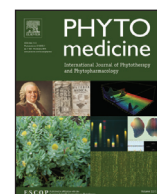


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## A *Myrtus communis* extract enriched in myrtucummulones and ursolic acid reduces resistance of *Propionibacterium acnes* biofilms to antibiotics used in acne vulgaris



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### ABSTRACT

**Background:** Recent works present evidence of *Propionibacterium acnes* growing as a biofilm in cutaneous follicles. This formation of clusters is now considered as an explanation for the *in vivo* resistance of *P. acnes* to the main antimicrobials prescribed in acne vulgaris.

**Purpose:** Our objective was to explore this hypothesis and propose a new therapeutic approach focusing on anti-biofilm activity of Myrtacine<sup>®</sup> New Generation (Mediterranean Myrtle extract–Botanical Expertise P. Fabre) alone or combined with antibiotics.

**Methods/Results:** Using *in vitro* models able to promote the growth of adhered bacteria, the loss of sensitivity of *P. acnes* biofilms (48 h) towards erythromycin and clindamycin was checked considering either sensitive or resistant strains. In the same time, the activity of Myrtacine<sup>®</sup> New Generation against biofilm formation and mature biofilm (48 h) was evaluated. Using a dynamic model of biofilm formation, we noted an inhibition of biofilm formation (addition of Myrtacine<sup>®</sup> New Generation at T 0) and a significant effect on mature biofilm (48 h) for 5 min of contact. This effect was also checked using the static model of biofilm formation for Myrtacine<sup>®</sup> New Generation concentrations ranging from 0.03% to 0.0001%. A significant, dose-dependent anti-biofilm effect was observed and notable even at a concentration lower than the active concentration on planktonic cells, *i.e.* 0.001%. Finally, the interest of the combination of Myrtacine<sup>®</sup> New Generation with antibiotics was explored. An enhanced efficacy was noted when erythromycin (1000 mg/l) or clindamycin (500 mg/l) was added to 0.001% Myrtacine<sup>®</sup>, leading to significant differences in comparison to each compound used alone.

**Conclusion:** The efficiency of Myrtacine<sup>®</sup> New Generation on *P. acnes* biofilm alone or combined with antibiotics was demonstrated and can lead to consider it as a potent adjunctive product efficient during the antibiotic course for acne vulgaris treatment.

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### Introduction

*Propionibacterium acnes* is a Gram positive bacillus that is part of the human ecosystems (Grice and Segre 2011). *P. acnes* resides in pilosebaceous follicles of the skin, colonizing the lipid-rich sebaceous glands. As an opportunist, *P. acnes* is well recognized as being involved in the inflammation process of acne vulgaris (Burkhardt and Gottwald 2003; Li et al. 2014), a common skin disorder that

affects most individuals at some point in their lives, and other chronic diseases. More recently, it has become recognized as the cause of foreign-body infections (Portillo et al. 2013) by devices such as breast implants (Del Pozo et al. 2009), neurosurgical shunts (Conen et al. 2008), cardiovascular devices (Delahaye et al. 2005), ocular implants, internal fracture fixation devices, spinal hardware, and prosthetic joints (Levy et al. 2013; Piper et al. 2009; Portillo et al. 2013).

At first, *P. acnes* was considered to be susceptible to a wide range of antibiotics, including clindamycin,  $\beta$ -lactams and quinolones (Hoeffler et al. 1976). However, in the last decade, the rate of antibiotic-resistant bacteria has increased, especially for

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macrolides and tetracyclines (Dumont-Wallon et al. 2010; Ozolins et al. 2004) but also rifampicin (Furustrand Taffin et al. 2015). Although *P. acnes* virulence is reported to be low, recent studies using genomic approaches and *in situ* observations have allowed a better understanding of this pathogen's importance in chronic and recurrent infections, focusing on the ability of *P. acnes* to produce a biofilm on implant devices (Holmberg et al. 2009), and also in acne (Jahns and Alexeyev 2014; Jahns et al. 2012). The loss of sensitivity of microorganisms in a biofilm to the main antimicrobials could explain both treatment failures and the fact that no optimal treatment regimen of *P. acnes* biofilm infections has yet been defined, in either implant-associated infections (Furustrand Taffin et al. 2015) or acne (Coenye et al. 2007).

In 2009, James et al. reviewed the current acne treatments, and underlined significant side effects of isotretinoin and rising antibiotic resistance (James et al. 2009). Recent advances in the pathogenesis of acne and inflammatory mechanisms and the role of *P. acnes* as biofilms led to new targets being considered. New molecules with potent *P. acnes* anti-biofilm efficacy were thus investigated. Some plant extracts or their active components have already been described as anti-biofilm even at subinhibitory concentrations (Coenye et al. 2012). Studies of the pharmacological properties of *Myrtus communis* L. (Myrtaceae) demonstrated its antibacterial efficiency against *P. acnes* strains that may be linked to myrtucommulones (Alipour et al. 2014; Fiorini-Puybaret 2011).

The present study continues along these lines by studying the efficacy of an isopropyl acetate extract prepared from leaves of *M. communis* (Myrtacine® New Generation), previously demonstrated to be effective on *P. acnes* planktonic cells (Alipour et al. 2014; Fiorini-Puybaret 2011), in combating *P. acnes* biofilm. For this purpose, we validated *in vitro* biofilm models (dynamic or static) in which growth of adherent *P. acnes*, but not of planktonic cells, was promoted. Myrtacine® New Generation was tested alone or in combination with the most current topical antibiotics used in acne treatment (erythromycin and clindamycin). The interest of this extract and its association with antibiotics was checked on *P. acnes* strains presenting sensitivity or acquired resistance to erythromycin and clindamycin.

## Materials and methods

### Plant material

Leaves of *M. communis* were collected in Morocco in 2008 (Region around Fes). Identification of this plant was confirmed by Dr. Jean Gabriel Fouche (Institut de Recherche Pierre Fabre). A voucher specimen (N°16280) has been deposited at the Conservatoire Botanique Pierre Fabre, (Cambounet-sur-Sor, France).

