1. Corrections should be marked with the Adobe Annotation & Comment Tools below:

2. To save your proof corrections, click the 'Publish Comments' button. Publishing your comments saves the marked up version of your proof to a centralized location in Wiley’s Online Proofing System. Corrections don’t have to be marked in one sitting – you can publish corrections and log back in at a later time to add more.

3. When your proof review is complete we recommend you download a copy of your annotated proof for reference in any future correspondence concerning the article before publication. You can do this by clicking on the icon to the right of the ‘Publish Comments’ button and selecting ‘Save as Archive Copy...’.

4. When your proof review is complete and you are ready to send corrections to the publisher click the ‘Complete Proof Review’ button that appears above the proof in your web browser window. Do not click the ‘Finalize/Complete Proof Review’ button without replying to any author queries found on the last page of your proof. Incomplete proof reviews will cause a delay in publication. **Note: Once you click ‘Finalize/Complete Proof Review’ you will not be able to mark any further comments or corrections.**

**Firefox, Chrome, Safari Users**
If your PDF article proof opens in any PDF viewer other than Adobe Reader or Adobe Acrobat, you will not be able to mark corrections and query responses, nor save them. To mark and save corrections, please follow these instructions to disable the built-in browser PDF viewers in Firefox, Chrome, and Safari so the PDF article proof opens in Adobe within a Firefox or Chrome browser window.
Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:

This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We’ve picked out some of these tools below:

1. **Replace (Ins) Tool** – for replacing text.

   Strikes a line through text and opens up a text box where replacement text can be entered.

   **How to use it**
   - Highlight a word or sentence.
   - Click on the Replace (Ins) icon in the Annotations section.
   - Type the replacement text into the blue box that appears.

2. **Strikethrough (Del) Tool** – for deleting text.

   Strikes a red line through text that is to be deleted.

   **How to use it**
   - Highlight a word or sentence.
   - Click on the Strikethrough (Del) icon in the Annotations section.

3. **Add note to text** Tool – for highlighting a section to be changed to bold or italic.

   Highlights text in yellow and opens up a text box where comments can be entered.

   **How to use it**
   - Highlight the relevant section of text.
   - Click on the Add note to text icon in the Annotations section.
   - Type instruction on what should be changed regarding the text into the yellow box that appears.

4. **Add sticky note** Tool – for making notes at specific points in the text.

   Marks a point in the proof where a comment needs to be highlighted.

   **How to use it**
   - Click on the Add sticky note icon in the Annotations section.
   - Click at the point in the proof where the comment should be inserted.
   - Type the comment into the yellow box that appears.
5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate place in the text.

How to use it
- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you’d like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

END

6. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks. Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.

How to use it
- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.
ORIGINIAL ARTICLE

Essential oils and metal ions as alternative antimicrobial agents: a focus on tea tree oil and silver

Wan L Low¹, Ken Kenward¹, Stephen T Britland¹,², Mohd CIM Amin³ & Claire Martin¹,²

¹ School of Pharmacy, University of Wolverhampton, Wolverhampton, UK
² Research Institute in Healthcare Science, Faculty of Science and Engineering, University of Wolverhampton, Wolverhampton, UK
³ Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia

Key words
Antimicrobial; Silver; Tea tree oil; Wound infection

Correspondence to
C Martin
School of Pharmacy
Faculty of Science and Engineering
University of Wolverhampton
Wulfruna Street
Wolverhampton WV1 1LY
UK
E-mail: claire.martin2@wlv.ac.uk

Abstract
The increasing occurrence of hospital-acquired infections and the emerging problems posed by antibiotic-resistant microbial strains have both contributed to the escalating cost of treatment. The presence of infection at the wound site can potentially stall the healing process at the inflammatory stage, leading to the development of a chronic wound. Traditional wound treatment regimes can no longer cope with the complications posed by antibiotic-resistant strains; hence, there is a need to explore the use of alternative antimicrobial agents. Pre-antibiotic compounds, including heavy metal ions and essential oils, have been re-investigated for their potential use as effective antimicrobial agents. Essential oils have potent antimicrobial, antifungal, antiviral, anti-inflammatory, antioxidant and other beneficial therapeutic properties. Similarly, heavy metal ions have also been used as disinfecting agents because of their broad spectrum activities. Both of these alternative antimicrobials interact with many different intracellular components, thereby resulting in the disruption of vital cell functions and eventually cell death. This review will discuss the application of essential oils and heavy metal ions, particularly tea tree oil and silver ions, as alternative antimicrobial agents for the treatment of chronic, infected wounds.

Key Messages
• the use of broad spectrum, pre-antibiotic era antimicrobial agents, such as essential oils and metal ions, may be an alternative approach to tackling the growing problem of drug-resistant wound infections
• the aim of this review is to consider the evidence for the use of silver and tea tree oil (TTO) in tackling chronic wound infections, such as those associated with diabetic foot and burns
• the broad spectrum, multi-target mechanism of action of silver and TTO indicates that there may be a role for them in a clinical setting to both reduce the microbial load within the wound bed and speed up the healing process
• the broad spectrum, multi-target mechanism of action of silver and TTO indicates that there may be a role for them in a clinical setting to both reduce the microbial load within the wound bed and speed up the healing process

Introduction
The use of metal ions and essential oils as antimicrobial agents is of particular interest in topical wound management. Management of chronic wounds, such as varicose skin ulcers and burns, aims to induce rapid healing and minimise the extent of scarring. Infection of such wounds can not only delay the healing process but also lead to the development of a chronic wound with increased potential to develop into a systemic infection. Topical application of antimicrobial agents is common as effective concentrations may be difficult to achieve with systemic drugs as the effects of the wound trauma may impede delivery of the agent into the wound (1,2). The reduced concentration may also create selective pressure for antibiotic resistance.

