The chemicals (in water) were screened for their potential antibacterial activities *in vitro* against *E. coli*, *Bacillus sp.*, *Staphylococcus sp.*, *Streptococcus pyogenes* and antifungal activities against *Candida sp.*, (obtained from the American Type Culture Collection – ATCC). Antimicrobial activity was measured as a minimal inhibitory concentration (MIC) with Mueller Hinton Agar (MHA). Serial two-fold dilutions of chemicals, ranging from 125 to 1000 μ g/mL, were prepared. After inoculation of microorganisms on MHA plates with certain tested chemical concentrations, plates were incubated at 37 °C for bacteria and at 30 °C for *Candida sp.* for 24 and 48 h, respectively. Plates containing sterile water were used as controls. Minimum three repetitions were run for each assay. The MIC value was determined as the lowest concentration of chemicals, that completely inhibited bacterial and *Candida sp.* growth after 24 h or 48 h incubation.

3. Results

Optical density (OD) measurements of bacteria culture were the basic values for cytotoxic effect assessment and expressed as percent of growth inhibition (GI) values. The potency of cytotoxic effect of analysed chemicals was calculated on the base of the comparison of the doses of chemicals which caused 20% of inhibition of GI values (Fig. 2).

Obtained data revealed the strongest cytotoxic potency for rosmarinic acid and their salts at concentration of 10^{-5} mg/mL, Na

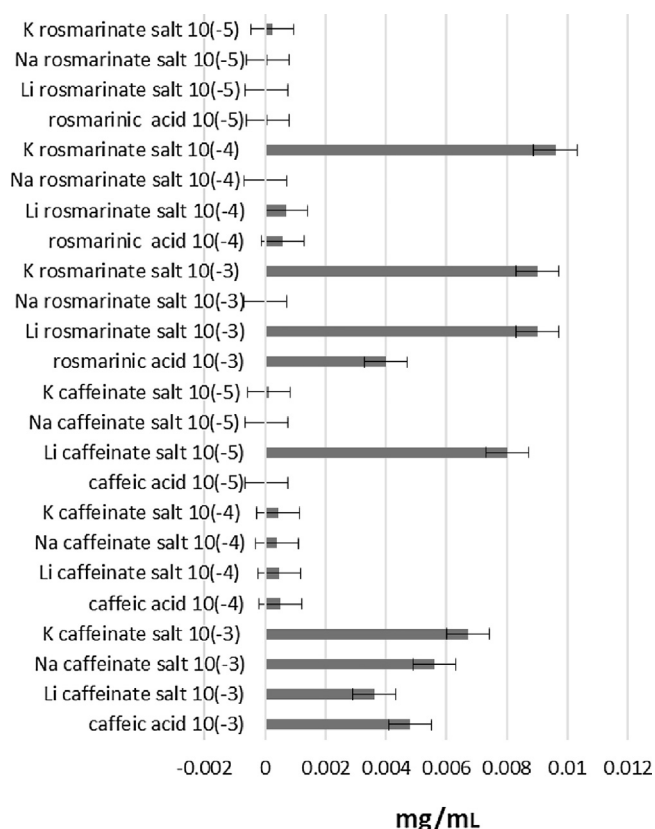


Fig. 2. The comparison of the values of tested chemicals doses (mg/mL) which caused 20% of inhibition of GI values of bacteria. Mean values $\pm$ SD; n = 8.

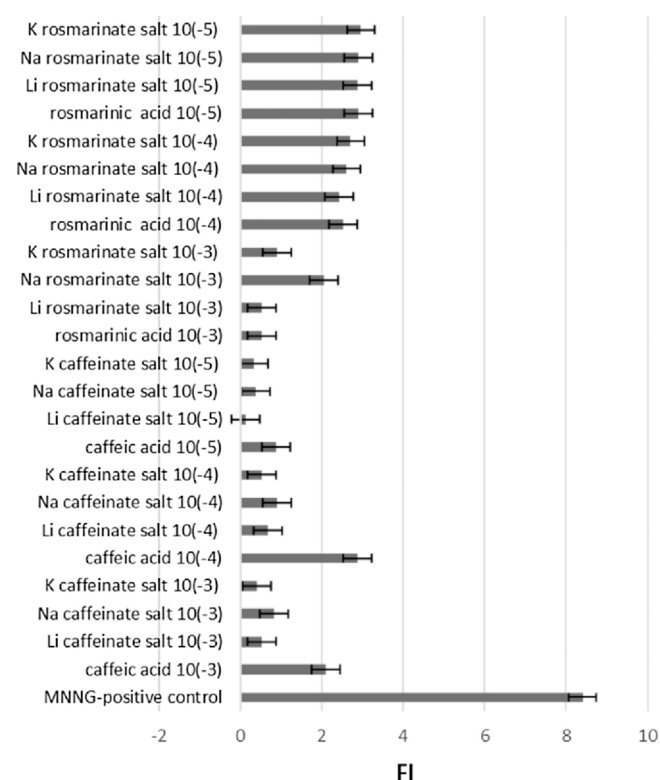


Fig. 3. The fold increase (FI) of SFI value normalized with control for *E. coli recA: gfpmut2* 24 h treatment with caffeic, rosmarinic acids and their salts with Li, Na and K. Mean values $\pm$ SD; n = 8.

