1 **Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with**

2 **eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of**

3 *Staphylococcus epidermidis*

4

5 **Short running title:** Antimicrobial activity of chlorhexidine and essential oils

6

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15 **Key words**: Essential oils, chlorhexidine, synergism, skin antisepsis, antimicrobial

16 activity

17

18   **Synopsis:**

19   *Objectives:* Effective skin antisepsis and disinfection of medical devices are key factors

20   in preventing many healthcare acquired infections associated with skin microorganisms,

21   particularly, *Staphylococcus epidermidis*. The aim of this study was to investigate the

22   antimicrobial efficacy of chlorhexidine digluconate (CHG), a widely used antiseptic in

23   clinical practice, alone and in combination with tea tree oil (TTO), eucalyptus oil (EO)

24   and thymol against planktonic and biofilm cultures of *S. epidermidis*.

25   *Methods*: Antimicrobial susceptibility assays against *S.epidermidis* in a suspension and in

26   a biofilm mode of growth were performed with broth microdilution and ATP

27   bioluminescence methods respectively. Synergy of antimicrobial agents was evaluated

28   with checkerboard assay.

29   *Results*: Chlorhexidine exhibited antimicrobial activity against *S. epidermidis* in both

30   suspension and biofilm [Minimum inhibitory concentration (MIC) 2–8 mg/ L]. Of the

31   essential oils thymol exhibited the greatest antimicrobial efficacy (MIC 0.5–4 g/ L)

32   against *S. epidermidis* in suspension and biofilm followed by TTO (MIC 2–16 g/ L) and

33   EO (4–64 g/ L). Minimum inhibitory concentrations of CHG and EO were reduced

34   against *S. epidermidis* biofilm when in combination (MIC 8 reduced to 0.25-1 mg/ L and

35   32-64 reduced to 4 g/L for CHG and EO respectively). Furthermore, the combination of

36   EO with CHG demonstrated synergistic activity against *S. epidermidis* biofilm with a

37   fractional inhibitory concentration index (FICI) of <0.5.

38   *Conclusion*: The results from this study suggest there may be a role for essential oils, in

39   particular EO, for improved skin antisepsis when combined with CHG.

40 **Introduction**: Incision of human skin is a common practice in the clinical setting, for

41 example during surgery, when taking blood or inserting intravascular devices. Adequate

42 skin antisepsis is therefore essential in avoidance of healthcare associated infections

43 (HAI) which may occur post incision, commonly from resident microorganisms located

44 within the skin, particularly *S. epidermidis*.[1, 2] *Staphylococcus epidermidis* is common on

45 both the surface of human skin and also within the deeper layers where it may exist as

46 microcolonies, which, like bacterial biofilms, exhibit increased resistance to

47 antimicrobials including antiseptics.[3] Unfortunately, HAI do arise following incision of

48 skin[4,5] and are likely to be associated with increased prevalence of microbial resistance to

49 antibiotics and antiseptics and inadequate skin antisepsis which encompasses both the

50 contact time between skin and antiseptic prior to incision and permeation of the antiseptic

51 within the skin.[6-10] Chlorhexidine is one of the most widely used antimicrobials within

52 clinical practice for skin antisepsis and is currently recommended within the Evidence-

53 Based Practice in Infection control (EPIC)[11] and Healthcare Infection Control Practices

54 Advisory Committee (HICPAC)[12] guidelines. However, its antimicrobial efficacy is

55 significantly reduced by factors including pH and organic matter.[13] Therefore additional

56 strategies for skin antisepsis or improvement of existing methods need to be considered.

