EUGENOL INDUCES DAMAGE OF BACTERIAL AND FUNGAL ENVELOPE

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Abstract. Eugenol, the phenolic major component of clove essential oil, was used in this study to elucidate its antimicrobial mechanism against the yeast; *Saccharomyces cerevisiae*, gram positive bacteria; *Bacillus subtilis* and gram negative bacteria; *Escherichia coli*. For all organisms tested, the treatment with this phenolic major component reduced the cellular viability by inducing the release of substances absorbing at 260 nm. This supposes that cell lethality was a consequence of cellular lysis. In addition, scanning electron microscopy analysis revealed that the envelope of all treated cells by eugenol was significantly damaged.

Key words: Eugenol, *S. cerevisiae, B. subtilis, E. coli*, Mechanism of action.

Introduction

The antimicrobial activity of essential oils (EO) has been widely described in several studies [1, 9, 10, 11]. This activity of EO is mainly due to their high content in phenolic derivatives [7, 18, 20, 22].

In the present work, we sought to elucidate the antimicrobial mechanism of eugenol; the phenolic major component of clove essential oil on *Saccharomyces cerevisiae, Bacillus subtilis* and *Escherichia coli,* in order to determine how these component act on the cell envelope of yeast and bacteria cells. In this respect, the antimicrobial activity of eugenol was investigated using two approaches: 260 nm absorbing of released cytosolic compounds coupled with cellular mortality and scanning electron microscope (SEM).

Material and methods

Microorganisms

S. cerevisiae strain (SB36–85) was isolated from Baker's yeast in our laboratory and identified using standard yeast determination procedures [12].

E. coli strain (APL 87/1) was isolated from a hen affected by collibacillosis. It was identified at the Avian Pathology laboratory of the Institut Agronomique et Vétérinaire Hassan II, Rabat- Morocco.

B. subtilis strain (APL 87/35) was isolated from poultry meat in the same laboratory.

Culture media

Glucose-YNB (0.67 % yeast nitrogen base and 0.5 % glucose) was used for yeast culture. M9 medium [14] was used for bacterial culture.

The washing medium used was Phosphate Buffer Saline PBS (8 g/l NaCl; 0.2 g/l KCl; 1.13 g/l Na_2HPO_4 2 H_2O and 0.2 g/l KH_2PO_4).

Preparation of washed yeast and bacteria cells

S. cerevisiae cells were grown in 200 ml glucose YNB for 18 h on a shaker at 30°C. The cell cultures were washed twice in PBS by centrifugation 10 min at 12000 x g at 4°C.

The bacteria were grown overnight at 37°C in 200 ml under aeration in minimal medium M9 with initial pH 7.2-7.4. The cells were

harvested at 400 g for 25 min at 4°C. For all organisms, the supernatant was discarded and the cells were resuspended in PBS. This operation was repeated twice.

Antimicrobial agent

Eugenol purchased from Sigma (2001) is the major constituent of clove oil (90 % of total oil) [4]. Eugenol was dispersed in sterile 0.2 % agar suspension [17].

Determination of the minimal inhibitory concentrations (MIC) and minimal lethal concentrations (MLC)

The MIC and MLC were determined in triplicate in liquid medium by the contact of eugenol with yeast cells at 30°C or bacteria at 37°C for 24 hours according to the method improved in our laboratory by Remmal et al [16].

Estimation of the yeast cells or bacterial cytosol release

The release of cytosolic material absorbing at 260 nm from yeast or bacteria treated with eugenol was performed on aliquots of 1.5 ml of cells suspension in PBS.

Two group treatments were considered: (a) Control cells suspended in PBS (yeast or bacteria cells) and (b) Cells suspended for one hour in PBS containing various concentrations of eugenol ranging from 1.5 mM to 12 mM.

Correction was made for the absorption of the suspending liquids containing the same concentration of eugenol after two minutes contact with bacteria or yeast cells at 260 nm in Beckman UV spectrophotometer.

Counting of viable yeast cells

The number of viable cells following the phenolic component treatment was determined on YPG-agar plates for yeast cells and M_9 agar for bacteria using the drop count method described by Courvalin et al. [8].

Scanning electron microscopy (SEM)

After one hour contact with the MLC concentrations of eugenol, the cells were prefixed in 2 % glutaraldehyde for one hour at 4°C. Post–fixation was done using a 2 % osmium tetroxyd solution during 30 minutes at 4°C. After each fixation, the cells were washed twice with PBS. The cells were then dried at a critical point (Balzers CPD 010) in liquid CO_2 under 95 bar pressure. The samples were gold covered by cathodic spraying (Edwards S 150 B). Finally, the samples were examined as described with the scanning electron microscope (Stereoscann 360, Cambridge) [2].

Statistical test

Student's test was used to determine the significance of the observed differences [19].

