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Antimicrobial and Resistance Modulatory Activity of *Alpinia katsumadai* Seed Phenolic Extract, Essential Oil and Post-Distillation Extract

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Summary

Antimicrobial resistance of food-related bacterial pathogens is becoming a serious problem, especially after the emergence of multidrug-resistant strains. To overcome this problem, new and effective antimicrobials or resistance modulators are highly needed and plant kingdom represents a valuable source of these compounds. We investigated antimicrobial and resistance modulatory activity of the phenolic extract, essential oil and post-distillation extract of *Alpinia katsumadai* seeds against *Campylobacter jejuni* and *Staphylococcus aureus*. Among the tested plant formulations, *A. katsumadai* seed phenolic extract and post-distillation extract showed moderate antimicrobial activity against *C. jejuni*, while *S. aureus* was more resistant. When evaluating resistance modulatory potential of *A. katsumadai* phenolic extract, essential oil and post-distillation extract in *C. jejuni* against ciprofloxacin, erythromycin, triclosan, bile salts and ethidium bromide, plant formulations exhibited modulatory activity in combination with all antimicrobials. Modulation of resistance was more strain- and antimicrobial-specific in *S. aureus*, but very efficient in the case of reduced resistance to bile salts. Essential oil from *A. katsumadai* seeds efficiently increased intracellular ethidium bromide accumulation and was thus confirmed as potential inhibitor of efflux pumps in *C. jejuni* and *S. aureus*.

Key words: antimicrobial activity, antimicrobial resistance, resistance modulation, *Alpinia katsumadai* seed extracts, essential oil, efflux inhibition

Introduction

Despite many recent technological advances in the food industry, which have contributed to increased safety in the food supply chain, we are still facing high incidence of foodborne illnesses. Only in 2011, there were 69 553 human cases originating from foodborne zoonotic outbreaks in the EU (*1*). Ninety-three of them were fatal. In the same year, *Campylobacter*, as the most prevalent foodborne zoonotic agent, caused 220 209 registered, mostly sporadic human illnesses and it is assumed that the real burden is even much higher (*1*). Besides emerging and reemerging food-related zoonotic agents, the non-zoonotic pathogens are also transmitted by foods. Together with their high incidence and the increasing resistance against antibiotics and other antimicrobials used in the food chain (*2*,*3*), microbial pathogens compromise food safety, especially with the emergence of multidrug-resistant strains (*4*).

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Search for alternative antimicrobials derived from plants seems to be a viable solution for mitigation of resistance (*5*). In addition to searching for new antimicrobially active plant formulations, the strategies for combating antibiotic resistance are focused also on resistance modulators. These are not necessarily antimicrobially active, but can decrease the resistance of pathogens when administered together with other antimicrobials. The microbial sensitization by resistance modulators is mostly due to efflux inhibition, increased membrane permeability, increased porin production or change in porin profile (*6*).

Plant extracts rich in phenolic compounds (phenolic extracts), and essential oils have long been shown to possess antimicrobial activity and were frequently studied and reviewed (*7–10*). They are especially interesting as they are generally recognized as safe and have a potential to be used as preservatives in food products. *Alpinia katsumadai* Hayata (syn. *A. katsumadae* Hayata; Zingiberaceae) is widely used in traditional Chinese medicine as an anti-emetic remedy and to increase the appetite, but also in animal feed, to facilitate rapid growth of domestic animals (*11,12*). In our study we have focused on investigating the bioactivity of *A. katsumadai* extract, essential oil and also waste material that remains after the essential oil production. Such residual materials are often disposed of and may present an environmental problem. Their high phenolic content and the potential to provide an economically feasible source of natural antioxidants and antimicrobials is unused (*13*).

In our study phenolic extract, essential oil and post-distillation extract of *A. katsumadai* seeds were tested against drug-sensitive and resistant strains of Gram-negative *Campylobacter jejuni* and Gram-positive *Staphylococcus aureus*, and their potential to modulate the resistance of these pathogens against different antimicrobials was evaluated. Additionally, intracellular accumulation of ethidium bromide was tested to confirm efflux inhibition with the plant formulation, which was most efficient in resistance modulation.

Materials and Methods

Bacterial strains, growth conditions and antibiotic resistance

All bacterial strains were stored at –80 °C. *Campylobacter* was grown on Columbia blood agar (Oxoid, Basingstoke, UK) at 42 °C for 24 h under microaerobic conditions. *Staphylococcus* was grown on Mueller-Hinton agar (MHA, Oxoid) at 37 °C for 24 h. For the antimicrobial activity assays, cultures were suspended in Mueller-Hinton broth (MHB, Oxoid) to 10^5–10^6 CFU/mL. Antibiotic-sensitive and -resistant strains of both species were included in testing. Their resistance against five or more medically important antibiotics was determined by broth microdilution method (Sensititre® TREK Diagnostic Systems Inc., Thermo Scientific, Independence, OH, USA) or CellTiter-Blue® reagent (Promega, Madison, WI, USA) and automated fluorescence signal detection (*14*).

