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ORIGINAL CONTRIBUTION

Interaction between the antibacterial compound, oleuropein, and model membranes

Jorge Casas-Sanchez · M. Asuncion Alsina · Mathias K. Herrlein · Concepció Mestres

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Abstract Oleuropein, a secoiridoid glycoside extracted from the olive tree, *Olea europaea L*., has been described as showing antibacterial properties. However, the exact mechanism of these antimicrobial properties is not yet well understood. In the present study, we have studied the interaction of oleuropein with phosphatidylglycerol (PG) as a model membrane for *Staphylococcus aureus* (*S. aureus*) (Gram-positive bacteria) and phosphatidylethanolamine and *Escherichia coli* (*E. coli*) lipid extract as a model membrane for *E. coli* (Gram-negative bacteria). The study has been carried out using monolayers as model membranes and using kinetics at constant area and compression isotherms with Brewster angle microscopy (BAM) observations. The results show that oleuropein interacts in higher extent with PG monolayers, which is related with its stronger antibacterial effect against Gram-positive bacteria. The effects on the membrane are probably produced at the cell surface because oleuropein did not form stable mixed monolayers with the lipids assayed at the air/water interface.

Keywords Oleuropein · Surface activity · Langmuir monolayers · Compression isotherm · Brewster angle microscopy (BAM) · *Staphylococcus aureus* · *Escherichia coli*

J. Casas-Sanchez · M. A. Alsina · C. Mestres (✉)
Department of Physicochemistry, Faculty of Pharmacy,
Universidad de Barcelona,
Av. Joan XXIII s.n.,
08028 Barcelona, Spain
e-mail: cmestresm@ub.edu

J. Casas-Sanchez · M. K. Herrlein
Procter and Gamble Service GmbH,
Sulzbacherstrasse 40,
65823 Schwalbach am Taunus, Germany

Introduction

The search of new molecules with antimicrobial properties is an ongoing process due to limitations such as adverse effects or development of resistance of previously sensible microorganisms to the drugs already in use.

Products of natural origin have been always a source of all kinds of drugs with different pharmacological properties, including antimicrobials, and at the present time, the interest for those products in therapeutics is increasing. In this group, we find oleuropein, a water-soluble phenolic monoterpenoid glycoside (Fig. 1), extracted from the leaves and drupes of the olive tree (*Olea europaea L.*), being present especially in high amounts (60–90 mg/g dry weight) in the leaves [1]. Oleuropein belongs to a very specific group of coumarin-like compounds, called secoiridoids, which are abundant in Oleaceae, Gentianaceae, Cornaleae, and many other plants. Iridoids and secoiridoids are compounds, usually glycosidically bound, and produced from the secondary metabolism of terpenes as precursors of various indole alkaloids. The secoiridoids in Oleaceae are usually derived from the oleoside type of glucosides (oleosides), which are characterized by an exocyclic 8,9-olefinic functionality, a combination of elenolic acid and a glucosidic residue. Oleuropein is an ester of 2′-(3′,4′-dihydroxyphenyl)-ethanol (hydroxytyrosol) and the oleosidic skeleton common to the secoiridoid glucosides of Oleaceae [1].

Oleuropein has shown high antimicrobial activity against both Gram-negative and Gram-positive bacteria [2–4] as well as mycoplasma [5]. Phenolic structures similar to oleuropein seem to produce its antibacterial effect by damaging the bacterial membrane, disrupting cell peptidoglycans, or both of them. Different authors have used biophysical assays to study the interaction between oleuropein and membrane lipids [6]; however, the exact mechanism of antimicrobial

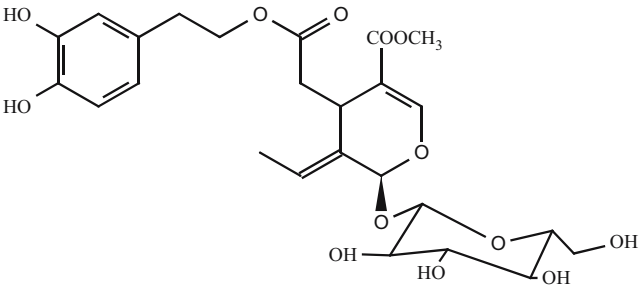

Fig. 1 Oleuropein chemical structure

activity of oleuropein is still not completely established. A better knowledge of this mechanism would be of great interest for the future use of this product and some related substances as antimicrobial agents.

In the present work, we have studied the surface activity of oleuropein and its interaction with lipid monolayers. Monolayers are a simple approach that can give invaluable information at the molecular scale and have been broadly used in studies of interaction between molecules with antimicrobial properties and membranes [7, 8].

The lipids chosen in our study have been (1) phosphatidylglycerol (PG) as the major component of Gram-positive bacteria (*Staphylococcus aureus*) membrane, and we used (2) phosphatidylethanolamine (PE) as well as (3) *Escherichia coli* total lipid extract that also contains small amounts of PG and cardiolipin [PE (70–90%) + PG (around 10%) + cardiolipin (3%)] to study the interaction with Gram-negative (*E. coli*) bacteria. The interaction with the membrane components has been studied through kinetics at constant area and compression isotherms.

Experimental methods

Materials

Ultrapure water was produced by deionization and nanopure purification coupled to a Milli-Q purification system (Milli-Q system, Millipore) up to a resistivity of 18.2 MΩ cm. Chloroform proanalysis was from Merck (Poole, Dorset, UK). Oleuropein was purchased from Extrasynthese (Genay, France) and was further purified by reverse-phase high-performance liquid chromatography. *E. coli* total polar lipid extract [PE (70–90%) + PG (around 10%) + cardiolipin (3%)], L-α-PE, and L-α-PG (egg, chicken–sodium salt) were obtained from Avanti Polar Lipids.

