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SPECTROSCOPIC INVESTIGATION OF ANTI-BACTERIAL AGENTS ACTIVITY

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ABSTRACT. FTIR spectroscopy has been widely used in recent time as a efficient tool to biomedical investigations. In this study we examined the potential of FTIR microspectroscopy as analytical instrument for early evaluation of anti-bacterial therapy efficiency. For this purpose, the effect of propolis (PE) on the development of bacterial infection in cell culture was examined. PE is a natural honeybee product with a potent anti-bacterial activity. Our results show early (2h post treatment) unique and significant spectral indicators for successful treatment with PE. Some of these biomarkers demonstrate different trends in gram (-) compared to gram (+) bacteria. It seems that FTIR spectroscopy can be used as an effective analytical tool for an early evaluation of the efficiency of the anti-bacterial effect of PE and probably other used drugs.

Keywords: *FTIR microscopy; Bacteria; Propolis*

INTRODUCTION

Bacteria are considered as one of the major causes of human and animal serious and dangerous infections. Although antibiotics are known as the most effective antibacterial drugs, bacteria can develop resistant mutants to the used antibiotics and, in fact, several bacterial mutants are known today to be resistant to all available antibiotics. Therefore, both the search for new anti-bacterial drugs and early evaluation techniques for the used drug efficiency are highly essential and guarantee for future effective treatment [1,2]. Most commercially available identification systems based on physiological (morphology, growth temperature, etc.) and nutritional (media composition, sugar assimilation, enzymatic tests, etc.) characteristics [3] are time consuming and not always very specific.

The detection and identification of microorganisms by spectroscopic techniques promises to be of a great value because of their sensitivity, rapidity, low expenses and simplicity. This together with the large information already known about spectral peaks obtained from FTIR spectra of living

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cells, make FTIR spectroscopy as an attractive technique for detection and identification of pathogens. FTIR spectroscopy was used successfully for identification of different kinds microorganisms [1, 4, 5], cancer cells, cells infected with viruses [6 - 10]. It was also used lately for the evaluation of antiviral drugs efficiency [11].

Propolis (PE), a natural product produced by honeybee and based on resins collected by bees from certain trees and plants, has been used for thousands of years in folk medicine for several purposes. The most important group of compounds of PE(in terms of amount and biochemical activity) are the flavonoids, which are thought to play a significant role in its bioactivities [12-14]. Between its various bioactivities, this product showed impressive antimicrobial activity mainly against gram positive bacteria [15, 16].

In the present study we examined the potential of FTIR microscopy as rapid and efficient analytical method for early evaluation of PE anti-bacterial activity against gram positive [gram (+)] and gram negative [gram (-)] bacteria.

RESULTS AND DISCUSSION

Effect of PE on gram (+) and gram (-) bacteria growth.

Different gram (-) and gram (+) bacteria were grown in the presence and absence of various concentrations of *PE* in L. Broth (LB) medium at 37C for 24h. The obtained results show that *PE* at a concentration of 0.2% completely inhibited the growth of the examined gram (+) bacteria whereas it hadn't any inhibitory effect on all examined gram(-) bacteria. At higher concentrations it had only partial inhibitory effect on the gram (-) bacteria (Table 1). It can be seen also from Figure 1 that *PE* was able to inhibit completely the growth of gram (+) bacteria as early as 2h post-treatment.

Table 1. Effect of *PE* treatment on the growth of various gram (-) and (+) bacteria. Equal amounts (10^3 bacteria/ml) of gram (-) and (+) bacteria were grown in 2ml LB medium in the presence or absence of different concentrations of *PE* at 37°C for 24h. At the end of the incubation period, the amounts of bacteria were evaluated. Results are means ± SD (n=5); the SD for these means was negligible.