Results

MIC and MLC of eugenol against *S. cerevisiae*, *E. coli* and *B. subtilis*

The MIC and MLC for eugenol are presented in table 1.

Table 1. MIC and MLC of eugenol with *S. cerevisiae*, *E. coli* or *B. subtilis* in liquid medium

	S. cerevisiae	***E. coli***	***B. subtilis***
MIC	2 mM	3 mM	2 mM
MLC	3 mM	6 mM	3 mM

Mortality of cells treated with a range of eugenol concentrations

Figure 1 shows that for *S. cerevisiae,* the number of viable cells decreased following treatment with eugenol at concentrations ranging between 1.5 mM and 6 mM, while below 1.5 mM, no significant mortality was shown. Total mortality was observed for concentration higher than MLC. Concerning *E. coli,* Figure 2 also shows that the number of viable bacteria decreased slightly below 3 mM and dropped rapidly before 6 mM beyond which the mortality was total. Regarding *B. subtilis,* Figure 3 shows that cellular

mortality was more rapid than *E. Coli* since 3 mM concentration was enough to kill 100 % of bacteria cells.

Lysis of yeast and bacteria cells treated with a range of eugenol concentrations

In an attempt to explain how the cells died, we evaluated the lysis of yeast cells, Gram- and Gram+ bacteria treated with a range concentration of eugenol by measuring the release of substances absorbing at 260nm. Figures1, 2 and 3 show that the release of cellular content increased according to eugenol concentration. This release was thus obtained with concomitant mortality.

Scanning electron microscope (SEM) observations of yeast cells treated with eugenol.

Given that this phenolic component treatment of yeast cells and bacteria led to the release of cytosolic compounds, we investigated its action on the cellular surface. The electron micrographs obtained from scanning microscopy analysis showed that the treatment of *S. cerevisiae* cells with eugenol generated an important morphological damages compared to control (Micrograph 1a and 1b). Concerning *B. subtilis,* the envelope of treated bacteria also presented cellular

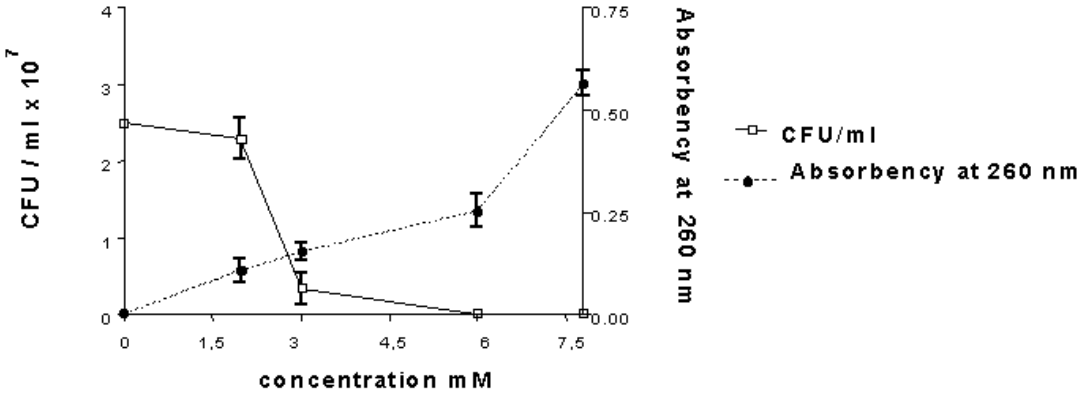


Figure 1. The effect of eugenol concentration on cellular mortality and the release of 260 nm absorbing material from *S. cerevisiae*

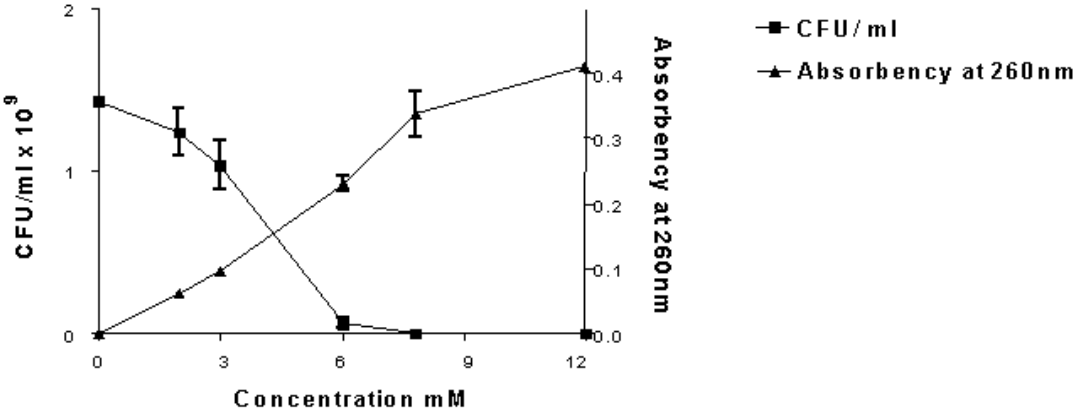


Figure 2. The effect of eugenol concentration on cellular mortality and the release of 260 nm absorbing material from *E. coli*

