Research Article

Antimicrobial activities of caffeic acid phenethyl ester

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Abstract

Caffeic acid phenethyl ester (CAPE) is considered as one of the most active components of Propolis extract (PE), a natural product obtained from beehives. PE comprises a complex of chemicals and has been found to have various biological activities. The aim of the present study is to assess the antibacterial activities CAPE has against various gram positive [gram (+)] and gram negative [gram (-)] bacteria and try to elucidate its mechanism of action. Bacteria were grown in the presence of various doses of CAPE and examined at different periods of time for their growth, both by absorbance (OD) measurement and colony assay. The results show that CAPE significantly inhibited the growth of most examined gram (+) bacteria while having only a slight inhibitory effect on most tested gram (-) bacteria. Our results also show that continuous treatment of gram (+) bacteria with CAPE for at least 6h caused irreversible inhibition of the bacterial growth (bacteriocidic effect); however, treatment for shorter periods of time caused only a stopping of bacterial growth (bacteriostatic effect). It seems that these effects were caused, at least partially, as a result of disruptions of the treated bacterial outer and plasma membranes. There is no significant synergistic effect between CAPE and ampicillin, although an additive effect has been found.

Introduction

The use of natural products as medicines for the treatment of various diseases has had a long history. Traditional healers, throughout history, have acquired detailed knowledge regarding the use of medicinal plants (Abel & Busia 2005).

It has been estimated that at least 25% of the active compounds present in currently prescribed synthetic drugs were first identified in natural sources. In this regard, the investigation of natural products and their potential therapeutic properties is essential (Halberstein 2005). Increasing efforts are currently being devoted towards novel applications for natural products and their derivatives for treating human diseases.

PE is obtained from beehives and based on resins produced by honeybees from certain trees and plants, bee's wax and their secretions (Onlen *et al.* 2007). Scientific research has revealed various biological activities of PE such as antibacterial (Huang *et al.* 2006, Velazquez *et al.* 2007), antiviral (Drago *et al.* 2007, Huleihel & Eshanu 2002, Orsolić & Basić 2005, Salomão *et al.* 2008, Viuda-Martos *et al.* 2008, Yao *et al.* 2004), antioxidant (Ahn *et al.* 2007, Kerem *et al.* 2006), antifungal (Quiroga *et al.* 2006, Silici & Koc 2006), antiinflamatory (Harris *et al.* 2006, Wu *et al.* 2006), antitumor (Akao *et al.* 2003) and other activities (de Rezende *et al.* 2008, Kosalec *et al.* 2005, Viuda-Martos *et al.* 2008). During the last few years, PE has also been used in the food industry as an additive as well as in beverages and nutritional supplements to enhance health and prevent diseases (Velazquez *et al.* 2007). In addition, previous studies have reported antibacterial activity of PE mainly against gram (+) but not gram (-) bacteria (Velazquez *et al.* 2007).

The chemical composition of PE is complex and has not been completely elucidated. Mainly, differences in the composition of PE have been found to depend on its origin but also among topical samples, depending on the local flora at the site of collection

(Salomão *et al.* 2008). Nevertheless, it is known that the most important group of compounds - in terms of amount and biochemical activity - is the flavonoids, which are thought to play a significant role in its biological activities (Viuda-Martos *et al.* 2008).

The most active and studied component of PE is caffeic acid phenethyl ester (CAPE), which is known to be a potent inhibitor of activation of NF-kB (Natarajan *et al.* 1996). A major part of CAPE bioactivities is thought to be related to NF-kB inhibition (Marquez *et al.* 2004, Song *et al.* 2002, Yang *et al.* 2005). CAPE has also been reported to be a potent anti -inflammatory and antioxidant agent and possess several antiviral, antibacterial and antifungal properties (Alici *et al.* 2015, Ilhan *et al.* 1999, Tolba *et al.* 2013, Velazquez *et al.* 2007).

In this work, we tested the inhibitory effects of an aqueous extract of CAPE *in vitro* against different gram (+) and gram (-) bacteria, trying to elucidate its antibacterial mechanism of action.

Materials and Methods

CAPE

CAPE was purchased as a powder from Sigma-Aldrich Corporation, USA. A stock solution of this product was prepared by dissolving it in dimethyl sulfoxide (DMSO) and then making the appropriate concentrations for examining its activity by dilution with bacterial growth medium (LB medium).

Bacteria

In the present study, the following gram (-) bacteria were used: *Escherichia (E.) coli, Serratia (S.) marcescens, Pseudomonas (P.) aeruginosa, Haemophilus (H.) influenza, Pseudomonas, Shegella, Salmonella (S.) entenidis, Neisseria* and *Klebsiella*. The gram (+) bacteria used were the following: *Staphylococcus (Staph.) aureus, Micrococcus, Streptococcus (S.) olysgalactiae, Streptococcus (S.) mitis, Bacillus (B.) subtilis, B. cereus, B. megaterium* and *B. thuringiensis.*

All the above bacterial strains were supplied by Dr Valentina Pavlov from the microbiology department in our institute. All bacteria were grown on Nutrient Agar (Difco) at 37 °C.

