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Antibacterial efficacy of essential oils of three aromatic plants in combination with povidone-iodine against *Staphylococcus aureus* grown in biofilm cultures

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ABSTRACT

Staphylococcus aureus, one of the opportunistic species responsible for clinical and / or bacteriological infections may react with biotic and abiotic surfaces and produces biofilm. In this form the bacteria become somewhat vulnerable to various physical and / or chemical antimicrobial agents. The aim of this study was to investigate the antibacterial efficacy of PVI, alone and in combination with essential oils (EO) of three aromatic plants, *Eucalyptus globulus*, *Thymus capitatus*, and *Rosmarinus officinalis*, against biofilms of four clinical isolates of *Staphylococcus aureus* from intravenous catheter associated infections. These strains isolated at the university hospital of Tlemcen, were characterized and identified, in a previous study, as strongly adherent using the microtiter plate method. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations of different essential oils sole and in combination with PVI for studied strains grown in biofilm, were determined by microbroth dilution method. The antibacterial effect of EOs and PVI, alone and in combination, against biofilm-forming staphylococci, was also demonstrated. EO of *E. globulus* was more effective, in comparaison with the two other EOs. In addition, the antibacterial effects of the three EOs studied in combination with PVI were deemed synergistic against the biofilm of all tested strains, with a fractional inhibitory concentration index (FICI) <0.5. Accordingly, we could suggest the use of PVI in combination with EOs, especially of *E. globulus* ; since this EO was proved to be more efficacious in enhancing the antibacterial efficacy of PVI against biofilm of *S. aureus*.

INTRODUCTION

Biofilms are thought to be the predominant growth mode for bacteria in natural environments, and increasing evidence implicates them as a cause of human infections (Costerton *et al.*, 1999; Parsek and Singh, 2003). Several researches have shown that biofilm cells can exhibit an increased resistance to biocides, antimicrobials, and host defense mechanisms in comparison to planktonic cells (Parsek and Singh, 2003; Anwar *et al.*, 1989). Therefore, Biofilm-associated infections are of particular concern, since they remain difficult to control with standard antibiotics (Ceri *et al.*, 1999) or to clear by host defenses (Donlan and Costerton, 2002; Leid *et al.*, 2002; Shiau and Wu, 1998). *Staphylococcus aureus*, a common human pathogen, appear to be closely associated infections due to biofilms. Incision of human skin is common practice within the healthcare setting, for example during surgery and insertion of indwelling medical devices such as central venous catheters (Mack *et al.*, 2004; Worthington and Elliott, 2005), So skin insertion site disinfections is pivotal mainstay to avoid the risk of microbial colonization of catheters by opportunistic microorganisms such as *S. aureus* eventually present within the skin. The povidone or polyvidone iodine (PVI) is one of the widely used antiseptics in the clinical setting. It is is a complex of iodine and polyvinylpyrrolidone, such that it contains not less than 9 % and not more than 12 % available iodine calculated on a weight basis (USP, 1980).

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The PVI has a bactericidal action and is effective against a wide range of bacteria, protozoa viruses, fungi and even spore-forming bacteria (Rutala, 1996) his antiseptic efficacy is attributed primarily to the free or available iodine which it contains. The iodine is slowly released and delivered to the bacterial cell surface where it penetrates the cell membrane and inactivates key cytosolic proteins, fatty acids, and nucleotides (Durani and Leaper, 2008), it has rapid bactericidal activity, but activity is diminished shortly after contact with organic matter present in skin (Messager *et al.*, 2001, Reimer *et al.*, 2002) and no resistance has been determined.

To enhance the efficacy of PVI, the combined use with three different essential oils has been evaluated in this work for potential synergistic effects. It is known that PVI and EOs have different cellular targets and different mechanisms of action, These differences may prove beneficial when using these two antimicrobial in combination. Unfortunately, few studies have evaluated the activity of this antiseptic in combination with essential oils. Furthermore, essential oils have been shown to act as effective penetration enhancers, increasing permeation and improving retention of drugs within the skin (Biruss *et al.*, 2007; Fang *et al.*, 2004).

