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A screening of curcumin derivatives for antibacterial phototoxic effects Studies on curcumin and curcuminoids. XLIII

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Abstract: Curcumin, bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, has potential as a photosensitiser for photodynamic treatment of localised superficial infections in e.g., the mouth or skin. The aim of the present study was to evaluate the *in vitro* antibacterial phototoxic potential of a series of five curcumin derivatives. The gram-positive *Enterococcus faecalis* and the gram-negative *Escherichia coli* were used as bacterial models. The bacteria were exposed to curcuminoid preparations in two concentrations (2.5 and 25.0 μM) in combination with a constant irradiation dose (5 J/cm^2). The UV-VIS absorption spectrum of the curcuminoids was in the same range as curcumin, 300–500 nm. Compound 1 (dimethoxycurcumin; C1) and compound 3 (bisdemethoxycurcumin; C3) were strongly phototoxic towards *E. faecalis* (no surviving bacteria) and showed a lower but significant effect towards *E. coli* ($\leq$ 0.5 log reductions and 1 – 4 log reductions, respectively). Compound 2 (C2) and compound 4 (C4) in combination with blue light reduced the colony forming ability of *E. faecalis* ($\sim$1–4 log reductions). The phototoxic effect of the curcuminoids varied with concentration, and for compounds C1, C2 and C3 it was further influenced by the addition of polyethylene glycol 400 (PEG 400) to the preparations. 2,6 – Divanillylidenecyclohexanone (C5) showed very low phototoxic potential (<0.2 log reductions) under the conditions used in the present study. The addition of polyethylene glycol 400 (PEG 400) seemed to increase the solubility of compound C1, C3 and C5 in phosphate buffered saline (PBS). This investigation demonstrates the importance and influence of the substituents on the phenolic rings and the keto-enol moiety for the phototoxic potential of curcumin and its derivatives.

1. Introduction

In the search for new compounds with biological activity curcumin and derivatives thereof have attracted the interest of scientists. Curcuminoids are naturally occurring yellow compounds found in turmeric, a spice from the plant *Curcuma longa L.* Three different curcuminoids are present in the rhizomes of the plant, and the total amount and the ratio between them varies. Curcumin, bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is usually the major constituent and the most extensively studied curcuminoid. The other two naturally occurring curcuminoids dimethoxycurcumin and bisdemethoxycurcumin, lack one or two of the methoxy-groups present on the phenolic rings of curcumin. The naturally occurring curcuminoids exhibit strong light absorption at 420–430 nm in organic solvents ($\varepsilon \sim 55000$). They are also fluorescent in organic media (Tønnesen et al. 1995). The curcuminoids have the ability to form inter- and intramolecular hydrogen bonds. These bonds will strongly influence the compounds' physicochemical properties in both ground- and excited states. Curcumin and similar derivatives exist in equilibrium between the diketo- and keto-enol forms; the keto-enol form is strongly favoured by intramolecular hydrogen bonding. The keto-enol moiety can theoretically be also involved in intermolecular hydrogenbonding and possibly interact with the solvent together with the phenolic groups. Intermolecular hydrogen bonding can be demonstrated by changes in the UV-VIS and fluorescence spectra of the curcuminoids in various organic solvents (Tønnesen et al. 1995).

Previous studies have focused on the *in vitro* phototoxic effect of curcumin in various aqueous preparations against gram-positive *Enterococcus faecalis* and gram-negative *Escherichia coli* bacteria (Haukvik et al. 2009). Curcumin in surfactant preparations showed its potential as a photosensitiser (PS) in antibacterial photodymanic therapy (aPDT) *in vitro*. The killing effect was shown to be dependent on curcumin concentration, radiant exposure, post-irradiation incubation time, bacteria species and pharmaceutical preparation. The exact mechanism by which curcumin causes light induced cell death has not yet been established, but it is generally accepted that a prerequisite for photosensitisation of a microbial cell is the binding of the PS to the outer membrane (Malik et al. 1992; Jori and Coppellotti 2007). Hydrogen bonding and charge delocalisation are important features for interactions with biomolecules at the bacteria outer walls (An and Friedman 1998). The interactions between the curcuminoids and the bacteria are affected by the positioning and type of substituents on the phenolic rings and the keto-enol moiety of the curcuminoids. Therefore, investigations of