Measurement of bacterial amount

The amounts of bacteria were evaluated by 2 different methods. Their optical density (OD) was examined by spectrophotometer at a wavelength of 620nm. This method gives an evaluation of the live and dead bacteria. The other method involved counting bacterial colonies, by plating raising dilutions of each bacteria on LB agar plates for 24h at 37°C and counting the number of the obtained colonies. This method only gives the number of live bacteria.

Antibacterial activity

In order to examine the antibacterial activity of the tested products, we grew the appropriate bacteria overnight and diluted them with LB medium to obtain about 10^4 colony-forming units (cfu)/mL, as estimated by optical density (OD_{630nm}) and plating on agar examinations. These suspensions were used as inoculates in the antimicrobial activity tests. Various concentrations of the tested material (CAPE) were added to the appropriate bacterial suspension with a final volume of 10ml. Following this, treated and untreated bacterial suspensions were incubated at 37°C in a shaking incubator. Thereafter, the following steps were undertaken at different time-points post-treatment: (a) the OD_{630nm} of these samples was measured and corrected by subtraction of the OD_{630nm} of PE or CAPE alone in sterile LB; (b) 0.5ml from each suspension was centrifuged at 2000*g* for 15 minutes, washed twice with sterile distilled water (ddH_2O), resuspended in 0.2ml LB, plated on agar plates, incubated at 37°C for 24h and the number of colonies was counted. The lowest concentrate of CAPE that prevented bacterial growth was considered to be the minimum inhibitory concentration (MIC).

Table 1. Minimum inhibitory concentration (MIC) of CAPE on different species of gram (+) and gram (-) bacteria.

Bacterial species	Gram (+/-)	MIC (mM)
S. aureus	+	48±2.5
S. olysgalactiae	+	48±3.1
S. mitis	+	55±2.8
B. thuringiensi	+	250±10.5
(B.) subtilis	+	70±3.2
B. cereus	+	96±2.8
B. megaterium	+	50±2.6
Micrococcus	+	55±2.6
E. coli	-	254±15.1
S. marcescens	-	400±23.7
P. aeruginosa	-	500±25.1
H. influenza	-	200±12.1
Pseudomonas	-	400±26.1
S. entenidis	-	60±2.70
Neisseria	-	500±24.3
Klebsiella	-	50±3.10

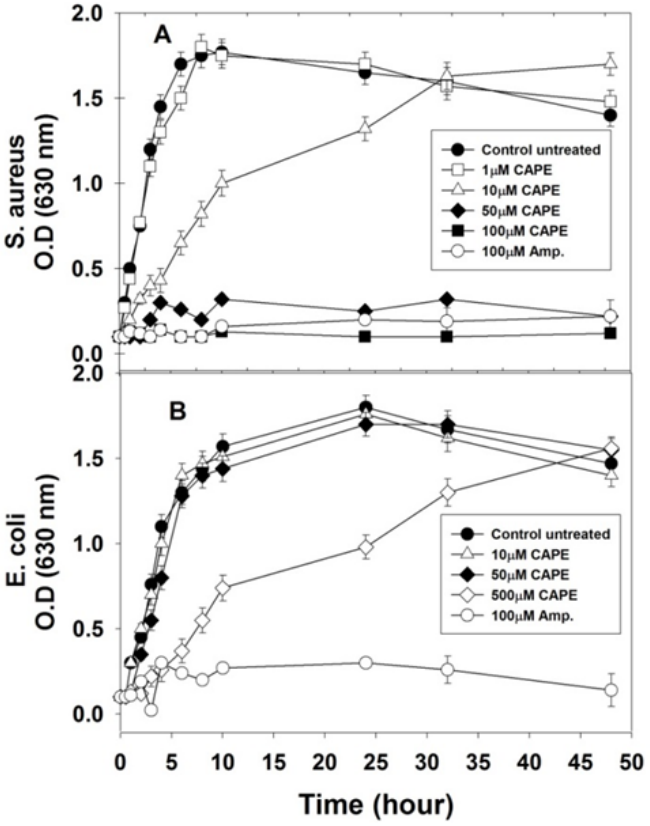

Figure 1. Effect of CAPE on Gram (+) and Gram (-) bacteria. Various concentrations of CAPE were added to 0.1 OD suspensions of *S. aureus* (A) and *E. coli* (B) and incubated at 37°C with shaking. In parallel, the appropriate suspensions were treated with 100 μg/ml of ampicillin as a positive control. At different time-points post-treatment, the amount of the bacteria was determined by measuring their OD_{630nm}. The results are means ± SD (n=5).

Combined antibacterial activity with ampicillin

In the present study we examined possible synergistic or additive activity between CAPE and ampicillin, as a representative antibiotic against different gram (+) and gram (-) bacteria, according to Bonapace *et al.* (2002). Briefly, overnight working bacterial broth cultures were diluted in LB medium together with 10% serial dilutions of CAPE and different ampicillin combinations, so as to give about 10^4 cfu/mL. Samples without treatment were used as controls. The cultures were incubated for 24h at a 37°C incubator with shaking. MICs were determined for each tested bacterial strain and the interaction between CAPE and ampicillin was calculated by the fractional inhibitory concentration (FIC) index of the combination. The FIC for each antibacterial agent was calculated as the ratio of the MIC of the agent in combination (with the other tested agent) to its MIC alone. In addition, the FIC index equals the FIC of CAPE plus the FIC of ampicillin. The interaction between the two agents was considered as synergy when the FIC index was ≤ 0.5, additivity/no interaction when the FIC index was between 0.5 to 4 and antagonism when the FIC index >4.