57 The antimicrobial efficacy of essential oils has been known for several years and

58 many studies have demonstrated activity against bacteria, fungi and viruses.[14] More

59 recently, in the light of increased antimicrobial resistance within the clinical setting, the

60 potential of essential oils for the prevention and treatment of infection has been

61 researched in several studies.[15-19] Indeed, TTO has recently been shown to be more

62 effective than CHG at clearing superficial skin sites and lesions colonised with

63   methicillin resistant *S. aureus.*[17] In addition, preliminary research within our laboratory

64   has demonstrated that several essential oils (EO, TTO, thymol, lavender, juniper and

65   citronella) have antimicrobial efficacy against a wide range of microorganisms associated

66   with HAI.[20] Many of the essential oils are thought to disrupt cell membranes by the

67   lipophilic compounds contained within the essential oil, such as terpenes. However,

68   essential oils are mixtures of many heterogeneous compounds, and the main components

69   responsible for their antimicrobial activity and the mode of their activity are not well

70   understood.[14] Furthermore, essential oils have been shown to act as effective penetration

71   enhancers, increasing permeation and improving retention of drugs within the skin.[21,22]

72   The aim of this study was to investigate the antimicrobial efficacy of aqueous

73   CHG alone and in combination with TTO, EO and thymol against planktonic and biofilm

74   cultures of *S. epidermidis*.

75

Materials and methods

Materials

Congo red agar for demonstrating slime production in the test strain *S.epidermidis* was

prepared by mixing 0.4 g of Congo red (Hopkins and Williams Ltd, Essex, UK), 25 g

sucrose (Fisher Scientific, Leics, UK) and 5 g of agar No.1 (Oxoid, Basingstoke, UK)

with 490 mL of brain heart infusion (BHI) (Oxoid) and sterilised according to the

manufacturers' recommendations. Mueller-Hinton agar (MHA) and Mueller-Hinton broth

(MHB) (Oxoid) were also prepared and sterilised in line with the manufacturers'

recommendations. Phosphate buffered saline (PBS), aqueous CHG (20% in water), TTO

(40.2% terpinen-4-ol and 3.5% cineole), EO (82.9% cineole), thymol (>99.5%) and

dimethylsulphoxide (DMSO) were purchased from Sigma-Aldrich (Dorset, UK) and

glucose from Fisher Scientific. White walled, clear bottom, tissue culture treated 96-well

microtitre plate were from Corning Incorporated (NY, USA) and clear, round bottom 96-

well plates from Barloworld Scientific (Staffordshire, UK).

Microorganisms

Staphylococcus epidermidis RP62A [23] and a clinical isolate of *S.epidermidis*, TK1,

(University Hospital Birmingham NHS Trust, Birmingham, UK) were stored on

MicroBank beads (Pro-Lab Diagnostics, Cheshire, UK) at -70˚C until required.

98   *Preparation of antimicrobial agents*

99   Aqueous CHG was diluted with MHB to obtain a stock solution of 512 mg/ L. Thymol,

100   TTO and EO were diluted with MHB to obtain stock solutions of 512 g/ L. Five percent

101   (v/v) DMSO was added to the essential oil stock solutions to enhance the solubility of the

102   oils in solution.

103

104   *Preparation of S.epidermidis inoculum for suspension assay*

105   Suspensions of *S.epidermidis* for the suspension assays were prepared by inoculating 10

106   identical colonies of overnight cultures of *S.epidermidis* from MHA into sterile PBS. The

107   bacterial concentration was adjusted to 1×10^8 cfu/ mL by diluting the culture with sterile

108   PBS and measuring the optical density at 570 nm. The suspensions were further diluted

109   with MHB to obtain inocula containing 1×10^6 cfu/ mL.

110

111   *Determination of MIC and MBC of CHG, TTO, EO and thymol against S.epidermidis in*

112   *suspension*

113   Minimum inhibitory concentration of aqueous CHG and TTO, EO and thymol were

114   determined using a broth microdilution assay in line with CLSI (formerly NCCLS)

115   guidelines.[24] The antimicrobial activity of 5 % (v/v) DMSO was also studied on a

116   separate microtitre plate alongside the assay. Each well containing 100 μL of

117   antimicrobial agent was inoculated with 100 μL of *S.epidermidis* suspension containing

118   1×10^6 cfu/ mL. Following 24 h incubation in air at 37˚C, the wells were inspected for

119   microbial growth and the MIC was defined as the lowest concentration which did not

120   show visual growth. Controls containing antimicrobial agent in broth and broth with

121 inocula were also included. Minimum bactericidal concentrations were determined by

122 removing the total volume (200 µL) from each of the clear wells and mixing with 20 mL

123 of cooled molten MHA, which was then allowed to set. Plates were incubated in air at 37˚

124 C for 24 h. Minimum bactericidal concentration was defined as the first plate yielding no

125 growth. The assay was performed in triplicate.