Preparation of plant formulations

Alpinia katsumadai seeds were bought from a commercial source (Cat. no. 680381, Plantasia, Oberndorf/Salzburg, Austria) and extracted with 96 % ethanol for 24 h at room temperature to obtain an extract rich in phenolic compounds which was dried by gradual pressure decrease at 45 °C using rotavapor. Part of the extract was suspended in water and hydrodistilled for 2 h using Clevenger-type apparatus to obtain the essential oil. The remaining material after hydrodistillation was freeze-dried and dissolved in ethanol for further testing as post-distillation extract.

Total phenolic content and DPPH radical-scavenging assay

The total content of phenolic compounds (TPC) of tested formulations was determined spectrophotometrically using Folin-Ciocalteu reagent (*15*). The reaction mixture contained 100 μL of 0.1 % phenolic extract or post-distillation extract diluted in methanol, 500 μL of 0.2 M Folin-Ciocalteu reagent (Fluka Chemicals, AG, Buchs, Switzerland), and 400 μL of 10 % aqueous Na_2CO_3 solution (Lach-Ner, Brno, Czech Republic). The absorbance was measured spectrophotometrically (Agilent Technologies, Santa Clara, CA, USA) at 760 nm after a 30-minute incubation in the dark. The results are expressed as mg of gallic acid equivalents (GAE) per g of tested dried material.

The radical-scavenging potential of the tested plant formulations was evaluated using the DPPH assay (*16*), with some modifications. Briefly, the samples were dissolved in methanol and tested at four concentrations ranging from 3.75 to 150 μg/mL. The reaction mixtures contained 50 μL of the sample dilution and 150 μL of 50 μM DPPH dissolved in methanol. Spectrophotometric absorbance at 535 nm was measured after 30 min, against methanol as a blank on a Wallac 1420 Victor2 multilabel counter (PerkinElmer, Waltham, MA, USA). The RSC was calculated using the following equation:

$$RSC=[(A_c-A_s)/A_c]\cdot 100 \qquad /1/$$

where A_s is the absorbance of the sample, and A_c is the absorbance of the control.

Antimicrobial activity assay

Antimicrobial activity was evaluated using broth microdilution assay in microtiter plates as previously described using CellTiterBlue® kit (Promega) (*14*). After 24-hour incubation under species-specific growth conditions, viability was measured based on the intensity of the fluorescence signal with a microplate reader (Tecan, Mannedorf/Zurich, Switzerland). Minimal inhibitory concentration (MIC) was determined as the lowest concentration of the tested antimicrobial at which no fluorescence signal was detected. Negative control, growth control and dimethyl sulphoxide (DMSO) control were included and each assay was repeated in triplicate.

Resistance modulatory activity assay

The resistance modulatory assay was performed by the same principle as the antimicrobial activity assay with the addition of the tested modulators in concentrations of half MIC value into the medium.

Ethidium bromide accumulation assay

Ethidium bromide (EtBr) accumulation assay (*17*) was carried out to evaluate the potential efflux inhibitory activity of essential oil at 0.5 and 0.25 MICs of essential oil on *C. jejuni* NCTC 11168 and *S. aureus* 5.3. Additionally, 100 μg/mL of verapamil were tested as a positive efflux inhibitor reference. The overnight cultures were resuspended in MHB to achieve $A_{600\ nm}$=0.2 and incubated for 4 h at 37 °C (*S. aureus*) or 42 °C (*C. jejuni*). Further on, the cells were washed and resuspended in phosphate buffered saline (PBS) to achieve $A_{600\ nm}$=0.2. Plant formulations and EtBr at final concentration of 0.5 μg/mL were added to the culture in black microtitre plates (100 μL per well). Intracellular EtBr accumulation kinetics was measured at 500 nm excitation and 608 nm emission wavelength with a microplate reader (Tecan) for 1 h. Culture with EtBr, but without inhibitor was used as a baseline accumulation control and EtBr alone as blank. Three independent experiments with three replicates were carried out.

Statistical analysis

The results were statistically analyzed using the SPSS® software, v. 21 (IBM Corp., Armonk, NY, USA). Comparisons of the group mean values and the significances of the differences between the groups were verified by one-way ANOVA. Pearson coefficients were calculated for the correlations between different variables. The results were considered significant when $p \leq 0.05$.