Surface activity

Measurements were performed in a Langmuir film balance (NIMA type 601 series 060D) equipped with a platinum Wilhelmy plate. A polytetrafluoroethylene (Teflon®) trough (volume of 20 ml) was used. Increasing volumes of a stock solution of oleuropein in water (1 mg/ml) were injected into water pH 6.0 under constant stirring. The adsorption of the secoiridoid at the air/water interface is therefore monitored by following the increase in surface pressure as a function of time under continuing stirring of the subphase.

Kinetics at constant area

To study the insertion of oleuropein into lipid monolayers mimicking the membranes of different microorganisms, we used the same equipment described above. The aim of this study was to determine the behavior of the lipid monolayer when oleuropein is dissolved in the subphase. For this purpose, lipid monolayers were spread from chloroformic solutions at the air/water interface; 25 min was allowed for evaporation of the volatile solvent and complete monolayer extension before injecting 25 μl of 1 mg/ml aqueous solution of oleuropein into the subphase. The injection was performed through a side sample hole. Therefore, in this experiment, the surface pressure (π) variation was registered through time. The subphase employed was water pH 6.0. At the concentrations assayed, oleuropein was completely soluble in water as its partition coefficient (K_p) in water is 0.0006 [9].

Compression isotherms

Pressure–area isotherms were carried out in the same instrument using a trough measuring 80×8×0.4 cm. Phospholipid monolayers were formed, spreading chloroform solutions onto water subphase, pH 6.0 alone, or with oleuropein. After allowing 25 min for evaporation of the solvent and stabilization of the monolayer, it was compressed with an area reduction rate of 20 mm^2/min.

For mixed monolayers, mixtures of different proportions of oleuropein + PG or oleuropein + *E. coli* lipid extract

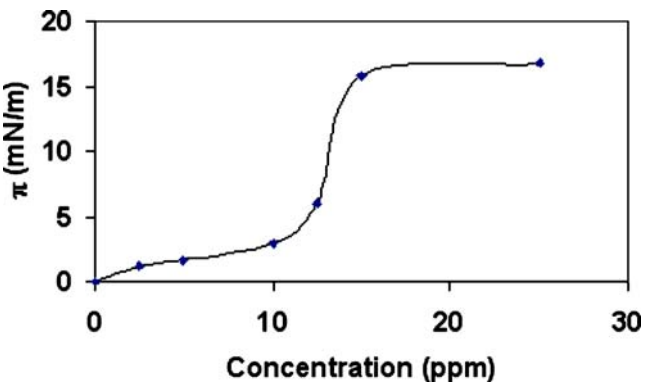

Fig. 2 Oleuropein surface activity at different concentrations. Subphase water pH 6.0

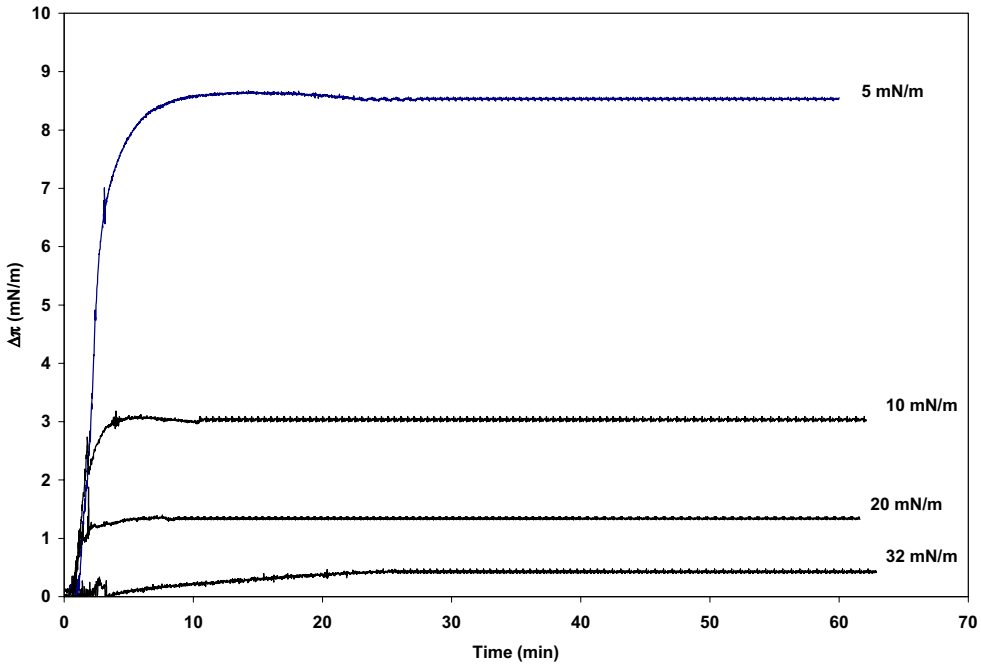

Fig. 3 Kinetics at constant area for *E. coli* lipid extract monolayers at different initial pressures. Oleuropein concentration in the subphase: 12 ppm

dissolved on chloroform/methanol (2:1) were spread on water subphase and compressed after 25 min.

Each run was repeated three times, and the accuracy of the measurements was ±0.01 nm^2/molec.

Brewster angle microscopy

The different topographic structures of the monolayer were observed with a commercial Brewster angle microscope (BAM), manufactured by NFT (Göttingen, Germany), mounted on the NIMA Langmuir film balance previously described. This model uses a class IIIb laser of 30 mW, emitting p-polarized light of λ=660 nm. BAM examined films containing no added fluorescent probes. The air–monolayer–water interface was illuminated at 53.1° by a laser beam polarized in the plane of incidence (p-polarized), and a lens system focused an image onto a charge-coupled device (CCD) camera. The CCD camera converts the reflectivity signal from the sample into a video image. Principles, and a detailed description of the BAM technique, have been described elsewhere [10, 11]. All the experiments were carried out at a temperature of 21±1 °C.

