Synthetic cinnamates as potential antimicrobial agents

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Abstract

This study deals with synthesis of methyl cinnamate, butyl cinnamate, and *p*-methoxy methyl cinnamate and testing of their *in vitro* antimicrobial activity. Antimicrobial activity was examined towards 29 microorganisms using microdilution method. It is shown that antimicrobial activity of methyl cinnamate and *p*-methoxy methyl cinnamate was better than that of butyl cinnamate. *Sarcina lutea*, *Bacillus subtilis* ATCC 6633, *B. subtilis* and *B. subtilis* IP 5832 (probiotic) were the most sensitive bacteria. It is established that *p*-methoxymethyl cinnamate can be a new, potential anti-*Staphylococcus aureus* agent with minimum inhibitory concentration of 62.5 μg/ml. Methyl cinnamate and *p*-methoxy methyl cinnamate inhibited the growth of *Aspergillus restrictus*, *A. flavus* and *A. fumigatus* in the concentration range from 62.5 to 250 μg/ml.

Keywords: methyl cinnamate, butyl cinnamate, *p*-methoxy methyl cinnamate, antimicrobial activity, bacteria, fungi.

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Cinnamates can be found in nature as secondary metabolites widely distributed in the plant kingdom. They are synthesized by plants and, actually, represent components of essential oils. Numerous studies have demonstrated that essential oils with methyl cinnamate as one of the main components, exhibit different kind of biological activity, such as antibacterial, antifungal, antithrombotic, anti-inflammatory and antioxidative activity [1–5].

Besides naturally occurring, the cinnamates can be synthesized using different methods: esterification of corresponding cinnamic acids with alcohols [6], Wittig reaction [7], Reformatsky reaction [8], Heck reaction [9], as well as other methods [10]. Due to the characteristic flavour and fragrance, as well as high boiling point and stability, synthetic methyl, ethyl- and butyl cinnamate are widely used in food industry, especially for beverages and baked goods. Council of Europe [11] included methyl cinnamate in the group of substances safe for use in foodstuffs. Application of cinnamic acid esters in cosmetic and pharmaceutical industry is also significant. Ethyl cinnamate is used especially in the manufacture of fine products, due to its sweet, fruity and strong cinnamon odour with hints of amber and vanilla. In the perfume industry it can be used as flavour fixative agent. Butyl cinnamate is used in the manufacturing of fine fragrances, decorative cosmetics, shampoos, toilet soaps and other toiletries, as well as in non-cosmetic products such as household cleaners and detergents [12,13].

The cinnamic acid derivatives showed different biological activities like antioxidant, antidiabetic, heaptoprotective, anti-inflammatory, cytotoxic, etc. [10]. According to *in vivo* observations, methyl cinnamate is not irritating to skin and mucous membrane [14]. Studies of mutagenicity and genotoxicity have shown that in rec assay with *Bacillus subtilis* strains H17 (rec^+) and M45 (rec^-), a dose of 20 μg/disk methyl cinnamate produced no genotoxic effects, while an *in vitro* assay in Chinese hamster ovary cells showed that methyl cinnamate, at concentrations below 100 μM, did not have any cytogenetic effect [14] .

Cinnamates have wide use in different areas of industry, especially in food industry. However, there is limited information about antimicrobial activity of cinnamates as synthesized compounds, therefore, the aims of this work were to synthesize and to estimate the antimicrobial effects of methyl cinnamate, butyl cinnamate, and *p*-methoxy methyl cinnamate on broad panel of bacteria and fungi.

MATERIALS AND METHODS

General

Methyl, butyl and *p*-methoxy methyl cinnamates were synthesized using the palladium-catalyzed phosphine-free Heck reaction. Different bases and ionic liquids [15,16] were used (Figure 1).