Results and Discussion

Phenolic extract, essential oil and the post-distillation extract of *A. katsumadai* seeds were tested for their antimicrobial activity against five *C. jejuni* and five *S. aureus* isolates. Among each tested bacterial species, antibiotic-sensitive and -resistant strains, including multidrug-resistant ones, were included (Table 1; *18*).

Statistically significant differences between the antimicrobial activities of different plant formulations were observed ($p<0.0001$). According to the average MICs for *Campylobacter* (275 μg/mL), *A. katsumadai* phenolic extract was the most efficient antimicrobial plant formulation, followed by *A. katsumadai* post-distillation extract. Generally, plant formulations were more effective against *C. jejuni* than against *S. aureus* (Table 1). We could not confirm any difference between the antimicrobial activities of the tested plant formulations against antibiotic-sensitive or resistant strains. They were active also against multidrug-resistant strains (Table 1).

Besides direct antimicrobial activity, we also tested and confirmed remarkable resistance modulatory activity of *A. katsumadai* extract, essential oil and post-distillation extract in *C. jejuni*, as well as in *S. aureus* (Table 2). *A. katsumadai* seed ethanol extract was confirmed for the first time to modulate antibiotic resistance in *Campylobacter* in our recent work (*12*). Here we comparatively investigated the resistance modulatory potential of additional *A. katsumadai* formulations, essential oil and post-distillation extract, and the activity of all three formulations in *S. aureus*, including MRSA strains (Tables 1 and 2). They were tested as modulators in concentrations half of their MIC on each individual strain of *C. jejuni* and *S. aureus* and in combination with antimicrobials, *i.e.* two antibiotics (ciprofloxacin and erythromycin), triclosan, bile salts and ethidium bromide. The cut-off value of signifi-

Table 1. Antimicrobial activity of *Alpinia katsumadai* plant formulations against antibiotic-sensitive and -resistant *Campylobacter jejuni* and *Staphylococcus aureus* strains, including multidrug-resistant ones

	MIC/(μg/mL)								
C. jejuni									
	PE	PDE	EO	ERY	CIP	TET	GEN	STR	CHL
NCTC 11168	250	250	1000	0.25 (S)	0.25 (S)	0.25 (S)	0.25 (S)	2 (S)	<2 (S)
ATCC 33560	125	250	1000	1 (S)	0.25 (S)	0.5 (S)	1 (S)	4 (R)	4 (S)
375/06	500	500	2000	128 (R)	32 (R)	0.25 (S)	0.5 (S)	<1 (S)	<2 (S)
573/03	250	250	4000	1 (S)	32 (R)	2 (S)	0.25 (S)	<1 (S)	<2 (S)
K49/4	250	1000	2000	0.5 (S)	1 (R)	0.25 (S)	0.25 (S)	2 (S)	<2 (S)
S. aureus									
	PE	PDE	EO	ERY	CIP	TET	GEN	PEN	MET
5.1	1000	1000	250	0.25 (S)	2 (R)	32 (R)	>16 (R)	>16	(R)
5.2	1000	1000	2000	>1024 (R)	0.25 (S)	<0.5 (S)	<0.25 (S)	>16	(S)
5.3	1000	1000	4000	0.5 (S)	0.5 (S)	<0.5 (S)	<0.25 (S)	<0.06	(S)
5.5	250	500	2000	0.5 (S)	64 (R)	>32 (R)	<0.25 (S)	0.008	(R)
5.6	1000	500	4000	0.25 (S)	0.5 (S)	8 (R)	<0.25 (S)	0.5	(S)

MIC=minimal inhibitory concentration; PE=*A. katsumadai* phenolic extract, PDE=*A. katsumadai* post-distillation extract, EO=*A. katsumadai* essential oil; ERY=erythromycin, CIP=ciprofloxacin, TET=tetracycline, GEN=gentamicin, STR=streptomycin, CHL=chloramphenicol, PEN=penicillin, MET=methicillin; R=resistant, S=sensitive. Resistant phenotypes were determined according to EUCAST breakpoints (*18*), where these are defined

cant resistance modulation was set at more than twofold decrease in the MIC of the tested antimicrobial and was referred to as modulation factor (MF, Table 2). When testing the post-distillation extract and essential oil as modulators at half of the MIC on *C. jejuni*, we found that the mean MF values were 34 and 78, respectively, considering combinations with all antimicrobials. Testing the phenolic extract, essential oil and post-distillation extract in *S. aureus* resulted in mean MFs of 63, 40 and 22, respectively. According to this, the essential oil was the best modulator in *C. jejuni*, while in *S. aureus* the most effective formulation was the phenolic extract, followed by