The current problems posed by increasing antibiotic resistance in gram-positive bacteria (methicillin-resistant Staphylococcus aureus, MRSA, Vancomycin-resistant enterococci, VRE) and gram-negative bacteria (New Delhi metallo-β-lactamase-1 (NDM-1) positive Escherichia coli, multidrug-resistant Acinetobacter baumannii, ciprofloxacin-resistant Pseudomonas aeruginosa) (3,4) have renewed interest in pre-antibiotic antibacterial agents, such as...
metal ions and essential oil [tea tree oil (TTO)] (5,6). Alternative, non-antibiotic-based treatments are attractive because of their decreased side effects compared with synthetic drugs and their multiple target sites within the microorganisms, which may contribute to reduced development of resistant strains (7). Despite their usefulness and long history of use, these agents are mainly restricted to topical applications against infected wounds, skin burns, ulcers and fungal infections of the skin. Despite their effectiveness in treating topical infections, the complete healing of wounds, especially slow/non-healing wounds, may require repeated application or use of high concentrations that may have adverse effects on the patient. Current developments in wound treatment focus on approaches that will allow reduced concentration (using combined agents) or delivery via controlled release delivery systems to minimise potential side effects whilst maintaining bioavailability to achieving therapeutic effects (8–11).

The useful properties of alternative antimicrobial agents, together with advances in drug-delivery technologies, may be able to enhance and expand the medical applications of these agents (9,10,12). Combining these alternative antimicrobial agents with advanced drug-delivery systems aims to:

- Promote bioavailability of agent at microbiocidal concentrations.
- Reduce drug concentration to enhance safety and practicality of application.
- Minimise scarring and promote wound-healing processes.
- Reduce discomfort and pain in consideration of the patient’s psychological needs.
- Decrease the frequency of dressing changes.

These aims would increase convenience, provide less opportunity for infection and/or reinfection of the wounds and ultimately reduce treatment costs.

**Microorganisms and wound management**

In healthy individuals, the skin supports a natural microflora comprising a balanced community of microorganisms, which rarely cause infection. However, a disturbance to the normal ratio of microflora or an exposure of subcutaneous tissue because of trauma may result in the pathogenic invasion by these microorganisms (7). Colonisation of wounds by these opportunistic pathogens is usually polymicrobial (12). The diversity and proliferation of the pathogens is influenced by various factors including the type, depth and location of the trauma as well as the host immune system response (7). The presence of microorganisms at a wound site does not confirm infection (13). Infection only occurs when the host immune system can no longer cope with the virulence factors expressed by the colonising microorganisms, thus triggering a series of systemic responses that delay the healing process (7,13). The increasing occurrence of hospital infections and widespread emergence of resistant microorganisms contribute to escalating treatment costs. In addition, hypersensitivity reactions to antibiotics and the lack of access to new treatments within the health care industry makes the provision of sufficient support and care for patients difficult. Modern lifestyles that frequently lack physical activity increase the possibility of developing various life-long (interconnected) health conditions, for example, diabetes, obesity and hypertension in old age. These underlying health conditions may influence the complexity and severity of wound healing. In addition, the growing size and longevity of the elderly population has increased the prevalence of wounds associated with these conditions, including slow/non-healing ulcers. The improvement in medical facilities has increased the number of patients surviving from complicated wounds, such as those caused by burns. Although rates of survival have improved, severely burned patients usually require extended stays in hospital, suffer from lowered immunity and extensive loss of skin. Such patients are prone to infection by both common wound pathogens as well as antibiotic-resistant microorganisms, which may further complicate the treatment regime (1).

The nature of burn wounds, and varicose ulcers in particular, may involve relatively lengthy treatment with antibiotics, which carries the attendant risk of selecting drug-resistant bacteria. Various approaches have been conducted to find the best method to treat and overcome this problem. The increasing incidence and broadenning spectrum of pathogens resistant to antibiotics has refocused scientific interest on the use of alternative antimicrobial compounds (6,14). Alternative, non-antibiotic-based treatments are attractive because of their decreased side effects compared with synthetic drugs and their multiple target sites within the microorganisms, which may contribute to reduced development of resistant strains (7). Despite their usefulness and long history of use, these agents are mainly restricted to topical applications against infected wounds, skin burns, ulcers and fungal infections of the skin.

**Microorganisms and the wound environment**

In healthy individuals, the skin supports a natural microflora comprising a balanced community of microorganisms, which rarely cause infection. However, a disturbance to the normal ratio of microflora or an exposure of subcutaneous tissue because of trauma may result in pathogenic polymicrobial invasion by these microorganisms (7). The diversity and proliferation of the pathogens is influenced by various factors, including the type, depth and location of the trauma as well as the host immune system response (7). The presence of microorganisms at a wound site does not confirm infection (13). Infection only occurs when the host immune system can no longer cope with the virulence factors expressed by the colonising microorganisms, thus triggering a series of systemic responses that delay the healing process (7,13). Besides patient microflora, other sources of infection include those acquired directly or indirectly via air, other infected patients, health care workers, contaminated medical devices, hospital environment and external sources, such as visitors (15). Cutaneous wounds offer a favourable (moist, warm, nutritious) environment to support bacterial growth and proliferation. Heavy microbial infection (above critical colonisation) retards wound healing by increasing the bio-burden at wound sites, which stalls the normal process at the inflammatory phase. When acute wounds become infected and reach critical colonisation by pathogenic microorganisms, the stimulated
pro-inflammatory environment (because of microbial production of toxins, proteases or pro-inflammatory molecules) will stop the process of wound healing, and the site develops into a chronic wound (7,10,12,16).

**Alternative antimicrobial agents**

Although conventional antibiotics are regarded as effective antimicrobial agents, there is concern about their side effects and the increasing incidence of microbial resistance to them (17,2). Antimicrobial agents are only effective until resistant strains of the target microorganisms begin to emerge (6,13).

With conventional antibiotics, the emergence of resistance is mainly because of their action against a single target. This has led to re-examination of the use of other antimicrobial agents, such as metal ions and plant extracts, which often attack multiple target sites (6,17,19,20).

