Heat treatment enhances the antimicrobial activity of (+)-Catechin when combined with copper sulphate

Article

Accepted Version

Article Type: Original article

Heat treatment enhances the antimicrobial activity of (+)-Catechin when combined with copper sulphate

Running title: Antimicrobial activity of heat and Cu^{2+} treated (+)-Catechin

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Significance and Impact of Study:

Natural products attract considerable attention in the search for novel antimicrobials, prebiotics, and antioxidants. Enhanced biological activity of natural products has been demonstrated with chemical and heat treatment. This article extends the few publications on heat treatments of plant products and combinations with adjuncts, to raise antimicrobial

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/lam.12472

activity against pathogens such as *Staphylococcus aureus*. We demonstrated that heat treatment could increase the activity of (+)-Catechin, a weak antimicrobial flavanol found commonly in plants in the presence of copper sulphate. Heat treatment of readily-available resources merits consideration in the development of more potent substances for use in clinical settings and agriculture.

Abstract

The aim of the study was to compare the antimicrobial activities of freshly-made, heat-treated (HT), and 14 d stored (+)-Catechin solutions with (+)-catechin flavanol isomers in the presence of copper sulphate. (+)-Catechin activity was investigated when combined with different ratios of Cu^{2+}; 100°C heat treatment; autoclaving; and 14 d storage against *Staphylococcus aureus.* Cu^{2+}-(+)-Catechin complexation, isomer structure-activity relationships, and H_2O_2 generation were also investigated. Freshly-made, HT, and 14d stored flavanols showed no activity. Whilst combined Cu^{2+}-autoclaved (+)-Catechin and -HT(+)-Catechin activities were similar, HT(+)-Catechin was more active than either freshly-made (+)-catechin (generating more H_2O_2) or (-)-Epicatechin (though it generated less H_2O_2) or 14d-(+)-Catechin (which had similar activity to Cu^{2+} controls - though it generated more H_2O_2). When combined with Cu^{2+}, in terms of rates of activity, HT(+)-Catechin was lower than (-)-Epigallocatechin gallate and greater than freshly-made (+)-Catechin. Freshly-made and HT(+)-Catechin formed acidic complexes with Cu^{2+} as indicated by pH and UV-vis measurements although pH changes did not account for antimicrobial activity. Freshly-made and HT(+)-Catechin both formed Cu^{2+} complexes. The HT(+)-Catechin complex generated more H_2O_2 which could explain its higher antimicrobial activity.

Keywords: (+)-Catechin, heat activation, Cu^{2+} adjunct, structure-activity, antimicrobial

Introduction

Worldwide efforts are continuing to develop antimicrobial substances for use in food preparation areas, health settings, veterinary and agricultural contexts, aquaculture and water treatment systems to combat pathogens that affect humans, plants and animals of commercial and domestic importance, as well as stored food and fodder (Dukic and Ilic 1991; Hanuso *et al.* 2004; Franco *et al.* 2010; Cavaco *et al.* 2011; Espirito *et al.* 2011; Grube *et al.* 2011; Nedorostova *et al.* 2011).

Flavanols, including (+)-catechin are a subgroup of flavonoids that occur in many plants across the world (Taguri *et al.* 2004; Stapleton *et al.* 2004a; Stapleton *et al.* 2006). As antimicrobials, flavanols are active against Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus* and *Escherichia coli* (Taguri *et al.* 2004). *S. aureus* is a suitable model organism for research into antimicrobial agents as some strains show considerable resistance to antimicrobials and are of concern in both food and agricultural settings (Franco *et al.* 2010; Cavaco *et al.* 2011).

Previous studies have investigated the extent to which flavanols and their synthetic analogues generate antimicrobial activity as well as their mechanisms of action. Others have shown that flavanol activity can be enhanced by adjuncts, such as conventional antibiotics, vitamin C, and transition metal ions (Stapleton *et al.* 2004a, 2004b, 2006, 2007; Hatano *et al.* 2008; Holloway *et al.* 2012). Heating of some phenolics can also enhance antimicrobial activity (Kim *et al.* 2010). Therefore, autoclaving of extracts may provide a means of raising activity as well as sterilising them for subsequent use.

According to the literature the minimum inhibitory concentrations (MICs) of the common tea flavanols: (+)-Catechin, ((+)-Cat); (-)-Catechin, ((-)-Cat); (+)-Epicatechin, ((+)- EC); (-)-

Epicatechin, ((-)-EC); (-)-Epigallocatechin, ((-)-EGC); (-)-Epicatechin gallate, ((-)-ECg); and (-)-Epigallocatechin gallate, ((-)-EGCg), against different strains of *S. aureus* ranged between 0.025 and 256 mg ml^{-1} and averages for each flavanol were ranked as follows: (-)-EGCg < (-)-ECg < (-)-EGC < (-)-EC < (+)-Cat < (+)-EC < (-)-Cat (Takahashi *et al.* 1995; Akiyama *et al.* 2001; Gibbons *et al.* 2004; Roccaro *et al.* 2004; Stapleton *et al.* 2004a, 2006, 2007; Taguri *et al.* 2004, 2006).

(+)-Cat, which is a common but weak antimicrobial flavanol, is a suitable candidate for investigating the possibility of antimicrobial enhancement by heating and the addition of copper sulphate (Holloway *et al.* 2012). (+)-Cat lacks the features typically found in compounds with higher antimicrobial activities, i.e. three OH groups in the B ring, *cis*-stereochemistry at the C ring and a galloyl group at the C-3 position (Taguri *et al.* 2004; Stapleton *et al.* 2004a, 2006). In this report we present data that reveal the following: (i) the effects of heat treatment and storage of (+)-Cat solution in combination with copper sulphate on antimicrobial activity and (ii) effects of chemical structure on the activity of flavanol isomers.