Table 2. Resistance modulatory activity of *Alpinia katsumadai* plant formulations in *Campylobacter jejuni* and *Staphylococcus aureus*

	Ciprofloxacin		Erythromycin		Triclosan		Bile salts		EtBr	
	MIC/(μg/mL)	MF	MIC/(μg/mL)	MF	MIC/(μg/mL)	MF	MIC/(mg/mL)	MF	MIC/(μg/mL)	MF
C. jejuni										
NCTC 11168	0.25		0.25		32		32		2	
+PE*	0.063	**4**	0.03	**8**	n.d.	n.d.	8	**4**	0.5	**4**
+PDE	0.25	1	0.063	**4**	8	**4**	0.5	**>64**	2	2
+EO	<0.125	**>2**	0.004	**>64**	<0.5	**>64**	<0.125	**>256**	<0.078	**>256**
375/06	32		128		8		>2		1	
+PE*	16	2	64	2	n.d.	n.d.	4	**4**	0.25	**4**
+PDE	16	2	n.d.	n.d.	4	2	0.5	**>4**	0.5	2
+EO	<0.5	**>64**	n.d.	n.d.	<0.25	**>32**	<0.016	**>128**	<0.03	**>32**
K49/4	1		0.5		32		128		1	
+PE*	0.125	**8**	0.125	**4**	n.d.	n.d.	16	**8**	0.125	**8**
+PDE	<0.016	**>64**	<0.125	**>4**	<0.5	**>64**	<2	**>64**	<0.02	**>64**
+EO	<0.016	**>64**	<0.125	**>4**	<0.5	**>64**	<2	**>64**	<0.02	**>64**
573/03	32		1		16		8		0.5	
+PE*	16	2	0.25	**4**	n.d.	n.d.	4	2	0.125	**4**
+PDE	<2	**>16**	<0.5	**>2**	<0.25	**>256**	<0.25	**>32**	0.5	1
+EO	<2	**>16**	<0.5	**>2**	<0.25	**>256**	<0.25	**>32**	<0.02	**>32**
S. aureus										
5.1	2		0.25		0.12		4		64	
+PE	<0.03	**>64**	<0.03	**>8**	<0.001	**>128**	<0.06	**>128**	16	**4**
+PDE	2	1	2	1	0.12	1	0. 26	**16**	32	2
+EO	1	2	1	2	>0.12	<1	1	**4**	8	**8**
5.2	0.25		>1024		0.03		>4		8	
+PE	0.25	1	>1024	n.d.	0.12	0.25	<0.03	**>256**	8	1
+PDE	0.06	**4**	>1024	n.d.	<0.001	**>32**	0.51	**>16**	4	2
+EO	1	0.25	1024	>1	<0.001	**>32**	0.26	**>32**	8	1
5.3	0.5		0.5		0.002		>4		2	
+PE	0.25	2	0.06	**8**	<0.0001	**>32**	<0.03	**>256**	<0.03	**>64**
+PDE	0.25	2	<0.008	**>64**	0.0001	**16**	<0.03	**>256**	<0.03	**>64**
+EO	0.25	2	0.125	**4**	0.0001	**16**	<0.03	**>256**	<0.03	**>64**
5.5	64		0.5		0.06		4		4	
+PE	32	2	0.25	2	0.06	1	2	2	2	2
+PDE	32	2	1	0.5	>0.06	<1	0.51	**8**	2	2
+EO	128	0.5	1	0.5	>0.06	<1	1	**4**	4	1
5.6	0.5		0.25		>1		>8		8	
+PE	0.125	**4**	0.125	2	<0.008	**>256**	<0.06	**>256**	2	**4**
+PDE	0.25	2	0.125	2	>1	n.d.	1	**>16**	4	2
+EO	0.008	**32**	<0.004	**>128**	<0.008	>256	1	**>16**	<0.06	**>128**

*The results were previously published with 0.25 MIC of modulators (*12*). 0.5 MIC=half of minimal inhibitory concentration, 0.25 MIC=quarter of minimal inhibitory concentration; n.d.=not determined; MF=modulation factor; PE=*A. katsumadai* phenolic extract, PDE=*A. katsumadai* post-distillation extract, EO=*A. katsumadai* essential oil; EtBr=ethidium bromide. Numbers in bold represent significant resistance modulatory activity (MF>2)