Gentian violet uptake by gram (+) and gram (-) bacteria

CAPE treated bacteria (1 ml) were centrifuged at 2000 rpm for 15 minutes at room temperature, washed twice with ddH_2O, resuspended in 1 ml of ddH_2O containing gentian violet (10μg/ml) and incubated for 10 min at 37°C under vigorous shaking. The cells were removed by centrifugation and the amount of gentian violet remaining in the supernatant was measured at 590 nm in a spectrophotometer.

Loss of 260nm absorbing material

The release of UV-absorbing materials was measured by a UV–VIS spectrophotometer. Overnight cultures of gram (+) and gram (-) bacteria were adjusted to an OD600 of 1.0. Bacterial cells were centrifuged at 2000 rpm for 15 minutes, the supernatant was discarded, and the resulting pellet was washed twice and then resuspended in 1 ml of PBS (pH 7.4). Different concentrations of CAPE were added to the bacterial suspensions. All samples were incubated at 37°C for different periods of time (2 or 6h) and centrifuged at 13,000rpm for 15 min, before the OD260 value of the supernatant was measured.

Fourier-transform infrared (FTIR) microscopy

The alteration in structural features of gram (+) and gram (-) bacteria at the molecular level upon treatment with PE or CAPE was analyzed by FTIR spectroscopy. Different doses of CAPE were added to the cell suspensions of the examined bacteria, from overnight cultures with an OD600 of 1.0. Treatment was performed for 6h at 37°C. The cells were then pelleted by centrifugation at 3000 rpm for 5 min and washed three times with distilled water. The bacterial pellet was resuspended in 100μl of distilled water and 1μl of the obtained suspension was placed on a certain area on a zinc sellenide crystal, air dried for 15min at room temperature (or for 5 min by air drying in a laminar flow) and examined by FTIR microscopy.

FTIR measurements were performed in the transmission mode with a liquid-nitrogen-cooled MCT detector of the FTIR microscope (Bruker IRScope II) coupled to an FTIR spectrometer (BRUKER EQUINOX model 55/S, OPUS software). The spectra were obtained in the wave number range of 600‑4000 cm-1. Spectral resolution was set at 4 cm-1. Baseline correction by the rubber band method and vector normalization was obtained for all the spectra by OPUS soft-

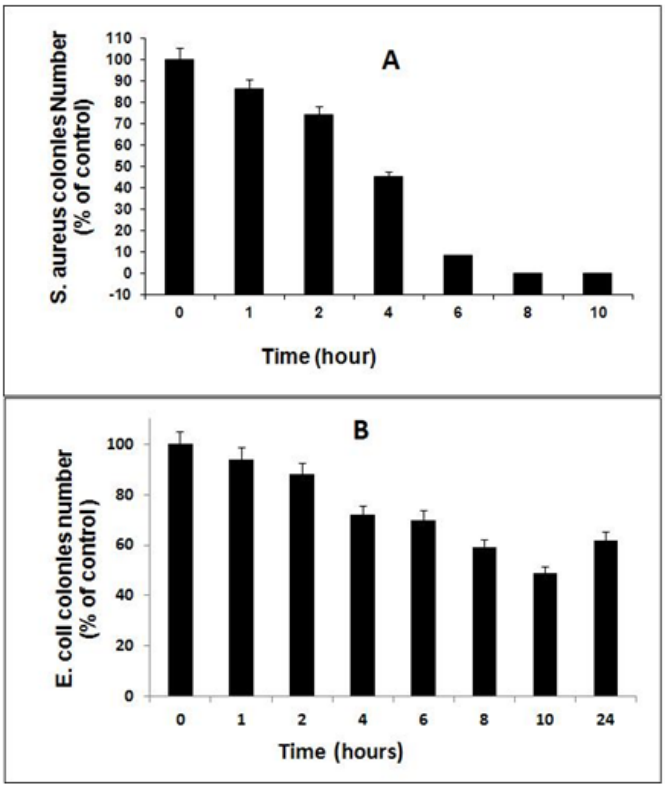

Figure 2. Effect of CAPE on bacteria colony growth. Suspensions (0.1 OD) of *S. aureus* (A) and *E. coli* (B) were treated with 50mM of CAPE and incubated at 37°C with shaking. At different time-points post-treatment, the amount of the living bacteria was determined by counting bacterial colonies. The results are means ± SD (n=5).

ware. Peak positions were determined by means of a second derivation method by OPUS software.

Results

Effect of CAPE on Gram (+) and Gram (-) bacteria

To determine the antibacterial activity of CAPE, we evaluated its effect on Gram (+) (*S. aureus, Micrococcus, S. olysgalactiae, S. mitis, B. subtilis, B. cereus, B. megaterium* and *B. thuringiensis*) and Gram (-) (*E. coli, S. marcescens, P. aeruginosa, H. influenza, Pseudomonas, Shegella, S. entenidis, Neisseria* and *Klebsiella*) bacteria.