Results and discussion

Surface activity

Increasing volumes of oleuropein solubilized in water at a concentration of 1 mg/ml were injected into water subphase pH 6.0, and the surface activity at the air/water interface was recorded. At low concentrations of oleuropein, the increase in the pressure values was low; at some point around 10 ppm, equivalent to 10 mg of oleuropein per liter, the pressure increased abruptly (Fig. 2). This behavior is typical of a cooperative effect between the molecules that reach the interface. Similar results have been found for other molecules, as the antimicrobial peptide Defensin A [7]. The sigmoid aspect of the pressure concentration indicates that the adsorption of the hydrophobic moiety of the molecule is the first step leading other molecules to the interface.

Maximum surface activity was achieved at 25 ppm; higher concentrations produced small increases in the pressure values. This is consistent with the formation of oleuropein aggregates at the interface, which are solubilized to the subphase, as has been noted for other substances with similar behavior [12].

Kinetics at constant area

The interactions of oleuropein with spread monolayers of PG, PE, and *E. coli* lipid extract were studied using subphase concentrations of 5 and 12 ppm. At 5 ppm, only

Table 1 Pressure increases after 1 h

	5 mN/m	10 mN/m	20 mN/m	32 mN/m
PG	12.3	9.2	2.5	0.1
PE	6.5	3.5	1.3	0.2
E. coli lipid extract	8.4	2.4	1.1	0.4

Subphase: oleuropein of 12 ppm

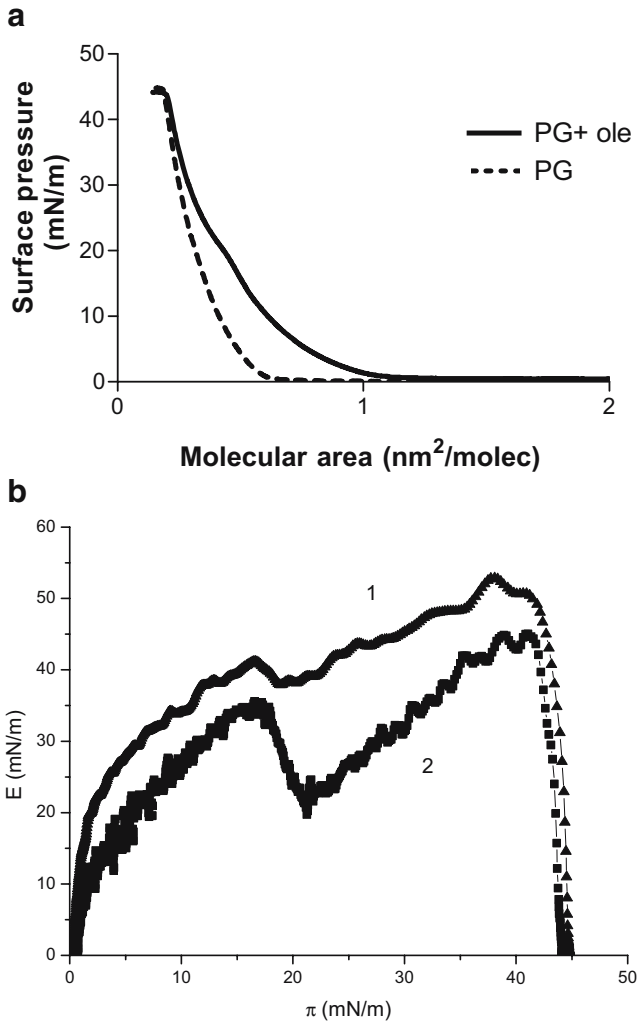

Fig. 4 **a** Compression isotherms of PG monolayers on water subphase pH 6.0 and subphase containing oleuropein 12 ppm. **b** Surface elasticity-surface pressure plots for PG monolayers on: *1* subphase water pH 6.0 and *2* subphase containing oleuropein 12 ppm

a slight interaction was detected at initial surface pressures of 5 and 10 mN/m. However, when the concentration of oleuropein in the subphase was higher (12 ppm), it was able to interact with all the lipids assayed, the results being similar for all of them (e.g., Fig. 3 shows the curves obtained for *E. coli* lipid extract). Oleuropein produces the maximum pressure increases in the first minutes after the injection. Interestingly, at low initial pressures (i.e., 5 and 10 mN/m) in PG monolayers, oleuropein is able to promote a higher change of surface pressure ($\Delta\pi$) than its surface activity (Table 1 vs Fig. 2). The interaction with PE monolayers was not as strong as the one recorded with PG monolayers at low initial pressures. Nevertheless, at 20 and 32 mN/m, the pressure increases in pressure values were similar to those with PG. The penetration in monolayers formed by polar lipid extract of *E. coli* was similar to the ones with PE; this is not strange considering that 65–70% of the phospholipids from the *E. coli* membrane are PE.

To compare better the interaction of oleuropein with the three monolayers assayed, we calculated the exclusion density form the representation of the increase in pressure values with respect to the lipid density [7]. The results obtained are similar for the three monolayers with no significant differences (PE is 0.021 molec/nm^2, PG is 0.018 molec/nm^2, and *E. coli* lipid extract is 0.016 molec/nm^2). However, it has to be noted that these monolayer density values are equivalent to pressures of 32.93 mN/m for PG, 27.53 mN/m for PE, and 34.06 mN/m for *E. coli* lipid extract. Therefore, even at low concentrations, oleuropein is able to interact with these lipid monolayers at pressures equivalent to those of biological membranes [12]. It also has to be noted that the degree of incorporation of oleuropein in *E. coli* lipid extract is slightly higher than PE alone. So it can be concluded that the presence of PG and/or cardiolipin in the monolayer has an enhancing effect in the interaction.