The application of essential oils to reduce bacterial growth and prevent decay is not a new idea. Plants synthesise aromatic secondary metabolites to protect against predation and prevent colonisation by plant pathogens (19). These aromatic compounds are divided into classes, including essential oils (primarily phenolics and/or terpenoids), alkaloids, lectins, poly peptides and polyacetylenes, all of which have different mechanisms of antimicrobial activity (24). Some examples of essential oils or plant extracts commonly used for their antimicrobial properties are TTO, ylang ylang, betel pepper, manuka, eucalyptus, arnica, lemon verbena, rosemary, green tea extract and cadendula (18,19). Although extensively practiced since ancient times, the use of natural extracts from plants as antimicrobial compounds declined after the development of synthetic antibiotics.

Metals (especially heavy metals) were used as disinfecting agents since ancient times. Silver, copper and gold, for example, have been used to treat diseases, disinfect wounds and water. Examples of metals commonly used in these applications include zinc, iron, bismuth, cobalt, magnesium, titanium, copper and the more extensively used heavy metal, silver.

Despite having useful properties, treatment using metals is limited because excessive concentrations of metals, especially heavy metals, are toxic to human cells. Amongst the heavy metals, silver has a long history of use as an antimicrobial agent because of its relatively lower toxicity to human cells (21,22).

Recently, in response to issues surrounding antibiotic resistance, topical application of silver compounds has increased in popularity (23).

**Plant products as antimicrobial agent**

Plants with medicinal properties have been used for the treatment of various diseases both in traditional and modern medicine. When faced with microbial invasion or attack leading to infection, plants have their own defence mechanisms, which rely on the production of compounds that interfere with the cellular and intracellular structure of microorganisms (24). These antimicrobial plant compounds that show effective antimicrobial activity are often secondary metabolites formed in aromatic plants. These aromatic compounds give the plants their characteristic strong odour (24) that can repel insects or herbivores. In addition, certain compounds give plants pigment or flavour; these may be irritants to other organisms and may flow out from injured plants to prevent colonisation by microorganisms (25,26). These secondary metabolites are mainly present as volatile compounds such as phenols or their oxygen-substituted derivatives, which are categorised into five major classes detailed in Table 1 (19). All plant organs, including buds, flowers, leaves, stems, seeds, fruits, twigs and branches, are able to synthesise these compounds.

The amount of active compound present in botanical extracts varies depending on the main adaptation of the plant to its environment, harvesting period, the extraction process, dehydration procedures, purification and storage methods (20). The extraction of secondary metabolites from plants is usually by distillation (water or steam) to produce a volatile essential oil. Essential oils are aromatic compounds synthesised by plants as secondary metabolites and are well known for their antibacterial, antifungal and antiviral properties (6,24), in addition to anti-cancer, anti-diabetic, anti-inflammatory and antioxidant activities (24,27). Essential oils are multi-component compounds, usually with terpenes and their derivatives (terpenoids) as the major components. After extraction, they present as clear, almost colourless volatile liquids, soluble in organic solvents and lipids in addition to some hydrophilic components (24).

Because of the versatility and wide-ranging properties of essential oils, they are used not only in the pharmaceutical industry but also incorporated into the cosmetics, agriculture, sanitation, disinfection, food preservation and manufacturing industries (20,24). Examples of the various uses of essential oils are summarised in Table 2.

**Tea tree essential oil as an antimicrobial**

**History, background of use and production of TTO**

Bundjalung Aborigines of northern New South Wales traditionally used crushed tea tree leaves as a treatment for coughs and colds (inhalation), a herbal infusion for sore throats and also sprinkled them directly on skin wounds to promote healing (26). The use of TTO as an antimicrobial agent became a common practice after Penfold published reports on its antimicrobial properties in the 1920s and 1930s (28). In modern society, the useful properties of TTO are made commercially available, either as the essential oil per se or alternatively formulated into various products, including antiseptic creams, soaps, shampoo, anti-acne creams, toothpaste and household cleaning agents.

TTO is recognised as having broad spectrum antibacterial, antifungal, antiviral, antinecoplasmal and antiprotozoal activity as well as anti-inflammatory and anti-cancer properties (28,29). There are over one hundred different components in whole TTO, of which its major component, terpinen-4-ol, is primarily responsible for its active antimicrobial properties (26). TTO is extracted from the leaves and terminal branches of the Melaleuca alternifolia plant via steam distillation and condensed to yield a pale yellow oil (28). The potential variation in the composition of TTO has lead to the classification of six chemotypes of *M. alternifolia*, based on the amount of terpinen-4-ol (one type), terpinolene (one type) and 1,8-cineole (four types) (28). Commercially acceptable grade
Table 1  Summary of the major classes of active antimicrobial compounds from plants [summarised from references (19,24,34,95–98)]

<table>
<thead>
<tr>
<th>Class and description</th>
<th>Subclass</th>
<th>Example</th>
<th>Mechanism</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolics</td>
<td>Simple phenol and phenolic acid</td>
<td>Catechol, epicatechin and caffeic acid</td>
<td>Substrate deprivation and membrane disruption</td>
<td><img src="quinoine.png" alt="Caffeic acid" /></td>
</tr>
<tr>
<td></td>
<td>Phenolics</td>
<td>Simple phenol and phenolic acid</td>
<td>Catechol, epicatechin and caffeic acid</td>
<td>Substrate deprivation and membrane disruption</td>
</tr>
<tr>
<td></td>
<td>Quinone</td>
<td>Hypericin</td>
<td>Binds to adhesins, complex with cell wall and inactivate enzyme</td>
<td><img src="quinoine.png" alt="Hypericin" /></td>
</tr>
<tr>
<td></td>
<td>Flavonoids</td>
<td>Chrysin</td>
<td>Binds to adhesins</td>
<td><img src="quinoine.png" alt="Chrysin" /></td>
</tr>
<tr>
<td></td>
<td>Flavones</td>
<td>Abyssinone</td>
<td>Complexes with cell wall and inactivates enzymes</td>
<td><img src="quinoine.png" alt="Abyssinone" /></td>
</tr>
<tr>
<td></td>
<td>Tannins</td>
<td>Ellagitannin</td>
<td>Binds to proteins, adhesins, inhibits enzymes, causes substrate deprivation, complexes with cell wall, membrane disruption and metal ion complexation</td>
<td><img src="quinoine.png" alt="Ellagitannin" /></td>
</tr>
</tbody>
</table>