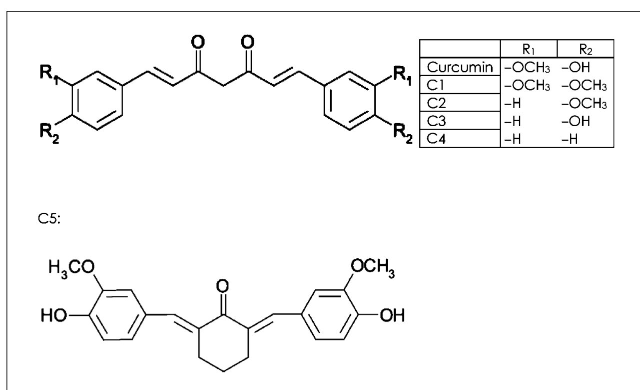

Fig. 1: Structure of the curcuminoids investigated in the present work (C1 to C5). The structure of curcumin is indicated as a reference

the curcuminoids in the present study may gain insight into the importance of the various substituents for the phototoxic effect of curcumin on bacteria. In the present work only pure synthetic curcuminoids were used. The curcuminoid samples were prepared from stock solutions in ethanol; in phosphate buffered saline (PBS), pH 6.1, with or without the presence of 2.5% v/v polyethylene glycol 400 (PEG 400). Polyethylene glycols are stable, water-soluble and hydrophilic polymers of ethylene oxide, frequently used as excipients in topical pharmaceutical formulations (Rowe et al. 2003). PEG 400 was previously shown to have some solubilising effect on curcumin in aqueous solutions, but at the same time this compound can reduce the phototoxic potential of curcumin against *E. coli* (Haukvik et al. 2010). This effect was probably due to hydrophobic interactions and hydrogen bonding between curcumin and PEG 400, reducing the availability of the curcumin molecules for the bacteria. Hence, uptake and/or adsorption to the outer bacterial wall were reduced resulting in less killing of the bacteria. It was therefore of interest to investigate how PEG 400 influences the UV-VIS absorption spectra and the antibacterial phototoxic effect of other curcuminoids. To optimize the stability of the curcuminiods and eliminate the effect of hydrolytic degradation products on the bacterial cells, the preparations of curcuminoids used in the present study were made in PBS, pH 6.1. However, for the final dilution of the bacterial suspensions a saline buffer of pH 7.0 was applied.

In the present study the aim was to investigate the *in vitro* phototoxic effect of five curcuminoids (Fig. 1) using the same bacterial models as previous studies (*E. faecalis* and *E. coli)* with or without the presence of PEG 400. By comparing the results to those previously obtained for curcumin (Haukvik et al. 2009; Haukvik et al. 2010) valuable knowledge of the phototoxicity of curcumin could be obtained.

2. Investigations and results

2.1. UV-VIS spectra

The compounds in the investigated preparations absorbed radiation in the UV-visible range of the spectrum (Fig. 2 A – E). The wavelength of the absorption maximum varied from 387 nm for C4, 396 nm and 397 nm for C5 and C2, respectively, to 416 nm and 421 nm for C1 and C3, respectively. The addition of 2.5% v/v PEG 400 to the solutions caused an increase (C1, C3 and C5) or decrease (C4) in the absorption maximum of the compounds. Further, the addition of PEG 400 produced a minor red shift in the absorption maximum of the compounds C1 (9 nm), C4 (4 nm) and C3 (2 nm), while no shift was observed in the case of C2 and C5. The emission spectrum of the irradiation source in the range 400 nm to 700 nm is presented in Fig. 2 F. The maximum irradiance was at 450 nm. There was a spectral overlap between the absorption spectrum of the curcuminoids in the investigated preparations and the emission spectrum of the irradiation source.

2.2. Phototoxicity experiments

All the curcuminoids tested in the present study demonstrated a significant phototoxic effect against *E. faecalis* (Figs. 3–6) under the given experimental conditions. The compounds C1, C3, C4 and C5 were added to the bacteria in the same concentration. The observed efficacy was in the following order of ascending bacterial survival: C3 > C1 > C4 > C5. The results were corrected for the variation in spectral overlap between the absorption of each curcuminoid preparation and the emis-