Results and discussion

Screening of tea flavanols

The antimicrobial activity of freshly-made (+)-Cat was compared to that of four different types of flavanols found in green tea: **(-)-**EC, **(-)-**EGC, **(-)-**ECg, **(-)** EGCg, at final concentrations between 214 nmol l^{-1} and 2135 μmol l^{-1}. There were no effects on *S. aureus* viability (results not shown). However, in the presence of 2175 μmol l^{-1} (f.c.) copper sulphate flavanol activity decreased in the order: (-)-EGCg = (-)-EGC > (-)-ECg > (-)-EC > (+)-Cat. The pattern of activity followed the general MIC trend reported in literature without the addition of copper sulphate described above (Table 1).

**Position of Table 1*

Having established that (+)-Cat could be activated by 2175 μmol l^{-1} (f.c.) copper sulphate, several copper sulphate concentrations were investigated to establish whether a particular molar ratio of Cu^{2+}: (+)-Cat was optimal.

Studies using freshly-made (+)-Cat plus different copper sulphate concentrations

Samples containing progressively higher levels of copper sulphate ranging from 53.5 to 3424 μmol l^{-1} (f.c.) combined with a fixed (+)-Cat concentration of 214 μmol l^{-1} (f.c.), resulted in progressively lower viabilities with no pronounced affect seen at any particular molar ratio. (+)-Cat treatment on its own similar viabilities as buffer solutions resulted in similar viabilities as buffer values. These results extend previous findings that (+)-Cat-Cu^{2+} combinations can damage bacteria (Holloway *et al.* 2012).

Appearance and activities of heat-treated (HT) and 14 d stored (+)-Cat solutions

A 1 mmol l^{-1} freshly-made (+)-Cat solution changed after heating for 10 min at 100°C, from a clear, colourless to a clear, pale golden-coloured solution which suggested oxidation. Heating for a further 20 min caused no further visible changes. Preliminary studies with nuclear magnetic resonance (NMR) to identify any newly-formed reaction products within HT(+)-Cat solution (10 min at 100°C) suggested that less than 20% of the (+)-Cat had been converted into new products following heat treatment (spectra not shown). If this estimation is correct, this amount was sufficient to produce a difference in antimicrobial activity against *S. aureus* when it was subsequently combined with copper sulphate and compared to freshly-made (+)-Cat with copper sulphate (Fig. 1). A previous study also with (+)-Cat solutions identified biologically active yellow substances as (+)-Cat derivatives after heat treatment (Es-Safi *et al.* 2003).

In the susceptibility assays, freshly-made, HT, and 14 d stored (+)-Cat solutions when tested alone showed similar antimicrobial activity to buffer solutions. (+)-Cat solution HT for 10 or 30 min and subsequently combined with equimolar copper sulphate showed similar activities to each other, but activities were greater than freshly-made (+)-Cat plus copper sulphate solutions (Table 2).

**Position of Table 2*

Autoclaving freshly-made (+)-Cat raised its antimicrobial activity to that seen with HT (+)-Cat when subsequently tested in combination with copper sulphate whereas HT (+)-Cat activity was not raised when tested in the same way. In both cases the results were similar to freshly-made and HT (+)-Cat combinations. However, when freshly-made (+)-Cat or HT (+)-Cat solutions were each combined with copper sulphate and subsequently autoclaved, antimicrobial activity was reduced (Table 2). Such lowered antimicrobial activity shown by autoclaved combinations of the different (+)-Cat solutions with copper sulphate suggested that the level of activity of antimicrobial components within the combinations had been reduced by autoclaving, possibly by chemical interactions.

Three different methods of heating (+)-Cat solutions were compared for their effects against *S. aureus*. Heat-treatment at 100°C for 10 min proved most effective and there was no further increase of antimicrobial activity with heating for 30 min, or by autoclaving at 121°C for 15 min when subsequently assayed (Table 2). Autoclaving of freshly-made or HT(+)-Cat in the presence of copper sulphate produced a dark brown precipitate and lower antimicrobial activities, which was likely to have been caused by an insoluble Cu^{2+}-phenolic complex.

Freshly-made (+)-Cat solution stored in the dark for 14 d at room temperature (*c.* 20°C) contained a suspended pale brown precipitate. The viability of cells exposed to 14d stored 214 µmol l^{-1} (f.c.) (+)-Cat was found to be the same as freshly-made 214 µmol l^{-1} (f.c.) (+)-

Cat and buffer controls. 14d stored (+)-Cat with copper sulphate showed a similar effect to copper sulphate controls which indicated no enhancement by the adjunct (Table 2).

Comparing activities of freshly-made and HT (+)-Cat to its flavanol isomers

Following the finding that copper sulphate was capable of enhancing both freshly-made and HT (+)-Cat solutions, the possible mechanisms of action were explored in relation to the molecular features of flavanols. The activities of freshly-made and 10 min HT(+)-Cat solutions were compared to other freshly-made flavanol solutions to investigate effects of stereoisometry and epimerisation on viability. The following solutions were investigated: freshly-made solutions of (+)-Cat, (-)-Cat, (-)-EC, (+)-EC, HT(+)-Cat and 14d stored (+)-Cat solutions were combined with equimolar 214 μmol l^{-1} (f.c.) copper sulphate (Fig. 1).