Compression isotherms

Effect of oleuropein on the compression isotherms of lipid monolayers

The results obtained in the kinetics previously discussed show that there are interactions between oleuropein and the lipids assayed. However, sometimes, kinetics at constant area is not a very sensitive method to evaluate hydrophobic interactions. Therefore, to obtain more information about those interactions, we used another method based on the monolayer technique: compression isotherms using surface

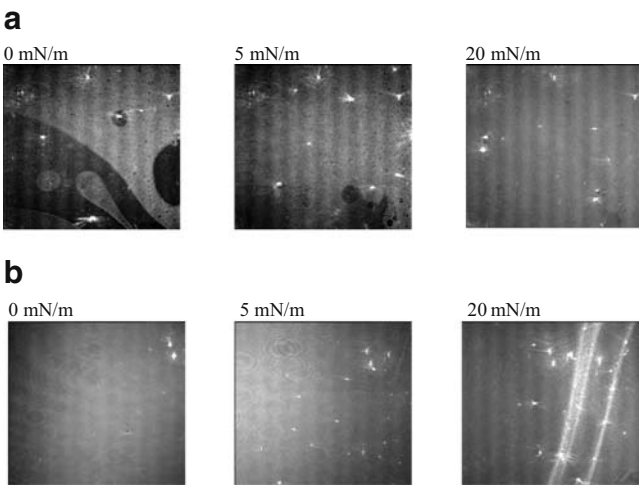

Fig. 5 Representative BAM images of PG monolayers at different surface pressures. The visual field is 570×570 μm. **a** Subphase water. **b** Subphase water + oleuropein 12 ppm

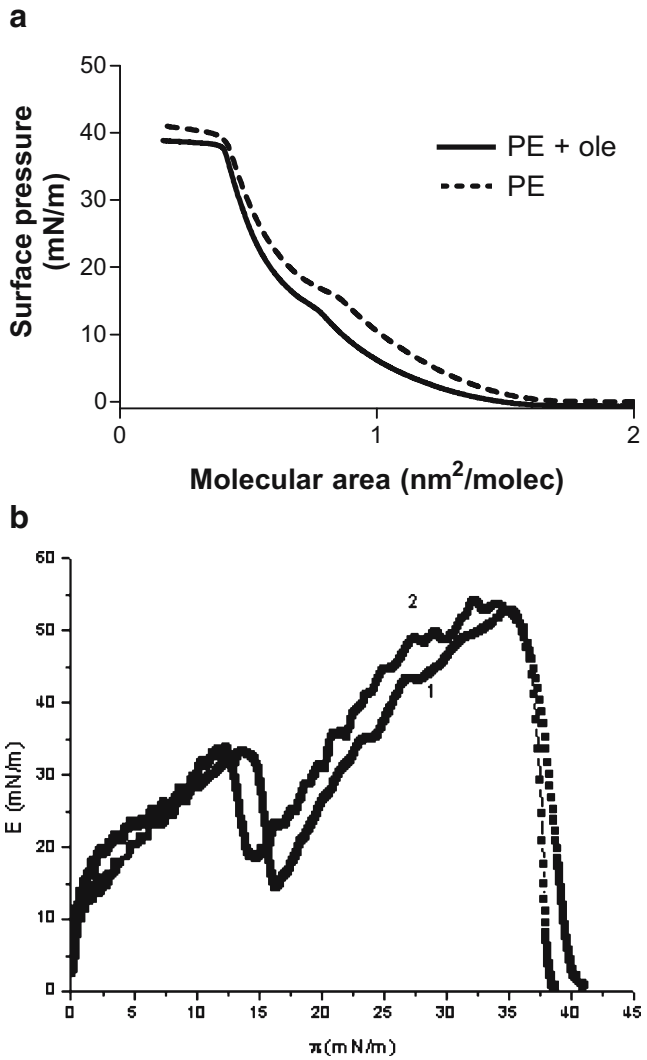

Fig. 6 **a** Compression isotherms of PE on water subphase pH 6.0 and subphase containing oleuropein of 12 ppm. **b** Surface elasticity-surface pressure plots for PE monolayers on: *1* subphase water pH 6.0 and *2* subphase containing oleuropein 12 ppm

pressure (π)–area (A) isotherms complemented with Brewster angle microscopy images. The interactions between the lipids and oleuropein were studied by dissolving the latter into the subphase and monitoring differences in the shape of the compression isotherm of the lipid film with and without oleuropein.

The π–A isotherm of PG showed rather condensed character with a collapse pressure of 45 mN/m, similar to the one obtained for dipalmitoyl phosphatidyl glycerol (DPPG) with acidic subphases [13]. Figure 4a shows the isotherms obtained after spreading 25 μl of 1 mg/ml of a PG–chloroform solution on the water subphase and in the presence of 12 ppm of oleuropein. Compression isotherms of PG spread on oleuropein-containing subphase showed that for all the values of surface pressures until it reaches the collapse, the area per phospholipid molecule was larger than those observed in the absence of oleuropein, suggesting that oleuropein is at least partly incorporated into the lipid monolayers. This point was confirmed by the analysis of the surface elasticity, $E=-(\mathrm{d}\pi/\mathrm{dln}A)_T$, as function of surface pressure. Surface elasticity was calculated from isotherms by graphical difference and was plotted against surface pressure in Fig. 4b. It can be observed that the presence of oleuropein in the subphase is accompanied by slight decrease in the surface elasticity of the monolayer.

To have a better understanding of this process, we proceeded to take BAM images of PG films compressed on subphase with and without oleuropein. Images of the π–A isotherm taken for PG films on water (Fig. 5a) showed at 0 mN/m a nonhomogeneous formation. When pressure increases, the monolayer becomes more regular till achieving the collapse. On the other hand, when oleuropein was present in the subphase (Fig. 5b), a stable monolayer could be seen from the beginning, showing that oleuropein and PG form an homogenous structure, which is compatible with the incorporation of oleuropein to the film. BAM images also showed a number of bright domains immersed in the expanded phase (gray zone) (this phenomenon has been observed by other authors [14]) and are formed of condensed domains of the phospholipid.