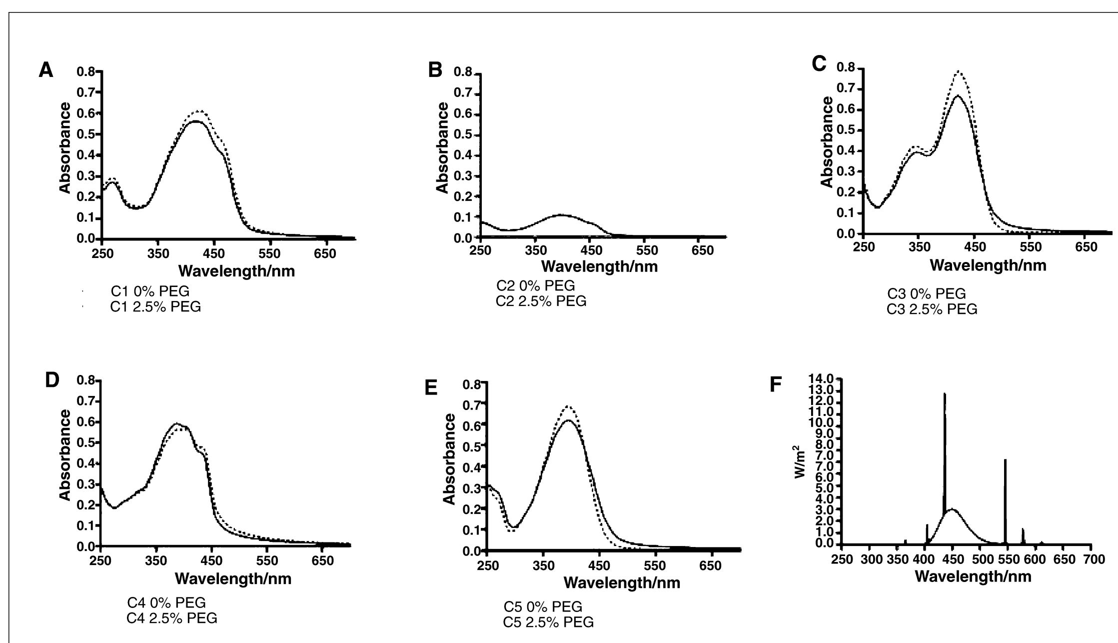

Fig. 2: UV-VIS absorption spectra of the curcuminoids dissolved in PBS without or with 2.5% v/v PEG 400 (A–E) compared to the emission spectrum of the irradiation source (F). A: compound C1 (25 μM); B: compound C2 (62.50 μl stock solution in 5 ml PBS); C: compound C3 (25 μM); D: compound C4 (25 μM); E: compound C5 (25 μM)

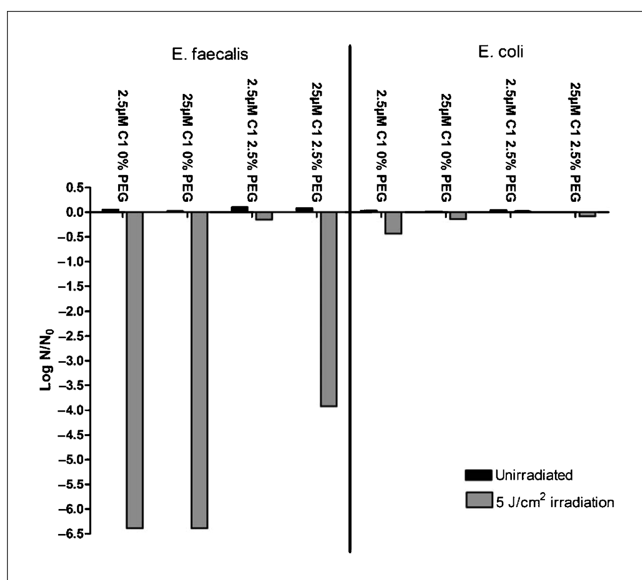

Fig. 3: Phototoxic effect of 2.5 μM and 25 μM C1 in PBS without or in the presence of 2.5% v/v PEG 400 combined with radiant exposure of 5 J/cm^2 against *E. coli* and *E. faecalis* (n = 12). N = number of CFU/ml on the exposed plates, N_0 = number of CFU/ml on the plates in the controls without PEG 400, C1 and irradiation.