rosmarinate salt (10^{-3} and 10^{-4} mg/mL) and for the lowest concentrations (10^{-5}) of caffeic acid and Na and K caffeinate salts and Li, Na and K caffeinate salts (10^{-4}).

Bacteria incubation with caffeic acid resulted in a significant stimulation of SFI ($S_{gfpexp.} = 187.70\%$) values for 10^{-4} mg/mL for 24 h of *E. coli recA: gfpmut2* treatment compared to the control sample (Table 1). Treatment of bacteria cells with rosmarinic acid and its salts with Li, Na and K significantly influenced the *gfp* expression in *E. coli K-12 recA: gfp mut2* at the whole applied concentrations, as compared to the caffeic acid and its salts. The stimulation of *gfp* expression was observed at concentrations of 10^{-4} and 10^{-5} mg/mL, with maximum point of stimulation for rosmarinic acid salt with K at concentration of 10^{-5} mg/mL, being $S_{gfpexp.} = 196.50\%$. Also, significant growth of SFI values were obtained for rosmarinic acid salts with Li and Na at concentrations of 10^{-5} mg/mL (with $S_{gfpexp.} = 187.24\%$ (Li), $S_{gfpexp.} = 190\%$ (Na), respectively). The dose-response results were expressed as fold increase (FI) of SFI normalized with control (Fig. 3). The highest FI values (above two-fold) were observed for rosmarinic acid and Li, Na and K rosmarinate salts, mainly at concentration of 10^{-4} and 10^{-5} mg/mL and for caffeic acid at concentrations of 10^{-3} and 10^{-4} mg/mL.

Following the recommendation of earlier authors [18–20], concerning the validation of sensitivity of assessment of *recA: gfp* genetic biosensing system in *E. coli K-12*, 4% acetone was tested as the negative control. In the case of this chemical there was no increased in FI values for 3 h and 24 h of incubation. Methyl-nitrosoguanidine (MNNG) – known genotoxin at the concentration of 50 μ M – was used as the positive control. For this analyte FI = 8.4 was obtained for 24 h incubation time and FI = 2.8 for 3 h of incubation. These results proved higher sensitivity of a *E. coli K-12 recA: gfp* biosensing system for MNNG than for an acetone stressor.

Antioxidant potency assessment on the base on DPPH assay showed better antioxidant properties for rosmarinic acid and its Li,

Table 1
SFI, S_{gfpexp} and FI values for 24 h treatment of *E. coli* K-12 *recA:gfp mut2* with caffeic and rosmarinic acids and their salts with Li, Na and K in comparison to the control sample. C – concentrations, MNNG – methylnitronitrosoguanidine.