© 2016 Medicalhelplines.com Inc and John Wiley & Sons Ltd

Uncorrected Proofs
Table 1 Continued

<table>
<thead>
<tr>
<th>Class and description</th>
<th>Subclass</th>
<th>Example</th>
<th>Mechanism</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarins</td>
<td></td>
<td>Warfarin</td>
<td>Interactions with eukaryotic DNA and has antiviral properties</td>
<td></td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Monoterpenes</td>
<td>Terpinen-4-ol</td>
<td>Membrane disruption</td>
<td></td>
</tr>
<tr>
<td>Alkaloids</td>
<td>N/A</td>
<td>Berberine and piperine</td>
<td>Intercalate into cell wall or DNA, differentially inhibit sterol and chitin biosynthesis</td>
<td></td>
</tr>
<tr>
<td>Polyacetylenes</td>
<td>N/A</td>
<td>Falcarinol</td>
<td>Mechanism unknown</td>
<td></td>
</tr>
<tr>
<td>Lectins and polypeptides</td>
<td>N/A</td>
<td>Mannose-specific agglutinin, fabatin, defensins and thionins</td>
<td>Inhibit adhesion and fusion interactions between virus and host cell. May form ion channels in microbial membranes or reduce adhesion of microbial proteins to host receptors via competitive inhibition</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Common essential oils extracted from plants and their medicinal and/or antimicrobial activity

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
<th>Active compound</th>
<th>Activity or therapeutic properties</th>
<th>Application/product</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aloe</td>
<td>Aloe barbadensis, Aloe vera</td>
<td>Anthraquinones (aloin, emodin and resistanol), β-sitosterol and gibberellins</td>
<td>• Antimicrobial • Increases wound healing • Induces hypoglycaemic effect • Lowers blood cholesterol</td>
<td>Used on burns and wounds • Treatment of psoriasis and genital herpes • Medicate insulin production</td>
<td>(98-102)</td>
</tr>
<tr>
<td>Arnica</td>
<td>Arnica montana</td>
<td>Sesquiterpene and lactones</td>
<td>• Anti-inflammatory</td>
<td>Applied to bruises and swelling • Osteoarthritis treatment</td>
<td>(104)</td>
</tr>
<tr>
<td>Basil</td>
<td>Ocimum basilicum</td>
<td>Linalool, methylchavicol, epi-α-cadinol, α-bergamotene, γ-cadinene and germacrene D</td>
<td>• Antimicrobial • Antioxidant</td>
<td>Antimicrobial activity in food preservation and packaging industry</td>
<td>(105,106)</td>
</tr>
<tr>
<td>Calendula (Marigold)</td>
<td>Calendula officinalis</td>
<td>Triterpenoid</td>
<td>• Antimicrobial • Anti-inflammatory • Anti-tumorogenic</td>
<td>Herbal antimicrobial mouthwash • Treatment against athletes foot, ringworm and candidal infections • Inhibits biofilm formation by clinical strains of Staphylococcus epidermidis • Active against common food spoilage microbes • Used to treat toothache and skin sores • Herbal antimicrobial mouthwash • Inhibits growth of food pathogen • Fragrant in pharmaceuticals, soaps, detergents and food • Natural pesticide/insecticide</td>
<td>(107-109)</td>
</tr>
<tr>
<td>Cinnamon</td>
<td>Cinnamomum zeylanicum</td>
<td>Cinnamaldehyde</td>
<td>• Analgesic • Antiseptic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clove</td>
<td>Syzygium aromaticum</td>
<td>Eugenol</td>
<td>• Antibacterial • Antifungal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eucalyptus</td>
<td>Eucalyptus globulus</td>
<td>1,8-cineole, Tannin</td>
<td>• Antimicrobial • Insect repellent • Skin penetration enhancer for drugs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Garlic</td>
<td>Allium sativum</td>
<td>Allicin</td>
<td>• Antimicrobial • Potential anti-cancer agent • Lowers cholesterol and triglycerides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green tea extract</td>
<td>Camellia sinensis</td>
<td>Catechins, tannins, caffeine</td>
<td>• Anti-inflammatory • Antimicrobial • Anti-cancer • Antioxidant</td>
<td>In vitro antimicrobial activity against Helicobacter pylori • Food preservative • Reduce risk of hypertension • Reduces dental caries • Lowers risk of Helicobacter infection</td>
<td>(27,100,118,119) (107-120,129)</td>
</tr>
</tbody>
</table>
of TTO are classed in the terpinen-4-ol chemotype and have to comply with ISO4730:2004 standards for 'Oil of Melaleuca, terpinen-4-ol type', which defines the maximum and minimum level for the 14 major components of the essential oil, including terpinen-4-ol (28).

Mode of action

The physicochemical properties of the oil include those from hydrophilic hydrocarbon compounds with sufficient lipophilicity and allow the oil to partition preferentially into biological membranes, causing bilayer expansion (28). Thus, TTO components diffuse easily through the hydrophobic lipid bilayer of the microbial cell membrane, causing disruption to integrity and function, increased fluidity, loss of permeability and inhibition of embedded membrane enzymes. Consequently, the cell loses essential metabolites and repair enzymes, ultimately resulting in cell death (28,30). The microbiocidal properties of active monoterpenes in particular can mainly be attributed to disruption of the cell membrane's barrier function; cells are thus unable to establish control over membrane-coupled energy-transducing processes, solute transport, regulation of metabolism and maintenance of turgor pressure (30). However, when a compound is highly lipophilic, its low solubility in aqueous media hinders its ability to contact with and permeabilise cell membranes (24).
Cox et al. (2000) examined membrane disruption of E. coli, S. aureus and Candida albicans by TTO via the leakage of potassium ions, materials that absorb at 280 nm (proteins) and uptake of fluorescent nucleic acid stain, propidium iodide (30). All three microorganisms showed decreased microbial viability (inhibition of respiration), increased uptake of propidium iodide and increased leakage of 280 nm absorbing material. Leakage of potassium ions was prominent in E. coli and S. aureus but less so in C. albicans (30,32). Carson et al. (2002) (33) assessed the release of 260 nm absorbing materials from S. aureus after treatment with whole TTO, terpinen-4-ol, 1,8-cineole and α-terpineol (29). These results showed significant leakage of nucleic acids, suggesting extensive damage to the cytoplasmic membrane.

