Antimicrobial Activity of Eucalyptus Tar

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Abstract

Wood tar is a chemically complex by-product of the wood distillation process and a potential economic liability for companies that utilise this process. Any accumulation of this substance must be stored properly on-site in order to limit any leaching of chemicals into the surrounding environment and release of odours into the atmosphere. Phenol, which can be derived from wood tar, was the first widely used antiseptic and disinfectant. In an attempt to find a use for eucalyptus tar, one type of wood tar, research was undertaken to determine its antimicrobial activity. Solvent-mediated extractions of the whole tar were chemically analysed and then tested against a variety of different microbes.

1.0 Introduction

By-products in many industries, such as mining and refining ores, production of steel and other resources in high demand, are becoming an increasing concern for the community. As a result of its effect on the environment, industries are under enormous economic and political pressure to find solutions to the accumulation of toxic by-products. It is not acceptable to allow harmful substances to leach into soil and waterways or to contaminate the air.

Wood tar is one such by-product that is causing environmental concerns for the community. It is produced when wood is carbonised to form charcoal. In the iron production industry, charcoal is used in some countries as an energy source for the conversion of iron ore to liquid iron or steel. In such a sizeable industry, it is no surprise that any accumulation of by-products can create a potentially massive economic liability for that business.

Until 1981, wood tar was still being produced on a large scale by a Western Australian company at Wundowie. During the years of government ownership (1948-1974), 9 000 tonnes of tar was accumulated on-site near Wundowie. In 1974, the business became privately owned and the environmental management of the accumulated tar became the new owner's responsibility. Since then the tar has been secured by a multi million dollar dam, which has prevented any adverse environmental impact. In the search for a long term solution, this company turned to historical uses of wood tar. The non-water soluble fraction of wood tar, also known as "Stockholm tar", is used as a hair treatment for psoriasis, in veterinary medicine to treat cuts on sheep caused by shearing, and is applied to horse's hooves to prevent or treat fungal and bacterial infections (Anon, 1985). Lister (1865) discovered that phenol (derived from wood tar) had excellent antimicrobial activity, leading the application of phenol as the first widely-used disinfectant and antiseptic.

Amen-Chen *et al.* (1997) used a variety of extraction methods to analyse the chemical composition of tar that was produced as a by-product of carbonising eucalyptus wood. They showed that eucalyptus tar, like many other types of wood tar, contained phenolic compounds such as cresol, catechol, syringol and guaiacol.

Based on this background, and a natural curiosity, Wundowie Foundry, acting on behlkf of current owners, has invested money into research to identify and investigate potential medical uses of eucalyptus tar.

2.0 Materials and Methods

2.1 Solvents

A variety of solvents with different polarities was selected for the extraction process. These included (in order of increasing polarity) hexane, diethyl ether, dichloromethane, ethyl acetate, acetone, methanol, ethanol and sterile distilled water (SDW).

2.2 Extraction Process

Previous characterisation of eucalyptus tar (Amen-Chen *et al.*, 1997), and other types of wood tar (Ogata and Baba, 1989), indicated that this substance contains many phenolic compounds which are relatively polar. Therefore, in order to separate the eucalpytus tar into simpler fractions which were easier to analyse, three relatively non-polar solvents (hexane, dichloromethane and ethyl acetate) were chosen for further analysis. A set ratio of solvent to tar was allowed to extract in a fume hood and, after 24 hours, 5mL volumes were aliquoted into sterile McCartney bottles. The solvent was then allowed to evaporate off, leaving behind a tar residue. This residue was resuspended in 1mL of SDW and is referred to as the aqueous fraction of that specific solvent extract. The remaining residue (after the aqueous fraction had been removed) was resuspended in 1mL of 100% ethanol. This is referred to as the ethanol fraction of the specific solvent extract.

Controls were prepared by dispensing a volume of solvent into a sterile McCartney bottle. After 24 hours, 5mL volumes were distributed into sterile McCartney bottles and the solvent was allowed to evaporate off. Aqueous and ethanol fractions of the control were prepared in a similar fashion to the eucalpytus tar extract.