Increasing evidence, plant essential oils and extracts have been used for a wide variety of purposes for many thousands of years (Jones, 1996) in particular; their antimicrobial activity has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Reynolds, 1996; Lis-Balchin and Deans., 1997).

The PVI is the primordial antiseptic used in the university hospital of Tlemcen; that why it was the purpose of this work to evaluate the efficacy of this antiseptic against biofilms of four clinical isolates of interest medical, *S. aureus*.

MATERIALS AND METHODS

Bacterial strains

The Clinical isolates of *S. aureus* derived from intravenous catheters in the hospital university of Tlemcen, were identified conventionally and characterized as highly adherent (forming biofilm) in a previous study (for more detailed information see Ghellai *et al.*, 2014). These strains were stored at -20 °C on nutrient broth 15% glycerol (Ausubel *et al.*, 1993; Baker and Bench, 1998) and on nutrient agar slopes at 4 °C until required. The strains used in the current study are shown in Table 1. *S.aureus* ATCC 25923, MRSA ATCC 43300 and MRSA ATCC 33591 Were used as reference strains in this study.

Table. 1: Codes and biotypes of Clinical isolates from intravenous catheters (data not shown here) at the hospital university of Tlemcen.

Strain Code	Strains	Biotype	Source/Unit
LMB20330	*S.aureus*	6335053	Pneumology service
LMB20338	*S.aureus*	6376153	Neonatology service
LMB20359	*S.aureus*	6336153	Neonatology service

Antimicrobial agents

Antiseptic

The skin antiseptic, Povidone iodine (PVI) in commercial preparations has usually a concentration of 10 % w/v in this study PVI used was purchased from Sigma-Aldrich and tested over a concentration range of 65.53 to 0.06 g/L

Essential oils

Essential oil Samples were obtained from fresh leaves of *Eucalyptus globulus*, *Thymus capitatus*, and *Rosmarinus officinalis*, collected in August 2012 from Tlemcen located in the north west of Algeria. Extraction was carried out by hydro-distillation for 2 h using a standard Clevenger-type apparatus recommended in the European Pharmacopoeia. Essential oils obtained were dehydrated with anhydrous sodium sulfate (Na_2SO_4) and stored in glass tubes at 4 °C away from the light until analysis. EOs were tested over a concentration range of 4mg/mL to 0.003 g/L.

Determination of MIC and MCB of EOs of *E. globulus* (EOE), *R. officinalis* (EOR), and *T. capitatus* (EOT), and PVI against strains in biofilm cultures

MICs and MBCs in this study were determined according to the works of Hendry et al. (2009). Wells of polypropylene microtiter plate containing *S. aureus* biofilms (data not shown here) were washed once with 200 µL of sterile phosphate buffered saline (PBS) to remove any unbound cells. Serial double dilutions of PVI and EOs were prepared in Mueller–Hinton broth (MHB) (PVI 65.53 to 0.06 mg/mL and EOs 4 to 0.03 mg/mL). For EOs, all tests were performed in MHB supplemented with 2% (v/v) Tween 80 to enhance the solubility of EOs. 200µL of each antimicrobial agent dilution was added to microtitre plate wells. The two last columns (11 and 12) served as control tests containing the biofilm in saline and MHB only. Plates were incubated in air at 37 °C for 24 h, antimicrobial solutions were removed then and the wells washed once with PBS. All wells were filled with 200 µL of PBS and the microtitre plate was sonicated at 50 Hz in a water bath for 30 min at room temperature.

Biofilm was retrieved from the wells by mixing the entire contents (200 µL) of of the clear wells with molten modified Lethen broth (MLB) 1% agar (MLB: 10g/L typtone, 5g/L beef extract, 1g/L sodium sulfite, 5g/L polysorbate 80 and 7g/L sodium chloride), cooled to 45°C. After incubation of the plates in air at 37°C for 24 h, MICs and MBCs were determined. On the Letheen broth 1% agar, MIC corresponds to the lowest concentration (in mg/mL) of the antibacterial agent showing growth below or equal to that of the control wells (biofilm treated with saline) and MBC was determined as the lowest concentration of antibacterial agent demonstrating no visible bacterial growth. The experience was realized in duplicate microtiter plates. The antibacterial efficacy of 2% (v/v) Tween 80 was simultaneously performed against biofilm-forming staphylococci on a separate microtiter plate.