The compression isotherms of PE show a kink around 15 mN/m (corresponding to the transition from liquid expanded to liquid condensed state) and a collapse pressure of 40 mN/m (Fig. 6a), as previously observed for different PE acyl derivates [15]. The presence of oleuropein in the subphase only produced a slight compression of the monolayer, with the elasticity values (Fig. 6b) denoting also a nonsignificant change. BAM images show at low pressure (Fig. 7a) some bright aggregates immersed in the

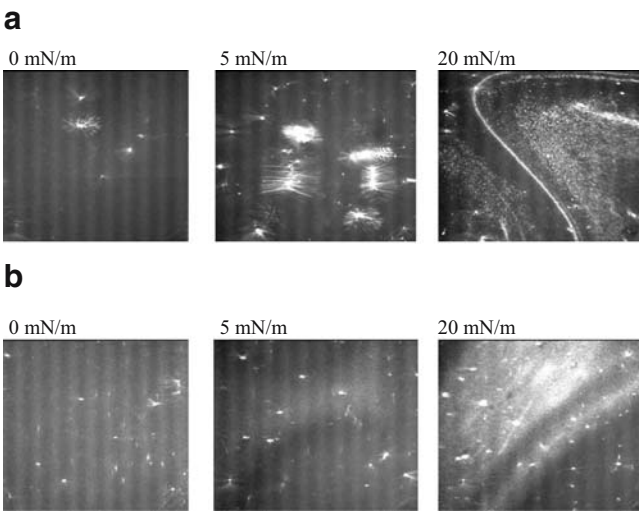

Fig. 7 Representative BAM images of PG monolayers at different surface pressures. The visual field is 570×570 μm. **a** Subphase water. **b** Subphase water + oleuropein 12 ppm

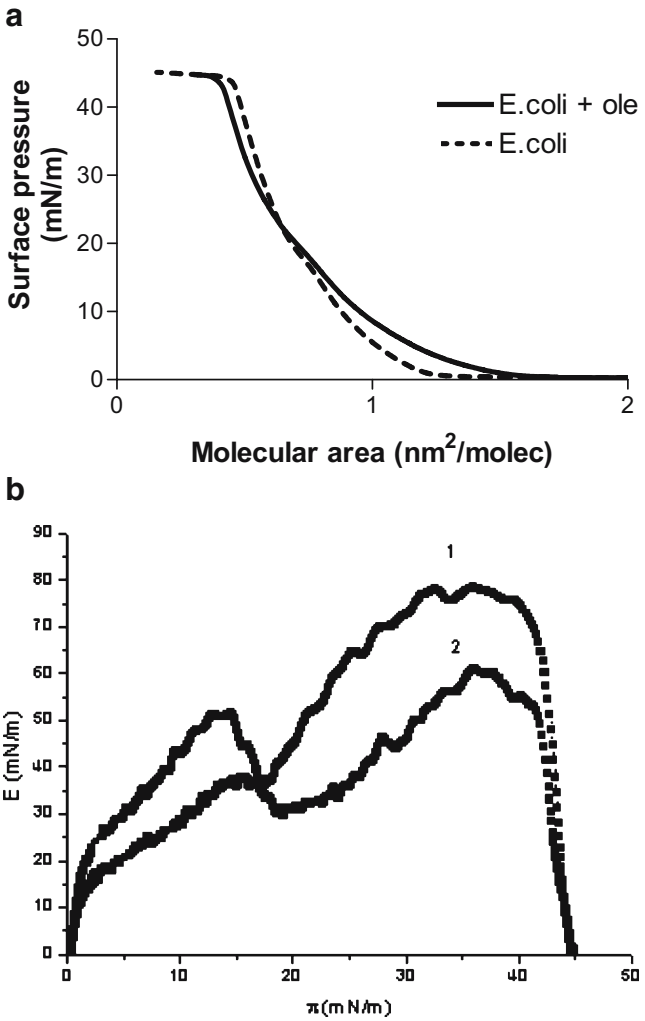

Fig. 8 **a** Compression isotherms of *E. coli* lipid extract on water subphase pH 6.0 and subphase containing oleuropein of 12 ppm. **b** Surface elasticity-surface pressure plots for *E. coli* monolayers on: *1* subphase water pH 6.0 and *2* subphase containing oleuropein 12 ppm

expanded phase (gray zone). As pressure increases, the domains grow in size and some of them have a dendritic shape, which has been related with the fact that the cohesion of the molecules are favored in a definite direction [9]. After the transition, the condensed domains undergo a process of aggregation that involves their fusion and enlargement until it reaches the collapse. When oleuropein is present in the subphase (Fig. 7b), it promotes a reduction in the area/molecule and the new isotherm run parallel to the one obtained in its absence, the collapse pressure being slightly reduced. In the images, microcondensations can be observed since low pressures; these condensations can be responsible for the area/molecule reduction observed. At all pressures studied, the images show a high degree of condensation, and the collapse region is visualized as broad stripes of high brightness.

In the study of *E. coli* lipid extract monolayer, the compression isotherm (Fig. 8a) showed a more expanded shape than PE, probably due to the presence of the other lipids in its composition. In addition, the presence of these lipids produces a smoothing effect on the phase change even though the collapse pressure does not change (45 mN/m). The images obtained using the BAM show a homogenous monolayer at low surface pressures, and bright domains are observed upon compression at higher pressures until the collapse is reached (Fig. 9a). When the isotherm was recorded with oleuropein of 12 ppm in the subphase (Fig. 8a), at low pressures, there is a small increase in the area/molecule. As the pressure values increase, the area/molecule of the isotherm becomes smaller than the one obtained without oleuropein, but the collapse pressure remains the same. The elasticity values showed a significant reduction (Fig. 8b).