The Heck reaction products were analyzed with GC–MS chromatography and ^{1}H-NMR spectroscopy. GLC analyses were obtained with an Agilent 6890N (G 1530N) instrument (Serial # CN10702033), with capillary apolar column. The ^{1}H-NMR spectra were run in

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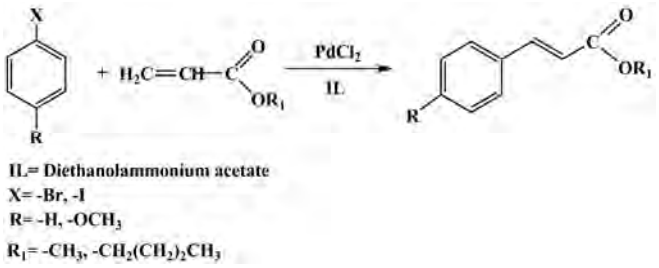

Figure 1. Synthesis of investigated cinnamates.

$CDCl_3$ on a Varian Gemini 200 MHz spectrometer. ^{1}H--NMR spectra are given in Supplementary material. The compounds $PdCl_2$, diethanolamine, aryliodide, olefins and amphotericin B were obtained from Aldrich Chemical Co, St. Louis, USA. Nutrient media, Mueller–Hinton broth was from Liofilchem, Roseto, Italy, while Sabouraud dextrose broth was from Torlak, Belgrade, Serbia. Doxycycline and fluconazole were from Galenika A.D., Belgrade, Serbia, and Pfizer Inc., New York, USA, respectively. Resazurin was from Alfa Aesar GmbH & Co, Karlsruhe, Germany.

In vitro antimicrobial assay

Test microorganisms

Antimicrobial activity was tested against 20 strains of bacteria and 9 strains of fungi. The list of tested microorganisms was presented in Tables 1 and 2. All clinical isolates of bacteria were a generous gift from the Institute of Public Health, Kragujevac, Serbia. The other microorganisms (the ATCC strains and fungi) were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac.

Suspension preparation

Bacterial and yeast suspensions were prepared by the direct colony method. The turbidity of initial suspension was adjusted by comparing with 0.5 McFarland's standard. When adjusted to the turbidity of the 0.5 McFarland's standard, bacteria suspension contains about 10^8 colonies forming units (CFU)/mL and suspension of yeast contains 10^6 CFU/mL. The initial suspensions were additionally diluted in 1:100 ratio in sterile 0.85% saline. The suspensions of fungal spores were prepared by gentle stripping of spore from agar slants with growing aspergilli. The resulting suspensions were 1:1000 diluted in sterile 0.85% saline.

Microdilution method

Antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) using microdilution method with resazurin [17]. The tested compounds were first dissolved in dimethyl sulfoxide (DMSO) (10% of total volume) and then into nutrient liquid medium (up to 100% of total volume). The stock concentrations of tested compounds were 2000 μg/mL. Next, serial twofold dilutions were made in a concentration range from 7.81 to 1000 μg/mL in sterile 96-well microtiter plates containing Mueller–Hinton broth for bacteria and Sabouraud dextrose broth for fungi. After that, 10 μL of diluted bacterial, yeast suspensions and suspensions of spores was added to appropriate wells. Finally, 10 μL resazurin solution, as an indicator of microbial growth, was added to each well inoculated with bacteria and yeasts. Resazurin is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated plates were incubated at 37 °C for 24 h for bacteria, 28 °C for 48 h for yeasts and 28 °C for 72 h for moulds. MIC was defined as the lowest concentration of tested substance that prevented resazurin color change from blue to pink. For moulds, MIC values of the tested substance were determined as the lowest concentration that visibly inhibited mycelia growth. Minimum microbicidal concentration was determined by plating 10 μL of samples from wells, where no indicator color change was recorded, on nutrient agar medium. At the end of the incubation period the lowest concentration with no growth (no colony) was defined as minimum microbicidal concentration (MMC).

Doxycycline, fluconazole and amphotericin B, dissolved in nutrient liquid medium, were used as a positive control. Solvent control test was performed to study an effect of 10% DMSO on the growth of microorganism. Also, in the experiment, the concentration of DMSO was subsequently decreased because of the twofold serial dilution assay, resulting in a working DMSO concentration of 5% or lower. Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

Statistical analysis

Data were analyzed using the Student's *t*-test and the one-way analysis of variance (ANOVA). In all cases p values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS package.