Antibacterial effect of EOs in combination with PVI against strains in biofilm cultures

Wells of polypropylene microtiter plate containing *S. aureus* biofilms were washed once with 200 µL of sterie PBS. 100µL of PVI solution was added in diminishing concentrations to the rows of the microtiter plate and 100µL of EO solution was added to the columns in diminishing concentrations. Final concentrations of the antibacterial agents ranged of 32.76 to 0.06 mg/mL for PVI solution and 4 to 0.003 mg/mL for EOs. Columns 11 and 12 contained biofilm in saline and MHB only. Microtiter plates were incubated for 24 h at 37 °C. MICs and MBCs of each antibacterial agent alone and in combination were determined as described previously. Synergistic, antagonistic or indifferent antibacterial activity of each one of the EOs studied in combination with PVI was assessed by the following formulae (White *et al.*, 1996):

Fractional inhibitory concentration (FIC) = MIC of antibacterial agent in combination/MIC of antibacterial agent alone

FIC index (FICI) = FIC of PVI +FIC of EO

Antagonistic antibacterial activity: FICI ≤ 4.0

Synergistic antibacterial activity: FICI ≤ 0.5

Indifferent antibacterial activity: 0.5<FICI≤4.0

RESULTS

It was the purpose of the current work to assess anti-bacterial activity of essential oils of *E. globulus*, *T. capitatus*, and *R. officinalis*, against biofilms of four clinical isolates of *S. aureus* from intra-venous catheter associated infections. The results of MICs and MBCs of the three Eos and PVI alone against the tested strains of *S. aureus* are shown in Table 2.

MICs of PVI alone were 4.09 g/L and 1.02 g/L for LMB20338 and *S.aureus* ATCC 25923. The other studied strains LMB20327, LMB20359 and MRSA (ATCC 43300) were similarly susceptible to PVI solution with identical MICs, 8.19 g/L. The MIC of PVI alone was higher (16.38 g/L) for LMB20330 and MRSA (ATCC 33591) strains. In general, minimum bactericidal concentration of PVI alone was identical (65.53 g/L) against all study organisms in sessile form, except for *S. aureus* (ATCC 25923) which was equal to 8.19 g/L.

The results of minimum inhibitory concentrations of EOs alone (Table 2) against biofilm of the studied strains of *S. aureus* were fluctuant between 0.25 to 2 g/L, while MBCs of almost all EOs were > 4 g/L against strains grown in biofilm.

According to the results obtained in this work, EOs of the aromatic plants used were shown to possess higher antibacterial activity compared with PVI solution. Moreover, it seems that antibacterial activity of EO of *E. globulus* is significantly more effective than the other EOs tested, against strains of *S. aureus* in biofilm. Furthermore, against some isolates, other essential oils tested are effective. MICs of the three Eos and PVI alone and when applied simultaneously against the tested strains of *S. aureus* are shown in Table 3. The determination of fractional inhibitory concentration index (FICI) of each EOs in combination with PVI against all strains of *S. aureus* grown in biofilm cultures implied that the EOE/PVI, EOR/PVI and EOT/PVI combinations were deemed synergistic with FICIs <0.5.

Table 2. MICs and MBCs of PVI and EOs against strains of *S. aureus* grown in biofilm cultures.