### Extraction

Myrtacine® New Generation is a lipophilic dry extract from leaves of *M. communis* produced by the Pierre Fabre Company (Fiorini-Puybaret 2011). It was prepared from dried powdered leaves using isopropyl acetate (ratio 1:10) at room temperature for 2 h. After filtration, the residue was treated a second time using the same method and the two fractions were pooled. After the removal of chlorophyll using activated charcoal (1% w/w) and essential oil by hydro-distillation, the filtrate was concentrated under vacuum at 40 °C. The residue was resuspended in water then dried by lyophilisation. Myrtacine® New Generation was obtained as a yellow powder with a yield of 2% w/w. The extract was stored in the dark at 4 °C.

The aqueous solutions tested were prepared extemporaneously with dimethylsulphoxide (maximum concentration 10%).

### Quantification of the ursolic acid and the myrtucommulones

HPLC analyses were carried out on a Merck/Hitachi LaChrom HPLC system comprising a L7420 PDA detector and a L7200 pump. Myrtucommulones B' (5-déméthylsemimyrtucommulone) S (semimyrtucommulone), IsoS (isosemimyrtucommulone), A (myrtucommulone A) and ursolic acid were titrated by analytical HPLC performed with a column Symmetry® C8 (Waters), 5 µm, 245 mm x 4.6 mm using a gradient with 0.1% aqueous trifluoroacetic acid 0.1% (eluent A) and a mixture of acetonitrile and trifluoroacetic acid 0.1% (eluent B) with the following program: 0 min: 78% B; 15–25 min: 100% B; 26–35 min: 78% B. Spectral UV data from the peaks were accumulated in the range 210–400 nm. Detection with the diode array was performed at 280 nm for myrtucommulones and 210 nm for ursolic acid. The temperature of the column was maintained at 20 ± 5 °C. The flow rate of the mobile phase was 1 ml/min. The injection volume was 10 µl and the samples were prepared in the mixture dichloromethane-ethanol: 1-1 (v/v). 2-methylanthraquinone (2-MAQ) was used as an external standard. The response factor of the myrtucommulone B' (3.1) and ursolic acid (12.7) relative to the 2-MAQ were defined with pure myrtucommulone B' and ursolic acid previously purified (unpublished work). Using these response factors the respective response factors of ursolic acid and myrtucommulones relative to myrtucommulone B' in the extract were calculated. Under these conditions the 2-methylanthraquinone, ursolic acid, myrtucommulones B', S, IsoS and A were eluted at 5.3 min, 9.9 min, 9.3 min, 10.4 min, 11.1 min and 21.9 min respectively (Suppl. Figs. 1 and 2, Supplementary content). Myrtucommulones and ursolic acid were quantified by HPLC. Their contents in the batch ES 120 used for this study were respectively 8.1% (w/w) and 20% w/w.

### Antibiotics

Erythromycin and clindamycin were obtained from Sigma Aldrich (Saint-Quentin Fallavier, France) and were dissolved in ethanol (5% final maximum concentration) or water respectively.

### Minimal medium for biofilm formation

In preliminary studies (Khalilzadeh et al. 2010; Samrakandi et al. 1997), we demonstrated that MBB (Modified Biofilm Broth) was able to promote the growth of adherent cells but not of planktonic ones. The selected minimal medium consisted of MgSO<sub>4</sub> 7H<sub>2</sub>O (0.2 g/l), FeSO<sub>4</sub> 7H<sub>2</sub>O (0.0005 g/l), anhydrous Na<sub>2</sub>HPO<sub>4</sub> (1.25 g/l), KH<sub>2</sub>PO<sub>4</sub> (0.5 g/l), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.1 g/l) and glucose (0.05 g/l). The ability of *P. acnes* to form a biofilm using this medium was checked in dynamic and static models.

### Selection of *P. acnes* strains

Reference strains (Institute Pasteur Collection, Paris, France) and cutaneous isolates (24 strains) from the lab collection and Pierre Fabre DermoCosmetics collection were screened for their susceptibility to erythromycin and clindamycin as planktonic cells. Strains were preserved in Eugon broth complemented with 10% glycerol at -80 °C. Before each experiment, two subcultures on Columbia sheep blood agar (bioMérieux, Craponne, France) were performed for 48 h at 36 °C, under anaerobic conditions (Anoxomat Mart system, Mart Microbiology B.V., Netherlands). MICs were determined by a broth dilution micromethod according to EUCAST and CASFM recommendations ([www.sfm-microbiologie.org](http://www.sfm-microbiologie.org)). Briefly, solutions under assay were diluted in microtitre plates in Mueller Hinton broth (bioMérieux, Craponne, France) supplemented by 10% foetal bovine serum (Dutscher, Brumath, France) to a final volume of 100 µl. Microbial suspensions were prepared in tryptone salt

solution at about  $10^8$  CFU/ml to obtain final inocula of  $10^6$  CFU/ml in wells. Microtitre plates were incubated for 48 h at 36 °C, under anaerobic conditions. The MIC was then defined as the concentration at which no macroscopic sign of cellular growth was detected in comparison with the control without antimicrobial compound (column 12). Column 11 was free of inoculum and checked the sterility conditions. The MBCs were determined by sub-cultivating on Columbia sheep blood agar plates after incubating as described above. The MBC was defined as the compound concentration at which no macroscopic sign of cellular growth was detected in comparison to the control without antimicrobial compound.

All the experiments were carried out in duplicate at each concentration. In order to ensure that dimethylsulphoxide and ethanol *per se* did not interfere with the antimicrobial activity of the products under assay, a control test was also carried out on inoculated broth supplemented with only dimethylsulphoxide and ethanol at the same concentration used in the assays.