**Position of Figure 1*

The following order of antimicrobial activity was found against *S. aureus* : HT(+)-Cat-Cu^{2+} > (-)-EC-Cu^{2+} > (+)-Cat-Cu^{2+} > (+)-EC-Cu^{2+} = (-)-Cat-Cu^{2+} > 14d (+)-Cat-Cu^{2+} = Cu^{2+} control (Fig. 1).

Stored (+)-Cat, (-)-Cat, or (+)-EC solutions, which were combined with equimolar copper sulphate, had similar activity compared to the copper sulphate control (Fig. 1). This agrees with the findings that (-)-Cat and (+)-EC were the least active of the tea flavanols against different strains of *S. aureus* (Stapleton *et al.* 2004). (+)-Cat and **(-)-**EC had greater antimicrobial effects in the presence of copper sulphate than Cu^{2+} controls, which suggested that these molecules possessed more favourable configurations at the C2 and C3 positions than their enantiomers. Isometry occurs at carbons 2 and 3 in the C ring where the 3',4'-dihydroxyphenyl and hydroxyl group can be found in two configurations giving rise to (+)-Cat and (-)-Cat or (-)-EC and (+)-EC. However, HT(+)-Cat solution had a greater effect on

antimicrobial activity in the presence of copper sulphate than freshly-made (+)-Cat or **(-)-**EC. This suggested that heating generated further active substances within the solution.

Hydrogen peroxide generation by test mixtures

A possible mechanism of action of the freshly-made flavanols that were investigated, as well as HT, and 14 d stored (+)-Cat solutions, combined with copper sulphate was investigated by measuring the generation of any antimicrobial H_2O_2 (Fig. 2).

**Position of Figure 2*

These solutions generated H_2O_2 in the following order: (-)-EC-Cu^{2+} > HT(+)-Cat-Cu^{2+} > 14d stored (+)-Cat-Cu^{2+} = (-)-Cat-Cu^{2+} > (+)-EC-Cu^{2+} > (+)-Cat-Cu^{2+} > Cu^{2+} control. Control buffer and flavanols on their own did not produce H_2O_2 (data not shown). H_2O_2 concentrations on their own cannot fully explain the observed effects, because some combinations, e.g. (-)-Cat-Cu^{2+} and 14 d stored (+)-Cat-Cu^{2+}, produced more H_2O_2 than the Cu^{2+} control but, their effects on viability were the same as the Cu^{2+} control. It is conceivable that flavanols attract Cu^{2+} ions to the cell envelope to varying degrees and this could explain the variable effect of the generated H_2O_2. Previous investigations have indeed shown that (+)-Cat and other flavanols generate H_2O_2 in the presence of copper sulphate, which accounted for their antimicrobial effects (Hoshino *et al*. 2000; Holloway *et al.* 2012).

pH measurements of (+)-Cat solutions

Measurements of pH were done at suspension assay concentrations before and after heating (+)-Cat solutions with/without addition of copper sulphate solutions. This information was needed to determine whether pH changes could independently account for test sample effects

on *S. aureus* viabilities. In each case the addition of either 214 µmol l^{-1} (f.c.) or 2175 µmol l^{-1} (f.c.) copper sulphate solution to freshly-made or HT 214 µmol l^{-1} (f.c.) (+)-Cat solutions (which tested alone showed pH mean values of 6.5 (±0.02) and 6.7 (±0.03) respectively) resulted in a drop of 0.7 - 0.8 pH units. Since HT(+)-Cat solution was found to be less acidic than freshly-made (+)-Cat solution this finding could reflect a reduction in the quantity of acidic components present following heat treatment e.g., caused by the hypothetical conversion of (+)-Cat to quinine methides (see below). Freshly-made or HT(+)-Cat added to both concentrations of copper sulphate resulted in a similar increase in acidification. This finding suggests the possibility of a limiting effect of the concentration of species within the (+)-Cat solutions on protonation in excess copper sulphate. Given that the interactions between (+)-Cat and copper sulphate reduced solution pH, it is possible that the lower pH may have made the cell membrane more susceptible to the effects of Cu^{2+} ions (Hoshino *et al*. 2000). Separate experiments with Ringer's solutions adjusted to pH values between 3.5 and 7.2, showed no pH effect on viability of *S. aureus* compared to buffer control values (data not shown).

UV-vis spectroscopy of freshly-made and HT (+)-Cat mixtures

UV-vis spectroscopy was used to probe the effect of copper sulphate on HT and freshly-made (+)-Cat solutions. Spectra of these solutions were also recorded at different molar ratios of copper sulphate with and without ethylenediamine tetra-acetic acid (EDTA). Freshly-made and HT (+)-Cat solutions gave similar absorption spectra but the freshly-made solution gave a greater absorption at *c.* 290 nm. Ringer's solution had little or no absorbance. Peak heights at *c.* 390 nm appeared to be positively linked to copper sulphate concentrations, whether added to freshly-made or HT(+)-Cat solutions. Copper sulphate controls with no (+)-Cat present showed no absorption at this wavelength. Freshly-made (+)-Cat with copper sulphate

gave higher absorbance values than the HT combinations whilst the addition of EDTA to (+)-Cat-Cu^{2+} samples removed the absorbance peaks at *c.* 390 nm. EDTA tested with freshly-made and HT (+)-Cat solutions in the absence of copper sulphate had no effect on the spectra (data not shown).