Applications

Preparations containing TTO are commonly used as antiseptic agents with antimicrobial, cleansing, healing and itch relieving properties (34). For example, creams containing 5% TTO have been used to treat acne and toenail onychomycosis. A 6% gel formulation was shown to have antihypertensive effects, and TTO has been used as an antiseptic agent in handwash soaps and mouthwashes as well as for the treatment of microbial infections such as folliculitis and vaginitis (34–36). Whole TTO essential oil applied over 12 days successfully and permanently removed skin warts, whereas salicylic acid (12% w/w) and lactic acid (4% w/w) only resulted in temporary removal of the warts (37). Treatment with 4% TTO nasal ointment together with a 5% TTO body wash performed better at eradicating MRSA from patients than 2% mupirocin nasal ointment together with 2% triclosan body wash (40). Similarly, a 10% TTO cream and 5% TTO body wash was more effective at clearing MRSA on skin compared with 4% chlorhexidine gluconate soap and 1% silver sulfadiazine cream (41). In contrast, treatment of MRSA in the nasal cavity alone, even with 10% TTO cream, showed only 47% eradication (41).

TTO has been widely used in the management of wounds because of its antimicrobial and therapeutic properties. Burnaid® is a commercial hydrogel dressing impregnated with TTO for the treatment of burns (42). Other studies have reported the enhancement of antimicrobial activity when using TTO in combination with other antimicrobial agents such as chlorhexidine (8,43), tobramycin (43) and silver ions (29).

Wound-healing benefits

In addition to antimicrobial activity, TTO also plays a role in wound healing and modulation of the inflammatory response (6,44,45). Water soluble components of TTO, especially terpinen-4-ol, contribute to inflammatory regulation by suppressing monococyte production of superoxide ions (45) as well as inflammatory-inducing mediators, for example, TNFα, IL-1β, IL-8, IL-10 and PGE2 (44). This in turn limits further production of other inflammatory cytokines (44) and reduces oxidative damage to cells (6), thereby enhancing wound healing. The aromatic vapours and analgesic properties of TTO may promote wound healing by providing temporary relief to patients (42,46). Burnaid reduces skin temperature at the burn site by approximately 2°C within 20 min, providing localised soothing and cooling effects (42), and may improve the patients’ ability to cope with the treatment. Using TTO to treat patients with malodorous skin ulcers showed a significant reduction of the malodour as well as of infection and pus secretion (46,47). The TTO compounds therefore improved the patient’s well-being by reducing social isolation associated with the malodour.

Resistance

Despite the popularity of TTO in many applications, concerns of microorganisms developing resistance have not been totally neglected. In vitro exposure of S. aureus to stepwise increasing concentrations of TTO resulted in a selection of TTO-resistant sub-populations (48).

Although there is little evidence of cross-resistance to conventional antibiotic-resistant strains with TTO (28), mutant strains of S. aureus with reduced susceptibility to household cleaning agents containing plant extracts were less susceptible to TTO (49). Based on the available data, the activity of TTO may not favour spontaneous development of resistance; however, it is still important to minimise exposure to sublethal concentrations to limit the possibility of resistance development (28,48).

TTO resistance is also noted in gram-negative bacteria because of the nature of the outer membrane, which is composed of lipopolysaccharide, proteins and phospholipids. This membrane provides a hydrophilic permeability barrier, which is an essential factor in the tolerance of P. aeruginosa to membrane-damaging agents, such as TTO (31).

Toxicity concerns

As with most drugs, overdose or extended exposure induces toxic side effects. Evidence from several reports has shown that toxicity of TTO when ingested is rarely, if ever, fatal (28). In general, the symptoms of oral toxicity of TTO vary according to age and dose ingested. Reported symptoms arise from effects on the central nervous system, resulting in changes in respiration rate, oxygen saturation levels, pupil reactivity, electrolyte and blood glucose concentration, development of systemic hypersensitivity, ataxia, muscle weakness, unconsciousness and hallucinations (50,51). Similar toxic symptoms including lack of coordination, muscle tremors, dehydration, hypothermic, ataxic effects and, in more severe cases, death have been observed in animals (52).

Dermal toxicity of TTO has been reported to cause irritation and allergic reactions (28). The localised cooling effect on treated burn wound sites may lead to triggering hypothermia when using TTO-based dressings on large areas of the skin (42).

Heavy metals as antimicrobial agents

Heavy metals have a density of at least 5 g/cm³ and are located centrally in the periodic table, along with all the other transition elements, because of their ability to form complexes via their partially filled d-orbitals (52). These d-orbitals allow the heavy
metals to accept electrons to form complexes, either by redox or other biochemical reactions in the cell. At low concentrations, some heavy metals may serve as essential trace elements to maintain normal cell function. However, at high concentrations, cell toxicity may result from the formation of unspecified complexes within the cell (53,54). Three classes of heavy metals have been proposed by Nies, 1999 (53):

i. low toxicity and play an important role as trace elements, for example, iron (Fe), molybdate (Mo), manganese (Mn),

ii. toxic but high importance as a trace element, for example, zinc (Zn), nickel (Ni), copper (Cu), vanadium (V), cobalt (Co), tungsten (W), chromium (Cr); and

iii. toxic with little or no beneficial action, for example, arsenic (As), silver (Ag), antimony (Sb), cadmium (Cd), mercury (Hg), lead (Pb) and uranium (U).

Amongst the various biological functions of heavy metals, their antimicrobial activity will be discussed in further detail. The sequence of antimicrobial toxicity is reported as follows: Ag > Hg > Cu > Cd > Cr > Pb > Co > Au > Zn > Fe > Mn > Mo > Sn (55). The mechanism of antimicrobial action of heavy metal cations varies because of differences in chemical characteristics and their effect on different biochemical pathways in the cell (Table 3). These effects result in disruption of the microorganism’s normal cell function, leading to irreversible damage and cell death (53-56).