#### Bactericidal activity on planktonic cells in MBB

To assess the bactericidal activity of Myrtacine® New Generation (0.03% to 0.0001% w/v) and antibiotics (erythromycin: 0.1 mg/l to 1000 mg/l; clindamycin: 10 mg/l to 500 mg/l) on non-growing planktonic cells in MBB,  $10^5$  CFU/ml were maintained under agitation (100 rpm) and anaerobic conditions at 36 °C for 72 h in the presence or absence of each product. Cell quantification was carried out by culture of samples (24 h, 48 h and 72 h). The samples were homogenized and serially diluted (10-fold dilutions), and 100  $\mu$ l of each dilution were spread on Columbia sheep blood agar and incubated at 36 °C under anaerobic conditions for 5 days.

#### Biofilm formation

##### Dynamic model

*P. acnes* biofilms were obtained by a previously described procedure (Samrakandi et al. 1997). The MBB was circulated through a sterile loop of Tygon® tube (Fischer Scientific SAS, Illkirch, France, inner diameter, 6.4 mm) at 100 ml/min. The loop was maintained at 30 °C and was connected to a discharge line and to a feeding tank (supply pump; feeding rate, 3 ml/min). After the bioreactor had been filled with the adhesion broth, the loop was inoculated with 5 ml of a bacterial suspension containing about  $10^8$  CFU/ml. The circulation pump was run for 30 min to allow the cells to spread around the loop before the supply pump was turned on. After 48 h, stabilized and reproducible populations of adherent and evacuated bacteria were obtained for the tested strain.

The adherent cells were recovered by scraping them off samples of the Tygon tube (2-cm pieces cut in half lengthwise) with a sterile cutter in 10 ml distilled water. The portions of the Tygon tube and the corresponding suspension were then dispersed for 1 min with a vortex mixer. Viable bacterial counts (log CFU/cm<sup>2</sup>) were determined by spreading of duplicate serial dilutions of homogenized samples on Columbia sheep blood agar. The agar plates were incubated anaerobically as described above. Results are expressed as log CFU/cm<sup>2</sup> and the quantification limit was considered to be 0.4 log. Planktonic population was also evaluated.

##### Static model

Bacterial suspensions of the tested strains were prepared and diluted in each tested medium in order to obtain a concentration of either  $10^5$  CFU/ml. Two millilitres of bacterial suspensions were added to the wells of 24-well polystyrene microplates (BD Falcon, San Jose, CA, USA). Microplates were incubated statically at 36 °C (to check *P. acnes* optimal culture and *in vivo* conditions), under anaerobiosis. During the course of incubation and when products were added, the medium was renewed, after two

gentle rinses, in order to eliminate non-adherent/released bacteria from the biofilm and favour sessile growth. Before biofilm collection, planktonic cells were sampled by pipetting directly in the bulk phase. After two successive rinses with 2 ml of sterile distilled water (SDW), 1 ml of SDW was added and the well was scraped with a sterilized spatula for 1 min in order to detach biofilm cells. Numerations of viable cells (adherent and planktonic) were performed as previously described. Results are expressed as log CFU *per well* and the quantification limit was considered to be 1.3 log.

#### Biofilm treatments

Myrtacine® New Generation concentrations were selected according to the maximum concentration in the marketed product and were between 0.1% and 0.0001% (w/v).

Antibiotic concentrations were defined by considering MIC/MBC values and lack of bactericidal activity on planktonic cells in MBB, *i.e.* erythromycin: 1000 and 500 mg/l for erythromycin resistant strains and 10 and 0.1 mg/l for erythromycin sensitive strains and 500, 100 and 10 mg/l for clindamycin.

Myrtacine® New Generation was first evaluated for (i) preventive treatment, which consisted of an addition of the product (0.001%, 0.01% and 0.1% w/v) at the time of inoculation of the dynamic biofilm model (T 0) and numeration of adherent and planktonic cells at 5 h, 24 h and 48 h and (ii) curative treatment, which consisted of an addition of the product (0.1%, 0.01% and 0.001% w/v) on a 48 h biofilm with circulation for 1 min and 5 min.

To explore the anti-biofilm activity of Myrtacine® New Generation and the interest of using it in association with antibiotics, we considered a treatment consisting of an addition of the products (Myrtacine® New Generation associated or not with antibiotics) to a 48 h biofilm for 24 h of contact using the static model.

#### Statistical analysis

Comparisons between data sets were performed using paired *t*-test and significant differences were defined for  $p < 0.05$  and  $p < 0.01$ .

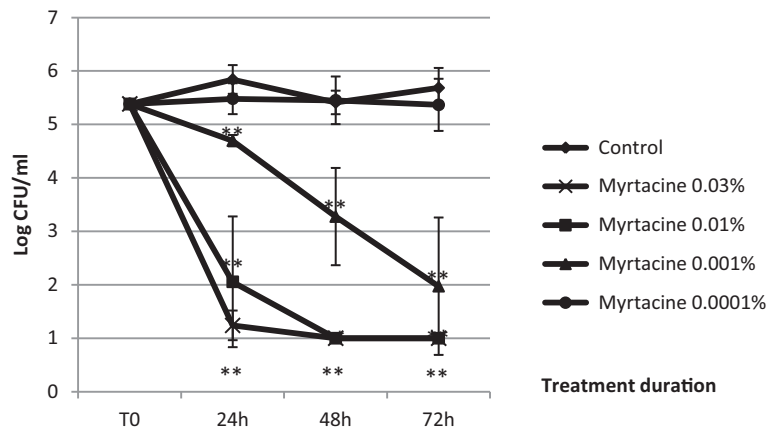
## Results

#### Selection of *P. acnes* strains

Table 1 presents the MICs and MBCs for the selected cutaneous isolates and the reference strains showing their level of susceptibility to erythromycin and clindamycin. *P. acnes* CIP 53.117T is sensitive to both molecules. *P. acnes* CIP 110.371 is described as resistant to erythromycin and also presents reduced susceptibility to clindamycin. Among wild cutaneous strains, the selection includes a sensitive strain (B872), and two strains characterized by resistance to erythromycin and clindamycin (R4 and R3.6). No