BAM images suggest that with the increase of surface pressure, the condensed domains increase, and at the same time, some different tones of gray can be seen in the background. Oleuropein seems to produce upon compression not only a compressing effect on the monolayer but also some kind of disorganization in its structure.

Mixed monolayers oleuropein + lipids

Oleuropein did not form monolayers at the air/water interface, probably due to its high solubility in water.

Mixed monolayers of oleuropein + PG or *E. coli* lipid extract containing increasing quantities of oleuropein were spread at the air/water interface and compressed. The

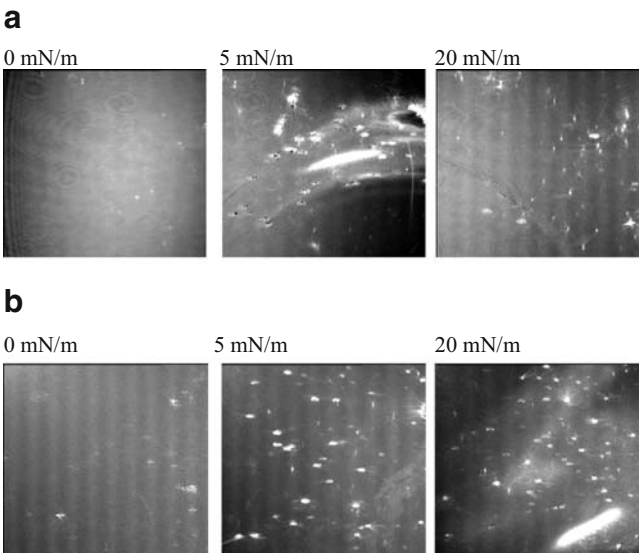

Fig. 9 Representative BAM images of PG monolayers at different surface pressures. The visual field is 570×570 μm. **a** Suphase water. **b** Subphase water + oleuropein 12 ppm

results obtained are shown in Fig. 10a (PG) and b (*E. coli* lipid extract). In both cases, it is clear that oleuropein dissolves into the subphase during the compression process. Isotherms shift to lower values of area/molecule upon increase of the oleuropein/lipid ratio due to the fact that only the lipid remains at the interface. This effect was confirmed by the BAM images: Monolayers with high content of lipid (e.g., PG 0.8/Ole 0.2) showed an expanded image at low pressures (Fig. 11a) where no bright domains could be observed, indicating that oleuropein did not diffuse into the lipid. Once the isotherm was compressed, condensed bright domains coexist between the liquid expanded phase (gray zones). When the proportion of oleuropein increases (Fig. 11b,c), it dissolves into the subphase, and only the lipid is visualized, forming bright-condensed domains in a water black background.