126

127 *Checkerboard assay to assess antimicrobial activity of CHG in combination with TTO,*

128 *EO and thymol against S.epidermidis in suspension*

129 The antimicrobial activity of aqueous CHG in combination with TTO, EO and thymol

130 was assessed in a suspension assay by the checkerboard method.[25] In brief, serial double-

131 dilutions of the antimicrobial compounds were prepared (256 to 1 g/L for EO and TTO,

132 64 to 0.25 g/ L for thymol and 64 to 0.5 mg/L for CHG). Fifty microlitres of each CHG

133 solution was added to the rows of a 96-well microtitre plate in diminishing concentrations

134 and 50 µL of the essential oil to the columns in diminishing concentrations. The wells

135 were then inoculated with 100 µL of *S.epidermidis* suspension containing $1x10^5$ cfu (the

136 final concentrations of EO and TTO ranged from 64 to 0.25 g/L, thymol 16 to 0.06 g/L

137 and CHG 16 to 0.125 mg/L). Columns 10, 11 and 12 served as controls containing MHB

138 and inoculum alone, and antimicrobial compounds separately with the inoculum. The

139 microtitre plates were incubated in air at 37˚ C for 24 h and MIC for both antimicrobial

140 compounds in combination was determined. To assess synergistic or antagonistic activity

141 of antimicrobial combinations, the fractional inhibitory concentration (FIC) and FIC

142 index (FICI) were determined using the following formulae:

143

144 $$FIC= \frac{\text{MIC of CHG or natural compound in combination}}{\text{MIC of CHG or natural compound alone}}$$

145

146

147 $$FICI= FIC \text{ of oil} + FIC \text{ of CHG}$$

148

149 FICI $\leq$ 0.5 were regarded as synergistic effect, values between 0.5 to 4.0 as

150 indifferent and over 4.0 as antagonistic activity. The assay was performed in

151 duplicate microtitre plates.

152

153 *Preparation of S.epidermidis biofilm*

154 The ability of *S.epidermidis* strains to produce slime was confirmed by culturing the

155 bacteria on Congo red agar.[26] The optimal conditions and incubation period for the

156 production of the bacterial biofilms were established in preliminary experiments over a

157 72 h period (data not shown). Bacterial biofilms were prepared by aliquoting 200 μL of

158 the bacterial suspension containing 1×10^5 cfu/ mL into the wells of white walled, clear

159 bottom, tissue culture treated 96-well microtitre plates. Four wells in the last column of

160 each plate were left blank to serve as bioluminescence negative control. Suspensions of

161 *S.epidermidis* were prepared in MHB supplemented with 2% (w/v) glucose. Microtitre

162 plates containing *S.epidermidis* suspensions were incubated in air at 37° C for 48 h (these

163 conditions demonstrated in preliminary experiments to be optimal conditions for

164 production of a confluent biofilm with approximately 5.5×10^6 cfu per well - data not

165 shown).

166

167 *Determination of MIC and MBC of CHG, TTO, EO and thymol against S.epidermidis in*

168 *biofilm*

169 Microtitre plates containing *S.epidermidis* biofilms were washed once with sterile PBS to

170 remove any unbound bacteria. Antimicrobial agents were diluted with MHB to obtain

171 CHG concentrations ranging from 128 mg/ L to 0.25 mg/ L, thymol 128 to 0.25 g/L and

172 EO and TTO 256 to 0.5 g/L. Two hundred and fifty microlitres of each antimicrobial

173 agent was added to each microtitre plate well. Columns 11 and 12 served as controls

174 containing the biofilm and saline alone and MHB alone without bacterial biofilm.