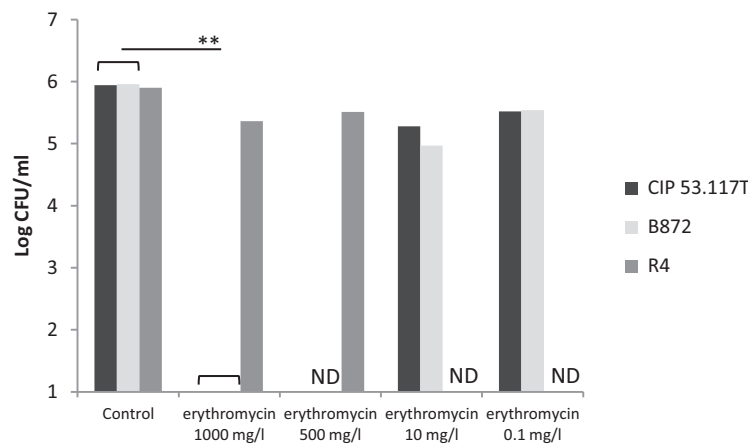
**Table 1**  
MIC/MBC values (mg/l) of erythromycin and clindamycin for the selected strains.

	Erythromycin		Clindamycin	
	MIC	MBC	MIC	MBC
<i>P. acnes</i> CIP 53.117T	0.016	0.016	0.03	0.03
<i>P. acnes</i> CIP 110.371	>500	>500	2	2
<i>P. acnes</i> B872	0.008	0.008	0.03	0.03
<i>P. acnes</i> R4	>500	>500	62.5	62.5
<i>P. acnes</i> R3.6	>500	>500	62.5	62.5



**Fig. 1.** Evaluation of bactericidal activity (log CFU/ml) of Myrtacine® new generation against planktonic eryS/clindaS (CIP 53.117T; B872) and eryR/clindaR (CIP 110.371; R4) strains in MBB according to the contact time (T 0 to T 72 h).

\*\* $p < 0.01$ .



**Fig. 2.** Evaluation of bactericidal activity (log CFU/ml) of erythromycin against planktonic eryS (CIP 53.117T and B872) and eryR (R4) strains in MBB after 24 h of contact. ND, not determined.

\*\* $p < 0.01$ .

antibacterial effect was noted for dimethylsulphoxide and ethanol at the highest final concentrations used (data not shown).

#### Bactericidal activity on planktonic cells in MBB

*P. acnes* strains were exposed to Myrtacine® (from 0.0001 to 0.03%) for 72 h. The evolution of bacterial concentrations according to the contact time and the Myrtacine® New Generation concentration in MBB is indicated in Fig. 1. A bactericidal effect can be noted starting from 24 h of contact with the highest concentrations (0.03%, 0.01% and 0.001% w/v). At 0.001% (w/v), a significant time-dependent reduction is observed but no clearance expected for *P. acnes* B872. The initial planktonic *P. acnes* population was preserved in the presence of 0.0001% Myrtacine® New Generation even after a 72 h of contact. Most importantly similar behaviours were observed among strains, regardless of their susceptibility to erythromycin or clindamycin according to MIC/MBC (Table 1) determination.

When strains were in contact with erythromycin in MBB for 24 h at 10 or 0.1 mg/l (Fig. 2), no bactericidal activity was noted even for susceptible strains (MBC  $\leq$  0.016 mg/l) while the 1000 mg/l concentration led to a total reduction of the strains. For the R4 resistant strain, no bactericidal effect was detected, even at the 1000 mg/l concentration.

Clindamycin had no bactericidal effect on the tested strains even at 500 mg/l (Fig. 3) and even on susceptible strains in the assay conditions.

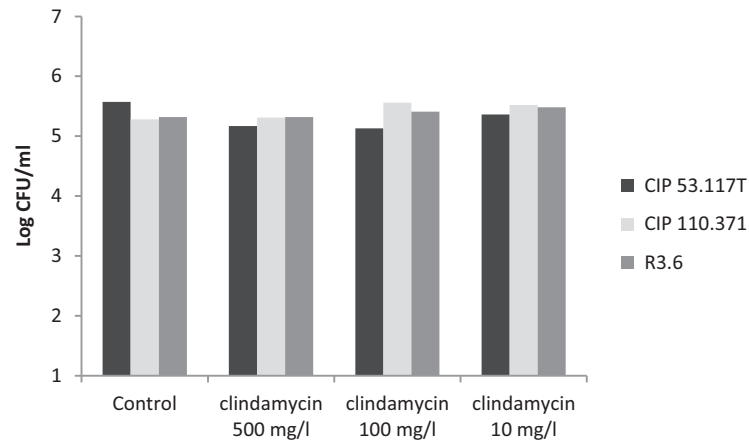
The preservation of *P. acnes* viability in MBB without cell proliferation was demonstrated by the CFU values for the control (Figs. 1–3).