	***PE* Concentration (%)**					
Bacterial strain	0		0.2		5	
	Bacteria Amount		Bacteria Amount		Bacteria Amount	
	(OD)	(No/	(OD)	(No\ml)	(OD)	(No/ml)
P. aeruginosa	1.31	$5.8x10^7$	0.95	$4x10^7$	0.74	$6x10^6$
S. enteridis	1.45	$6.8x10^8$	1.17	$8x10^7$	0.73	$1x10^7$
Micrococcus	1.01	$6.2x10^7$	0.20	$1.3x10^2$	0.01	0
B. subtilis	1.15	$2.5x10^8$	0.09	$1.7x10^2$	0.013	8

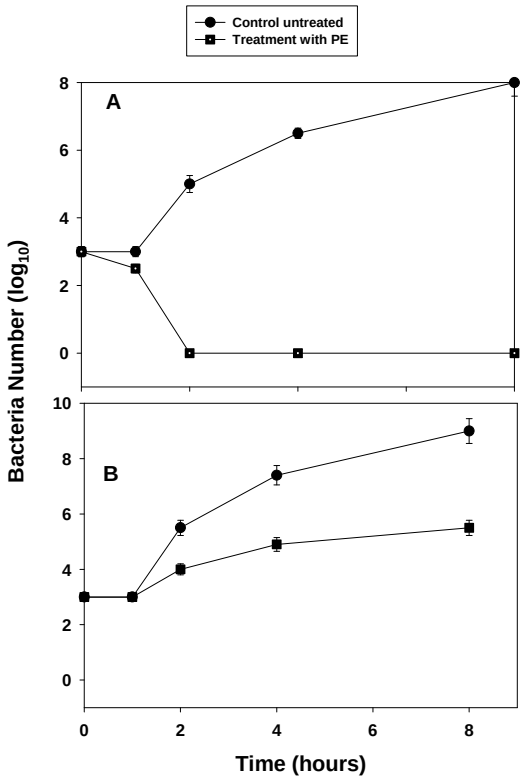

Figure 1. Effect of *PE* on the growth of Micrococcus [gram(+)](A) and P. aeruginosa [gram (-)] (B) bacteria. Micrococcus and P. aeruginosa bacteria were grown in LB medium containing 0.2% or 1% PE respectively and at various times post treatment. The number of living bacteria was evaluated by bacterial colonies counting as detailed in the "Experimental" section.

FTIR microscopy examination of gram (+) and gram (-) bacteria treated with PE

Similar aliquots (10^5 cells/ml) of the examined gram (+) or gram (-) bacterial strains were grown in LB medium in the presence or absence of 0.5 or 5% of *PE* respectively and examined by FTIR microscopy at 2h post-treatment. The obtained FTIR spectra of control untreated and *PE* treated Pseudomonas aeruginosa bacteria (as representative of gram (-) bacteria) and Micrococcus bacteria (as representative of gram (+) bacteria) share several bands as follow (Figure 2A, Figure 3A): the dominant bands at 1655 cm^{-1} and 1546 cm were attributed to protein amide I and II bands [17]. The shoulder at about^{-1} 1730 cm^{-1} was attributed to lipid C=O stretching vibrations [17]. The band at 1465 cm^{-1} was assigned to the CH_2 bending mode of the cell lipids. The bands at 1454 cm^{-1} and 1397 cm^{-1} were attributed to asymmetric and symmetric CH_3 bending modes of end ethyl groups and

branched methyl groups of proteins and lipids, respectively [18]. The peaks at 1237 cm^{-1} and 1082 cm^{-1} were attributed to asymmetric and symmetric stretching vibrations and phospholipids [19]. The peak at 1064 cm^{-1} resulted from the overlap of several bands, including absorption due to the vibrational modes of $–CH_2OH$ and the C–O stretching vibration coupled to the C-O bending mode of cell carbohydrates [20].The peak at 857 cm^{-1} was attributed to N-type sugars[20].