2.3 Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

The aqueous and ethanol fraction of each of the selected solvents were analysed by GC/MS. Library searches were performed in order to identify the chemical components of each of the fractions.

2.4 Susceptibility Testing

Susceptibilty testing was performed using the broth microdilution method as outlined in the National Committee for Clinical Laboratory Standards (2000), with the exception of *Streptococcus pyogenes* where Todd Hewitt broth was used as growth medium instead of Müeller Hinton broth with 2-5% lysed horse blood. Minimum inhibitory concentrations (MICs) and minimum bactericidal/fungicidal concentrations (MBCs/MFCs) were determined for the aqueous and ethanol fractions of the three selected solvents (and the controls) against two reference bacteria (*Escherichia coli* and *Staphylococcus aureus*) and two reference yeasts (*Candida albicans* and *Malassezia furfur*). Each test was repeated a minimum of three times or until a reproducible mode was determined.

Further susceptibility testing was performed using the aqueous fraction of both the dichloromethane and ethyl acetate extracts against a selection of Gram-negative (*Pseudomonas aueruginosa, Serratia marcescens* and *Klebsiella pneumoniae*) and Gram-positive (*Enterococcus faecalis, Micrococcus* spp., *St. pyogenes, S. epidermidis* and other coagulase-negative *Staphylococcus* spp. including *S. haemolyticus, S. hominis, S. saprophyticus, S. warneri* and *S. xylosus*) bacteria and yeasts (*C. glabrata, C. parapsilosis* and *M. sympodialis*). For each Gram-negative bacterium and yeast species that were chosen, one reference and two clinical strains

were tested. One reference and nine clinical strains of each Gram-positive bacterium listed above were also tested. Results from susceptibility testing the Gram-positive bacteria were used to determine a concentration that was growth inhibitory in 50% of strains (MIC50) and 90% of strains (MIC90).

3.0 Results and Discussion

3.1 Controls

Both the ethanol and aqueous control for all solvent extracts were chemically analysed using GC/MS. In every case, both controls showed the presence of little to no chemical components (see Figure 1). This supported the results from the susceptibility testing of the aqueous control. None of the microbes tested (see section 2.4) was susceptible to this control at every concentration tested. For this reason, the aqueous control was only tested once against all of the microbes used for further testing. On the other hand, all microbes that were tesed were susceptible to the ethanol control. As the GC/MS analysis showed that there were no chemicals in this control, the antimicrobial activity exhibited by this control was due to the presence of ethanol only.

3.2 Ethanol Fraction

The ethanol fraction of the three different extracts showed broad spectrum bactericidal and fungicidal activity. But this antimicrobial activity did not differ from the activity exhibited by the ethanol control ($P>0.05$). GC/MS analysis showed that each of the ethanol fractions contained a minute amount of some of the chemicals found in the corresponding aqueous fraction. From this information it is possible to deduce that the antimicrobial activity of the ethanol fraction is predominantly due to the activity of ethanol. For this reason the ethanol fraction was not included in further antimicrobial testing.

3.3 Aqueous Fraction of Hexane Extract

3.3.1 GC/MS Analysis

The aqueous fraction of the hexane extract was analysed using GC/MS (Figure 2). A library search was performed on this fraction and the largest peak was identified as syringol (49.80%). The relative abundance of syringol in this fraction was approximately 800 000.