#### Anti-biofilm activity

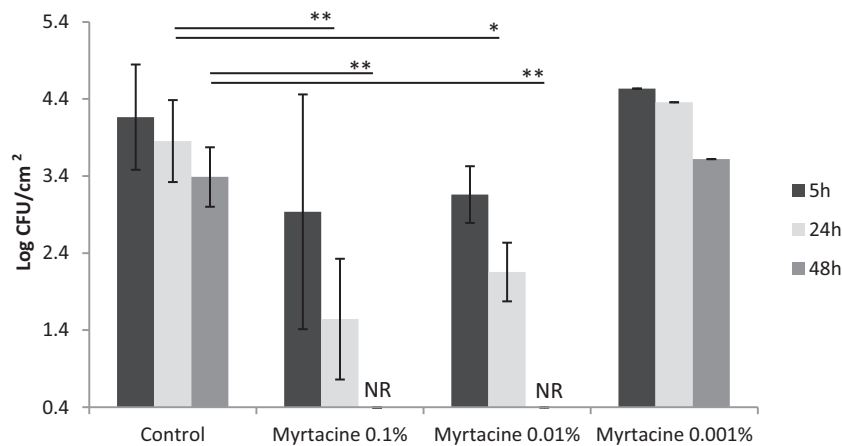
##### Dynamic model

**Activity of Myrtacine® New Generation from the adhesion step.** Fig. 4 presents the CFU numeration of adherent cells/cm<sup>2</sup> (*P. acnes* CIP 53.117T) when Myrtacine® New Generation (0.1%, 0.01% or 0.001% w/v) was added or not at T 0 in the broth medium. In such conditions, Myrtacine® New Generation induced a dose-dependent anti-biofilm effect decreasing from 0.1% to 0.001%, with no significant effect at the lowest concentration. This effect was also time-dependent, with a significant increase of activity from 24 h to 48 h of contact (0.1% and 0.01% w/v). At high Myrtacine® New Generation concentrations, a significant reduction in planktonic cells (cells released from the biofilm), of about 2 to 3 log, was also observed (data not shown).

**Activity of Myrtacine® New Generation on a mature biofilm.** Assays were performed on a 48 h *P. acnes* CIP 53.117T biofilm. Myrtacine® New Generation contact was achieved by the addition and circulation of solutions for 1 min and 5 min. After rinsing, the residual adherent cells were counted (Fig. 5). We observed a significant dose-range effect leading to a global reduction of the

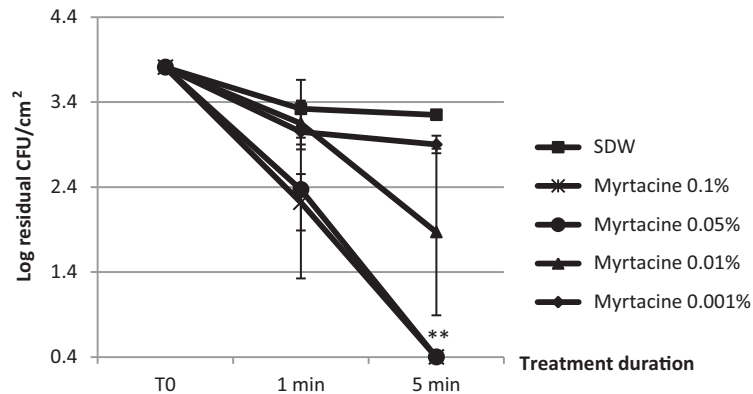


**Fig. 3.** Evaluation of bactericidal activity (log CFU/ml) of clindamycin against planktonic clindaS (CIP 53.117T) and clindaR (CIP 110.371 and R3.6) strains in MBB after 24 h of contact.



**Fig. 4.** Number of adherent cells (log CFU/cm<sup>2</sup>; n = 2; mean ± SD) when Myrtacine® new generation is added for T 0 (*P. acnes* CIP 53.117T, dynamic model) according to the contact time (5 h, 24 h and 48 h) and the concentration (0.1%, 0.01% and 0.001%). NR, no recovery.

\* p < 0.05 \*\*p < 0.01.



**Fig. 5.** Number of residual adherent cells (log CFU/cm<sup>2</sup>; n = 2; mean ± SD) after treatment of a 48 h biofilm (*P. acnes* CIP 53.117T; dynamic model) with Myrtacine® new generation according to the contact time (0 mn, 1 mn and 5 mn) and the concentration (0.1%, 0.01% and 0.001%). SDW = control.

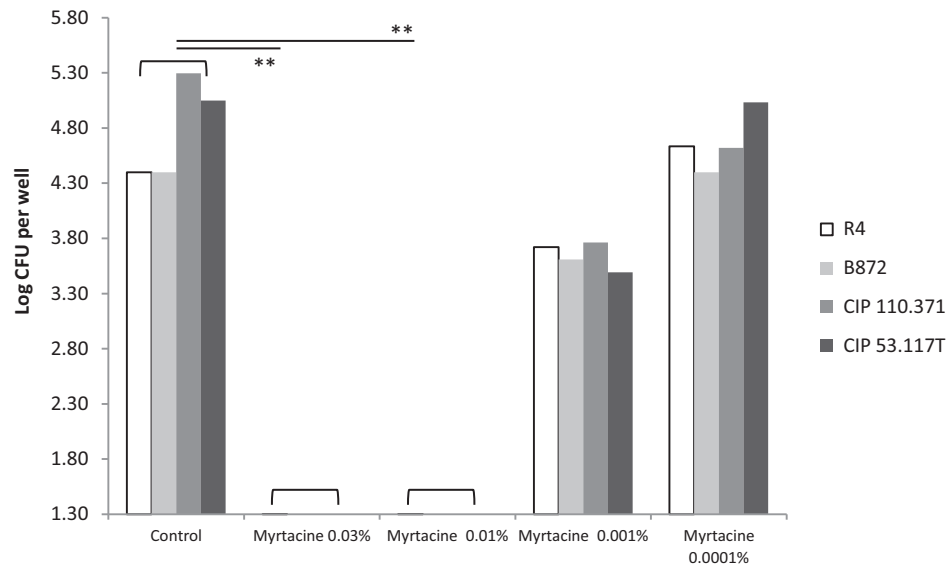
\*\* p < 0.01.

adherent population at the highest concentration 0.1% (w/v) compared to the control (addition and circulation of sterile distilled water only).