the essential oil and the post-distillation extract. However, these differences could not be confirmed as statistically significant in *C. jejuni* (p=0.071) nor in *S. aureus* (p=0.202). The mean modulation factors of all formulations in combination with individual antimicrobials ranged from 13 to 93, confirming the high modulation potential of *A. katsumadai* seed formulations in *C. jejuni*. The comparative analysis of modulatory activity of *A. katsumadai* formulations in *C. jejuni* confirmed them as equally efficient in combination with different antimicrobials, including bile salts (MF_{mean}>55, Table 2). This is important since the intestinal tract with the presence of bile salts is a natural environment and reservoir of *Campylobacter* sp., so resistance to bile salts is essential for *C. jejuni* survival and virulence potential. It was also shown that active efflux is one of the resistance mechanisms used by *C. jejuni* to resist the bactericidal effects of bile salts (*19,20*). With this work we confirmed *Alpinia* formulations to be very efficient in restoring *C. jejuni* sensitivity to bile salts (Table 2) and thus potentially influence its survival and infection capacity. On the other hand, in *S. aureus*, there were significant differences in modulation effects when formulations were combined with different antimicrobials (p=0.005). The strongest resistance modulation was achieved in combination with bile salts (MF_{mean}=101), followed by triclosan (MF_{mean}=57) and ethidium bromide (MF_{mean}=23). The weakest, yet still exceptional increase in sensitivity was obtained when plant formulations were used together with antibiotics, where mean modulation factor was 18 in the case of erythromycin and 10 in the case of ciprofloxacin. Phenolic extract and essential oil were able to restore resistance of *S. aureus* 5.1 against ciprofloxacin, while all of the tested plant formulations were able to reverse ciprofloxacin resistance in *C. jejuni* strain K49/4. Compared to other studies of resistance modulatory activity of rosemary extract, vine leaf extract and epigallocatechin gallate in *Campylobacter*, our plant formulations performed equally good or better, according to the modulation factors (*12,14*). This might also be due to the differences in concentrations of modulators used. In these previous studies the strain specificity of the resistance modulation had been observed and this phenomenon was to some degree observed also in our study, especially in *S. aureus*. Catechins and gallates had previously been investigated as resistance modulators in methicillin-resistant *S. aureus*. Among them, (–)-epicatechin gallates could significantly decrease the resistance to flucloxacillin, imipenem and meropenem (*21*).

There are several possible mechanisms of increasing susceptibility of bacteria to antibiotics and other antimicrobials. The most promising are focusing on increasing the antibiotic influx by destabilizing lipopolysaccharides in Gram-negative bacteria and increasing the membrane permeability, and blocking the efflux using efflux pump inhibitors (*6*).

In order to elucidate the mechanism of modulatory activity of *A. katsumadai* essential oil in *C. jejuni* and *S. aureus*, we have evaluated its potential to increase the accumulation of the common efflux pump substrate EtBr, which is an indicator of the efflux inhibition. The phenolic extract and post-distillation extract were excluded from the experiments because of the high autofluorescence. We compared the levels of EtBr accumulation in cultures treated with half and quarter MIC values of essential oil, relative to the untreated culture, to evaluate whether it can potentiate the intracellular EtBr accumulation. The known efflux pump inhibitor verapamil was included in the study as a positive reference. The results show significant (p<0.0001) increase in the EtBr accumulation in the presence of *A. katsumadai* essential oil, compared to untreated culture of *C. jejuni*, as well as *S. aureus* (Figs. 1 and 2). The accumulation of EtBr was 1.7-fold better in the presence of *A. katsumadai* essential oil in half of its MIC than in the presence of positive control, verapamil (Table 3). The accumulation of EtBr in the presence of

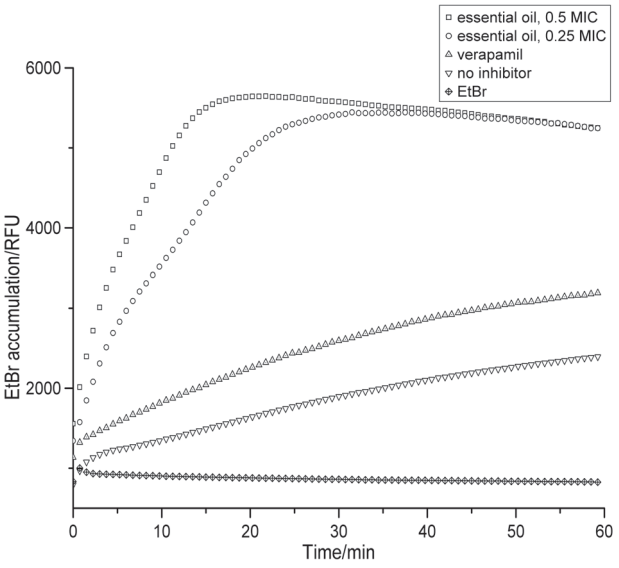

Fig 1. Ethidium bromide accumulation in *Campylobacter jejuni* NCTC 11168 in the presence of *Alpinia katsumadai* essential oil and verapamil. RFU=relative fluorescence units; 0.5 MIC=half of minimal inhibitory concentration, 0.25 MIC=quarter of minimal inhibitory concentration; EtBr=ethidium bromide