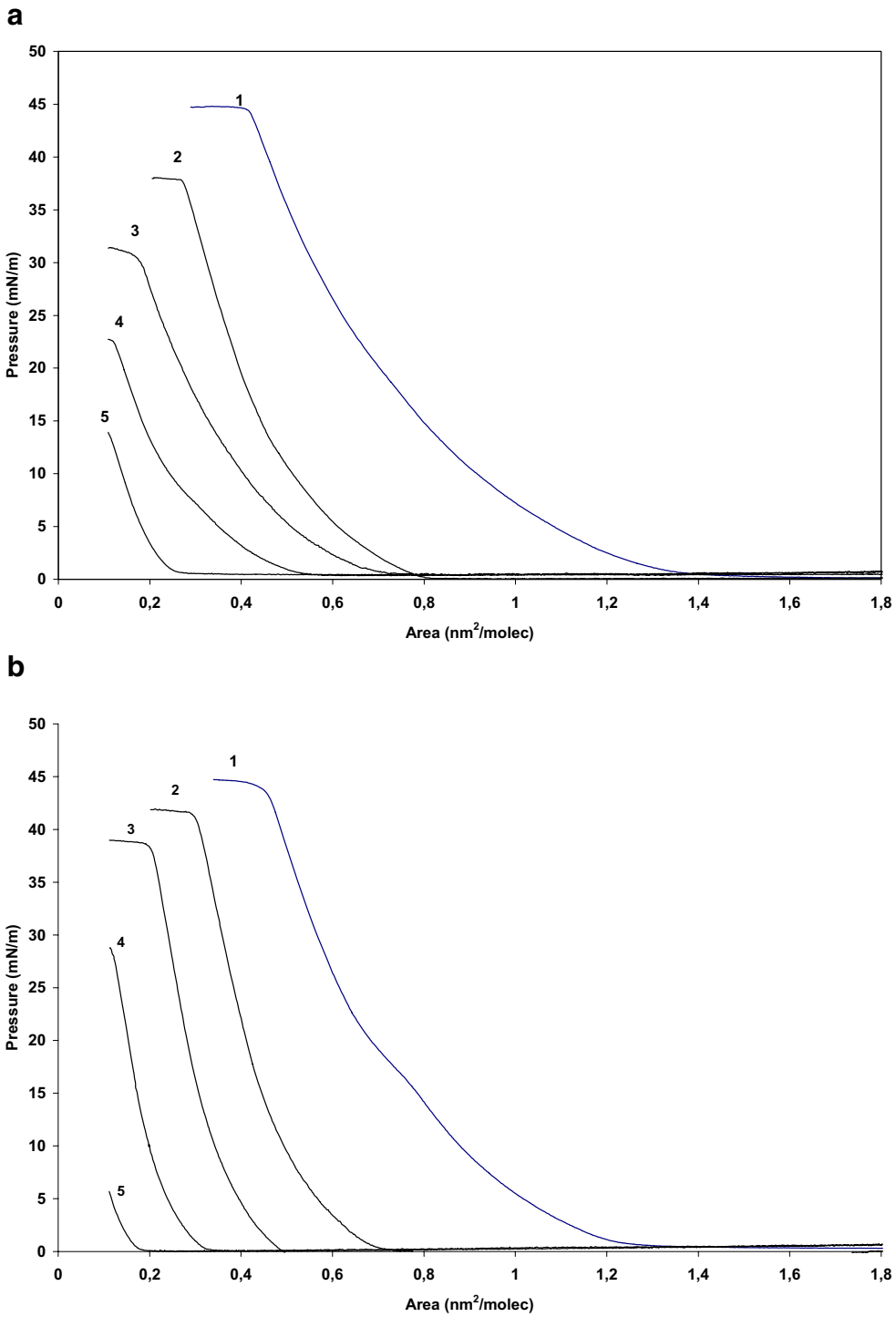

Fig. 10 π–A Langmuir isotherms of oleuropein/lipid mixed monolayers. *1* Lipid 1, *2* lipid 0.8/ole 0.2, *3* lipid 0.6/ole 0.4, *4* lipid 0.4/ole 0.6, *5* lipid 0.2/ole 0.8. **a** Lipid: PG. **b** Lipid: *E. coli* lipid extract

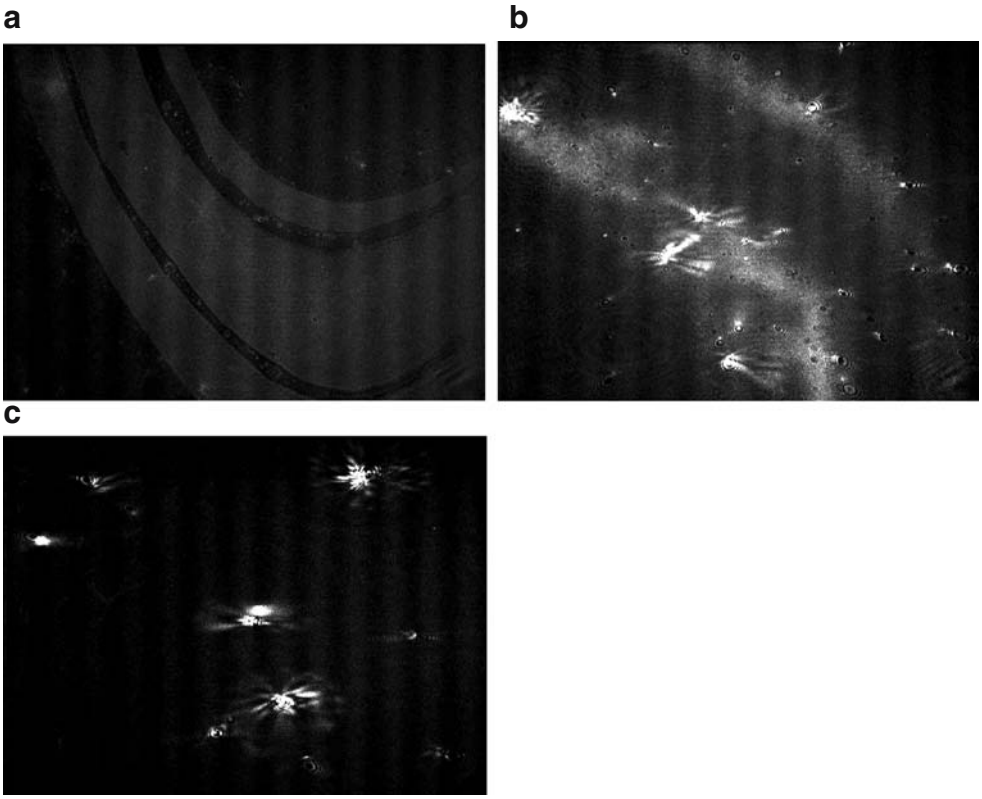

Fig. 11 BAM images for PG-oleuropein mixed monolayers. **a** PG 0.8/oleuropein 0.2 at 0 mN/m. **b** PG 0.8/oleuropein 0.2 at 15 mN/m. **c** PG 0.2/oleuropein 0.8 at 0 mN/m

Oleuropein showed interaction with lipid membranes of similar composition of those of *S. aureus* and *E. coli* at a concentration of 12 ppm; significantly lower than the minimum inhibitory concentration (MIC) for oleuropein was found by the different authors [2–4]. Oleuropein showed a moderate surface activity at the air/water interface due to its bivalent structure that shares hydrophilic and hydrophobic moieties. The increase of concentration leads to the formation of aggregates that get dissolved into the subphase. On the other hand, the concentration at which those aggregates are formed has been a limitation in the present study because it did not allow working at higher concentrations.

Kinetics at constant area showed that oleuropein presents some interaction with all the monolayers assayed even at high surface pressures. The low degree of interaction and that there are no great differences between the three monolayers could be indicative that these interactions are mainly hydrophobic, and these indicate an absence of specificity of oleuropein for the polar head group of the lipids assayed.

Compression isotherms showed that the interaction with PG monolayers is produced through the incorporation of oleuropein into the monolayer and that a compact structure is formed with a decrease in the elasticity. The strong effect of oleuropein on dimiristoyl phosphatidyl glycerol (DMPG) has already been detected using other techniques [6]. These authors suggested the formation of structures of high viscosity between oleuropein and DMPG bilayers at pH 5.5–7.4. These effects could promote the formation of aqueous pores and/or disruption effects in the bacterial membrane [16, 17].