The appearance of UV-vis spectra of HT(+)-Cat solution showed small absorbance peaks at *c.* 425 nm which suggested the possible formation of new substance/s. Possible derivatives of HT(+)-Cat solutions could include phenoxonium cations or quinone methides along with their reaction products, such as dimers and branched structures (Chen *et al.* 2010; Ferreira D, pers. comm). Higher activities of other HT polyphenols have been previously explained in terms of newly-formed reaction products (Es-Safi *et al.* 2003).

Antimicrobial kinetics of freshly-made and HT(+)-Cat compared to (-)-EGCg

(-)-EGCg is known as one of the most active antimicrobial flavanols found in tea extracts and therefore a useful substance with which to compare the antimicrobial activities of freshly-made and HT (+)-Cat (Taguri *et al.* 2006). The antimicrobial rates of action of freshly-made 214 μmol l^{-1} (f.c.) (+)-Cat, 214 μmol l^{-1} (f.c.) HT(+)-Cat, and 214 nmol l^{-1} (f.c.) EGCg, combined with 2175 μmol l^{-1} (f.c.) copper sulphate were compared to each other, and to a flavanol-free Cu^{2+} control over 15 min.

**Position of Figure 3*

The order of antimicrobial activities against *S. aureus* were as follows: (-)-EGCg-Cu^{2+} > HT(+)-Cat-Cu^{2+} > freshly-made (+)-Cat-Cu^{2+} > Cu^{2+} controls, with the greatest effect occurring within the first min of exposure (Fig. 3). It would appear that the greater antimicrobial effect in the kinetics experiments with copper sulphate combinations with HT compared to freshly-made (+)-Cat was possibly due to new chemical substances generated

during heating. These products appear to have formed complexes in the presence of copper sulphate. Previous investigations indicated that tea flavanols and flavanols complexed with Cu^{2+} intercalate with the cell membrane palisade causing disruption of normal cell function thus affecting growth and viability (Taguri *et al.* 2006; Stapleton et al. 2007). The rates of antimicrobial action shown in Fig. 3 suggested that the activities of all flavanols could be enhanced by Cu^{2+} (Hoshino *et al*. 2000). The antimicrobial effects of these mixtures largely occurred within the first minute, which suggested a rapid effect from a highly active substance such as H_2O_2.

Materials and methods

Bacterial strain, storage and growth

S. aureus NCTC 6751 purchased from Pro-Lab Diagnostics, Wirral, UK was grown in Brain Heart Infusion broth obtained from Oxoid Ltd., Basingstoke, UK and kept in cryogenic tubes sourced from Pro-Labs at -80ºC. Cells were revived on nutrient agar plates purchased from Oxoid. Microbial suspensions were prepared in Ringer's solution sourced from Oxoid to a turbidity equivalent to 0.5 McFarland (~1.5 x 10^8 colony-forming units (CFU) ml^{-1}). When these suspensions were added to test substances the subsequent dilution resulted in an initial viable count of ~1 x 10^8 CFU ml^{-1}.

Preparation of flavanols and adjuncts

Solutions of (+)-Cat and other flavanols: (-)-Cat, (-)-EC, (+)-EC, (-)-ECg, (-)-EGC, and (-)-EGCg purchased from Sigma Aldrich, Gillingham, UK were prepared using sterile, deionised water. These were used freshly-made or stored at -20ºC until use. To investigate whether the oxidation of (+)-Cat in solution would affect activity, a freshly-made aqueous (+)-Cat solution was exposed to the atmosphere and placed in the dark at *c*. 20ºC. A stock aqueous solution of

4.8 mmol l^{-1} copper sulphate was kept at 5°C for subsequent use. Lambda buffer adjusted to pH 7.2 was prepared as previously described by Stewart *et al*. (1998) with components sourced from Sigma Aldrich, and samples of Ringer's solution adjusted to different pH values, with 1 mol l^{-1} NaOH or 1 mol l^{-1} HCl, were used as controls (Stewart *et al*. 1998). Freshly-made and thawed solutions were stored in the dark and used at room temperature within 20 min. A stop solution to neutralise antimicrobial activity was freshly-made for each experiment using 2% (v/v) Tween®-80 obtained from Sigma Aldrich in pH 7.2 adjusted Lambda buffer (Stewart *et al*. 1998).

Heat treatment of (+)-Cat solutions

Freshly-made 1 mmol l^{-1} (+)-Cat solutions were rapidly brought to 100ºC and maintained at this temperature for 10 or 30 min. The original volume of the solutions was restored with cold, sterile deionised water before use or storage at -20ºC. Independent assays of the antimicrobial activities of freshly-made solutions of flavanols compared to thawed solutions showed no significant differences (data not shown). Freshly-made and 10 min HT(+)-Cat solutions were also autoclaved at 121°C for 15 min prior to cooling, use or storage at -20ºC. Freshly-made and 10 min HT(+)-Cat solutions were also autoclaved in the presence of added equimolar copper sulphate solution (final concentration (f.c.), 214 μmol l^{-1}).