175 Antimicrobial activity of 5% (v/v) DMSO against the bacterial biofilm was also tested on

176 a separate plate. Following incubation at 37˚C in air for 24 h, the wells were washed once

177 with sterile PBS and the microbial viability was determined using an ATP

178 bioluminescence assay (ViaLight MDA Bioassay kit, Cambrex, Berkshire, UK). In brief,

179 100 µL of Bactolyse was added with 100 µL of saline into each well and the plates

180 sonicated at 50 Hz for 30 min to release and lyse the cells of the bacterial biofilm. Fifty

181 microlitres of ATP-monitoring reagent was added to each well and luminescence

182 measured (Lucy 1, type 16 850 fluorescence measurer, Rosys Anthos Labtech

183 Instruments). Minimum inhibitory concentration was defined as the minimum

184 concentration of antimicrobial agent that inhibited further growth of the initial biofilm

185 (control well containing biofilm treated with saline) and MBC was defined as the

186 concentration that produced below or equal to the background level of luminescence

187 (empty well). The assay was performed in duplicate microtitre plates.

188

189 *Checkerboard assay to assess antimicrobial activity of CHG in combination with TTO,*

190 *EO and thymol against S.epidermidis in biofilm*

191 Microtitre plates containing *S.epidermidis* biofilms were washed once with sterile PBS to

192 remove any unbound bacteria. Antimicrobial agents were diluted with MHB as described

193 previously and 125 µL of each of the antimicrobial dilutions aliquoted into each well in

194 decreasing concentrations. Columns 10 and 11 contained biofilm and antimicrobial

195 compounds alone at various concentrations and column 12 served as control with biofilm

196 with saline and clear wells with saline. The plates were incubated in air at 37°C for 24 h

197 after which the wells were emptied and the FIC and FICI values determined by ATP

198 bioluminescence as described previously. The assay was performed in duplicate.

199

200 **Results**

201

202 *Determination of MIC and MBC of CHG, TTO, EO and thymol against S.epidermidis in*

203 *suspension and in biofilm*

204 Chlorhexidine digluconate, TTO, EO and thymol demonstrated antimicrobial activity

205 against *S.epidermidis* RP62A and *S.epidermidis* TK1 in both suspensions and biofilms

206 (Table 1). Minimum inhibitory concentration and MBC of CHG were 4-fold higher

207 against *S.epidermidis* growing in biofilm compared to cells in suspension (MIC 2 to 8

208 mg/ L and MBC 4 to 16 mg/ L). Of the three essential oils thymol was the most effective

209 with MIC ranging from 0.5 to 4 g/ L and MBC 2 to 16 g/ L. All antimicrobials tested

210 reduced the luminescence from the bacterial biofilms to below negligible levels with

211 concentrations of 16 mg/ L (CHG), 2-8 g/ L (thymol), 32-64 g/ L (TTO) and 256 g/ L

212 (EO). Five percent (v/v) DMSO, which was used as co-solvent in oil solutions, did not

213 show antimicrobial activity against *S.epidermidis* in biofilm or suspension.

214

215 *Checkerboard assay to assess antimicrobial activity of CHG in combination with TTO,*

216 *EO and thymol against S.epidermidis in suspension and in biofilm*

217 Combination of CHG with TTO, EO and thymol demonstrated indifferent activity against

218 *S.epidermidis* RP62A and TK1 when grown in suspension (Table 2). In biofilm, CHG in

219 combination with EO demonstrated synergistic activity against both strains of

220 *S.epidermidis*, with FICI of 0.156 and 0.188 for *S.epidermidis* RP62A and TK1

221 respectively (Table 3).

222

223 **Discussion:**

224

225 The aim of this study was to assess the antimicrobial efficacy of aqueous CHG and three

226 essential oils (TTO, EO and thymol) against the common skin microorganism,

227 *S.epidermidis*, and to determine the antimicrobial activity of CHG in combination with

228 the oils.

229   The results demonstrate that CHG, EO, TTO and thymol exhibit antimicrobial

230 activity against *S.epidermidis* when growing both in suspension and as a biofilm.