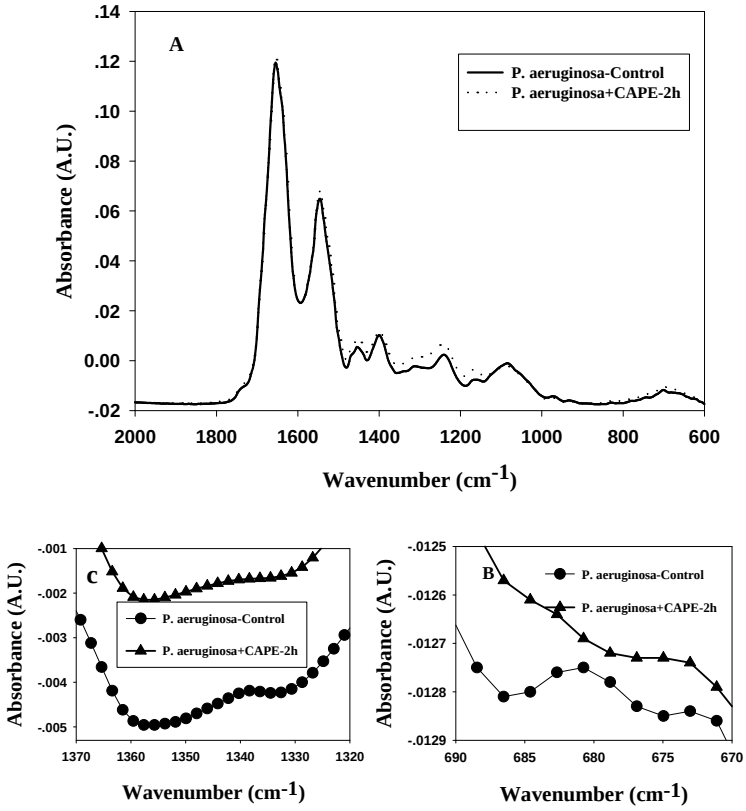

Figure 2. FTIR spectra of *P. aeruginosa* after 2h growth in medium with or without 5% *PE* at the regions (A) 600-2000 cm^{-1} (B) 674-686 cm^{-1} (C) 1330-1350 cm^{-1}.

The main consistent differences between the spectra of the control and the PE treated bacteria are at two specific spectral peaks:
1. Peak at 682 cm^{-1}: This peak represents NH bending of proteins [18]. A major decrease (about 25 folds) in the spectral absorption at this region was obtained at 2h post-treatment with *PE* in all examined gram (-) bacterial strains (Figure 2B). On the contrary, there was a considerable increase (about

8 folds) in all examined gram (+) bacterial strains in the spectral absorption at this region after treatment with *PE* (Figure 3B).

2. Peak at 1339 cm^{-1}: This peak which is assigned to adenosine (DNA) [21] highly (about 15 folds) decreases in gram (+) and almost don't change in gram (-) bacteria (Figs 2C, 3C).

Similar results were obtained with the other examined gram (+) and gram (-) bacteria (data not shown). The spectral discrepancies between normal and treated bacteria with *PE* at the various above peak areas of both gram (+) and gram (-) bacteria were statistically significant as assessed by t-Test ($P< 0.001$) and enough for identification of treated bacteria at very early stages of treatment.

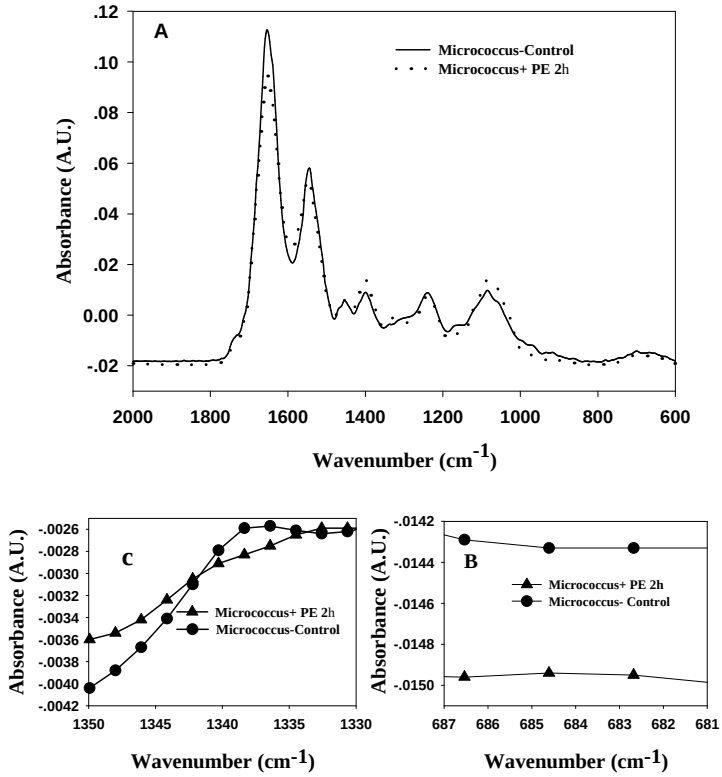

Figure 3. FTIR spectra of *Micrococcus* after 2h growth in medium with or without 5% PE at the regions (A) 600-2000 cm^{-1} (B) 680-686 cm^{-1} (C) 1330-1350 cm^{-1}.