**Static model**

**Myrtacine® New Generation anti-biofilm activity.** In static conditions, assays were performed by the introduction of Myrtacine® New Generation (0.03%, 0.01%, 0.001% and 0.0001% w/v) on a ma-

ture biofilm (48 h old) with a contact time of 24 h. In such conditions, a significant dose-dependent effect was observed, greater with the dynamic model, with a progressive loss of activity to the 0.0001% (w/v) concentration (Fig. 6). The assay was performed on 4 different strains and revealed similar behaviour regardless of their susceptibility to erythromycin and clindamycin. A total reduction of the planktonic viable population was noted at the highest concentrations tested (0.03% and 0.01% w/v, data not shown),



**Fig. 6.** Number of residual adherent cells (log CFU per well) for 4 *P. acnes* strains (*P. acnes* CIP 53.117T eryS/clindaS; *P. acnes* B872 eryS/clindaS; *P. acnes* CIP 110.371 eryR/clindaR; *P. acnes* R4 eryR/clindaR) after addition of Myrtacine® new generation (0.03%, 0.01%, 0.001% and 0.0001%) on a 48 h biofilm for a 24 h contact (static model). \*\*,  $p < 0.01$ .

in correlation with our previous results on planktonic cells in MBB (Fig. 1). This bactericidal effect decreased from 0.01% to 0.0001% (w/v).

#### Myrtacine® New Generation anti-biofilm activity when combined with antibiotics.

**Myrtacine® New Generation + erythromycin.** Assays were performed as indicated above, with a contact time of 24 h on a mature biofilm (48 h) for *P. acnes* CIP 53.117T (Fig. 7a) and *P. acnes* CIP 110.371 (Fig. 7b). The first observation was a dramatic loss of bactericidal activity of erythromycin alone on *P. acnes* biofilm regardless of the susceptibility of planktonic cells to erythromycin (MICs), with little or no reduction of adherent cells even at 1000 mg/l. The difference between susceptible and resistant strains was seen only on the planktonic population for the resistant strain (*P. acnes* CIP 110.371), significantly higher than for the sensitive strain.

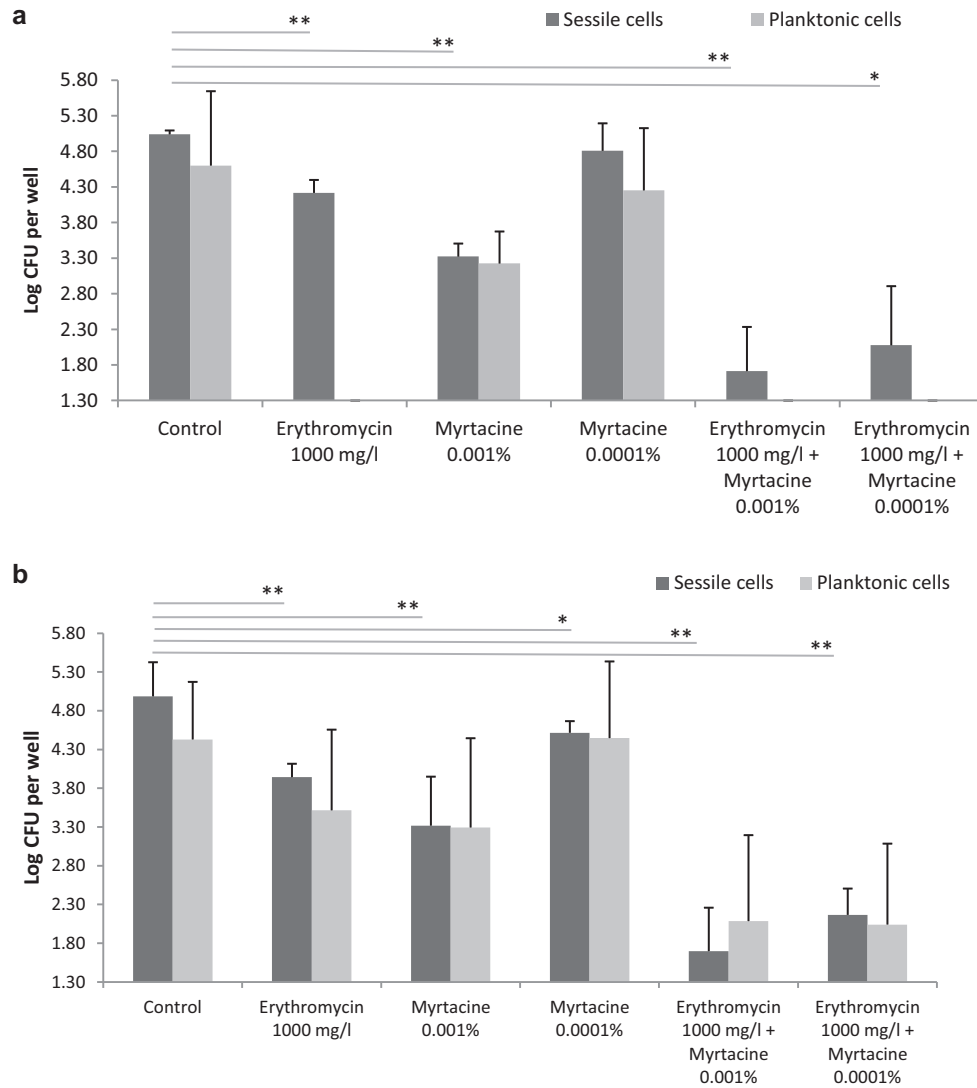
To detect a possible interaction between Myrtacine® New Generation and antibiotic, solutions of Myrtacine® New Generation (0.001% and 0.0001% (w/v)) were added to the biofilm after 48 h of biofilm formation for a 24 h contact time. When Myrtacine® New Generation was added to erythromycin at 1000 mg/l, a significant reduction of the sessile population was noted in comparison to each product alone, to a supplementary reduction of about 1.5 to 2 log. This effect concerned the 0.001% Myrtacine® concentration and also the 0.0001% one, which was considered as a non-active concentration when Myrtacine® New Generation was tested alone. This high enhancement of activity was also significant on the planktonic population, with detection of viable cells for the resistant strain (*P. acnes* CIP 110.371; Fig. 7b) and no detection for the susceptible strain (*P. acnes* CIP 53.117T; Fig. 7a).