The effects of oleuropein on PE monolayers were different; in this case, oleuropein produces a compacting effect on the film. These differences could be due to structural differences between PG and PE head group, as PE head group is known to be involved in intermolecular hydrogen-bounding interactions with PE adjacent molecules [18] that imply a higher hydration in PE. This major hydration has been related with a higher susceptibility to fuse. Therefore, the high repulsive forces between PE molecules could be a barrier for the incorporation of oleuropein into the monolayer. However, oleuropein could induce the formation of aggregates with PE molecules, and this aggregating effect is also detected in *E. coli* lipid extract monolayers, where PE is the major component. A possible explanation of this process could be that the presence of oleuropein reduces the repulsion forces between PE molecules. The presence of PG and cardiolipin in this extract has some influence, reducing the compacting effect of oleuropein on the monolayer. Therefore, oleuropein produces less disruption in the monolayer compared with PE alone.

The mixed monolayers showed that oleuropein is solubilized into the subphase and cannot mix with the lipids at the air/water interface. The glycosidic group of oleuropein seems to be the responsible of this low lipid solubility and has been related with its lower antimicrobial activity compared with hydroxytyrosol, which does not contain it [2].

Summing up, oleuropein has an important effect on PG monolayers, which correlates with its activity against *S. aureus*. The effects on *E. coli* would be less strong, which is not surprising as Gram-negative bacteria show lower sensitivity to certain antiseptics and antibiotics like oleuropein due to the more complex membrane composition. It seems clear that oleuropein antimicrobial actions, as pointed out by other authors, are likely exerted mainly at the cell surface [19, 20], through hydrophobic interactions between its elenoic hydrophobic moiety and the lipids, and do not depend on its lipid partitioning (Fig. 12a,b). Oleuropein is able to insert in some degree in the monolayer, especially in PG, and produces changes in it. The effects are not so clear for PE monolayers but some changes have been observed, whereas the presence of cardiolipin and PG in the *E. coli* lipid extract has only a small effect, perhaps reducing the compacting effect observed in PE.

More studies are under way to revel the exact mechanism of antimicrobial effect of this molecule using other techniques as fluorescence and other work conditions (subphase compositions) in view that the susceptibility of different bacteria to oleuropein is related to the culture media composition [2, 4].

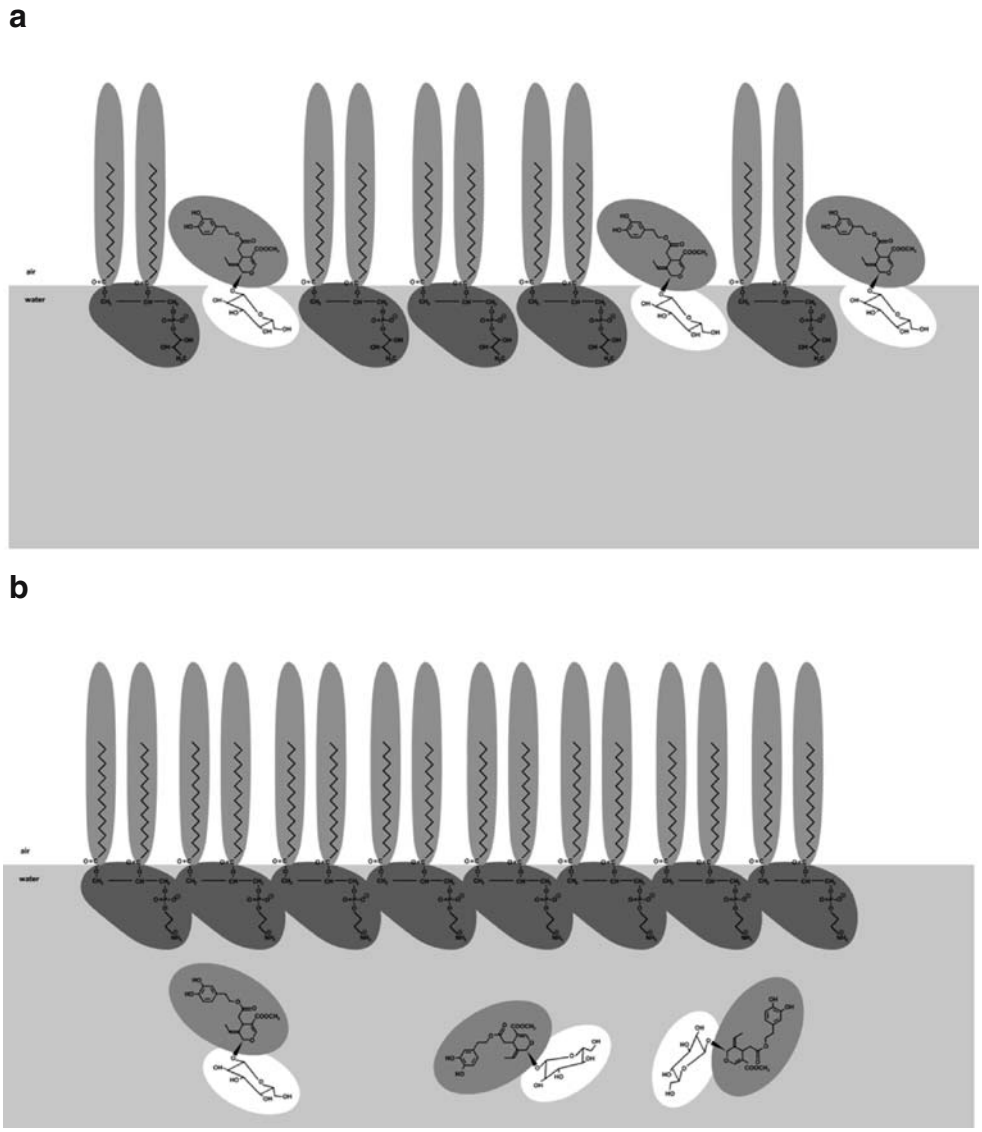

Fig. 12 Scheme of the possible orientation of oleuropein in the subphase and interaction with lipid monolayers. **a** PG. **b** PE

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