RESULTS

Synthesis of cinnamates

Methyl cinnamate (**MC**), butyl cinnamate (**BC**), and *p*-methoxy methyl cinnamate (**MMC**) were synthesized using the Heck reaction and subjected to investigation of their antimicrobial activity.

Antimicrobial activity

The results of *in vitro* testing of antibacterial and antifungal activities of cinnamates in relation to 29 species of microorganisms are shown in Tables 1 and 2. The tested compounds showed different degree of antimicrobial activity. *MIC* values were in range from 15.6 to >1000 μg/ml while MMC values were from 31.25 to >1000 μg/ml. It is found that **MC** and **MMC** exhibited better antimicrobial activity than BC ($p < 0.05$). Solvent control showed that 10% DMSO did not inhibit the growth of microorganism.

Regarding the tested bacteria, Gram-positive bacteria and probiotics were more sensitive than Gram negative bacteria ($p < 0.05$). The most sensitive bacteria were *Sarcina lutea*, *Bacillus subtilis* ATCC 6633, *B. subtilis* and *B. subtilis* IP 5832 (probiotic). In these cases, *MIC*s were 15.6 and 31.25 μg/ml, while *MMC*s were between 31.25 and 125 μg/ml. Moderate activity was shown against *S. lutea* ATCC 9341, *Staphylococcus aureus* and *Proteus mirabilis* (standard and clinical strain) with *MIC* at 62.5 and 125 μg/ml. The growth of *Enterococcus faecalis* ATCC 29212, *E. faecalis*, *Escherichia coli* ATCC 25922, *E. coli*, *Salmonella enterica*, *S. typhimurium*, *Lactobacillus rhamnosus* was not affected at least by one tested compounds.

Antifungal activity was a little bit better against moulds than yeasts, but not statistically significant ($p > 0.05$). *Aspergillus restrictus*, *A. fumigatus*, *A. flavus* have shown sensitivity at the lowest concentration (*MIC* = 250 μg/ml for **MC** and *MIC* = 62.5 and 125 μg/ml for **MMC**).

DISCUSSION

In vitro antibacterial and antifungal activity of cinnamates were tested against a panel of microorganisms including human pathogenic bacteria, yeasts, moulds, probiotics in order to evaluate broad-spectrum antimicrobial activity. Significant results were obtained for bacteria *S. lutea* and *B. subtilis.* These bacteria are important as contaminants because they are ubiquitous in the environment, particulary in soil and air. Therefore, they find their way easily into food products or colonize skin, gastrointestinal and respiratory tracts

*Table 1. Antibacterial activity (μg/ml) of methyl cinnamate (**MC**), butyl cinnamate (**BC**) and p-methoxy methyl cinnamate (**MMC**)*