Antimicrobial assays

Antimicrobial activity was defined as an inhibitory effect on cells caused by exposure to test samples which resulted in reduced numbers of CFUs. The activity of samples was measured by antimicrobial suspension assays and viable cell counts as follows. Aliquots of 330 μL 1 mmol l^{-1} (+)-Cat or other flavanol were added to 700 μL adjunct samples. Following mixing, 30 μL was removed and mixtures were then allowed to stand in the dark for 10 min prior to

the addition of microbial suspension. 500 µL of bacterial suspension was added which resulted in a final (+)-Cat concentration of 214 µM. In standard assays, after 30 minutes incubation in dark conditions at room temperature and subsequent mixing, a 150 µL sample was removed and transferred to an equal volume of stop solution, prepared as described above, to neutralise the action of the putative antimicrobial mixture. In kinetic studies samples were removed and stopped as above immediately after the addition of micro-organisms and at regular intervals up to 15 min. Following mixing, 20 µL aliquots were transferred to nutrient agar plates for spreading either directly, or following serial dilution in Ringer's solution. Where necessary, volumes of 50 µL to 1000 µL were plated to reduce the detection limit of progressively efficacious samples following incubation. All plates were incubated aerobically for 24 hours at 37 °C. Each assay was conducted in triplicate (Stewart *et al*. 1998; Taguri *et al.* 2006; Holloway *et al.* 2011, 2012).

Aliquots of (+)-Cat, (-)-EC, (-)-ECg, (-)-EGC, and (-)-EGCg (1-10 mmol l^{-1} additions) were assayed on their own and in combination with copper sulphate (4.8 mmol l^{-1} additions). Freshly-made (+)-Cat samples of 1 mmol l^{-1} (giving an f.c., 214 µmol l^{-1}) were also tested with molar ratios of copper sulphate concentrations that ranged from 53.5 to 3434 µmol l^{-1} (f.c.). (+)-Cat (1 mmol l^{-1}) solutions HT at 100°C for 10 or 30 min were assayed alone. The activities of 1 mmol l^{-1} (-)-EC, (+)-EC, and (-)-Cat were compared to freshly-made, 14d stored and HT(+)-Cat solutions in the presence of equimolar 472 µmol l^{-1} (214 µmol l^{-1}, f.c.) copper sulphate.

To investigate the effect of an alternative form of heat-treatment, autoclaved freshly-made (+)-Cat solution (1 mmol l^{-1}) and HT(+)-Cat solutions (1 mmol l^{-1} ; 100°C for 10 min were added to equimolar copper sulphate (214 µmol l^{-1} f.c.) or Ringer's solution and activities tested against *S. aureus*. The same concentrations of freshly-made and 10 min HT (+)-Cat solutions autoclaved together with equimolar copper sulphate solution were also tested

against *S. aureus*. Rates of antimicrobial action of freshly-made and 10 min HT 1 mmol l^{-1} (+)-Cat and 1 μmol l^{-1} (-)-EGCg were compared in the presence of copper sulphate (4.8 mmol l^{-1} addition) against *S. aureus*.

Ringer's solution was added to 4.8 mmol l^{-1} copper sulphate resulting in final concentrations between 53.5 to 3434 μmol l^{-1} which acted as additional controls to Lambda buffer in the assays.

Measurement of hydrogen peroxide generation

Antimicrobial mixtures were tested for the presence of H_2O_2 as previous investigations had reported that catalase removed the antimicrobial activity of flavanol-Cu^{2+} mixtures (Hoshino *et al.* 2000; Holloway *et al.* 2012). Therefore, freshly-made 1 mmol l^{-1} flavanol isomers of (+)-Cat as well as 10 min HT and 14d stored (+)-Cat were tested for H_2O_2 generation in the presence of equimolar copper sulphate (214 μmol l^{-1}, f.c.). H_2O_2 was measured according to Lespinas *et al.* (1989) with modifications described previously (Holloway *et al.* 2012). A linear calibration curve was obtained between 0.05-0.5% after diluting a 5% (v/v) aqueous H_2O_2 solution purchased from Sigma Aldrich and kept at 5°C. Samples of the different (+)-Cat isomers, the 10 min HT(+)-Cat solution and the 14d stored (+)-Cat solutions were assayed (as 1 mmol l^{-1} additions) in the presence of equimolar copper sulphate (214 μmol l^{-1}, f.c.). Copper sulphate was also assayed alone without flavanols.

pH of samples

To investigate the possibility that changes in cell viability were wholly or partly brought about by sample acidity, the pH values of tested samples were measured and independent experiments conducted using Ringer's solution adjusted to pH between 3.5-7.2 to investigate any antimicrobial effects arising from the pH of the tested samples. In addition to the above

assays, pH was measured in sample mixtures in order to check the effects of reagent combinations, e.g. between copper sulphate and (+)-Cat. At least three readings were taken of each mixture.

UV-vis spectroscopy of freshly-made and HT (+)-Cat-Cu^{2+} solutions

To investigate the possibility that (+)-Cat formed complexes with Cu^{2+} in solution, spectral changes of (+)-Ccat solutions following heating at 100°C for 10 min were monitored between 190–900 nm with combined Cu^{2+} using a Varian Cary 300 Bio UV-Visible Spectrophotometer. Spectra were recorded for single component solutions and mixtures at final concentrations as used in antimicrobial assays compared to deionised water. The solutions investigated were: Ringer's solution, 214 μmol l^{-1} freshly-made or 10 min HT(+)-Cat each with 214 μmol l^{-1}, 856 μmol l^{-1}, and 1712 μmol l^{-1} copper sulphate (f.c.). The same combinations were also investigated in the presence of EDTA purchased from Sigma Aldrich, UK using 2.5 x the final concentration of copper sulphate (Brown *et al. et al.* 1988). Freshly-made and 10 min HT(+)-Cat solutions were also tested with 535 μmol l^{-1} EDTA (f.c.) in the absence of copper sulphate. As a further control 214 μmol l^{-1} copper sulphate (f.c.) was tested with 535 μmol l^{-1} EDTA. Freshly-made samples were prepared on at least three separate occasions and diluted 1 in 4 with Ringer's solution prior to analysis in order for reliable readings to be made.