Various concentrations of the tested material (CAPE) were added to the appropriate bacterial suspension (0.1 OD) and incubated at 37°C with shaking. At various time-points post-treatment the amount of the bacteria was determined by both measuring their OD_{630nm} and counting growing colonies.

CAPE showed strong antibacterial activity against most of the gram (+) bacteria examined, in a concentration dependent manner (Table 1 and Figure 1A, C), while it only had a slight and weak antibacterial activity against most of the gram (-) bacteria examined (Table 1 and Figure 1B, D). However, CAPE showed weak antibacterial activity against the gram (+) bacteria *B. Thuringiensi* and a strong activity against the gram (-) bacteria *S. Entenidis* and *Klebsiella* (Table 1).

It can also be seen that continuous treatment with 50mM of CAPE caused a complete inhibition of all gram (+) bacterial growth up to the end of the experiment (48h post-treatment), while continuous treatment with lower doses (10mM of CAPE) caused a slower growth of gram (+) bacteria compared to the untreated control bacteria, as determined by measuring their OD_{630nm} (Figure 1A, C). However, continuous treatment with even 500mM of CAPE only caused a reduction in the growth rate of all gram (-) bacteria examined, as can be seen in the representative results in Figure 1B and D.

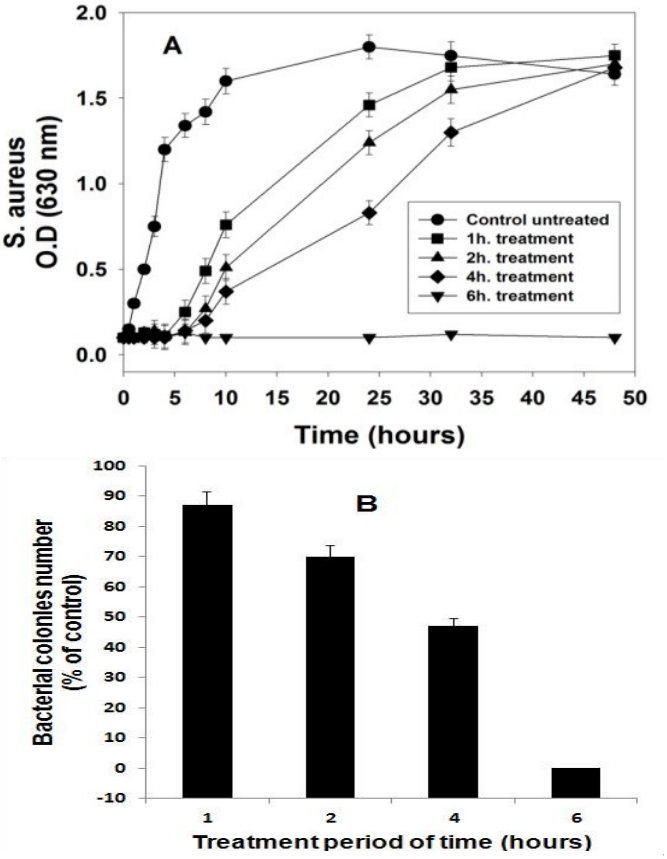

Figure 3. Effect of CAPE treatment termination on bacterial growth. Suspensions (0.1 OD) of *S. aureus* were treated with 50 mM CAPE and at different time-points treatment was terminated by exchanging the bacterial culture medium with a fresh medium free of the examined products. These bacterial cultures were incubated at 37°C with shaking and their growth was examined at different time-points post-treatment by measuring their absorbance (OD) (A) and by counting growing colonies (colony assay) (B). The results are means ± SD (n=5).

When the amount of living bacteria was determined by counting bacterial colonies, at different time-points post-treatment with 50mM of CAPE, it was found that treatment for up to 4h caused a significant decrease in the number of the gram (+) bacteria colonies, compared to the untreated control (Figure 2A). This result is mainly attributed to the increase in the number of the control bacteria, while the number of the treated bacteria did not change significantly over this period of time. In addition, the size of the treated bacterial colonies was significantly smaller than that of the controls. However, treatment for 6h or more caused complete death of all bacteria treated (Figure 2A). Treatment of the gram (-) bacteria with 500mM of CAPE reduced the number of bacterial colonies as a function of treatment time (Figure 2B).