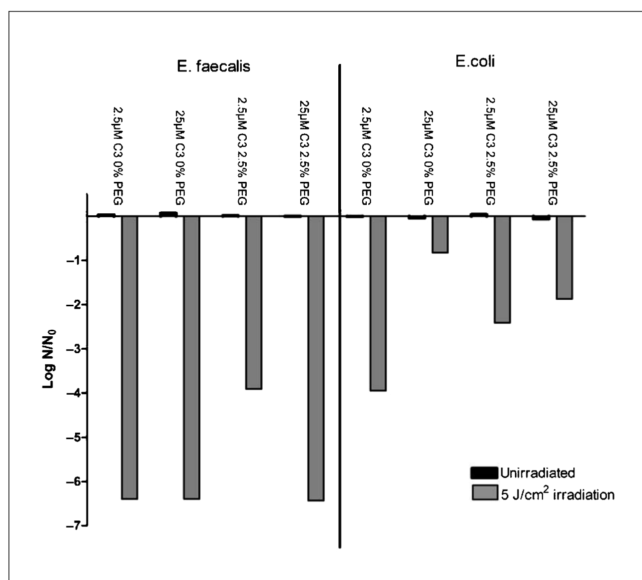

Fig. 5: Phototoxic effect of 2.5 μM and 25 μM C3 in PBS without or in the presence of 2.5% v/v PEG 400 combined with radiant exposure of 5 J/cm^2 against *E. coli* and *E. faecalis* (n = 12). N = number of CFU/ml on the exposed plates, N_0 = number of CFU/ml on the plates in the controls without PEG 400, C3 and irradiation.

sion of the irradiation source and compared on a normalized scale. C2 was used in a lower concentration due to its low solubility in ethanol. Nevertheless, it showed better efficacy than C4 and C5 against *E. faecalis* both before and after normalization. C5 had a significant but very low phototoxic effect (Log N/N_0 >0.2) against *E. faecalis* (data not shown). The gram-negative bacterium *E. coli* was less sensitive to the treatment. The compound C3 demonstrated, however, a substantial phototoxic effect against *E. coli* (Fig. 5), while C1 showed moderate antibacterial effect (Fig. 3).The compounds C2, C4 and C5 showed no significant phototoxic effect against *E. coli* under the conditions used in the present study (Fig 4 and 6, data for C5 not shown). The observed antibacterial phototoxic effect was dependent on the concentration of the curcuminoid in the preparation and/or the presence of PEG 400 (Table). Further, the curcuminoid concentrations used in the present study did not induce toxicity in the absence of light to any of the bacteria employed.

3. Discussion

The percent of "bacterial killing" caused by a given concentration of a PS gives only an approximate indication of the relative effectiveness of the sensitiser. One essential aspect in the study of photochemical and photobiological responses is the number of molecules available for light absorption (Pooler and Valenzeno 1982) and the number of photons absorbed. Thus, in order to compare the photobiological potential of different compounds the observed effect should always be normalized

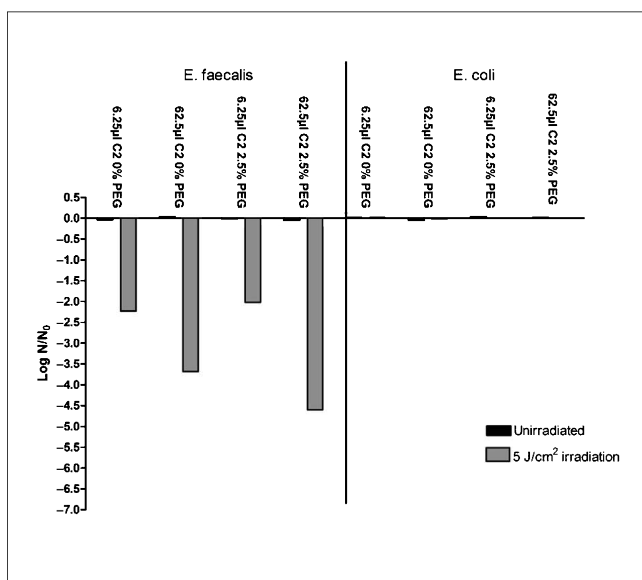

Fig. 4: Phototoxic effect of 6.25 μL and 62.5 μL C2 stock solution in 5 ml PBS without or in the presence of 2.5% v/v PEG 400 combined with radiant exposure of 5 J/cm^2 against *E. coli* and *E. faecalis* (n = 12). N = number of CFU/ml on the exposed plates, N_0 = number of CFU/ml on the plates in the controls without PEG 400, C2 and irradiation.