Species	**MC**		**BC**		**MMC**		Doxycycline	
	MIC	*MMC*	*MIC*	*MMC*	*MIC*	*MMC*	*MIC*	*MMC*
Gram-positive bacteria								
Sarcina lutea ATCC 9341	125	250	n.t.	n.t.	62.5	250	< 0.45	7.81
Sarcina lutea	31.25	125	>1000	>1000	15.6	62.5	< 0.45	3.75
Enterococcus faecalis ATCC 29212	1000	>1000	>1000	>1000	>1000	>1000	7.81	62.5
Enterococcus faecalis	>1000	>1000	>1000	>1000	>1000	>1000	7.81	62.5
Bacillus subtilis ATCC 6633	31.25	125	>1000	>1000	15.6	31.25	1.95	31.25
Bacillus subtilis	31.25	62.5	250	>1000	31.25	31.25	0.11	1.95
Staphylococcus aureus ATCC 25923	500	>1000	>1000	>1000	1000	>1000	0.22	3.75
Staphylococcus aureus	125	1000	>1000	>1000	62.5	1000	0.45	7.81
Gram-negative bacteria								
Escherichia coli ATCC 25922	>1000	>1000	>1000	>1000	>1000	>1000	15.62	31.25
Escherichia coli	1000	>1000	>1000	>1000	>1000	>1000	7.81	15.63
P. aeruginosa ATCC 27853	1000	>1000	>1000	>1000	1000	>1000	62.5	125
Pseudomonas aeruginosa	1000	>1000	250	>1000	1000	>1000	250	> 250
Proteus mirabilis ATCC 12453	250	500	>1000	>1000	125	500	15.62	62.5
Proteus mirabilis	500	1000	>1000	>1000	125	500	250	> 250
Salmonella enterica	1000	>1000	>1000	>1000	>1000	>1000	15.62	31.25
Salmonella typhimurium	1000	>1000	>1000	>1000	>1000	>1000	15.62	125
Probiotics								
Lactobacillus rhamnosus	>1000	>1000	31.25	62.5	>1000	>1000	7.81	31.25
Lactobacillus plantarum	250	500	>1000	>1000	250	250	0.45	7.81
Bifidobacterium animalis subsp. *lactis*	500	500	31.25	62.5	1000	>1000	31.25	62.5
Bacillus subtilis IP 5832	125	500	250	500	31.25	125	1.95	15.63

*Table 2. Antifungal activity (μg/ml) of methyl cinnamate (**MC**), butyl cinnamate (**BC**) and p-methoxy methyl cinnamate (**MMC**)*

Species	**MC**		**BC**		**MMC**		Fluconazole		Amphotericin B	
	MIC	*MMC*	*MIC*	*MMC*	*MIC*	*MMC*	*MIC*	*MMC*	*MIC*	*MMC*
Yeasts										
*Candida albicans*ATCC 10231	1000	> 1000	1000	> 1000	500	1000	31.25	1000	0.49	1.95
Candida albicans	1000	1000	1000	> 1000	500	1000	62.5	1000	0.98	1.95
Rhodotorula sp.	500	1000	500	1000	1000	1000	62.5	1000	3.9	3.9
Saccharomyces boulardii	250	500	n.t.	n.t.	250	500	31.25	1000	n.t.	n.t.
Moulds										
Aspergillus niger ATCC 16404	500	> 1000	> 1000	> 1000	500	> 1000	62.5	62.5	0.98	1.95
Aspergillus niger	500	> 1000	1000	> 1000	500	> 1000	500	1000	0.98	0.98
Aspergillus restrictus	250	1000	500	> 1000	125	250	500	1000	0.98	1.95
Aspergillus fumigatus	250	1000	1000	> 1000	125	250	500	1000	3.9	3.9
Aspergillus flavus	250	1000	1000	> 1000	62.5	1000	1000	1000	0.98	15.63

of people causing food poisoning or human infection. Also, *S. aureus* was inhibited by **MMC** at low concentration. This compound can be a new, potential anti-*S. aureus* agent since this bacterium has been developing resistance to common antibiotics. Majority of Gram-negative bacteria showed moderate sensitivity to tested cinnamates. It is known that Gram-negative bacteria, in contrast to Gram-positive bacteria, contain the outer membrane which serves as a permeability barrier and prevent the entry of noxious compounds including antibacterial compounds. With respect to activity of positive control, a broad-spectrum antibiotic doxycycline, the activity of tested compounds was lower. But, if we take into consideration the results of other studies about antibacterial activity of naturally occurring cinnamates or synthesized cinnamates, it can be concluded that *MIC* values below 1000 μg/ml indicate significant activity of cinnamates and their potential application as antibacterial agents [1–3,18,19]. Narasimhan *et al*. [18] synthesized and evaluated antimicrobial activity of series of esters, substituted derivatives and amides of cinnamic acid. The compounds, isobutyl cinnamate and dibromo cinnamic acid, were the most effective substances displaying growth inhibition of all the tested microorganisms. The authors observed that the removal of double bond in side chain of cinnamic acid was effected with –OH and –Br group. Also, the results have shown that addition of halogens to the side chain caused remarkable increase in growth inhibitory effect of cinnamic acid, whereas addition of hydroxy groups to the side chain double bond did not remarkably enhance the antimicrobial activity. Venkateswarlu *et al*. [19] tested derivatives of polyhydroxy cinnamic acid among which butyl hydroxy cinnamates showed high antibacterial and low antifungal activity. The most sensitive bacterium was *B. subtilis,* similarly with results in our study.