231 However, the concentration of essential oils required to achieve the same level of growth

232 inhibition as CHG is several orders of magnitude higher (g/ L for essential oils compared

233 to mg/ L for CHG). Thymol showed increased activity against *S.epidermidis* growing in

234 biofilm compared with planktonic cells. This is unusual as biofilms are considered to be

235   more resistant to antimicrobial agents compared to planktonic cells.[3] Partitioning of oil,

236   especially pure compound thymol, in oil suspension as well as in extracellular matrix in

237   bacterial biofilm, may alter thymol activity. In a previous study by Nostro *et al*.,[27] only

238   small differences between biofilm and planktonic cultures susceptibility to thymol was

239   demonstrated. Furthermore, in the study by Al-Shuneigat *et al*.,[15] staphylococci in a

240   biofilm mode of growth demonstrated increased susceptibility to an essential oil based

241   formulation compared with planktonic cells, which concurs with our findings in relation

242   to thymol. Thymol is a phenolic compound that has both hydrophilic and hydrophobic

243   properties which may enhance diffusion of this compound in a biofilm and allow its

244   access to bacterial cells where it alters the permeability of plasma membranes.[27]

245   Combining CHG with TTO, EO and thymol did not improve its antimicrobial

246   activity against *S.epidermidis* TK1 and RP62A strains during their planktonic phase of

247   growth, however, reductions in CHG concentrations required to inhibit growth of both

248   *S.epidermidis* strains in biofilm were observed. Of the three essential oils used in this

249   investigation, EO demonstrated the best potential for combination with CHG. Synergistic

250   activity between EO and CHG was demonstrated against biofilms of both strains of *S.*

251   *epidermidis* (FICI 0.19 and 0.16 for TK1 and RP62A respectively). To our knowledge,

252   this is the first report of synergism between EO and CHG.

253   Previous research that has investigated the synergistic activity of an essential oil

254   and an antimicrobial agent has suggested that the synergism may be due to their action on

255   both different[28] or similar targets on the bacterial cell (i.e. cell membranes).[29] Eucalyptus

256   oil and its main component 1,8-cineole are thought to act on the plasma membranes, the

257   same target as CHG. However, TTO (and its main antimicrobial component terpinen-4-

258 ol) and thymol also have lipophilic properties and target cellular membranes without

259 showing synergy in combination with CHG. Therefore, the interaction of EO and CHG

260 requires further studies to establish the mode of action of the potential synergism. It is

261 possible that not only one component is involved in the synergistic interaction between

262 EO and CHG, but a mixture of several components. Moreover, it has been suggested that

263 cationic CHG diffusion in the biofilm is hindered by the negatively charged extracellular

264 matrix, changing the physicochemical properties of the extracellular matrix and its

265 tertiary structure.[30] Chlorhexidine is likely to remain in the aqueous phase in the oil

266 suspension (Log P of CHG 0.037 [31]). Both EO, which consists of several heterogeneous

267 compounds, and CHG have hydrophilic and hydrophobic properties, and it may be

268 possible that they alter ionic interactions in extracellular matrix of the biofilm, as well as

269 act on the same target on the bacterial cell. However, further studies are needed to

270 establish the mode of action of EO and CHG in combination.

271   The use of essential oils for the prevention and treatment of infection has been

272 gaining popularity within the research field over the past decade.[19,32-34] Furthermore, the

273 antimicrobial activity of TTO [16,17], thymol [27] and EO [35] has been reported against several

274 important pathogens. However, there has been little research to assess the efficacy of

275 essential oils in combination with CHG against *S. epidermidis,* which is the major

276 microorganism associated with skin related HAI. Chlorhexidine is widely used as a skin

277 antiseptic within the clinical setting and is the recommended antimicrobial within the

278 EPIC and HICPAC guidelines.[11,12] However, infection rates associated with surgical

279 incision of the skin remain high.[4] Thus the current strategies adopted for skin antisepsis

280 need to be considered with a view for improvement.

281   The antimicrobial activity of CHG alone *in vivo* is reported as being

282   bacteriostatic[36] and may be one factor which contributes to the survival of *S. epidermidis*

283   within the skin following antisepsis and its association with subsequent infection.