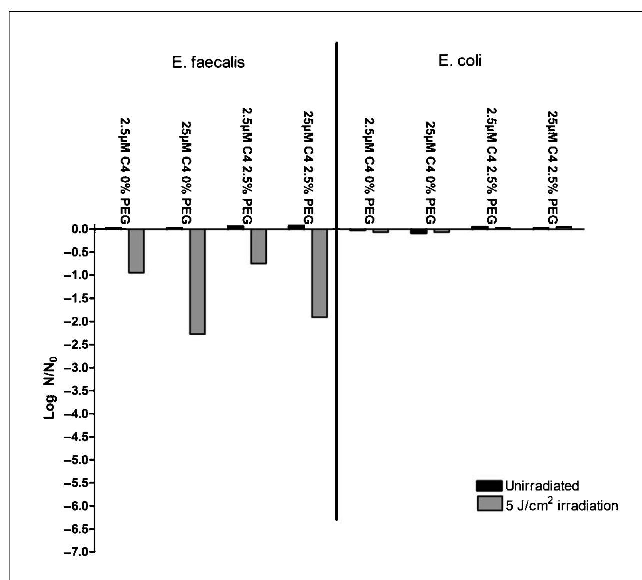

Fig. 6: Phototoxic effect of 2.5 μM and 25 μM C4 in PBS without or in the presence of 2.5% v/v PEG 400 combined with radiant exposure of 5 J/cm^2 against *E. coli* and *E. faecalis* (n = 12). N = number of CFU/ml on the exposed plates, N_0 = number of CFU/ml on the plates in the controls without PEG 400, C4 and irradiation.

Table: The influence of increased concentration of the curcuminoids ([C_n]) and the addition of PEG 400 ([PEG]) on the antibacterial phototoxic effect of the curcumin derivatives C1–C5

	E.faecalis		E.coli	
Compound (n)	Increased [C_n]	Increased [PEG]	Increased [C_n]	Increased [PEG]
C1	↑ for 2.5% PEG No survival CFU for 0% PEG	↓	↓ for 0% PEG ↑ for 2.5% PEG	↓ for 2.5 μM No significant difference for 25 μM
C2	↑	↑ for 62.5 μl No significant difference for 6.25 μl	–	–
C3	↑ for 2.5% PEG No survival CFU for 0% PEG	↓ for 2.5 μM No survival CFU for 25 μM	↓ for 0% PEG No significant difference for 2.5% PEG	↓ for 2.5 μM ↑ for 25 μM
C4	↑	No significant difference	–	–
C5	↑	No significant difference	–	–

↑ Significantly higher phototoxic effect ↓ Significantly lower phototoxic effect - no significant phototoxic effect CFU = colony forming units

to the number of photons absorbed by the individual sensitiser. The sum of photons absorbed at each emission wavelength of the irradiation source gives the total number of photons absorbed by the chromophore. Strictly, to correct for any transmittance change in the solutions upon addition of the bacteria the UV-VIS spectrum of the curcuminoid preparations should have been recorded immediately before irradiation, but after the addition and incubation with the bacteria. This was not performed for practical reasons. Furthermore, the various curcuminoids may not undergo identical photochemical reactions and therefore different photodegradation- and bacteria end-products may result which may alter the sample transmittance during the experiment. This has not been taken into account in the present discussion.