**Myrtacine® New Generation + clindamycin.** Following the same experiments, sessile *P. acnes* was exposed to clindamycin in combination with Myrtacine® New Generation. Both sensitive and resistance strains (Fig. 8a) CIP 53.117T and (8b) CIP 110.371 were very resistant to clindamycin alone (500 mg/l) even on the planktonic population (more than 3 log CFU per well after treatment). This last observation confirmed previous results on planktonic cells in MBB (Fig. 3). When Myrtacine® New Generation 0.001% (w/v) was combined with clindamycin, we observed a marked improvement in anti-biofilm activity (reduction gain 1.5 log) on both strains

with little or no detection of residual planktonic cells. For the 0.0001%/500 mg/l association, the effect was not higher than that of each product alone.

## Discussion

*P. acnes* is frequently considered as highly susceptible to a wide range of antibiotics, including  $\beta$ -lactams, quinolones, macrolides, clindamycin and rifampicin, even if its resistance is increasing, especially to clindamycin and erythromycin. At the moment, infections linked to this opportunistic pathogen, such as invasive infections associated with implants but also acne vulgaris, require prolonged antibiotic treatment (Achermann et al. 2014) without guaranty of efficiency. Among the virulence factors described, the ability of *P. acnes* to form biofilm has recently been considered as a major explanation for antibiotic susceptibility loss and implicated in the inflammation process (Li et al. 2014). *P. acnes* biofilm formation in follicles is actually well described in healthy subjects as well as in those with acne vulgaris (Jahns and Alexeyev 2014) with a higher prevalence for the last group (Jahns et al. 2012). In such conditions, the main antimicrobial treatment in acne vulgaris, including erythromycin, tetracyclines, clindamycin, and also salicylic acid or benzoyl peroxide were described as being unable to totally reduce *P. acnes* biofilm *in vitro* (Coenye et al. 2007). The present results confirm the low activity of erythromycin and clindamycin on *P. acnes* biofilm even at high concentrations, leading to similar reduction of biofilm populations regardless of the strain susceptibility defined by MIC determination. Assays performed on MBB confirmed that, in conditions that prevent planktonic cell proliferation, clindamycin has no bactericidal effect, even on sensitive strains and at high concentration (500 mg/l). Similar observations have been reported by Furustrand Tafin et al. (2012) with low MICs but high MBCs underlying the only bacteriostatic effect of clindamycin and low activity on *P. acnes* biofilm. Besides, erythromycin is effective at 1000 mg/l but only against sensitive strains. Despite differences between the two antibiotics bactericidal effect on non-growing cells, *P. acnes* biofilms are always resistant. These results are in accordance with previous experiments performed by Coenye et al. (2007) using microtitre plate crystal violet assay. The



**Fig. 7.** Number of residual adherent and planktonic cells (log CFU per well;  $n = 3$ ; mean  $\pm$  SD) when Myrtacine<sup>®</sup> and erythromycin are combined or not on a 48 h biofilm after a 24 h contact according to the concentrations on (a) *P. acnes* CIP 53.117T (eryS/clindaS) and (b) *P. acnes* CIP 110.371 (eryR/clindaR) (static model).

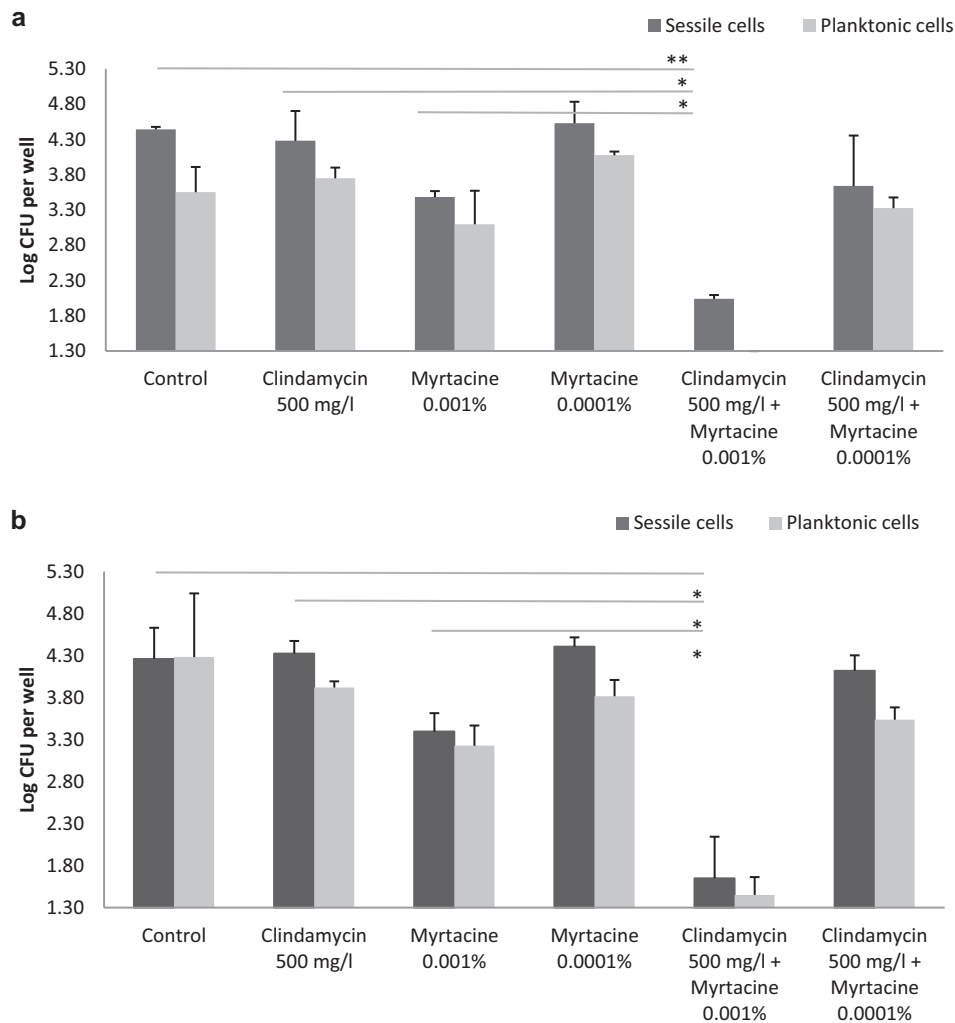
\* $p < 0.05$  \*\* $p < 0.01$ .

authors noted the low activity of erythromycin and clindamycin, even at high concentrations, of 0.5% and 1% (w/v) respectively.