Chemicals	Control sample SFI $\pm$ SD	C (mg/mL)	Salts with:			
			-SFI $\pm$ SD	Li SFI $\pm$ SD	Na SFI $\pm$ SD	K SFI $\pm$ SD
Caffeic acid	2582.40 $\pm$ 356.88	10 ⁽⁻³⁾	5417.70 $\pm$ 160.30 ^a $S_{gfpexp} = 109.8$	3954.40 $\pm$ 77.70 ^a $S_{gfpexp} = 53.13$	4694.30 $\pm$ 187.87 ^{ac} $S_{gfpexp} = 81.8$	3636.00 $\pm$ 109.57 ^{abd} $S_{gfpexp} = 40.8$
	2582.40 $\pm$ 356.88	10 ⁽⁻⁴⁾	7429.36 $\pm$ 591.44 ^a $S_{gfpexp} = 187.70$	4329.20 $\pm$ 702.44 ^a $S_{gfpexp} = 67.64$	4933.30 $\pm$ 445.52 ^a $S_{gfpexp} = 91.03$	3933.33 $\pm$ 127.00 ^{abd} $S_{gfpexp} = 52.32$
	2582.40 $\pm$ 356.88	10 ⁽⁻⁵⁾	4827.60 $\pm$ 145.15 ^a $S_{gfpexp} = 86.94$	2922.28 $\pm$ 124.94 ^b $S_{gfpexp} = 13.20$	3540.31 $\pm$ 113.21 ^{abc} $S_{gfpexp} = 37.10$	3472.84 $\pm$ 231.13 ^{abc} $S_{gfpexp} = 34.50$
IC ₅₀	–	–	0.870 $\pm$ 0.052	0.825 $\pm$ 0.01	0.712 $\pm$ 0.0008 ^b	0.728 $\pm$ 0.01 ^b
Rosmarinic acid	2582.40 $\pm$ 356.88	10 ⁽⁻³⁾	3943.55 $\pm$ 83.81 ^a $S_{gfpexp} = 52.71$	3954.40 $\pm$ 77.70 ^a $S_{gfpexp} = 53.13$	5281.23 $\pm$ 178.55 ^{abc} $S_{gfpexp} = 104.51$	4895.60 $\pm$ 166.17 ^{abc} $S_{gfpexp} = 89.60$
	2582.40 $\pm$ 356.88	10 ⁽⁻⁴⁾	6508.40 $\pm$ 198.26 ^a $S_{gfpexp} = 152.03$	6276.46 $\pm$ 124.58 ^a $S_{gfpexp} = 143.05$	6741.47 $\pm$ 272.01 ^a $S_{gfpexp} = 161.05$	7017.32 $\pm$ 185.21 ^{ab} $S_{gfpexp} = 171.74$
	2582.40 $\pm$ 356.88	10 ⁽⁻⁵⁾	7472.00 $\pm$ 187.22 ^a $S_{gfpexp} = 190.$	7417.62 $\pm$ 289.16 ^a $S_{gfpexp} = 187.24$	7508.30 $\pm$ 251.87 ^a $S_{gfpexp} = 190.$	7656.63 $\pm$ 274.13 ^a $S_{gfpexp} = 196.5$
IC ₅₀	–	–	0.387 $\pm$ 0.001 ^A	0.363 $\pm$ 0.009 ^A	0.355 $\pm$ 0.02 ^{bA}	0.366 $\pm$ 0.007 ^A
MNNG – positive control	21692.16 $\pm$ 896.54 $S_{gfpexp} = 840$	50 μ M	–	–	–	–

Mean values $\pm$ SD; n = 8 and n = 4; a – significantly different from control ($p < 0.05$); b – significant difference of Li, Na and K salts from acids ($p < 0.05$); c – significant difference of Na and K salts from Li salts group ($p < 0.05$); d – significant difference of K salts from Li and Na salts group ($p < 0.05$); A – significant difference of caffeic acid and Li, Na and K caffeinate salts group ($p < 0.05$).

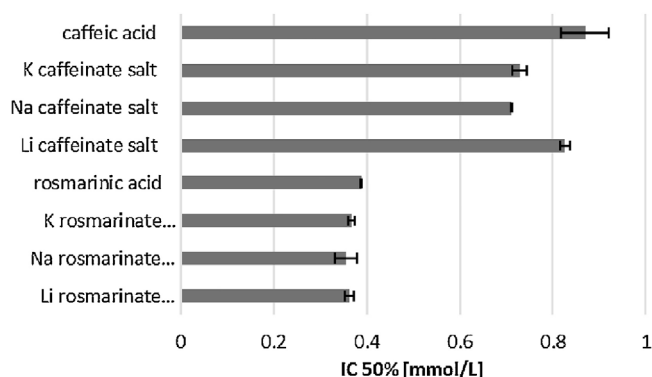


Fig. 4. Values of IC₅₀ parameter for antioxidant DPPH test. Mean values $\pm$ SD; n = 4.

Na and K salts than for caffeic acid and their salts. Caffeic acid Li, Na and K salts slightly modulated acid antioxidant activity. This tendency has not been noticed for rosmarinic acid (Fig. 4).

The MIC values of tested chemicals against microorganisms are shown in Table 2. The caffeic acid and their salts with Li and Na

exhibited better antimicrobial activity against *E. coli* (MIC = 250 μ g/mL) than caffeic acid complex with K (MIC >500 μ g/mL). Caffeinate Li, Na and K salts had MIC values ranging from 250 to >1000 μ g/mL against the tested microorganisms; the highest sensitivity was observed in the case of *E. coli* with the MIC value ranging from 250 to >500 μ g/mL for all chemicals. The rosmarinic acid showed the highest antimicrobial activity against *E. coli* with MIC = 250 μ g/mL. The MIC values of rosmarinic acid and their salts with Li, Na and K were >500 μ g/mL against all tested microorganisms. MIC values were above the values of positive control with kanamycin with all analysed chemicals and microorganisms for *E. coli*, *Bacillus* sp., *Staphylococcus epidermidis*, *Streptococcus pyogenes* (MIC = 100 μ g/mL) and for *Candida* sp. (MIC = 200 μ g/mL).