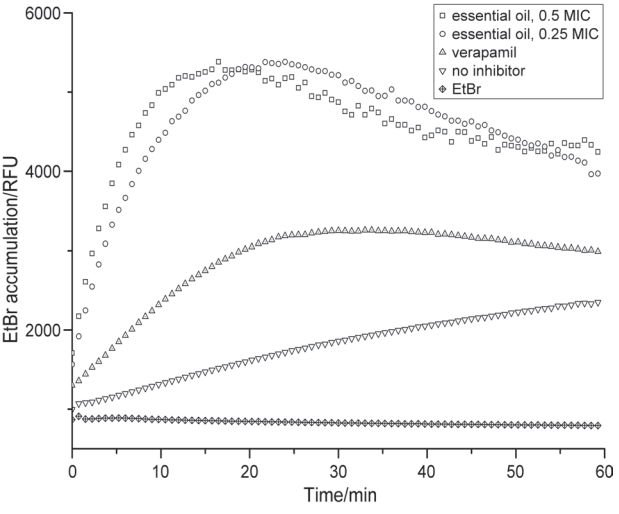

Fig. 2. Ethidium bromide accumulation in *Staphylococcus aureus* 5.3 in the presence of *Alpinia katsumadai* essential oil and verapamil. RFU=relative fluorescence units; 0.5 MIC=half of minimal inhibitory concentration, 0.25 MIC=quarter of minimal inhibitory concentration; EtBr=ethidium bromide

Table 3. Ethidium bromide accumulation in *Campylobacter jejuni* and *Staphylococcus aureus*

Plant formulation	EtBr accumulation*/RFU	
	C. jejuni	*S. aureus*
essential oil, 0.5 MIC	5303±39	4300±52
essential oil, 0.25 MIC	5298±31	4211±141
verapamil	3128±41	3045±35
no inhibitor	2335±39	2291±8
EtBr control	832±3	797±2
	N=9	*N*=9

*Ethidium bromide accumulation was calculated from the measurements during the last 10 minutes of each assay. The results are expressed as mean values±standard deviations. The differences between accumulation when essential oil or verapamil were added are statistically significant ($p<0.0001$), compared to the culture without added inhibitor. RFU=relative fluorescence units; 0.5 MIC=half of minimal inhibitory concentration, 0.25 MIC=quarter of minimal inhibitory concentration; EtBr=ethidium bromide

essential oil in half of MIC started to slowly decrease in *C. jejuni* after reaching the maximum accumulation after 15 min, whereas the accumulation in the presence of verapamil increased linearly and did not reach the plateau after 60 min (Fig. 1). Similar mean accumulation of EtBr in the presence of verapamil was achieved in *S. aureus*, but it reached the plateau after 25 min and then the accumulation started to decrease slowly. The same trend of EtBr accumulation as in *C. jejuni* in the presence of essential oil was observed in *S. aureus*, but the mean accumulation in the last 10 min was by 19 % lower than in *C. jejuni*. The mean EtBr accumulation values in the presence of essential oil in quarter of MIC were almost the same as in the presence of half of MIC (Table 3), only the time needed to reach the maximum accumulation was different (Figs. 1 and 2). Gradual decrease in EtBr accumulation after reaching the plateau might be due to the facilitation of EtBr influx by the essential oil and insufficient capacity to block the efflux.

Compounds from *A. katsumadai* had previously been confirmed to increase the EtBr accumulation in *Mycobacterium smegmatis* (*22*). Recently, several triterpenoids isolated from *Momordica balsamina* and coumarins from *Mesua ferrea* exhibited efflux-inhibitory activity in *S. aureus* (*23,24*); however, the activity was not directly comparable, due to the different experiment settings.