Statistical Analysis

All test samples were assayed in triplicate in each case. Data were processed using Microsoft Excel and expressed as means ± standard error of the mean of independent measurements. Further MS Excel processing was used to calculate the Student independent t-test with

symmetrical dispersion of data. Statistically significant values were defined as $P < 0.05$ or $P < 0.01$.

Acknowledgements

The authors wish to thank Professor D. Ferreira of the University of Mississippi, USA, for valuable discussions.

Conflict of Interest

No conflict of interest declared.

References

Akiyama H., Fujii, K., Yamasaki, O., Oono, T. and Iwatsuki, K. (2001) Antibacterial action of several tannins against *Staphylococcus aureus. J Antimicrob Chemoth* **48,** 487-91.

Brown, J.E., Khodr, H., Hider, R.C. and Rice-Evans, A. (1988) Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. *Biochem J* **330,** 1173-1178.

Cavaco, L.M., Hasman, H. and Aarestrup, F.M. (2011) Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance. *Vet Microbiol* **150,** 344-348.

Chen, S.S., Peng, H.M. and Webster, R.D. (2010) Infrared and UV-vis spectra of phenoxonium cations produced during the oxidation of phenols with structures similar to vitamin E. *Electrochim Acta* **55,** 8863-8869.

Dukic, D. and Ilic, Z (1991) Effects of manuring a meadow with pig manure on the occurrence of bacteria in hay. *Mikrobiologija (Zemun)* **28,** 161-167.

Espirito Santo, C., Lam, E.W., Elowsky, C.G., Quarenta, D., Domaille, D.W., Chang, C.J. and Grass, G. (2011) Bacterial killing by dry metallic copper surfaces. *Appl Environ Microb* **77,** 794-802.

Es-Safi, N-E., Cheynier, V. and Moutournet, M. (2003) Effect of copper oxidation of catechin in a model solution system. *Int J Food Sci Technol* **38,** 153-163.

Franco, W., Hsu, W.Y. and Simmone, A.H. (2010) Survival of *Salmonella* and *Staphylococcus aureus* in mexican red salsa in a food service setting. *J Food Protect* **73,** 1116-1120.

Gibbons, S., Moser, E. and Kaatz, G.W. (2004) Catechin gallates inhibit multidrug resistance (MDR) in *Staphylococcus aureus*. *Planta Med* **70,** 1240-1242.

Grube, M., Furnkranz, M., Zitzenbacher, S., Huss, H. and Berg, G. (2011) Emerging multi-pathogen disease caused by *Didymella bryoniae* and pathogenic bacteria on Styrian oil pumpkin. *Eur J Plant Pathol* **131,** 539-548.

Hanus, O., Frelich, J., Vyletelova, M., Roubal, P., Vorlicek, Z. and Jedleska, R. (2004) Technologically difficult, pathogenic and food risky bacterial contamination of raw milk and other materials from dairy cow herds. *Czech J Anim Sci* **49,** 489-499.

Hatano, T., Tsugawa, M., Kusuda, M., Taniguchi, S., Yoshida, T., Shiota, S. and Tsuchiya, T. (2008) Enhancement of antibacterial effects of epigallocatechin gallate using ascorbic acid, *Phytochemistry* **69,** 3111-3116.

Holloway, A., Gould, S.W.J., Fielder, M.D., Naughton, D.P. and Kelly, A.K. (2011) Enhancement of antibacterial activities of whole and sub-fractionated white tea by addition of copper (II) sulphate and vitamin C against *Staphylococcus aureus*; a mechanistic approach. *BMC Compl Altern Med*, **11,** 115-124.

Holloway, A.C., Mueller-Harvey, I., Gould, S.W.J., Fielder, M.D., Naughton, D.P. and Kelly, A.K. (2012) The effect of copper(II), iron(II) sulphate, and vitamin C combinations on the weak antimicrobial activity of (+)-catechin against *Staphylococcus aureus* and other microbes. *Metallomics* **4,** 1280-1286.

Hoshino, N., Kimura, T., Hayakawa, F., Yamaji, A. and Ando, T. (2000) Bactericidal activity of catechin-Cu^{2+} complexes against *Staphylococcus aureus* compared with *Escherichia coli.* *Lett Appl Microbiol* **131,** 213-217.

Kim, T.J., Silva, J.L., Kim, M.K. and Jung, Y.S. (2010) Enhanced antioxidant capacity and antimicrobial activity of tannic acid by thermal processing. *Food Chem* **118,** 740-746.

Lebedev, V.S., Voldina, L.A., Deinega, E.Y., and Fedorov, Y.I. (2005) Structural modifications on the surface of *Escherichia coli* bacteria and copper-induced permeability of plasma membrane. *Biofizika* **50**, 107-113.

Nedorostova, L., Kloucek, P., Urbanova, K., Kokoska, L., Smid, J., Urban, J., Valetrova, I. and Stolcova, M. (2011) Antibacterial effect of essential oil vapours against different strains of *Staphylococcus aureus*, including MRSA. *Flavour Frag J* **26,** 403-407.

Roccaro, A.S., Blanco, A.R., Giuliano, F., Rusciano, D. and Enea, V. (2004) Epigallocatechin-gallate enhances the activity of tetracycline in staphylococci by inhibiting its efflux from bacterial cells. *Antimicrob Agents Ch* **48,** 1968-1973.