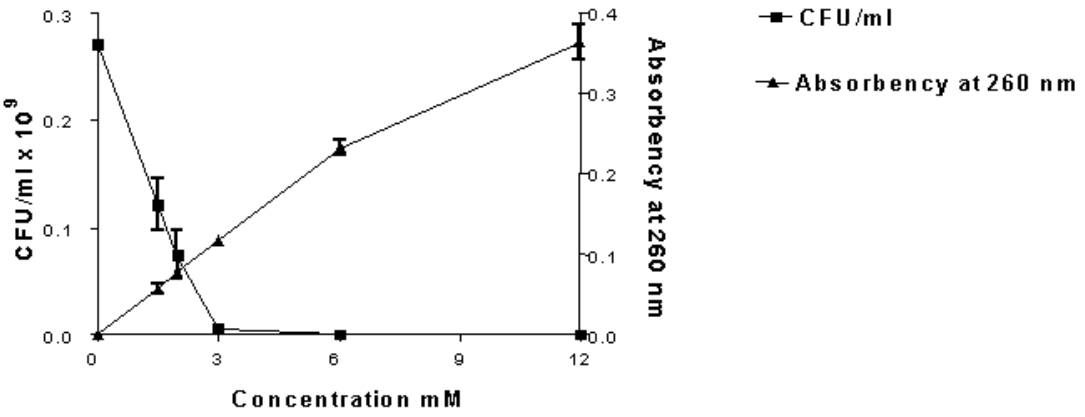


Figure 3. The effect of eugenol concentration on cellular mortality and the release of 260 nm absorbing material from *B. subtilis.*

deformity (Micrograph 2a and 2b). For *E. coli*, the aspect of the envelope of treated cells is different from that of untreated control since treated ones presented many holes at the envelope level (Micrograph 3a and 3b).

Discussion

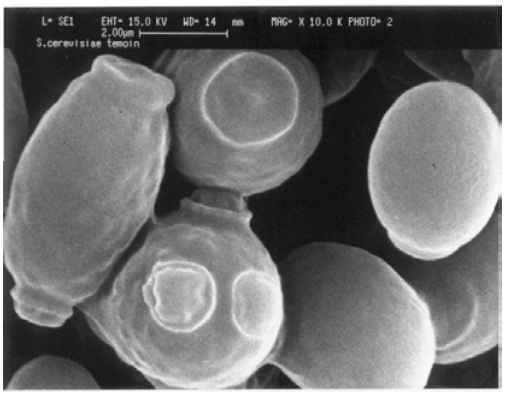
Antimicrobial activity of EO phenolic components has been extensively investigated [3, 7, 22].

For the determination of the minimal inhibitory concentration and the minimal lethal concentration, eugenol was dispersed in 0.2 % agar solution to avoid the use of solvents or detergents known to significantly decrease the antimicrobial activity of EO [16]. The MIC and the MLC of eugenol against the microorganisms tested have been proved to be as low as those obtained by other authors [3, 16, 23]. The determination of MIC and MLC allowed us to assess by colony counts the concentration of eugenol necessary to induce the cells death at ranging concentrations of these agents.

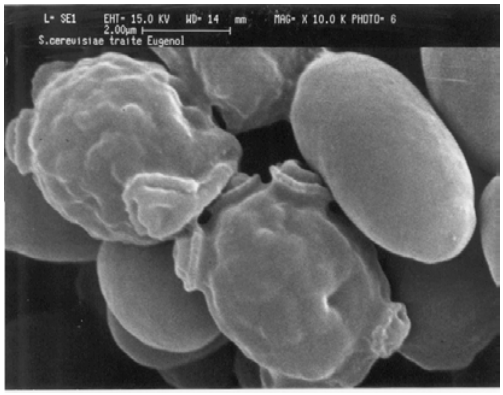
For all organism cells tested, the number of the viable cells remained almost constant at low concentrations of eugenol but markedly decreased with the MIC. The release of 260 nm absorbing material increased in linear manner according to the ranging concentrations of this phenolic component which led us to conclude that the mortality cells was a consequence of cellular lysis. These results corroborate with the previous works using whole essential oils [5, 6, 9, 15].