Metal cations interact with ionisable intracellular groups such as carboxylates and phosphates in the lipopolysaccharide layer of gram-negative bacterial cells, peptidoglycan and teichoic acids of gram-positive bacterial cells (57). Metal cations may be incorporated into the cell membrane, causing loss of fluidity, followed by further transport into the cell cytoplasm to inhibit vital biochemical processes, hence disturbing the normal growth (53-58). This transport is usually achieved via an unspecified system using a chemiosmotic gradient between the cell and its environment or, alternatively, an ATP-dependant specific transport system (53-54).

Within the cytoplasm, metal cations may affect various cell processes (52); for example, they may bind to sulphhydryl or thiol groups, leading to inhibition of various enzymes (52). Some examples of toxicity activities of metal cations towards microorganisms are detailed in Table 3.

Despite their usefulness as antimicrobial compounds, the intensity of exposure (duration and concentration applied) should be carefully considered. Cobalt, cadmium and mercury are too toxic to be used clinically as antimicrobial agents. Copper, zinc and silver are less toxic to human cells. These are often incorporated into antiseptic creams or cleaning agents as well as surfaces of medical devices surfaces, including hospital taps and door handles (59,60). Examples of applications using heavy metal ions and their mechanism of action are detailed in Table 4.

### Table 3: Examples of heavy metals with antimicrobial properties

<table>
<thead>
<tr>
<th>Metal cations</th>
<th>Activity towards microorganism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium, Cd²⁺</td>
<td>Reacts with sulphhydryl groups on various intracellular proteins</td>
<td>(130)</td>
</tr>
<tr>
<td>Copper, Cu²⁺</td>
<td>Membrane-bound copper ions may undergo Cu(I) to Cu(II) redox cycle, catalysing formation of highly toxic hydroperoxide radicals</td>
<td>(68,137,138)</td>
</tr>
<tr>
<td>Cobalt, Co²⁺</td>
<td>Competes with zinc for the active site of urease, thus inhibiting growth of Helicobacter pylori</td>
<td>(139)</td>
</tr>
<tr>
<td>Silver, Ag⁺</td>
<td>Interacts with intracellular components to suppress expression of enzymes and proteins essential for ATP production and condensation DNA, thus impairing replication</td>
<td>(69)</td>
</tr>
<tr>
<td>Zinc, Zn²⁺</td>
<td>Inhibits nutrient uptake, acid production and glycolysis in oral pathogens and rhinoviral replication. Interferes with protein transfer</td>
<td>(60,140,141)</td>
</tr>
</tbody>
</table>

Silver as an antimicrobial

**History and background of the uses of silver**

The antimicrobial properties of silver, including its use as an active water disinfectant, have been documented since 1000 B.C. (61). The use of silver as an antimicrobial has been reported since ancient Greek and Roman times, in which silverware was used to store perishable food and drinks. By the 19th century, the use of silver ions, Ag⁺ (as silver nitrate), in medical applications became more widespread, with records of it being used to treat venereal diseases, salivary gland fistulae, bone and periosteal abscesses and removal of granulation tissue prior to epithelialisation (61-64). After the discovery of penicillin and the rapid expansion in the number and use of antibiotics (64,61,63), the antimicrobial use of silver declined. Recent widespread emergence of antibiotic-resistant microorganisms has resulted in Ag⁺-based agents regaining popularity as their multi-target action in microbial cells is less likely to lead to silver resistance (55,64).

Silver in its non-ionised form is an inert metal that does not react with human cells (65). Compared with other heavy metals, Ag⁺ has relatively low toxicity towards human cells at concentrations that are still highly effective against microbial pathogens (66-68).

The reactivity of Ag⁺, even at concentrations as low as 10⁻⁹ to 10⁻⁶ mol/l (equivalent to 0-0.00108–0.108 ppm), has shown broad spectrum antibacterial, antiviral, antiprotoszoal and anti-fungal activity (62). In addition, the antimicrobial activity and low toxicity of silver towards human cells is also accompanied by wound-healing properties (62). Silver is able to treat infections while enhancing wound healing, which could be especially useful in the management of severe wounds such as burns and slow/non-healing ulcers.
The use of pre-antibiotic antimicrobials in the treatment of chronic wound infections

W. L. Low et al.

Table 4 Applications of heavy metals with lower toxicity to human cells

<table>
<thead>
<tr>
<th>Heavy metal ions</th>
<th>Antimicrobial mechanism and uses</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper, Cu²⁺</td>
<td>• Inhibition of bacterial growth or bactericidal activity at 50–250 ppm.</td>
<td>[50,142–145]</td>
</tr>
<tr>
<td></td>
<td>• Substitutes essential ions, blocks protein functional groups, inactivates enzymes,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>weakens membrane integrity and produces hydrogen peroxide-free radicals when</td>
<td></td>
</tr>
<tr>
<td></td>
<td>membrane bound.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• For example, antimicrobial hand rub, coated contact surfaces (copper toilet seats, brass</td>
<td></td>
</tr>
<tr>
<td></td>
<td>taps, door handles, door push plates) and cleaning materials (ultra-microfibre cloths and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mops).</td>
<td></td>
</tr>
<tr>
<td>Silver, Ag⁺</td>
<td>• Microbicidal activity at 0–0.5 ppm in phosphate-buffered saline or at &gt;50–60.5 ppm in</td>
<td>[5,23,60,146,147]</td>
</tr>
<tr>
<td></td>
<td>complex biological fluids.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Interact strongly with thiol groups, inhibits bacterial enzymes, interferes with electron</td>
<td></td>
</tr>
<tr>
<td></td>
<td>transport, binds to DNA, thus inhibiting normal cell replication.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Formulated as creams and incorporated into dressings for the treatment of burns and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>wound infections. Also incorporated into polyalkenoate dental cements, textiles,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>water filters, kitchen appliances and medical devices (theater).</td>
<td></td>
</tr>
<tr>
<td>Zinc, Zn²⁺</td>
<td>• Inhibits replication of rhinovirus at &lt;0.1 mmol/l (equivalent to ≤539 ppm). At 0.01–0.1</td>
<td>[60,140,141,146,148]</td>
</tr>
<tr>
<td></td>
<td>mM (equivalent to 0–539 ppm), inhibits acid production by oral plaque bacteria, effective</td>
<td></td>
</tr>
<tr>
<td></td>
<td>against plaque and gingivitis when combined with trichlosan. Inhibits nutrient uptake,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>proton transfer and sugar transport.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Incorporated into polyalkenoate dental cements, stainless steel surface coatings, air</td>
<td></td>
</tr>
<tr>
<td></td>
<td>conditioning ventilation, intake and exhaust ducts.</td>
<td></td>
</tr>
</tbody>
</table>