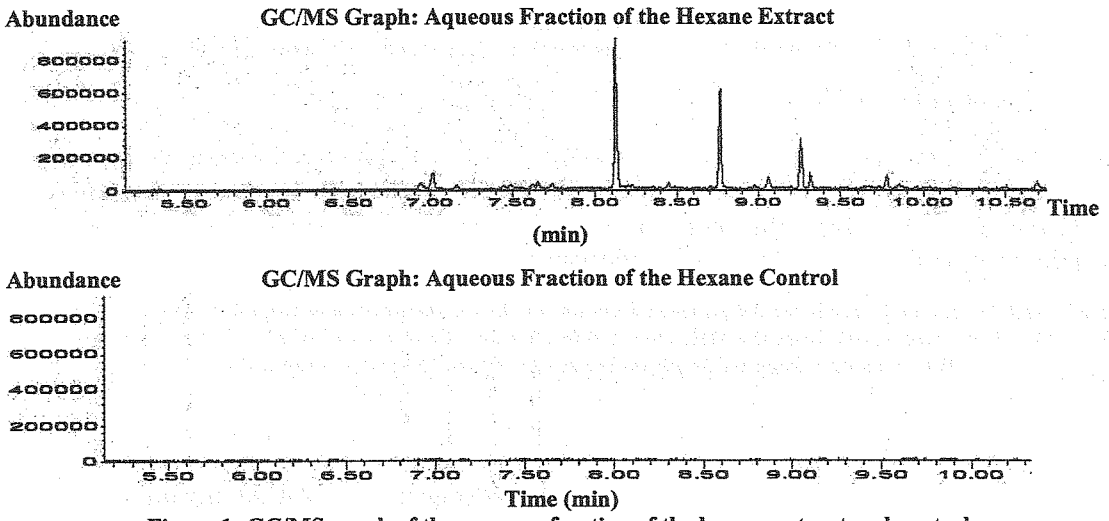

Figure 1: GC/MS graph of the aqueous fraction of the hexane extract and control.

3.3.2 Susceptibility Testing

The aqueous fraction of the hexane extract was tested against two reference bacteria and two reference yeasts (see section 2.4). This fraction only exhibited activity against *S. aureus* at the highest concentration that was tested (Table 1). It is possible that this fraction could also be active against *E. coli, C. albicans* and *M. furfur* but due to the low intial concentration it was too dilute to exhibit any activity against these more resistance microbes. For this reason there was no further testing of this extract.

Table 1: MICs for the aqueous fraction for three different extractions against four reference microbes.

	Solvent		
Organism	Dichloromethane (mg/mL)	Ethyl Acetate (mg/mL)	Hexane (mg/mL)
S. aureus	0.0120	0.0492	0.0520
E. coli	0.1923	0.7871	> 0.4166
C. alibcans	0.3847	1.5743	> 0.4166
*M. furfur**	0.3847	0.7871	> 0.4166

* MBC concentrations shown.

3.4 Aqueous Fraction of Dichloromethane and Ethyl Acetate Extract

3.4.1 GC/MS analysis

The aqueous fraction of the dichloromethane extract was analysed using GC/MS (Figure 2). A library search was performed on the aqueous fraction and all of the components identified were phenolic in nature. These included 2,6-dimethoxyphenol (19.32%), 1,2-benzenediol (10.42%) and 4-methoxy-1,2-benzenediol (3.62%). GC/MS analysis of the aqueous fraction of the ethyl actetate fraction showed a similar graph to that shown in Figure 2. A library search showed the presence of phenolic compounds such as 2,6-dimethoxyphenol (14.46%), 1,2-benzenediol (13.21%) and 3-methoxy-1,2-benzenediol (4.47%). As the chemical composition of the dichloromethane and ethyl acetate extracts were similar, they will be discussed together.

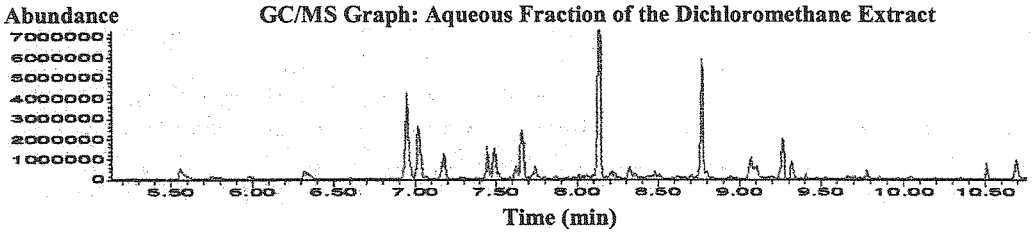

Figure 2: GC/MS graph of the aqueous fraction of the dichloromethane extract.