4. Discussion

Many pharmaceutical agents with high biological activity have been discovered by screening of natural products extracted from plants. Plants as a natural source of active substances offer great opportunity to evaluate new chemical classes of anticancer agents as well as to study novel and potentially relevant mechanisms of action [9]. Some authors showed, that plant natural phenolic

Table 2
MIC values (μ g/ml) of tested chemicals against microorganisms.

Chemicals	<i>E. coli</i> (Gram-)	<i>Bacillus</i> sp. (Gram +)	<i>Staphylococcus epidermidis</i> (Gram +)	<i>Streptococcus pyogenes</i> (Gram +)	<i>Candida</i> sp. (Gram +)
Caffeic acid	250	>500	>500	>500	>1000
Li	250	>1000	>500	>1000	>500
Na	250	>1000	>500	>500	>500
K	>500	>1000	>500	>500	>1000
Rosmarinic acid	250	>500	>500	>500	>500
Li	>500	>500	>500	>500	>500
Na	>500	>500	>500	>500	>500
K	>500	>500	>500	>500	>500
Kanamycin (positive control)	100	100	100	100	200

compounds as caffeic and rosmarinic acids have antiproliferative and cytotoxic effects and can be potentially use as chemopreventive agents [1–4,9].

In this work we analysed cyto-, genotoxic effects, antioxidant potency and antimicrobial activity of caffeic and rosmarinic acids and their salts with Li, Na and K as potential substances with anticancer properties. *E. coli* strain with plasmid transcriptional fusion of *recA:gfp* gene was model microorganism applied. *RecA:gfp* genetic construct is one of the best scientifically proofed promoter construct from SOS bacteria regulon with well-known genotoxic sensitivity to anticancer and candidate drugs, and to different chemicals with pharmacological potency. Green fluorescent protein gene (*gfp*) has also been used widely as a reporter in cell-based fluorescence reporter gene assay (RGAs) of drug candidates [11–20].

According to the results the dose-response was not significant. Oppositely, lower concentrations exhibited stronger GFP response after rosmarinic acids and their salts treatment. Also, the highest cytotoxic effects were obtained for the lowest concentrations of rosmarinic and caffeic acids and their salts (without Li caffeine salt). The phenomenon that some chemicals have unexpected and potent effects at very low doses is so-called non-monotonic response and is not fully explained and for each substance and strain of bacteria should be considered individually. Sandyik et al., explained that the low concentration of toxins (in this case phenolic acids and their salts) are too low to activate the stress signaling and transcription of chaperone proteins. However, these concentrations are sufficient to exert a destructive effect on cells, eg in chemical processes such as ROS generation. At higher concentrations, SOS signaling pathways are activated to protect against the effects of stressors [28,29].

Rosmarinic acid activates apoptosis mainly by mitochondrial pathway (independently from the Fas/Fas), but in the presence of Lck kinase. Depolarization of the mitochondrial membrane and release of cytochrome C probably corresponds to intensive ROS generation [30–32]. Rosmarinic acid can also inhibits STAT5 (signal transducer and activator of transcription 5) phosphorylation and COX-2 (induced cyclooxygenase 2) expression [31,32]. Interestingly, in macrophages, this acid inhibits the formation of reactive oxygen and nitrogen forms by inhibiting $\text{I}\kappa\text{-}\beta$ (the protein product of NFKBIA gene), but on the other hand it is believed that the generation of free radicals by rosmarinic acid that determines its antimicrobial and proapoptotic potency [32]. The properties of caffeic and rosmarinic acids for the reduction of metals present in the cell may be responsible for the intensive generation of ROS including hydrogen peroxide and hydroxyl radical responsible for the formation of DNA bases [33]. Moreover, DNA damage is a major cause of both genotoxic and cytotoxic effects of the both acids. In our results genotoxic effects seemed to be independent of the salt ions. The caffeic acid salts with Li, Na and K showed reduced genotoxic effect in comparison to the caffeic acid while increased cytotoxic effect than that of caffeic acid. It is known, that caffeic acid induces apoptosis by the Fas/FasL pathway [34]. It can also affects the activity of Bcl-2 (apoptosis inhibitor) proteins by activating apoptosis in the mitochondrial pathway [35]. In addition to its antioxidant activity, caffeic acid can generate ROS which, in high concentrations, damage the cell structure and direct it to the apoptosis pathway [36]. Moreover, caffeic acid inhibits the G0/G1 cell cycle [37] and the catalytic activity of some proteins, such as MMP-9, as well as their transcription by inhibiting transcription factors such as NF- κ B. The presence of the nucleophilic catecholate causes the generation of H_2O_2 , which perform signaling functions in the cell, for example, participate in the induction of Nrf2 transcription factor and transport it to the nucleus [38].