Strain	compound	MIC (g/L)	MBC (g/L)
LMB20327	PVI	8.19	65.53
	EOE	1	>4
	EOR	1	>4
	EOT	1	>4
LMB20330	PVI	16.38	65.53
	EOE	0.5	1
	EOR	2	>4
	EOT	0.5	>2
LMB20338	PVI	4.09	65.53
	EOE	0.25	>4
	EOR	0.25	>4
	EOT	0.5	>4
LMB20359	PVI	8.19	65.53
	EOE	0.5	>4
	EOR	1	4
	EOT	0.5	>4
S.aureus ATCC 25923	PVI	1.02	8.19
	EOE	1	>4
	EOR	0.25	>4
	EOT	2	>4
MRSA ATCC 43300	PVI	8.19	65.53
	EOE	1	>4
	EOR	2	2
	EOT	0.5	4
MRSA ATCC 33591	PVI	16.38	65.53
	EOE	1	>4
	EOR	2	>4
	EOT	0.5	2

Table. 3: FICIs determination of EOs in combination with PVI against strains of *S. aureus* grown in biofilm cultures .

Strain in biofilm culture	Antibacterial agent	MIC of PVI (g/L)		FIC of PVI	MIC of EO (g/L)		FIC of EO	FICI	Result
		combined	alone		combined	alone			
LMB20327	EOE+PVI	0.06	8.19	0.007	0.03	1	0.03	0.037	Synergy
	EOR+PVI	0.06	8.19	0.007	0.03	1	0.03	0.037	Synergy
	EOT+PVI	0.06	8.19	0.007	0.03	1	0.03	0.037	Synergy
LMB20330	EOE+PVI	0.06	4.09	0.014	0.03	0.25	0.12	0.134	Synergy
	EOR+PVI	0.06	4.09	0.014	0.03	0.50	0.06	0.074	Synergy
	EOT+PVI	0.06	4.09	0.014	0.03	0.25	0.12	0.134	Synergy
LMB20338	EOE+PVI	0.06	4.09	0.014	0.03	0.25	0.12	0.135	Synergy
	EOR+PVI	0.06	4.09	0.014	0.03	0.25	0.12	0.135	Synergy
	EOT+PVI	0.06	4.09	0.014	0.03	0.5	0.06	0.075	Synergy
LMB20359	EOE+PVI	0.06	4.09	0.014	0.03	0.12	0.25	0.264	Synergy
	EOR+PVI	0.06	4.09	0.014	0.03	0.50	0.06	0.074	Synergy
	EOT+PVI	0.06	4.09	0.014	0.03	0.25	0.12	0.134	Synergy
S.aureus ATCC 25923	EOE+PVI	0.06	1.02	0.058	0.03	1	0.03	0.092	Synergy
	EOR+PVI	0.06	1.02	0.058	0.03	0.25	0.12	0.182	Synergy
	EOT+PVI	0.06	1.02	0.058	0.03	2	0.01	0.072	Synergy
MRSA ATCC 43300	EOE+PVI	0.06	4.09	0.014	0.03	0.12	0.25	0.264	Synergy
	EOR+PVI	0.06	4.09	0.014	0.03	0.50	0.06	0.074	Synergy
	EOT+PVI	0.06	4.09	0.014	0.03	0.25	0.12	0.134	Synergy
MRSA ATCC 33591	EOE+PVI	0.06	8.19	0.007	0.03	0.25	0.12	0.127	Synergy
	EOR+PVI	0.06	8.19	0.007	0.03	0.25	0.12	0.127	Synergy
	EOT+PVI	0.06	8.19	0.007	0.03	0.25	0.12	0.127	Synergy

DISCUSSION

The assessment of specific antibacterial activity against biofilm of *S. aureus* is an important parameter for establishment of an appropriate preventive or curative antimicrobial therapy, as well as MIC and MBC determination against cells in biofilm cultures (Reiter *et al.*, 2012). In addition, drugs targeting pathogenesis via biofilm inhibition could have great therapeutic value (Hobby *et al.*, 2012)