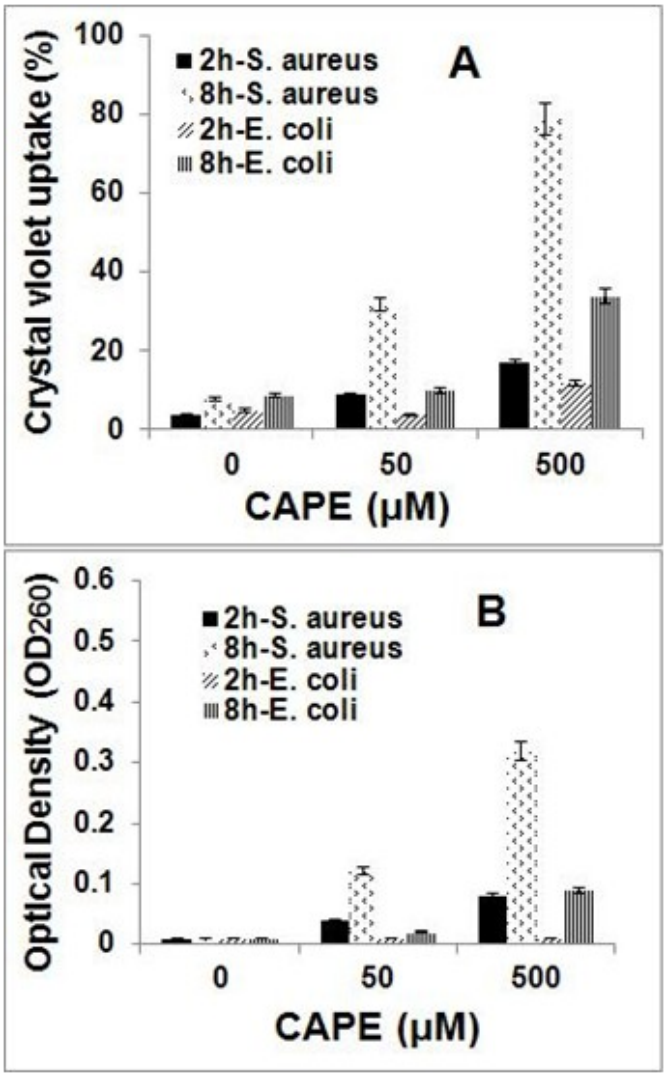

Figure 4. Effect of CAPE treatment on gentian violet uptake and on 260nm absorbing material loss by gram (+) and gram (-) bacteria. 1ml suspensions (0.5 OD) of *S. aureus* and *E. coli* were treated with different doses of CAPE for different periods of time (2 or 8h), then: (A) the bacteria were centrifuged at 2000 g for 15 minutes, bacterial pellets were washed twice with ddH2O, resuspended in 1ml of ddH2O containing gentian violet (10μg/ml) and incubated for 10 min at 37°C under vigorous shaking. The bacteria were removed by centrifugation and the amount of gentian violet remaining in the supernatant was measured at 590 nm in a spectrophotometer; (B) the bacteria were pelleted as above and the OD260 value of the supernatant was measured. The results are means ± SD (n=5).

Effect of CAPE treatment termination on bacterial growth

Trying to examine the antibacterial mechanism of CAPE, different gram (+) bacteria were treated with 50 mM CAPE, and at different time-points the treatment was terminated by exchanging the bacterial culture medium with a fresh medium, free of the examined products. These bacterial cultures were incubated at 37°C in a shaking incubator and their growth was examined at different time-points. The results obtained, either by OD measurement (Figure 3A and B) or colonies counting (Figure 3C) showed that continuous treatment of the bacterial culture with PE or CAPE for up to 4h only caused a delay of about 5h in the growth of the bacteria, which was followed by a moderate growth compared to the untreated control cultures. However, continuous treatment for 6h caused complete death of the bacteria without any recovery, even 2 days after the termination of the treatment.

Effect of CAPE treatment on bacterial outer membrane (OM) permeability

The effect of CAPE treatment on OM permeability of gram (+) and gram (-) bacteria to crystal violet was examined. It is known that gentian violet poorly penetrates the intact bacterial OM (Devi *et al.* 2010); therefore, it is possible to examine the effect of CAPE treatment on OM permeability by examining the effect on gentian violet uptake by the treated bacteria. Different gram (+) and gram (-) bacteria were treated with various doses of CAPE for 2 or 8h; the bacteria were the centrifuged, resuspended in 1 ml of ddH_2O containing gentian violet (10μg/ml) and incubated for 10 min at 37°C, under vigorous shaking as described in the "Materials and Methods" section. The gentian violet uptake was determined by measuring the remaining amount in the supernatant. The gentian violet uptake of the control untreated gram (+) and gram (-) bacteria was 4-10% of the input; however, it increased dramatically in gram (+) bacteria in a dose dependent mater, reaching over 95% uptake at the higher doses after 8h of treatment with CAPE (Figure 4A). Also, in the case of gram (-) bacteria there was a gradual and significant increase in the gentian violet uptake at the higher doses of CAPE (Figure 4A), although this increase was profoundly lower compared to the gram (+) bacteria. It

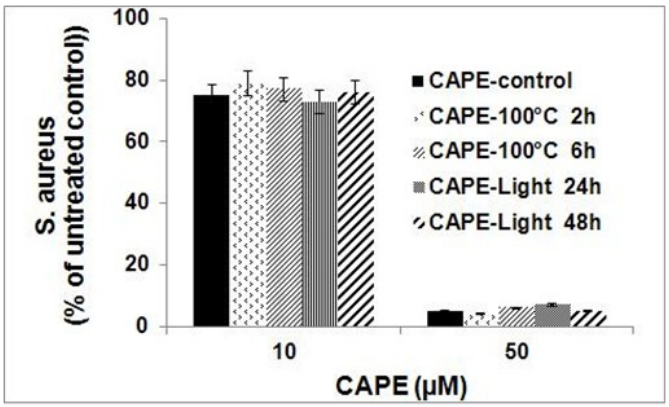

Figure 5. Effect of heat and light on CAPE antibacterial activities. Different doses of CAPE were incubated at 100C° or continuously exposed to a 100w lamp light for different periods of time. They were then added to 0.1 OD suspensions of *S. aureus*, incubated at 37°C with shaking and bacterial growth was examined at different time-points post-treatment by measuring their absorbance (OD). The results are means ± SD (n=5).

should also be noted that a short treatment of 2h with CAPE caused a moderate increase in the gentian violet uptake in gram (+) and gram (-) bacteria.