284   Furthermore, sub-inhibitory concentrations of chlorhexidine may increase a biofilm-mode

285   of growth of staphylococci,[37] which may reduce the efficacy of skin antisepsis if

286   inappropriate levels of antiseptic are used. The synergistic action of CHG in combination

287   with EO may therefore be one way forward for enhancing both skin antisepsis and

288   potentially disinfection of hard surfaces. The environment in the healthcare setting

289   contributes to the spread of pathogens and transfer of microorganisms between patients

290   and healthcare workers.[38] Microorganisms may reside on surfaces in aggregates

291   embedded in a biofilm rendering them less susceptible to cleaning and disinfection.

292   Furthermore, many medical devices such as central venous catheter hubs and needleless

293   connectors also become colonised with microorganisms capable of producing a biofilm.[39]

294   At present chlorhexidine-based compounds or isopropyl alcohol are commonly used for

295   disinfecting these medical devices prior to use. The synergistic activity between CHG and

296   EO in combination may therefore be of benefit in the clinical setting, for example, in

297   improved skin antisepsis and the elimination of *S.epidermidis* existing as microcolonies

298   which are likely to exhibit increased resistance to CHG alone, and also potentially hard

299   surface disinfection. However, whilst much of the research data advocates the potential

300   use of essential oils in the clinical setting for preventing and treating infection there is

301   little information regarding safety in relation to their use, which needs to be taken into

302   consideration. Therefore further studies are warranted.

303

Transparency declarations

None to declare.

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461 Table 1. MIC and MBC of aqueous chlorhexidine digluconate, eucalyptus oil, tea tree oil and thymol against clinical TK1 and RP62A
462 strains of *S.epidermidis* in suspension and in biofilm.
463

Strain	Compound	MIC (g/ L for oils, mg/ L for CHG)		MBC (g/ L for oils, mg/ L for CHG)	
		Suspension	Biofilm	Suspension	Biofilm
RP62A	Eucalyptus	4	32	64	256
	Tea tree oil	2	16	4	64
	Thymol	4	0.5	16	2
	CHG	2	8	4	16
TK1	Eucalyptus	8	64	32	256
	Tea tree oil	16	16	64	32
	Thymol	0.5	0.5	4	8
	CHG	2	8	4	16

464

465 Table 2. Antimicrobial activity of aqueous chlorhexidine digluconate, eucalyptus oil, tea tree oil and thymol against clinical TK1 and
466 RP62A strains of *S.epidermidis* growing in suspension.
467

Strain	Combination	MIC of oil (g/L) in combination/ alone	FIC of oil	MIC of CHG(mg/L) in combination/ alone	FIC of CHG	FICI	Result
RP62A	CHG + Eucalyptus	4 / 4	1	2/ 2	1	2	Indifference
	CHG + Tea tree oil	2 / 2	1	2/ 2	1	2	Indifference
	CHG + Thymol	1/ 4	0.25	2/ 2	1	1.25	Indifference
TK1	CHG + Eucalyptus	8 / 8	1	2 / 2	1	2	Indifference
	CHG + Tea tree oil	16 / 16	1	2 / 2	1	2	Indifference
	CHG + Thymol	0.25 / 0.5	0.5	2 / 2	1	1.5	Indifference

468

469   Table 3. Antimicrobial activity of aqueous chlorhexidine digluconate, eucalyptus oil, tea tree oil and thymol against clinical TK1 and

470   RP62A strains of *S.epidermidis* growing in biofilm.

471

Strain	Combination	MIC of oil (g/L) in combination/ alone	FIC of oil	MIC of CHG(mg/L) in combination/ alone	FIC of CHG	FICI	Result
RP62A	CHG + Eucalyptus	4 / 32	0.125	0.25/ 8	0.031	0.156	Synergy
	CHG + Tea tree oil	4 / 16	0.25	4/ 8	0.5	0.75	Indifference
	CHG + Thymol	0.5 / 0.5	1	8/ 8	1	2	Indifference
TK1	CHG + Eucalyptus	4 / 64	0.063	1 / 8	0.125	0.188	Synergy
	CHG + Tea tree oil	16 / 16	1	8 / 8	1	2	Indifference
	CHG + Thymol	0.25 / 0.5	0.5	4 / 8	0.5	1	Indifference

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