C2 and C4 in PBS in combination with light showed reduced effect on the colony forming units (CFU) of *E. faecalis* (Fig. 4 and 6). The effect increased with increasing concentration of the compounds. The comparison of the photokilling effect of C2 with that of curcumin in previous results could not be performed due to relatively low solubility of the compound in ethanol. Compound C4 showed a smaller reduction in colony forming units (CFU) in *E. faecalis*, compared to curcumin (Haukvik et al. 2010), even at ten times higher concentration of C4 and ten times higher radiant exposure. C4 has no substituents on the aromatic rings while C2 has methoxy-groups in the meta position, but lacks the hydroxyl groups of curcumin (Fig. 1). The photoeffect of C1 and C3 in PBS against *E. faecalis* was similar to the effect previously observed for curcumin (Haukvik et al. 2009). Exposure of these compounds resulted in no surviving bacteria, corresponding to 6 log reductions. Furthermore, C1 and C3 demonstrated some killing effect against the gram-negative bacteria *E. coli* in combination with a light dose of 5 J/cm^2. The higher efficiency seen for the lowest concentrations tested (2.5 μM versus 25.0 μM), reflects the results previously reported for curcumin in combination with twice the light dose (Haukvik et al. 2010). The potential of curcumin and C3 as photosensitisers in aPDT are in the same range when comparing the results obtained so far. The effect of the compounds was dependent on curcuminoid concentration and probably irradiation dose (tested for curcumin only; Haukvik et al. 2010).Optimization of concentration and radiant exposure for both compounds must be performed for a more thorough comparison. C1 was less effective than curcumin and C3 against *E. coli* (Fig. 3 and 5) (Haukvik et al. 2010). The only structural difference between C3 and curcumin is the lack of the methoxy groups in the meta position on the phenolic rings of C3. In C1 the hydroxyl groups in para position are replaced by methoxy groups compared to curcumin (Fig. 1). Compound C5 differs from curcumin in that the keto-enol moiety is replaced by a cyclohexanone group, while the substituents on the phenolic ring are identical to curcumin (Fig. 1). The very low phototoxic effect of this compound (2.2) demonstrated the importance of the keto-enol group for the phototherapeutic action of curcumin. The difference in sensitivity between gram-positive and gram-negative bacteria towards aPDT using curcuminoids as photosensitisers corresponds to previously reported results for curcumin in various aqueous preparations (Haukvik et al. 2009). The variation in sensitivity is probably due to the differences in the outer wall of gram-positive and gram-negative bacteria. Gram-positive bacteria are regarded as "easy targets" in aPDT as their membrane barrier consists of a relatively permeable peptidoglycan layer which offers little protection. In contrast, the outer lipopolysaccharide (LPS) layer of gram-negative bacteria is electrostatically linked to divalent cations and produces a very efficient barrier against lipophilic compounds (Hamblin and Hasan 2004). C1 and C3 might be taken up and/or adhere to the surface of *E. coli* and *E. faecalis* in a more efficient way than compound C2 and C4 due to differences in hydrogen bonding properties, polarity and/or ability to form hydrophobic interactions with the bacteria wall.

The difference in phototoxic effects observed in this study may be due to other reaction pathways than those obtained for curcumin or to less uptake and/or adsorption to the bacterial surface or a combination of both. The compounds formed during the photoactivation of curcumin that are responsible for the toxic effect have been suggested to include oxygen species such as singlet oxygen (Tønnesen et al. 1987), hydroxyl radical (Chignell et al. 1994; Tønnesen et al. 1992) or hydrogen peroxide (Dahl et al. 1989). Alternatively, curcumin radicals have been proposed as either the phototoxic species or long-lived transient reactants capable of interacting with oxygen to generate the toxic species without further irradiation (Dahl et al. 1994). Similar mechanisms as outlined for curcumin are likely to be (at least partly) responsible for the effects observed in the present study. The enolised diketone system in curcumin appears to be the part of the molecule most heavily involved in the scavenging of oxygen radicals (Tønnesen et al. 1992). The conformation of this moiety is important for the photoreactivity of curcumin (Nardo et al. 2008) and is possibly involved in the formation of phototoxic compounds. Intramolecular hydrogen bonding is assumed to influence on photophysical characteristics of the curcuminoids. The major non-radiative pathway is postulated to be that of excited-state intramolecular proton transfer between the hydroxyl- and the keto-group of curcumin present in the closed cis enol tautomeric form (Nardo et al. 2008, 2009). Therefore, any changes in the aromatic substituents, and thereby the intramolecular hydrogen bonding properties of the

curcuminoids may change the excited state deactivation pathways. Further, intermolecular hydrogen bonding between the aromatic substituents and the surroundings may also be involved in the photochemical processes of curcuminoids as such intermolecular bondings may stabilize the excited state (Tønnesen et al. 1995; Caselli et al. 2010).

The exact bacterial uptake and/or adherence mechanism of the curcuminoids is not known. However, it is generally accepted that a prerequisite for photosensitisation of a microbial cell is the binding of the PS to the outer membrane (Malik et al. 1992; Jori and Coppellotti 2007). The phenolic groups will be essential for interactions between curcuminoids and biological membranes in cases where hydrogen bonding plays an important role. The results of the present study demonstrated that the aromatic substituents apparently are involved in the uptake and/or adherence of the curcuminoids to the gram-negative bacteria. A further comparison of phototoxic effects between curcumin and C3 against *E. coli* may give valuable information about the interaction caused by hydrogen bonding between curcuminoids and bacterial membranes. The differences in hydrogen bonding capacity between curcumin and C3 has previously been postulated by Tønnesen et al. (1995): The presence of a methoxy group next to the phenolic group will make the hydroxyl group in curcumin a stronger hydrogen bond acceptor than the hydroxyl group in C3, the latter is more likely to be a hydrogen bond donor. Hence, curcumin is most likely to interact with sites possessing hydrogen bond donor properties while C3 will have greater attraction for hydrogen bond acceptor binding sites. C1 can act both as a hydrogen-bond acceptor and donor. The fact that C3 has a larger phototoxic effect than C1 may be due to the ability of the former to interact with the bacteria wall through hydrogen bonding.