Effect of CAPE on release of 260nm absorbing materials from gram (+) and gram (-) bacteria

Different gram (+) and gram (-) bacteria (1 OD) were treated with various amounts of CAPE for 2 or 8h. The bacteria were then centrifuged and the OD260 values of the supernatants were measured. It is known that measuring the release of UV-absorbing materials is an index of cell lysis (Zhou *et al.* 2008). Our results showed that an 8h treatment of gram (+) bacteria with CAPE caused a significant increase in the OD (from 0.01 up to 0.3) while it resulted in a significantly lower increase in the case of gram (-) bacteria (from 0.01 up to 0.09) (Figure 4B).

Effect of heat treatment or light exposure on CAPE antibacterial activity

PE is rich with aromatic and flavonoid components which are, at least in part, sensitive to photo-oxidation that might affect their antibacterial activity. Two main theories have been put forward to explain the antibacterial capacity of honey and probably of PE (Viuda-Martos *et al.* 2008). The first one assumes that it is due to the action of hydrogen peroxide that is produced by glucose oxidase in the presence of light and heat (Dustmann 1979), while the other one states that it is a nonperoxide activity that is independent of light and heat (Roth *et al.* 1986).

In this regard, we sought to examine the effect of high temperatures and light exposure on the antibacterial activity of CAPE. Different doses of CAPE were exposed to a high temperature or light for different periods of time and their antibacterial activity against gram (+) bacteria was examined. The results showed that neither incubation of CAPE in 100°C for 2 or 6h nor exposure to light for 24 or 48h had an effect on its antibacterial activity (Figure 5).

Combined antibacterial activity with ampicillin

We examined possible synergistic or additive activity between CAPE and ampicillin, as a representative antibiotic against different gram (+) and gram (-) bacteria. Our results, presented in Table 2, show that the FIC indices of CAPE plus ampicillin against all examined bacteria were above 0.5. Therefore, it seems that these combinations had only additive activity against the different tested bacteria without synergistic activity.

Spectral changes in gram (+) and gram (-) bacteria treated with CAPE

FTIR spectroscopy can be used to detect and monitor characteristic changes in molecular compositions and structures of living cells, providing a wealth of qualitative and quantitative information about a given sample. The infrared spectrum of any compound is known to give a unique "finger print" (Naumann *et al.* 1991). In addition, the large information already known about spectral peaks obtained from FTIR spectra of living cells (Diem *et al.* 1999) make FTIR spectroscopy an attractive technique for detection and identification of pathogens. This technique has been previously used for the detection and characterization of cancer cells (Erukhimovitch *et al.* 2002), cells infected with viruses

Table 2. Minimum inhibitory concentration (MIC), fractional inhibitory concentration (FIC) and FIC index of CAPE and ampicillin against different gram (+) and gram (-) bacteria.

Bacteria	MIC			FIC		FIC index
	CAPE (µM)	Amp (µg/ml)	CAPE + Amp	CAPE	Amp	
S. aureus	48	64	16 + 24	0.33	0.37	0.70
B. cereus	96	64	30 + 32	0.31	0.50	0.81
E. coli	254	48	96 + 30	0.38	0.62	1.00
Neisseria	500	96	180 + 50	0.36	0.52	0.88