Since the phenolic compounds are often identified as main active biomolecules in plant formulations (*25*), we have determined the total phenolic content (TPC) of the phenolic extract and post-distillation extract (Table 3) in order to see whether the TPC correlates with their bioactivity. Essential oil was not expected to be rich in phenols and therefore it was not tested. The TPC of *A. katsumadai* phenolic extract was 24.7 μg/mL, which is lower than in its post-distillation extract (39.9 μg/mL). However, the antimicrobial activity of the phenolic extract was better than of the post-distillation extract in all bacterial targets (Table 1), which indicates that some valuable antimicrobially active compounds have been lost or deactivated during hydrodistillation. A similar result was observed in the analysis of resistance modulation. The phenolic extract had the strongest potential to decrease the resistance in *S. aureus*, followed by the essential oil and the post-distillation extract. In *C. jejuni*, *A. katsumadai* essential oil was the best modulator. Post-distillation extract was compared to the phenolic extract and the essential oil and it was concluded that it is the least effective modulator, although still showing very good resistance modulation in both, *S. aureus* as well as in *C. jejuni*. This indicates the loss of some bioactive compounds during the hydrodistillation of the extract. Since both, phenolic extract and essential oil, had valuable resistance modulatory activities, it is possible that different compounds, other than phenolics, are responsible for the activity.

Compared to other plant extracts, also from medicinal plants, formulations in our study had comparable or higher phenolic content (*25–27*). Plant extracts rich in phenolic compounds often possess good radical scavenging potential (*25*); therefore, the DPPH radical scavenging of the tested plant formulations was determined. As seen in Table 4, the post-distillation extract, which had a higher TPC, was also a more effective radical scavenger (IC_{50}=14.5 μg/mL), compared to the phenolic extract (IC_{50}=64.7 μg/mL).

Table 4. Total phenolic content (TPC) and DPPH radical scavenging potential of *Alpinia katsumadai* plant formulations

Plant formulation	TPC	DPPH
	mg of GAE per g of d.m.	IC_{50}/(μg/mL)
phenolic extract	24.7±0.2	64.7±6.5
post-distillation extract	39.9±0.03	14.5±1.5
essential oil	n.d.	DPPH inhibition <50 %

GAE=gallic acid equivalents; d.m.=dried material; n.d.=not determined

All of the tested formulations originate from a plant that is used traditionally as an anti-emetic remedy, culinary spice and to increase the appetite, as well as in animal feed to increase growth. They also contain compounds that are generally recognized as safe or are approved as food additives by the FDA (*8*), which is why they have a good potential to be exploited as natural antimicrobial and antioxidative preservatives in food products. However, more research needs to be done to evaluate their sensorial impacts, activity in the real food model (*28*), and to prove that they do not cause any negative side-effects, like allergic reactions and irritations (*8*).

Conclusions

Alpinia katsumadai seed phenolic extract, essential oil and post-distillation extract showed moderate antimicrobial activity against *Campylobacter jejuni*, while they were less efficient against *Staphylococcus aureus*. However, all tested plant formulations were confirmed as good modulators of *C. jejuni* and *S. aureus* resistance against vari-

ous antimicrobials, including antibiotics and bile salts. Modulatory activity of *A. katsumadai* essential oil was confirmed to be at least partly due to the efflux pump inhibition, when tested with ethidium bromide accumulation assay. All tested plant formulations have a potential to be used as natural preservatives and functional additives in food products.