Myrtacine<sup>®</sup> New Generation is a lipophilic extract of the leaves of *M. communis* 6 times richer in myrtucommulones and ursolic acid than the ethanolic extract Myrtacine<sup>®</sup> (Fiorini-Puybaret 2011). These active markers showed anti-inflammatory and antibacterial *P. acnes* activities (Fiorini-Puybaret 2011). The batch ES120 used for this study contained 8.07% (w/w) of myrtucommulones and in particular 3.08% (w/w) of B', 1.76% (w/w) of S, 3.25% (w/w) of isoS and 0.32% (w/w) of A.

The first interest of Myrtacine<sup>®</sup> New Generation is its antimicrobial activity on *P. acnes* planktonic cells and also on biofilms. The antibacterial activity of traditional herbal medicines or extracts, especially against *P. acnes* has already been demonstrated, as well as their anti-inflammatory effects (Fu et al. 2012; Niyomkam et al. 2010; Sharma et al. 2013). Myrtle (*M. communis*) is one of the medicinal herbs found worldwide that is used against a large number of diseases, including skin diseases, because of its antioxidant, antiviral, antibacterial and antifungal properties (Alipour et al. 2014; Fiorini-Puybaret 2011). Some of the main biologically

active components are described as antioxidant without antibacterial activity, like ursolic acid (Sharma et al. 2013). Many other compounds are able to express antimicrobial activity, like limonene and, above all, myrtucommulones (Fiorini-Puybaret 2011). These natural acylphloroglucinols were described first as antimicrobial agents with low MICs against Gram-positive bacteria (Appendino et al. 2006) and, more recently, as anti-inflammatory molecules *in vitro* and *in vivo* (Rossi et al. 2009). This is the first report indicating that extract from *M. communis* was able to inhibit biofilm formation and reduce structured *P. acnes* biofilm in a concentration range of 0.1% to 0.001% (w/v) (i.e. 1000 to 10 mg/l), corresponding to MIC values observed on planktonic cells (Fiorini-Puybaret 2011) and to the bactericidal concentrations obtained on planktonic cells in a medium preventing planktonic growth (MBB). The effect appears to depend significantly on concentration and contact time. A concentration of 0.01% reduces planktonic MBB population by 3 to 4 log, and biofilm population (static or dynamic model) after 48 h of contact by 4 log. A partial disintegration of a 48 h biofilm (dynamic model) was observed at this concentration from 1 to 5 min of contact.



**Fig. 8.** Number of residual adherent and planktonic cells (log CFU per well;  $n = 2$ ; mean  $\pm$  SD) when Myrtacine<sup>®</sup> and clindamycin are combined or not on a 48 h biofilm after a 24 h contact according to the concentrations on (a) *P. acnes* CIP 53.117T (eryS/clindaS) and (b) *P. acnes* CIP 110.371 (eryR/clindaR) (static model). \* $p < 0.05$  \*\* $p < 0.01$ .

The loss of susceptibility of microorganisms in the form of biofilm, including *P. acnes*, to the main antimicrobial agents or treatments has a worldwide importance and, for this purpose alone, Myrtacine<sup>®</sup> New Generation can be considered as an original antimicrobial agent.

Considering this specific activity against *P. acnes* biofilm and the current antibiotic use in acne vulgaris treatment, the interest of combining erythromycin (1000 mg/l) or clindamycin (500 mg/l) with Myrtacine<sup>®</sup> New Generation was checked at concentrations under the accepted active concentration i.e. 0.001% and 0.0001% on a 48 h biofilm (static model). In such conditions, the reduction of adherent and planktonic populations (released from the biofilm) is significantly more important for the combinations than for each compound alone. The addition of Myrtacine<sup>®</sup> New Generation at 0.001% w/v (little or no activity alone) restores the susceptibility of erythromycin- or clindamycin-sensitive strains but, more importantly, is effective against antibiotic-resistant strains. In the latter case, the planktonic population released from the biofilm is not as dramatically reduced as for sensitive strains. This highlights a marked improvement of the effect certainly linked not only to a biofilm deconstruction effect, with population return to planktonic status, but also to other mechanisms and possibly multi-targets effects as other botanical products (Gretsch, 2011; Radalovic et al. 2013) which need further investigation.

## Conclusion

In conclusion, this is the first demonstration of the activity of a Myrtle extract, Myrtacine<sup>®</sup> New Generation, as a preventive or curative agent against *P. acnes* biofilm and as a potent adjunctive product efficient during the antibiotic course for acne vulgaris treatment. Considering the increasing percentages of *P. acnes* strains resistant to erythromycin and clindamycin, the efficiency of Myrtle extract on *P. acnes* biofilm alone or combined with antibiotics has to be considered to control *P. acnes* populations in acneic patients.

## Conflict of interest

Christel Fiorini-Puybaret and Philippe Joulia are members of the Laboratory for vegetable products, Pierre Fabre Research Institute, Pierre Fabre R&D Center, and were concerned by Myrtle extract preparation and characterization; Joëlle Luc is member of the Microbiology Lab., Pierre Fabre DermoCosmetics R&D, and was concerned by *P. acnes* strains selection.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2015.11.016.

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