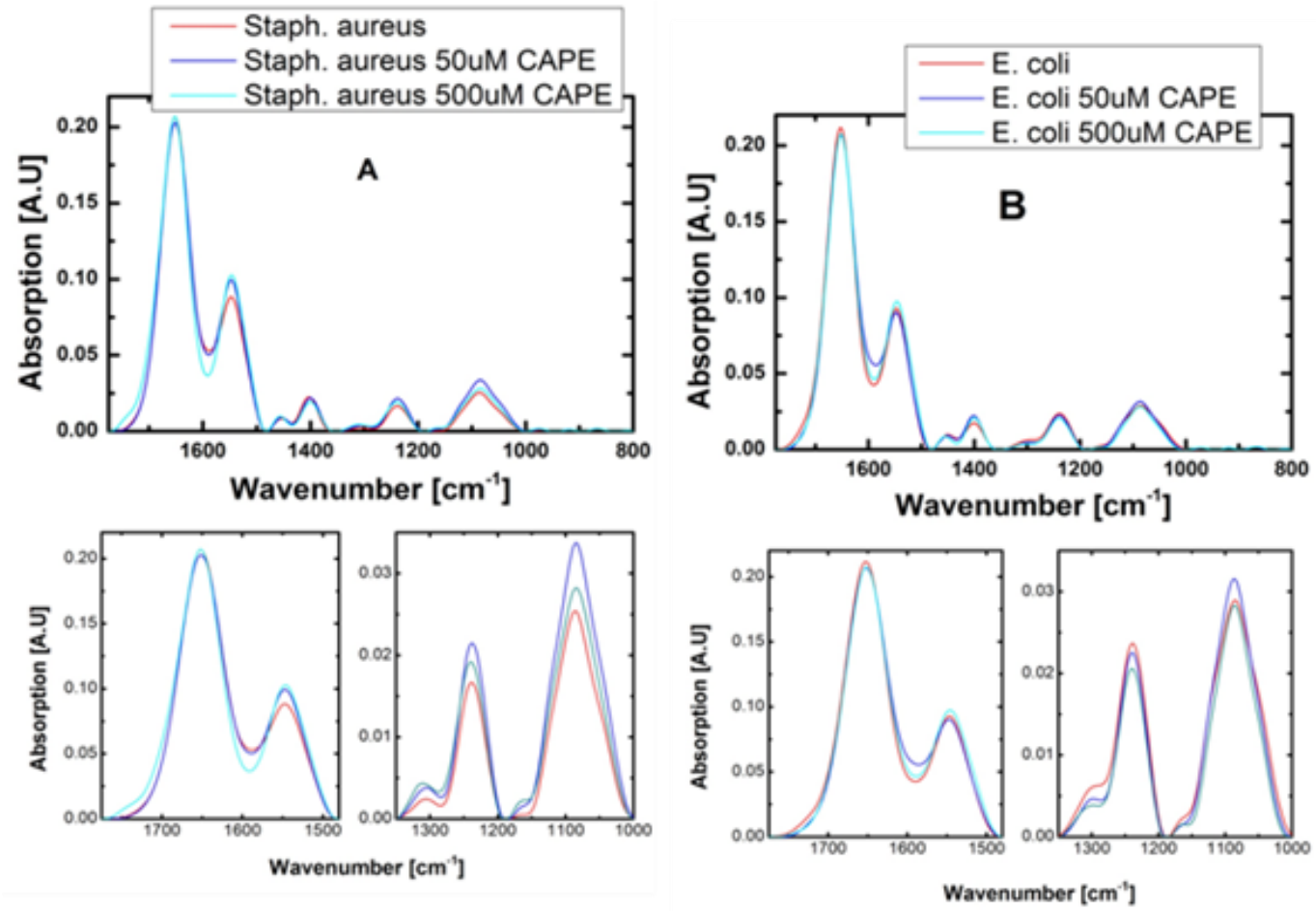

Figure 6. FTIR spectroscopy of gram (+) and gram (-) bacteria treated with CAPE. *S. aureus* and *E. coli* were treated with different doses of CAPE for 6h and examined by FTIR spectroscopy.

(Salman *et al.* 2002) and microorganisms (Erukhimovitch *et al.* 2002, Maquelin *et al.* 2003, Mariey *et al.* 2001).

In the present study, gram (+) and gram (-) bacteria were treated with different doses of PE for 6h and examined by FTIR spectroscopy. Significant spectral changes were observed in the frequency between 1700 and 1000cm^{-1} in CAPE treated samples of *S. aureus* (Figure 6A) compared to the slight spectral changes in the treated *E. coli* (Figure 6B), which may indicate an apparent deformation in the treated cells. These spectral changes were associated with changes in different components of the treated bacteria, such as proteins, polysaccharides, nucleic acids and lipids. There is a significant increase in frequency at 1100 and 1250 cm^{-1} of the CAPE-treated bacteria, which may indicate an alteration in polysaccharide and phosphate components. These differences may reflect changes in the content or distribution of the above components and possibly others.

Discussion

In the present study we examined the antibacterial activity of CAPE (one of the active components of PE) against various gram (+) and gram (-) bacteria. CAPE showed potent and impressive antibacterial activity against most tested gram (+) bacteria, while it only had moderate and partial activity against gram (-) bacteria. This observation is in complete agreement with previous studies which have shown that gram (+) bacteria are more susceptible to the antibacterial effect of PE or CAPE than gram (-) bacteria (Drago *et al.* 2007, Velazquez *et al.* 2007). In fact, various kinds of PE have been found to have multiple biological effects such as antiviral (Drago *et al.* 2007, Huleihel & Eshanu 2002, Orsolic & Basic 2005, Salomão *et al.* 2008, Viuda-Martos *et al.* 2008, Yao *et al.* 2004), antioxidant (Ahn *et al.* 2007, Kerem *et al.* 2006), antifungal (Quiroga *et al.* 2006, Silici & Koc 2006) and antibacterial (Huang *et al.* 2006, Velazquez *et al.* 2007) activities, mainly due to the presence of phenolic compounds and flavonoids (Viuda-Martos *et al.* 2008). Flavonoids, cin-

namic acids and their ester derivatives have been found to be the most abundant and effective antimicrobial compounds in PE (Diem *et al.* 1999, Dustmann 1979, Erukhimovitch *et al.* 2002, Fontana *et al.* 2004, Fujiwara *et al.* 1990, Isla *et al.* 2001, Maquelin *et al.* 2003, Mariey *et al.* 2001, Martos *et al.* 1997, Naumann *et al.* 1991, Pascual *et al.* 1994, Roth *et al.* 1986, Salman *et al.* 2002).