Stapleton, P.D., Shah, S., Anderson, J.C., Hara, Y., Hamilton-Miller J.M.T. and Taylor, P.W. (2004a) Modulation of beta-lactam resistance in *Staphylococcus aureus* by catechins and gallates. *Int J Antimicrob Ag* **23,** 462-467.

Stapleton, P.D., Shah, S., Hamilton-Miller, J.M.T., Hara, Y., Nagaoka, Y., Kumagai, A., Uesato, S. and Taylor, P.W. (2004b) Anti-*Staphylococcus aureus* and oxacillin resistance modulating capacity of 3-O-acyl-catechins. *Int J Antimicrob Ag* **24,** 374-380.

Stapleton, P.D., Shah, S., Hara, Y. and Taylor, P.W. (2006) Potentiation of catechin gallate-mediated sensitisation of *Staphylococcus aureus* to oxacillin by nongalloylated catechins. *Antimicrob Agents Ch* **50,** 752-755.

Stapleton, P.D., Shah, S., Ehlert, K., Hara, Y. and Taylor, P.W. (2007) The beta-lactam-resistance modifier (-)-epicatechin gallate alters the architecture of the cell wall of *Staphylococcus aureus. Microbiology* **153,** 2093-2103.

Stewart, G.S., Jassim, S.A., Denyer, S.P., Newby, P., Linley, K. and Dhir, V.K. (1998) The specific and sensitive detection of bacterial pathogens within 4 h using bacteriophage amplification. *J Appl Microbiol* **84,** 777-783.

Taguri, T., Tanaka, T. and Kouno, I. (2004) Antibacterial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biol Pharm Bull* **27,** 1965-1969.

Taguri, T., Tanaka, T. and Kouno, I. (2006) Antibacterial spectrum of plant polyphenols and extracts depending on hydroxyphenyl structure. *Biol Pharm Bull* **29,** 2226-2235.

Lespinas, F., Dupuy, G., Revol, F. and Aubry, C. (1989) Enzymic urea assay: a new colorimetric method based on hydrogen peroxide measurement. *Clin Chem* **35,** 58-662.

Takahashi, O., Cai, Z., Toda, M., Hara, Y. and Shimamura, T. (1995) Appearance of antibacterial activity of oxacillin against methicillin resistant *Staphylococcus aureus* (MRSA) in the presence of catechin. *Jpn J Infect Dis* **69,** 1126-1134.

Figure 1 Effects of fixed concentrations of flavanol isomers of (+)-Catechin (214 µmol l^{-1}) in the presence of equimolar copper sulphate tested against *S. aureus* NCTC 6751. The results show cell viability levels following exposure to: Lambda buffer and copper sulphate controls; Test substances, all containing copper sulphate plus (+)-Epicatechin, (+)-EC; or (-)-Catechin, (-)-Cat); or (+)-Catechin, (+)-Cat; or (-)-Epicatechin, (-)-EC; or heat-treated (+)-Catechin at 100°C for 10 min, HT(+)-Cat; or 14 d stored (+)-Catechin solution (stored at room temperature for 14 d in the dark), 14d(+)-Cat. Cells exposed to flavanol controls tested alone showed similar viabilities to that of the buffer control (data not shown). Cells (~1 x 10^8 CFU

ml^{-1}) were exposed to test substances for 30 min at *c.* 20°C. . Results are expressed as means and standard errors of triplicate experiments. Statistical differences (*$P < 0.05$, **$P < 0.01$) between control of copper sulphate tested alone and tested with combined flavanol ($n = 3$). Antimicrobial activity was defined as an inhibitory effect on cells caused by exposure to test samples which resulted in reduced numbers of CFUs.

Figure 2 Generation of H_2O_2 by fresh, HT, and 14d stored 214 µmol l^{-1} (f.c.) (+)-Catechin and fresh flavanol isomer solutions in the presence of equimolar copper sulphate. Results are expressed as means and standard errors of triplicate experiments. Statistical differences (*$P <$ 0.05, **$P < 0.01$) between control of copper sulphate tested alone (214 µmol l^{-1} f.c.) and tested with combined equimolar flavanol ($n = 3$). Differences in the levels of H_2O_2 generated between (+)-EC and (-)-Cat; 14d (+)-Cat and HT(+)-Cat; and HT(+)-Cat and (-)-EC all showed significance at the level of $P < 0.05$.

Figure 3 Effect of fresh and HT (+)-Catechin or (-)-Epigallocatechin gallate (EGCg) plus copper sulphate tested against *S. aureus*. Results are expressed as means of triplicate experiments. Statistical differences (*$P < 0.05$) between control of copper sulphate tested alone and tested with combined flavanol following 1 min of exposure ($n = 3$). Samples shown: lambda buffer, (circles); 2175 µmol l^{-1} (f.c.) copper sulphate alone, Cu^{2+} (filled squares); $Cu^{2+}$with added 214 µmol l^{-1} (f.c) freshly-made (+)-Catechin (filled triangles); or with HT-(+)-Catechin (diamonds); or with 214 nmol l^{-1} (f.c) (-)-EGCg (open triangles). Viabilities for flavanol controls were similar to buffer values (data not shown). Antimicrobial

activity was defined as an inhibitory effect on cells caused by exposure to test samples which resulted in reduced numbers of CFUs.

Table 1 Antimicrobial activities of five green tea flavanols tested in the presence of copper sulphate against *S. aureus* NCTC 6751.