According to several authors, there is a relationship between the antimicrobial activity and the chemical structures of the most abundant in the tested essential oil. Antibacterial activity of essential oils of *Eucalyptus* has been due to the components such as 1,8-cineole, citronellal, citronellol, citronellyl ecetate, p-cymene, eucamalol, limonene, linalool, β-pinene, γ-terpinene, α-terpinol, alloocimene and aromadendrene (Nezhad *et al.*, 2009). While, main constituents of the essential oil of *R. officinalis* are 1.8-Cineole, camphor, α -pinene, and camphene (Zaouali *et al.*, 2010). The antimicrobial activity of Rosmarinus against the tested microorganisms could be attributed to the presence of flavonoids, phenolic acids (caffeic, chorogenic and rosmarinic) and diterpenes (carnosol) which can be extracted with polar solvents, acetone, ethanol, methanol and water from the plant. Moreover, the antibacterial activity of this EO is also due to the high content of camphor and 1,8-cineole in the oil (Faixova and Faix, 2008; Rozman and Jersek, 2009). Faix, 2008; Rozman and Jersek, 2009). Carvacrol, p-cymene, γ-terpinene and β-caryophyllene are the main components of the essential oils of *T. capitatus*. (Bounatirou *et al.*, 2007) Carvacrol has been considered as a biocidal. It antimicrobial activity against biofilm formed by *S.aureus*, was investigated by Knowles et al. (2004). However, it was shown that the other constituents have also a good role as antimicrobial agents, as well as the minor components. There may be a synergistic effect between these components in the oil and carvacrol because, the essential oils are the mixture of many components. It is known that povidone–iodine has a wide-spectrum antimicrobial activity, is used as an antiseptic agent for the prevention of intravascular catheter-related infections and it is very efficient in the treatment of various types of wounds (O'Grady *et al.*, 2002, Fleischer and Reimer, 1997). However, antibiotic-resistant strains with increased resistance to antiseptics have been reported. Furthermore, many studies have evaluated the antimicrobial activity of PVI alone, but to our knowledge they are few reports about the activity of PVI in combination with EOs.

Generally, low concentrations of PVI (0.51 g/L) and EOs (0.12g/L), when used individually, were determined to have no effect on biofilm formation by the tested strains of *S.aureus*. At higher concentrations EOs (2 g/L) and PVI (56.53 g/L) were bactericidal for all tested strains in biofilm cultures. MIC values of EOs and PVI obtained in this study were lower than the MBC values, implying that the tested EOs and PVI were bacteriostatic agents at lower concentrations and bactericidal agents at higher concentrations. Our results were consistent with previous reports (Jarrar *et al.*, 2010; Suwanpimolkul *et al.*, 2008).

The interpretations of the activity of EOs of *E. globulus*, *R. officinalis* and *T. capitatus* combined with PVI produced a remarkable synergistic activity against the tested clinical isolates of *S. aureus* (Table 3). The results of the current study were in accordance with those shown by other works on the the antimicrobial activity of EOs of various Eucalyptus, Rosmarinus and Thymus species including *E. globulus*, *R. officinalis* and *T. capitatus* against biofilm of *S. aureus* (Ait-Ouazzou *et al.*, 2011., Elaissi *et al.*, 2011; Fit *et al.*, 2009; Celiktas *et al.*, 2007; Jarrar *et al.*, 2010). The MICs of EOs for biofilm-forming staphylococci were decreased from the range of (0.12-2.0) to 0.03 g/L when these EOs were combined with PVI solution at a concentration

corresponding to 0.06 g/L. Biofilm-forming bacteria is well known to exhibit increased resistance to antimicrobial compounds compared with bacteria in planktonic cultures, due in part to the physical barrier it create (Saginur *et al.*, 2006; Johansen *et al.*, 1997). The penetration into biofilms of Antiseptic solutions currently used is poor, such as povidone-iodine. Furthermore, cellular target of EOs is the cytoplasmic membrane. Whereas, key cytosolic proteins, fatty acids, and nucleotides are the main targets of PVI. Increasing evidence, these two antimicrobial coupounds have different mode of action. These differences may prove beneficial when using these 2 antimicrobial (EO/PVI) agents in combination.

CONCLUSION

The antimicrobial activity of the tested essential oils combined with PVI solution, against strains of *S. aureus* in biofilm cultures was superior to each antimicrobial agent alone. The application of these essential oils may provide a scientific ground in enhancing the antimicrobial effect of PVI against bacterial biofilms and accordingly prevention and treatment of nosocomial infections caused by, among others, *Staphylococcus aureus*.

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