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References

1. EFSA, The European Union report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011, *EFSA J. 11* (2013) 3129.
2. EFSA, The European union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2011, *EFSA J. 11* (2013) 3196.
3. A. Mavri, M. Kurinčič, S. Smole Možina, The prevalence of antibiotic and biocide resistance among *Campylobacter coli* and *Campylobacter jejuni* from different sources, *Food Technol. Biotechnol. 50* (2012) 371–376.
4. S. Smole Možina, M. Kurinčič, A. Klančnik, A. Mavri, *Campylobacter* and its multi-resistance in the food chain, *Trends Food Sci. Tech. 22* (2011) 91–98.
5. D. Savoia, Plant-derived antimicrobial compounds: Alternatives to antibiotics, *Future Microbiol. 7* (2012) 979–990.
6. J. Bolla, S. Alibert-Franco, J. Handzlik, J. Chevalier, A. Mahamoud, G. Boyer *et al.*, Strategies for bypassing the membrane barrier in multidrug resistant Gram-negative bacteria, *FEBS Lett. 585* (2011) 1682–1690.
7. M.M. Cowan, Plant products as antimicrobial agents, *Clin. Microbiol. Rev. 12* (1999) 564–582.
8. S. Burt, Essential oils: Their antibacterial properties and potential applications in foods – A review, *Int. J. Food Microbiol. 94* (2004) 223–253.
9. P.S. Negi, Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application, *Int. J. Food Microbiol. 156* (2012) 7–17.
10. A. Klančnik, S. Smole Možina, Q. Zhang, Anti-*Campylobacter* activities and resistance mechanisms of natural phenolic compounds in *Campylobacter, PLoS ONE, 7* (2012) e51800 (doi: 10.1371/journal.pone.0051800).
11. S.A. Zhang, Chinese herbal medicine feed additive for facilitating the rapid growth of domestic animals, Faming Zhuanli Shenqing. Cited in *CAPLUS CN 101683115 A 20100331* (2010).
12. A. Klančnik, B. Gröblacher, J. Kovač, F. Bucar, S. Smole Možina, Anti-*Campylobacter* and resistance-modifying activity of *Alpinia katsumadai* seed extracts, *J. Appl. Microbiol. 113* (2012) 1249–1962.
13. A. Moure, J.M. Cruz, D. Franco, J.M. Domínguez, J. Sineiro, H. Domínguez *et al.*, Natural antioxidants from residual sources, *Food Chem. 72* (2001) 145–171.
14. M. Kurinčič, A. Klančnik, S. Smole Možina, Epigallocatechin gallate as a modulator of *Campylobacter* resistance to macrolide antibiotics, *Int. J. Antimicrob. Agents, 40* (2012) 467–471.
15. G. Rieger, M. Müller, H. Guttenberger, F. Bucar, Influence of altitudinal variation on the content of phenolic compounds in wild populations of *Calluna vulgaris*, *Sambucus nigra*, and *Vaccinium myrtillus*, *J. Agric. Food Chem. 56* (2008) 9080–9086.
16. L. Fukumoto, G. Mazza, Assessing antioxidant and prooxidant activities of phenolic compounds, *J. Agric. Food Chem. 48* (2000) 3597–3604.
17. J. Lin, L.O. Michel, Q. Zhang, CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*, *Antimicrob. Agents Chemother. 46* (2002) 2124–2131.
18. Breakpoint Tables for Interpretation of MICs and Zone Diameters, v. 3.1, The European Committee on Antimicrobial Susceptibility Testing (EUCAST), ESCMID, Basel, Switzerland (2013) (http://www.eucast.org).
19. J. Lin, A. Martinez, Effect of efflux pump inhibitors on bile resistance and *in vivo* colonization of *Campylobacter jejuni*, *J. Antimicrob. Chemother. 58* (2006) 966–972.
20. A. Mavri, S. Smole Možina, Resistance to bile salt and sodium deoxycholate in macrolide- and fluoroquinolone-susceptible and resistant *Campylobacter jejuni* and *Campylobacter coli* strain, *Microb. Drug Resist. 19* (2013) 168–174.
21. P.D. Stapleton, S. Shah, J.C. Anderson, Y. Hara, J.M.T. Hamilton-Miller, P.W. Taylor, Modulation of β-lactam resistance in *Staphylococcus aureus* by catechins and gallates, *Int. J. Antimicrob. Agents, 23* (2004) 462–467.
22. B. Gröblacher, O. Kunert, F. Bucar, Compounds of *Alpinia katsumadai* as potential efflux inhibitors in *Mycobacterium smegmatis*, *Bioorg. Med. Chem. 20* (2012) 2701–2706.
23. C. Ramalhete, G. Spengler, A. Martins, M. Martins, M. Viveiros, S. Mulhovo *et al.*, Inhibition of efflux pumps in methicillin-resistant *Staphylococcus aureus* and *Enterococcus faecalis* resistant strains by triterpenoids from *Momordica balsamina*, *Int. J. Antimicrob. Agents, 37* (2011) 70–74.
24. S.K. Roy, N. Kumari, S. Pahwa, U.C. Agrahari, K.K. Bhutani, S.M. Jachak, H. Nandanwar, NorA efflux pump inhibitory activity of coumarins from *Mesua ferrea*, *Fitoterapia, 90* (2013) 140–150.
25. H. Li, C. Wong, K. Cheng, F. Chen, Antioxidant properties in vitro and total phenolic contents in methanol extracts from medicinal plants, *LWT-Food Sci. Technol. 41* (2008) 385–390.
26. V. Katalinić, S. Smole Možina, D. Skroza, I. Generalić, H. Abramovič, M. Miloš et al., Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 *Vitis vinifera* varieties grown in Dalmatia (Croatia), *Food Chem. 119* (2010) 715–723.
27. H. Abramovič, P. Terpinc, L. Generalić, D. Skroza, A. Klančnik, V. Katalinić, S. Smole Možina, Antioxidant and antimicrobial activity of extracts obtained from rosemary (*Rosmarinus officinalis*) and vine (*Vitis vinifera*) leaves, *Croat. J. Food Sci. Technol. 4* (2012) 1–8.
28. A. Klančnik, S. Piskernik, F. Bucar, D. Vučković, S. Smole Možina, B. Jeršek, Reduction of microbiological risk in minced meat by combination of natural antimicrobials, *J. Sci. Food Agric.* (in press; doi: 10.1002/jsfa.6621).