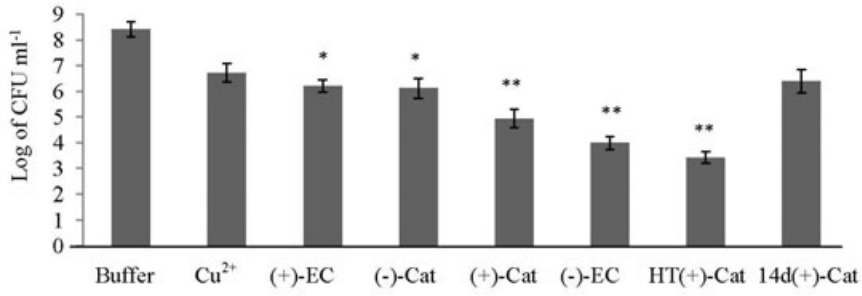
Flavanol tested	Final concentration of flavanol added to 2175 μmol l^{-1} (f.c.) $CuSO_4$ in assay mixture required to show a viability of *c.* 3 log CFU ml^{-1} (μmol l^{-1})
(+)-Catechin	1000
(-)-Epicatechin	500
(-)-Epicatechin gallate	150
(-)-Epigallocatechin	1
(-)-Epigallocatechin gallate	1

Cells (~1 x 10^8 CFU ml^{-1}) were exposed to test substances for 30 min at *c.* 20°C. Antimicrobial activity was defined as an inhibitory effect on cells caused by exposure to test samples which resulted in reduced numbers of CFUs.

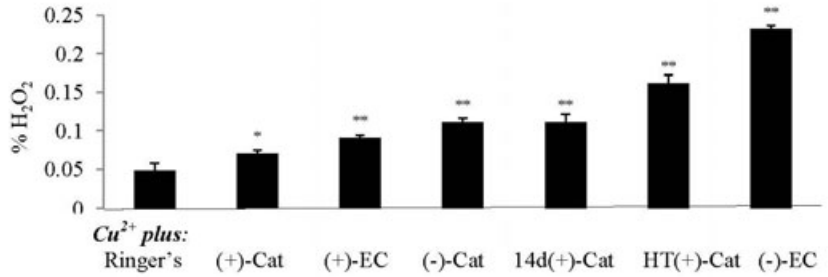
Table 2 Antimicrobial effects of freshly-made, heat treated (HT), autoclaved, and 14 d stored 214 µmol l^{-1} (f.c.) (+)-Catechin solutions alone and with equimolar copper sulphate tested against *S. aureus* NCTC 6751.

Sample tested	Log of CFU ml^{-1} (SEM)
Buffer	7.7 (±0.70)
Freshly-made (+)-Catechin	7.7 (±0.59)
(+)-Cat HT at 100°C 10 min	7.4 (±0.61)
(+)-Cat HT 100°C 30 min	7.6 (±0.33)
(+)-Cat autoclaved	7.2 (±0.45)
(+)-Cat stored for 14d	7.3 (±0.32)
Copper sulphate alone	6.0 (±0.56)
Freshly-made (+)-Cat + Cu^{2+}	4.9 (±0.50)**
Freshly-made (+)-Cat autoclaved + Cu^{2+}	4.3 (±0.42)**
Freshly-made (+)-Cat + Cu^{2+} autoclaved together	6.3 (±0.40)
(+)-Cat HT 100°C 10 min + Cu^{2+}	3.9 (±0.70)**
(+)-Cat HT 100°C 10 min autoclaved + Cu^{2+}	4.1 (±0.50)**
(+)-Cat HT 100°C 10 min + Cu^{2+} autoclaved together	5.1 (±0.47)
(+)-Cat HT 100°C 30 min + Cu^{2+}	4.0 (±0.70)**
(+)-Cat stored for 14d + Cu^{2+}	6.2 (±0.30)

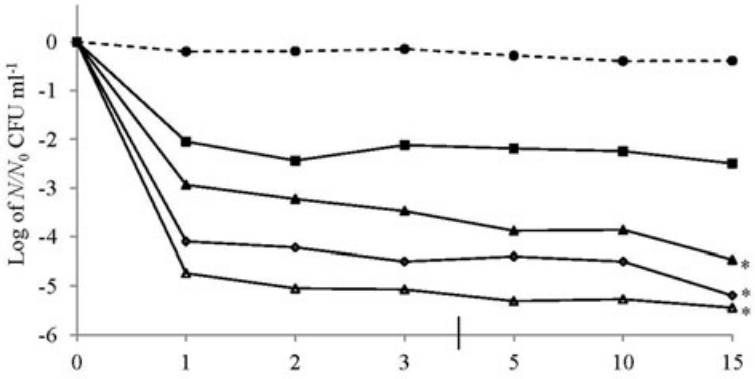
Cells (~1 x 10^8 CFU ml^{-1}) were exposed to test substances for 30 min at *c.* 20°C. Antimicrobial activity was defined as an inhibitory effect on cells caused by exposure to test samples which resulted in reduced numbers of CFUs. In each case assayed aliquots of (+)-Cat were equivalent to 214 µmol l^{-1} (f.c.). Results are expressed as means and standard errors of the mean of triplicate experiments. Statistical differences (**$P < 0.01$) between control of copper sulphate alone and test with combined flavanol ($n = 3$).



Copper sulphate tested alone or with added different flavanols



Copper sulphate tested with added Ringer's or freshly-made or HT or 14 d stored (+)-Catechin solution or fresh flavanol isomer



Time of removal of sample following mixing of inoculum with test agent/s (min)