DPPH assay (antioxidant assay) showed better antioxidant potency for rosmarinic acid and its Li, Na and K salts than for

caffeic acid and their salts. Our data presented no clear positive correlation between the antioxidant potency of caffeic and rosmarinic acids and their Li, Na and K salts and their cytotoxic effect. However, it seems that it was possible to notice a slight trend between antioxidant and cytotoxic potency of rosmarinic acid and their salts. Also, these compounds stronger influenced the expression level of the *gfp* gene. Phenolic compounds exhibit their antioxidant properties by the presence of characteristic groups, mainly phenylhydroxyl groups. They act as hydrogen donors that can react with ROS and RNS (reactive nitrogen species) to form a phenoxy radical [39]. However, the antioxidant activity of phenolic acids may follow other mechanisms, eg they can stabilize or relocate unpaired electrons, chelate transition metal ions, catalyze free radicals (oxidation reactions), and may act as oxidative inhibitors [40]. During complex or salt formation there are electronic charge distribution in the ligand. These changes were showed in our previous papers in the experimental way such as following methods: infrared spectroscopy (FT-IR), Raman (FT-R), nuclear magnetic resonance (^1H , ^{13}C NMR) and electronic absorption spectroscopy (UV-vis) as well as by quantum chemical calculations (DFT). Electronic charge distribution in molecule decides about its stability and reactivity including biological activity [24–26]. Rosmarinic acid has more hydroxyl groups than caffeic acid, which may explain differences in the ability to reduce the synthetic DPPH radical. In studies on the effect of both acids on the protection against hepatocyte oxidative damage, CA exhibits better protective properties than RA [39,40]. However, often the cytotoxic and genotoxic effects may be due to pro-oxidative properties. It turns out that both acids exhibit high prooxidation potentials due to the ability to reduce iron and copper ions present in the cell and generate hydroxyl radicals – the most reactive oxygen species [41]. Perhaps it could be one of the molecular mechanism of the stronger biological activity of lower doses of rosmarinic or caffeic acid and their salts.

Antimicrobial studies of caffeic acid and their Li and Na salts revealed the highest activity of tested chemicals against *E. coli*. In the case of other microorganisms the better antimicrobial action showed rosmarinic acid and their salts with Li, Na and K. In our previous projects we conducted antimicrobial tests of complexes of caffeic acid with manganese(II), copper(II) and cadmium(II). Our results shown, that caffeic acid inhibits the growth of *Escherichia coli* and *Proteus vulgaris* [29,30]. Moreover, the antimicrobial activity of CAPE (caffeic acid phenethyl ester – caffeic acid derivatives) was shown against *E. coli*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Haemophilus influenzae*. Additionally, CAPE is a valuable inhibitor of HIV-1 integrase; therefore it is believed as this polyphenol may be a potential agent in anti-HIV therapy [31]. Earlier authors experiments confirmed the bactericidal activities of rosmarinic acid against *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia*, and *Enterococcus faecalis* [26].

In the case of antimicrobial activity, the mechanisms of action of caffeic acid on selected bacterial strains have not yet been thoroughly explained [42], however, numerous reports have been published including studies on other polyphenolic compounds and phenolic acids [43–46]. It follows, that phenolic acids can inhibit the growth of bacteria due to pro-oxidative properties as well as by altering the hydrophobicity and charge on the surface of the cells, ultimately causing cellular cracking and formation and cytoplasmic deposition [47]. It seems that the main mechanism of action of rosmarinic acid is its ability to damage the cell membrane. It should be noted that *E. coli*, as a natural component of the bacterial flora of the digestive tract, has demonstrated the ability to metabolize coffee acid, which may explain the lower CA cytotoxic effect on this bacterium [48,49].

5. Conclusions

The results of the experiment show that there is no clear positive correlation between the antioxidant potency of caffeic and rosmarinic acids and their Li, Na and K salts and their cytotoxic effect. Used salts ions Li, Na and K do not significantly affect the antioxidant effect of natural phenolic compounds and they do not have a significant impact on the biological parameters such as cyto- and genotoxicity. Perhaps it is connected with the reaction environment including polarity of the solvent (water).

Conflict of interest

Authors declare no conflict of interests.

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