As can be seen from these results, the behavior of the spectral peaks is clearly different in gram (+) compared to gram (-) bacteria as a result of treatment with *PE*. This difference seems to be a direct consequence of the different biological effect of *PE* on these bacteria. As our results (Table 1)

showed, *PE* significantly inhibits the growth of gram (+) bacteria at low doses (0.2%), whereas it has only a slight inhibitory effect on the growth of gram (-) bacteria even at high concentrations (5%). The mechanism of the anti-bacterial activity of *PE* is still unclear.

The obtained spectral changes of the treated bacteria with *PE* are an evidence of chemical composition variations of these cells. These changes could be a result of changes in the metabolic activity of these bacteria. The increase in protein level observed in treated gram (+) bacteria may be resulted by over expression of some of the bacterial genes as a consequence of treatment with *PE*. The major reduction in the peak area at 1339cm^{-1}, which seems to be assigned to nucleic acids, in gram (+) bacteria may be due to the termination of the bacterial DNA synthesis and/ or its degradation induced by the treatment with *PE*.

CONCLUSIONS

The results presented in this study proved the potential of FTIR microscopy for rapid and reliable determination of anti-bacterial efficiency of *PE*. It can be seen that different spectral peaks over the FTIR spectra of the examined samples may be used as excellent indicative biomarkers for the efficiency of the used drugs at very early stages of the treatment. It seems that these above biomarkers can be used successfully for follow up after efficacy of *PE* and might be useful for evaluation and determination of its required doses.

The present study together with our and others previous studies strongly support the possibility of developing the FTIR microscopy a**s a** rapid and efficient analytical tool for early evaluation of the efficiency of drug treatment.

EXPERIMENTAL SECTION

Bacteria

In the present study we used the following gram (-) and gram (+) bacteria:
gram *(-) bacteria are* Pseudomonas aeruginosa and Salmonella entenidis,
gram *(+) bacteria are* Micrococcus and Bacillus subtitis. All used bacteria were grown on Nutrient Agar (Difco) at 37 °C.

Propolis (PE)

An aqueous extract of propolis (20% of row propolis) was diluted with RPMI culture medium to give the required concentrations (0.1, 1, 5, 10 or 20%). The pH of the diluted extract was adjusted to 7.1-7.2 with sodium borate. As a control, we used phosphate buffered saline (PBS) (pH 7.2) containing the same amount of sodium borate, which was necessary to adjust the pH of propolis extract.

Amount of bacteria measurement

The amounts of bacteria were evaluated both by 2 methods:
(a) Examining their optical density (OD) by spectrophotometer () at wave length 620nm. This method gives an evaluation of the live and dead bacteria.
(b) Bacterial colonies counting. 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 gives the number of only live bacteria.

Sample preparation.

Since ordinary glass slides exhibit strong absorption in the wavelength range of interest to us, we used zinc-sellenide crystals, which are highly transparent to IR radiation. The amount of the examined bacteria was determined by measuring it's optical density with a spectrophotometer. Similar amounts of the examined bacteria were pelleted from a medium containing bacteria by centrifugation at 1000 rpm for 2 min. Each pellet was washed twice with H_2O and resuspended with 20µl of H_2O and a drop of 1µl of the obtained suspension was placed on a certain area on the 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 spectra measurement.

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 mid-IR 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 were obtained for all the spectra by OPUS software. Peak positions were determined by means of a second derivation method by OPUS software. Since the samples to be analyzed were often heterogeneous, appropriate regions were chosen by FTIR microscopy so as to eliminate different impurities (salts, medium residuals, etc.). The aperture used in this study was 100µm, since this aperture gave the best signal/noise ratio. For each sample, the spectrum was taken as the average of five different measurements at various sites of the sample. Each experiment with each sample was repeated five times. It is important to mention that there were no significant differences in the spectra from various sites (SD did not exceed 0.005).

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