The observed increase in absorbance for compounds C1, C3 and C5 upon addition of PEG 400 is probably due to an increase in solubility of the curcuminoids in less polar media. The observation corresponds to results previously reported for curcumin in similar preparations (Haukvik et al. 2010). The substituents on the phenolic rings of compounds C1 – C3 were apparently involved in the interaction between PEG 400 and the curcuminoids as demonstrated by spectral changes upon addition of PEG 400 to the samples. This is in contrast to the absorption spectrum of C4 which did not change and has also previously been shown to be almost independent of the solvent properties (Tønnesen et al. 1995). This compound lacks the phenolic groups that can interact with the solvents through intermolecular hydrogen bonding. In this molecule, the keto – enol unit does not, or only weakly, interact with the actual solvents (Tønnesen et al. 1995). The addition of PEG 400 to the C1 and C3 preparations reduced the phototoxic effect against *E. faecalis* significantly. In contrast, the effect of C2 was significantly increased when the highest curcuminoid concentration was tested against *E. faecalis* (Table and Figs. 3, 4 and 5). Likewise, a reduction in photokilling of *E. coli* was observed after exposure of the bacteria to 2.5 μM C1 and C3 respectively, prepared in a 2.5% PEG 400 solution. On the other hand, the addition of 2.5% PEG 400 caused an increase in phototoxicity of C3 at a concentration of 25 μM compared to plain buffer. Similar variations in the phototoxic effect of curcumin on *E. coli* after the addition of PEG 400 were observed in an earlier study (Haukvik et al. 2010). The reduced phototoxic effect of 2.5 μM curcumin in the presence of PEG 400 was explained by interactions between curcumin and PEG 400 resulting in a low amount of free curcumin available for absorption/adsorption in the bacterial membrane at such a low curcumin concentration. This phenomenon may occur also with the other curcuminoids. At the highest C3 concentration the reduction in available molecules might be contradicted by an increase in aqueous solubility upon addition of co-solvent.

This study demonstrates that the antibacterial phototoxic potential of a series of curcuminoids varies with the substituents in the phenolic rings and also with the presence of the keto-enol moiety. All the curcuminoids tested were phototoxic to *E. faecalis,* although C5 very weakly. Furthermore, compounds C1 and C3 were photoactive against the more resistant *E. coli*. The effect was dependent on curcuminoid concentration and sample composition. C3 showed an antibacterial potential similar to curcumin, but further studies should be performed to confirm this preliminary observation. Moreover, PEG 400 had a solubilising effect on the curcuminoids in PBS, but the stability of the curcuminiods in the preparations should be further evaluated.

4. Experimental

4.1. Materials

Curcuminoids, C1 – C4, were synthesized and their purity and identity were confirmed as previously described (Haukvik et al. 2009, Tomren et al. 2007). C5 was purchased as 2,6 – divanillylidenecyclohexanone (Sigma-Aldrich, MO, USA) and used as received. Phosphate buffered saline (PBS; Lonza, Verviers, Belgium) was adjusted to pH 6.1 by adding HCl (Sigma-Aldrich) and filtered (0.22 μm; Millipore S.A.S., Molsheim, France) prior to use. All other chemicals were of analytical grade and used as received. The water was purified and deionised.

4.2. Preparation of samples

Stock solutions of the curcuminoids C1, C3, C4 and C5 (2 mM) were prepared in ethanol. Due to low solubility of C2 in ethanol the C2 stock solution was prepared as a saturated solution with a concentration below 2 mM. The solutions were stored in the refrigerator (+4 °C). Solutions of the curcuminoids C1, C3, C4 and C5 in final concentrations of 2.5 μM and 25.0 μM were prepared in PBS, pH 6.1 from the stock solutions without or with 2.5% (v/v) polyethylene glycol 400 (PEG 400; Aldrich). In order to keep the ethanol amount constant in all the experiments the C2 stock solution was filtered (0.22 μm) and added to the PBS with or without 2.5% PEG 400 in the same amount as the other curcuminoids (6.25 μl and 62.50 μl stock solution in 5 ml PBS). All curcuminoid preparations were made immediately before use and kept protected from light throughout the experiments.