Mode of action

Ag⁺ is classified as highly reactive moieties, which readily bind anions formed by electron donor groups containing sulphur (thiols), oxygen and nitrogen (69). Ag⁺ demonstrates broad spectrum antimicrobial activity at concentrations as low as 0.05 ppm in phosphate-buffered saline or between >50 and 60.5 ppm in complex organic biological fluids (5,70–72). Ag⁺ expresses its antimicrobial activity initially by binding to cell surface proteins and enzymes, resulting in morphological cellular changes, inhibition of cell replication (70,73,74) and impairment of solute and electron transport systems, leading to reduced production of vital cell components, such as ATP (75). Subsequent uptake of Ag⁺ into the cell cytoplasm, either via non-specific or substrate-specific transport systems, allows Ag⁺ to bind and interfere with the activity of essential intracellular enzymes and DNA (69,73,74).

In the presence of oxygen, Ag⁺ also promotes the generation of reactive oxygen species through the inhibition of respiratory enzymes, such as NADH dehydrogenase II (76), or by impairing superoxide-radical-scavenging enzymes, such as superoxide dismutase (77). Evidence also suggests that the antimicrobial activity of Ag⁺ may be a consequential result of its ability to bind to essential enzyme sulphydryls groups (thiols), thereby breaking these protein bonds (78). The binding of Ag⁺ to anionic groups, most notably disulfide, amino, imidazole, carbonyl and phosphate residues, results in intracellular and nuclear membrane (in eukaryotes) permeability changes as well as structural modifications of the cell wall (74,79,80). Ag⁺ may also bind to DNA bases, causing condensation and degenerative changes of DNA strands, leading to inhibition of cell replication and cell death (67,74). In general, the antimicrobial mechanism of action of Ag⁺ is described as a cascade of four steps (75):

- The Ag⁺ binds to receptors (especially in protein residues including sulphydryl, amino, imidazole, phosphate and carboxyl groups) on the microbial cell membrane, resulting in membrane damage.
- After entering the cytoplasm, Ag⁺ binds to other essential enzymes, restricting their activity and the production of vital metabolites.
- Binding to microbial DNA follows, thereby impairing cell replication.
- With weakened membrane structure and inhibition of cellular processes, vital components leak out; the bacterial cell cannot maintain normal function, resulting in cell death.

Applications

Formulations containing silver are commonly used to treat a variety of gram-positive and gram-negative bacteria as well as common highly antibiotic-resistant microorganisms such as P. aeruginosa (74,79). Silver-based pharmaceutical preparations, for example, silver sulfadiazine (Flamazine®), has been used for the treatment of burn wounds. It has been found that the antimicrobial activity of Ag⁺ depends not only on the amount of bioactive ions present but also on bioavailability, which is influenced by the physical and chemical form of silver, the affinity for moisture, rate of release and distribution (60,74,79,83). In the skin, silver may form a temporary reservoir by binding to tissues with an estimated half-life of 10–12 hours (74). Being a highly reactive species, Ag⁺ can readily bind to components present in the wound bed, for example, negatively charged proteins, RNA, DNA and chloride ions (5), thereby limiting the bioavailability of the ions. This ‘quenching’ effect of the host tissues may lead to the need for the application of higher and potentially damaging doses of Ag⁺ for effective treatment.
Recent advances in controlled delivery systems, which incorporate antimicrobial agents such as Ag+ into delivery devices or dressings, may play a role in overcoming the potential problems caused by the increased exposure and concentration of active antimicrobial agents. Some examples of silver-containing wound dressings available for current wound management applications include DuoDERM® (hydrocolloids), Aquacel® Ag (hydrofibre dressings containing antimicrobial silver ions), Tegaderm™ (films), Vaseline™ (gauze), Sorbsan® (alginates), Lyofoam® C (foam dressing) and Nu-Gel™ (hydrogels) (83, 84).

**Wound-healing benefits**

As discussed above, Ag+ helps to promote wound healing by reducing the bacterial load at wound site. Ag+ can also enhance wound healing directly by modulating the inflammatory response (85). At wound sites, Ag+ is taken into epithelial cells responsible for the regulation of tissue metal homeostasis, heavy metal detoxification and wound healing (5). This uptake induces the synthesis of low molecular weight, cysteine-rich, metal-binding proteins called metallothioneins (metallothioneins I and II). This activity of metallothioneins, which protect the healthy cells from the toxic effects of metals, is induced by several other xenobiogenic metals such as cadmium, gold and mercury (85). Metallothioneins also play an important role in promoting the uptake of key trace elements, such as zinc and copper, promoting RNA and DNA synthesis, cell proliferation, epithelialisation and tissue repair (5, 86). Rising zinc levels induced by the accumulation of Ag+ in wounds increased the activity of the zinc metalloenzymes, thus promoting cell proliferation and re-epithelialisation in rats (85).

During wound repair and the inflammatory response, matrix metalloproteinases (MMPs) are present within the wound. MMPs function to cause breakdown of the extracellular matrix, autolytic debridement, dissolution of basement membranes, growth promotion of capillary beds, re-epithelialisation and tissue remodelling (85). Hence, MMPs are essential in wound healing, but excessive levels degrade fibronectin and peptide growth factors needed for optimal re-epithelialisation (5, 86). Ag+ may form stable complexes with MMPs, thus down-regulating excessive localised inflammatory responses to promote wound healing (67, 85). Comparative studies revealed that patients had improved epithelialisation on the skin grafts when treated with nanocrystalline silver dressings in comparison to topical antibiotics (86). Results indicated that Acticoat™, a nanocrystalline silver-containing product, was effective as a dressing that reduced the wound bio-burden and altered the process of inflammation, thus facilitating the healing process (80, 88). The ability of Acticoat to provide prolonged sustained release of silver onto the wound site helps to avoid the development of resistance microorganisms while reducing potentially toxic side effects (80, 86).