These results allowed us to hypothesize about an action of eugenol on yeast and bacteria envelope. To verify this hypothesis, we submitted eugenol treated cells to scanning microscope observations. As expected, eugenol induced deformity in *S. cerevisiae* shape, which could explain the delay in response to the concentrations used. Concerning bacteria, eugenol exerts its bactericidal activity causing envelope damage. However, the type of damage generated was different; *E. coli* presented damage as holes in the envelope while *B. subtilis* showed damage as cell deformity. This difference may be explained by the fact that the envelopes of *E. coli* (gram- bacteria) and *B. subtilis* (gram+ bacteria) do not have the same structure.

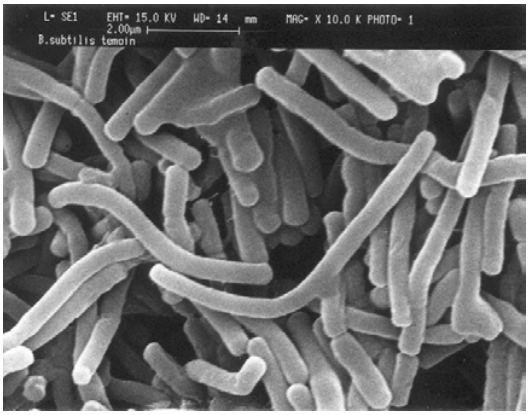
Recent investigations about the antimicrobial action of phenolic component of EO showed disruption of the bacterial and fungal membrane [13, 18, 21, 23]. All these reports suggest that the antimicrobial mechanism of eugenol is due to membrane damage. Our results assess a direct and a clear sight at the role of purified major phenolic component because the use of the whole EO doesn't allow the determination



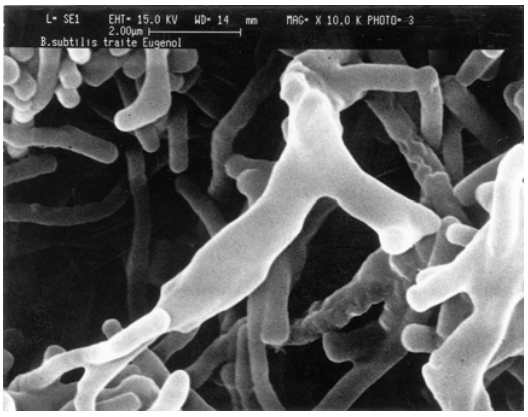
Micrograph 1a. Scanning electronic microscope of *S. cerevisiae* Untreated (Control)



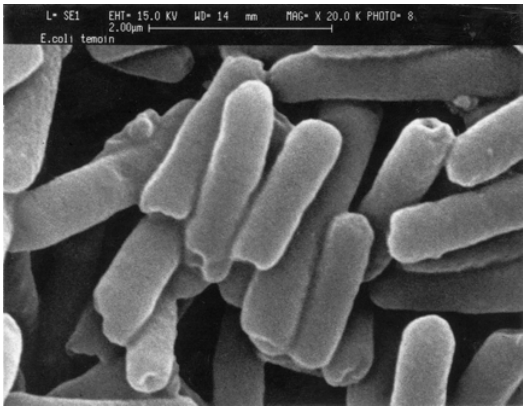
Micrograph 1b. Scanning electronic microscope of *S. cerevisiae* treated by Eugenol



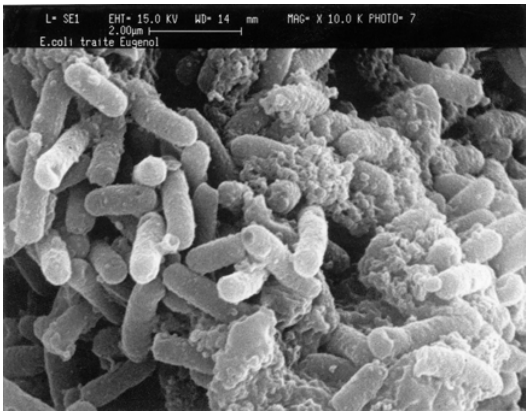
Micrograph 2a. Scanning electronic microscope of *B. subtilis* Untreated (Control)



Micrograph 2b. Scanning electronic microscope of *B. subtilis* treated by Eugenol



Micrograph 3a. Scanning electronic microscope of *E. coli* Untreated (Control)



Micrograph 3b. Scanning electronic microscope of *E. coli* treated by Eugenol

of the active principle on account of the complexity of its composition. SEM observations translated by an important surface alteration suggest a new antimicrobial mechanism of this major phenolic component of EO that affects not only the membrane but all the envelope of fungal and bacteria cells. Supplementary investigations to support this suggestion are on the way. The antifungal and antibacterial effects of this phenolic component are actually investigated *in vivo* on animal models in our laboratory. Results obtained are very encouraging (results not shown). So, more investigations are necessary to valorise essential oils having phenolic major component like oregano, thymus and other widespread Moroccan plants.

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