4.3. Absorption spectra

The UV-visible absorption spectra (190 – 700 nm) of the curcuminoids (62.5 μl stock solution in 5 ml PBS pH 6.1 without or with 2.5% PEG 400) were recorded immediately after preparation. The spectra were recorded on a spectrophotometer (Specord 200, Analytik Jena AG, Jena, Germany).

4.4. Incubation and irradiation

4.4.1. Microorganisms

Enterococcus faecalis (A197A) and *Escherichia coli* (ATCC 25922) were maintained by three times weekly subculture in tryptone soya broth (TSB; Oxoid Ltd., Bassingstoke, UK). The bacteria were incubated at 37 °C.

4.4.2. Irradiation and dosimetry

The light source was a light-polymerisation unit equipped with three fluorescent tubes emitting light in the wavelength range approximately 400–700 nm (Ralutec 9W/171) with an emission maximum in the blue range of the visible scale at 450 nm (Polylux PT, Dreve Dentamid GmbH, Unna, Germany). The mean irradiance of nine points at the cell dish level inside the irradiation chamber was determined to 17.3 mW/cm^2 (± 5%). The irradiance was kept constant throughout the experiments. Irradiation duration was 5 min corresponding to 5 J/cm^2 radiant exposure. Emission spectra were determined with a calibrated double monochromator spectroradiometer (model DTM300, Bentham Instruments Ltd., Reading, UK) and the irradiance was monitored at regular intervals with a UDT 271 radiometer (United Detector Technology, San Diego, CA, USA) calibrated towards the spectroradiometer. The radiometer was equipped with a probe sensitive in the blue (268 BLU) part of the spectrum.

4.4.3. Testing of phototoxicity

The bacterial test strains were grown overnight in nutrient broth at 37 °C. Aliquots of the overnight culture were diluted in PBS pH 6.1 and transferred to test tubes containing the curcuminoid solution to give a final bacteria

concentration of 10^6 CFU/ml. A volume of 1 ml from each test tube was transmitted to sterile 35 mm tissue culture dishes (Falcon; Becton, Dickinson, Bedford, MA, USA). The samples were incubated for 30 min in the dark at 37 °C to allow penetration of curcumin into the bacterial cells or interaction with the outer cell wall prior to irradiation. After incubation the samples were irradiated for a given time (4.4.2), diluted 40 times with PBS pH 7.0 and incubated in the dark for 1 h to allow for the reaction between possible toxic photoproducts and cellular targets. Aliquots of the bacterial suspensions were plated onto TSB agar with an automatic spiral plater (Whitley, Don Whitley Scientific LTD, Shipley, West Yorkshire, UK) to estimate the survival by counting the colony-forming ability. The colonies were counted by the use of a colony counter (Acolyte, Synbiosis Europe Office, Cambridge, UK) after 24 h of incubation at 37 °C. Unradiated and reference controls were included in the study. The bacterial survival was calculated as log N/N_0, where N is the number of CFU/ml on the exposed plates and N_0 is the number of CFU/ml on the plates in the absence of PEG 400, PS and irradiation. All bacterial tests were performed three times with four replicates in each experiment.

4.5. Calculations

The bacterial survival does not take into account the number of photons absorbed by each photosensitiser. Hence, the survivals were corrected for the total number of photons absorbed by each curcuminoid preparation to evaluate their relative potencies as photosensitisers and compared on a normalised scale:

$$\text{Bacterial survival}_n = \text{bacterial survival} / \sum (E\lambda \times A_l \times \lambda)$$

where bacterial survival_n is the normalised value, E_λ is the spectral irradiance ($Wm^{-2}nm^{-1}$) of the irradiation source, A_λ is the absorbance of the chromophore (curcuminoid) in the reaction medium and λ is the wavelength range (nm)(calculated from 330–700 nm) of the source. The sum of photons absorbed at each output wavelength from the lamp gives the total number of photons absorbed by the chromophore. The thickness of the irradiated samples was kept constant at 1 mm.

4.6. Statistics

Student's *t*-test for independent samples (two-tailed) was used to evaluate the results and estimate significant differences. A *P*-value less than 0.05 was chosen as statistically significant. Each value was expressed as the mean.

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