**Resistance**

While this multi-locus action of silver makes development of resistant microorganisms less likely, such resistance has been observed (87), and with the increasing use of silver, it may be a cause for concern (63, 88). The occurrence of resistance genes has been reported and may be chromosomal- or plasmid-born (89, 90). The identified silver resistance plasmid, pMG101, codes for periplasmic binding proteins (SilIE and SilIF) and a chemiosmotic efflux pump (SilCBA), which exports Ag+ donated from an ATPase efflux pump (SilP) via SilF (87, 89–91).

The initial mechanism of resistance may be because of periplasmic Ag+-binding proteins, SilIE, each of which binds five Ag+ in the periplasmic space. Synthesis of SilIE is stimulated by the presence of Ag+ during growth (82). Although SilIE has high affinity for Ag+, the actual release mechanism of the bound Ag+ has yet to be determined. It is possible that the Ag+ may be released to the cell exterior via the SilCBA protein trimmer. The SilCBA assembly functions as a transmembrane cation/proton antiporter, moving Ag+ from the cytoplasm directly to the cell exterior. It is classed as a member of the resistance, nodulation and cell division (RND) superfamily, sharing homologous sequences with similar resistance mechanisms in metal-resistant Alcaligenes and multi-drug resistance mechanisms in E. coli (87, 90, 91).

Silver resistance has been found in clinical isolates of E. coli, Enterobacter cloacae, Proteus mirabilis and Klebsiella pneumoniae in a burns unit. In addition, silver nitrate and SSD treatment has also been associated with resistance in Proteus spp., E. cloacae and miscellaneous enterobacteriaceae (92). Similarly, resistance to multiple antibiotics and Ag+ has been shown in Salmonella species (93). Laboratory exposure of clinical strains of E. coli to stepwise increases of silver nitrate or SSD has been shown to induce cross-resistance against both compounds (94).

A *sil* E gene has been detected in MRSA strains, isolated from dogs, and is ≥95% homologous with the *sil E* from plasmid, pMG101 (95). However, these isolates were still sensitive to treatment with silver-impregnated hydrofibres. The results from this study are compatible with those of Silver, 2003 and suggest that there is low prevalence of silver resistance in MRSA (82). The restricted occurrence of a single *sil E* gene encoding for resistance is not sufficient to induce significant resistance against silver (94).

**Toxicity concerns**

In common with many drug treatments, over exposure to the agents causes unwelcome side effects. Long-term topical exposure to high concentrations of Ag+ leads to a build up of Ag0 in the dermis, causing an irreversible blue-grey discoloration of the skin (argyria). This is particularly pronounced in areas exposed to sunlight, which accelerates the photoreduction and deposition of Ag0 (22, 65). Some patients treated with silver-containing dressings reported the occurrence of skin rashes, stinging and burning sensations when treated with silver-impregnated dressings (24). Other more serious problems associated with topical application can include disturbances in electrolyte concentration, resulting in hyponatraemia or hypochloraemia (75).

Despite the beneficial properties of various silver-based treatments, the potential toxicity and safety issues of silver use have to be carefully considered. With the increased availability
of formulations, administration of silver can be tailored to the patient’s condition, thus limiting the potential risk of side effects.

Conclusion
There has been increasing interest in the use of alternative, broad spectrum, pre-antibiotic antimicrobial agents, such as essential oils and metal ions, to address issues relating to increased antibiotic-resistant hospital infections. The versatility of alternative agents such as TTO and Ag⁺ against a wide range of different microorganisms because of their multiple target sites impedes the development of resistance and might be useful in improving the current wound treatment strategies. Despite the effectiveness of the agents, the potential development of side effects or toxicity to healthy host cells because of prolonged exposure at higher concentrations should be carefully monitored. Efforts to combine the use of these alternative antimicrobial agents with advances in targeted delivery techniques may help to address the issue of localised overloading and toxicity. Based on the current findings, which showed the efficacy and beneficial therapeutic properties of both Ag⁺ and TTO, the potential advantages of using these agents in wound treatment regimes should be explored further.

References
The use of pre-antibiotic antimicrobials in the treatment of chronic wound infections


Davies J. Microbes have the last word. A drastic re-evaluation of antimicrobial treatment is needed to overcome the threat of antibiotic-resistant bacteria. *EMBO Rep* 2007;8:616–21.


The use of pre-antibiotic antimicrobials in the treatment of chronic wound infections

W. L. Low


48. Bilia AR, Giomi M, Innocenti M, Gallori S, Vincieri FF. HPLC-DAD-ESI-MS analysis of the constituents of aqueous preparations of...
The use of pre-antibiotic antimicrobials in the treatment of chronic wound infections

W. L. Low et al.

QUERIES TO BE ANSWERED BY AUTHOR

IMPORTANT NOTE: Please mark your corrections and answers to these queries directly onto the proof at the relevant place. DO NOT mark your corrections on this query sheet.

Queries from the Copyeditor:

AQ1. Please confirm that given names (red) and surnames/family names (green) have been identified correctly
AQ2. As per journal style, Key Messages should be given in bullet list. Hence we have followed the same – please check.
AQ3. Table 1 images are poor quality, Kindly resupply
AQ4. Please provide supplier details (city, state if USA and country) for “Flamazine; DuoDERM; Aquacel; Tegaderm; Vaseline; Sorbsan; Lyofoam; Nu-Gel”.
AQ5. Reference “150” is not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.
AQ6. Reference “151” is not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.
AQ7. Reference “152” is not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.
AQ8. Reference “153” is not cited in the text. Please indicate where it should be cited; or delete from the reference list and renumber the references in the text and reference list.