Although our results didn't show any synergistic effect between CAPE and ampicillin, a significant additive effect between these agents can been seen (Table 2). These results may indicate that the mechanisms of action of CAPE and ampicillin are not related.

The exact mechanism of anti-bacterial activity of CAPE is still unclear. Our results showed that continuous treatment with CAPE for up to 6h caused a reversible antibacterial effect; however continuous treatment for longer periods of time caused irreversible inhibition of the bacterial growth (Figure 3). Also, the data presented in Figure 2 showed that 4h of treatment with CAPE did not affect the viability of the treated bacteria, while treatment for 6h or more caused complete bacterial death. It seems that this product has bacteriostatic effect during the first 5-6 h of treatment, while further treatment is required for a bacteriocidic effect.

Burdock (1998) attributes the antibacterial activity of PE to the aromatic acids and esters, while another study (Takaisi & Schilcher 1994) suggested it is due to flavonone pinocembrin, the flavonol galangin and CAPE, whose mechanism of action seems to rely on the inhibition of the bacterial RNA polymerase. Others have shown that other flavonoids, including galangin, can also have antibacterial activity (Cushnie & Lamb 2005). The mechanism of action involves degrading the bacterial cytoplasmic membrane, which causes a loss of potassium ions and further damage, leading to cell autolysis. Furthermore, quercetin, which is also found in PE, has been reported to increase membrane permeability and dissipate its potential, causing loss of the ATP synthesis capacity by the bacteria as well as dysfunction of membrane transport and mobility (Mirzoeva *et al.* 1997).

The effect of CAPE on the permeability of the bacterial outer membrane was assessed by the uptake of the crystal violet dye. Generally, crystal violet penetrates the outer membrane poorly, but it easily enters when the membrane is defective (Devi *et al.* 2010). Our results showed a dramatic enhancement in the uptake of crystal violet in gram (+) bacteria in a dose dependent mater, reaching over 95% uptake at the higher doses after 8h of treatment with CAPE (Figure 4A). In the case of gram (-) bacteria, there also was a significant increase in gentian violet uptake at the higher doses of CAPE (Figure 4A), even though this increase was much lower compared to gram (+) bacteria. After a 2h short treatment with CAPE, only a slight increase in the gentian violet uptake in gram (+) and gram (-) bacteria was observed. This shows that CAPE alters membrane permeability and makes the cells hyperpermeable to solutes. These results are in agreement with our results showing significantly higher antibacterial activity of this product against gram (+), as compared to gram (-) bacteria. Taken together, these results together with our findings that CAPE treatment for over 6h caused a bacteriocidic effect may indicate a dramatic irreversible disruption of the bacterial outer membrane, leading to bacterial death.

The escape of UV-absorbing substances from the cells is an index of membrane cell disruption and probably cell lysis and nonselective pore formation (Maisnier-Patin *et al.* 1996, Zhou *et al.* 2008). As seen in Figure 4B, treatment of *S. aureus* (a gram (+) strain) with CAPE caused a high leakage of intracellular components compared to a lower leakage from the treated gram (-) bacteria. These results suggest that the effect of CAPE on gram (+) and partially on gram (-) bacteria could be the formation of pores, or even a more severe disruption of the plasma membrane.

In addition, Scazzocchio *et al.* (2006) has reported that PE suppresses the expression of different bacterial virulence factors such as lipase and coagulase, while producing an evident suffering state of bacterial cells.

In this study, CAPE effectively inhibited the growth of most of the examined gram (+) bacteria, while they only had a limited effect on part of the gram (+) and on all tested gram (-) bacteria. This selective effect may be due to the presence of a capsule around these bacteria, which can prevent the penetration of the tested products into the bacteria. Therefore, at very high doses of the products, only small amounts of these products can penetrate into the capsular bacteria and partially affect their growth.

FTIR spectroscopy is a potential method used to study spectral changes in living cells (Erukhimovitch *et al.* 2002, Fontana *et al.* 2004, Fujiwara *et al.* 1990). Infrared spectra of microbial cells reflect the biochemical structure and composition of the cellular constituents such as water, fatty acids, proteins, polysaccharides and nucleic acids. In our study, major spectral variations were observed in the frequencies between 1800 and 1000cm^{-1}. More specifically, our results showed a significant increase in frequency at 1100cm^{-1} of the CAPE-treated bacteria. This may indicate an alteration in the polysaccharide content or distribution, probably of the bacterial envelope. This

result supports our findings showing a disruption of the bacterial outer membrane as a result of treatment with CAPE (Figure 4A). In addition, there was another notable increase in the frequency at 1250cm^{-1} of the CAPE-treated bacteria. This peak, which represents phosphate vibrations (Diem *et al.* 1999) may reflect significant changes in the phosphates. This could result from deformation in membrane phospholipids, suggesting that CAPE alters the macromolecular structures in the membrane, which further results in the complete loss of its integrity. These findings are in agreement with our results pointing towards formation of pores or even more severe disruption of the plasma membrane in CAPE-treated bacteria (Figure 4B).

Although the obtained results in this study provided a significant contribution for understanding the antibacterial mechanism of CAPE action, still further research is required for elucidating its exact mechanism of action.

Acknowledgements

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