Moulds are ubiquitous microorganisms with a great capacity to colonize many kinds of substrates. Also, they produce mycotoxins that can be teratogenic, carcinogenic. In this study, tested compounds **MC** and **MMC** showed marked inhibitory activity of the growth of *A. restrictus*, *A. flavus* and *A*. *fumigates*. However, that activity was lower than positive control amphotericin B. Amphotericin B was used as a control since it is a drug of choice for infection caused by *Aspergillus* species. *MIC*s of amphotericin B for most *Aspergillus* species are clustered between 0.5 and 2 μg/ml, so for tested *A. fumigatus* strain with *MIC*s above 2 μg/ml, the activity of amphotericin B is associated with a high probability of therapeutic failure [20]. Among the tested compounds, **MMC** was the most active against *A. fumigatus*. The activity of tested cinnamates against the bacteria and fungi may open the possibility of their use as a preservative in food industry or as antimicrobial agents in pharmaceutical industry.

CONCLUSIONS

In this study synthetic cinnamates: methyl cinnamate, butyl cinnamate and *p*-methoxy methyl cinnamate were tested for their *in vitro* antibacterial and antifungal activity. It is found that methyl cinnamate and *p*-methoxy methyl cinnamate have shown better antimicrobial activity than butyl cinnamate. These synthetic cinnamates could be potential antimicrobial agents against food spoilage and human pathogenic bacteria and fungi.

Supplementary material

^{1}H-NMR spectra of investigated compounds are available from corresponding author on request.

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IZVOD

SINTETISANI CINAMATI KAO POTENCIJALNI ANTIMIKROBNI AGENSI

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(Naučni rad)

Cinamati, osim što su prisutni u prirodi kao produkti sekundarnog metabolizma biljaka, mogu se i sintetisati primenom više različitih metoda. Poznato je da ispoljavaju brojna biološka delovanja, a i da se primenjuju u industriji. Stoga, ciljevi ovog radu su sinteza cinamata, metil cinamat, butil cinamat i *p*-metoksi metil cinamat, i testiranje njihove antimikrobne aktivnosti. Antimikrobna aktivnost je ispitana mikrodilucionom metodom u odnosu na 29 vrsta mikroorganizama. Testirana jedinjenja, metil cinamat i *p*-metoksi metil cinamat, su pokazala bolju aktivnost nego jedinjenje butilcinamat. Bakterije *Sarcina lutea*, *Bacillus subtilis* ATCC 6633, *B*. *subtilis* i *B*. *subtilis* IP 5832 (probiotik) su najosetljiviji mikroorganizmi sa minimalnim inhibitornim koncentracijama (*MIK*) od 15,6 do 31,25 μg/ml, dok su minimalne mikrobiocidne koncentracije (*MMK*) bile od 31,25 do 125 μg/ml. Na osnovu prikazane aktivnosti (*MIK* = 62,5 μg/ml), uočeno je da *p*-metoksi metil cinamat može da predstavlja budući, potencijalni agens protiv infekcija izazvanih bakterijom *Staphylococcus aureus*. Testirana jedinjenja metil cinamat i *p*-metoksi metil cinamat su inhibirala rast gljiva, proizvođača mikotoksina, *Aspergillus restrictus*, *A. flavus* i *A. fumigates* u koncentracijama od 62,5 do 250 μg/ml. Prikazani rezultati potvrđuju antimikrobnu aktivnost testiranih cinamata i ukazuju na njihovu primenu u borbi protiv štetnih bakterija i gljiva.

Ključne reči: Metil cinamat • Butil cinamat • *p*-Metoksi metil cinamat • Antimikrobna aktivnost • Bakterije • Gljive