3.4.2 Susceptibility Testing

Further testing of these fractions against a wider variety of microbes showed a consistent increase in activity against Gram-positive bacteria (data not shown), specifically *Staphylococcus* spp. (Table 2). By taking the mode of action of phenol into account, the increase in susceptibility of Gram-positive bacteria is understandable.

Table 2: MIC90 (mg/mL) values for the aqueous fraction for the dichloromethane and ethyl acetate extracts. Each MIC90 was calculated from the MIC from 1 reference and 9 clinical strains of the bacteria (except other coagulase negative *Staphylococcus* spp. where 15 strains where used).

Organism	Solvent	
	Dichloromethane MIC90 (mg/mL)	Ethyl acetate MIC90 (mg/mL)
S. aureus	0.0481	0.3936
S. epidermidis	0.0240	0.0984
Other coagulase negative *Staphylococcus* spp.	0.0962*	0.7871*

Micrococcus spp.	0.3847	1.5743
Ent. faecalis	0.1923	0.7871
St. pyogenes	0.0962	0.7871

* Increase in MIC90 due to increased resistance exhibited by *S. saprophyticus*.

At high concentrations phenol disrupts the cell wall allowing penetration of the chemical into the cell causing precipitation of cellular proteins (Block, 1991). Penetration of the cell is essential and thus it is understandable that Gram-positive bacteria are more susceptible to the actions of phenol. Gram-negative bacteria are generally more protected from this action due to the presence of lipid-containing cell membrane components (Block, 1991). Taking this into account, it is interesting that *Micrococcus* spp., which is phenotypically similar to *Staphylococcus*, were less susceptible to all of the extracts tested (Table 2).

3.5 Positive Control

Biogram (16.5 % w/v o-phenylphenol) and Dettol (4.8 % w/v chloroxylenol) were tested as positive controls against *E. coli, S. aureus* and *C. albicans*. Accurate MICs and MBCs were determined for each microbe and compared to the MIC and MBC values for the dichloromethane and ethyl acetate extract. Both of the wood tar extracts were significantly more active ($P<0.05$) than both positive controls against *S. aureus*. This was interesting as it has been shown that halogenated phenols, such as chloroxylenol, exhibit a greater antimicrobial activity than those that are not halogenated (Sutler, 1941).

4.0 Conclusions

Although GC/MS analysis of the three solvent extracts was successful in identifying some of the many chemical components of eucalyptus tar, additional research needs to be carried out in order to confirm the active component or components of each extract. Both the aqueous fraction of the dichloromethane and ethyl acetate extracts exhibited broad spectrum bactericidal and fungicidal activity. Both of these extracts displayed a statistically significant ($P< 0.05$) increase in antimicrobial activity against *Staphylococcus* spp. compared to the other Gram-positive bacteria tested. Further research into the increased susceptibility of *Staphylococcus* spp. needs to be undertaken. This will aid in a better understanding of why solvent extracts of eucalyptus tar are specifically anti-staphylococcal.

5.0 Acknowledgements

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6.0 References

Anonymous (1985), *Industrial Charcoal Making*, [Online], Food and Agriculture organisation of the United Nations. Avaliable from: http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/X5555E/X5555E00.htm [accessed on March 20, 2005]

Amen-Chen, C., Pakdel, H. & Roy, C. (1997), Separation of Phenols From Eucalyptus Wood Tar. *Biomass & Bioenergy*, **13**(1-2) pp. 25-37.

Block, S. (1991), *Disinfection, sterilisation and preservation*, Lea and Febiger, Philadelphia.

National Committee for Clinical Laboratory Standards, (2000). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard*. Document M7-A5; 5th Edition.

Ogata, N. & Baba, T. (1989), Analysis of Beechwood Creosote by Gas Chromatography-Mass Spectrometry and High-Performance Liquid-Chromatography. *Research Communications in Chemical Pathology and Pharmacology*, **66**(3) pp. 411-23.

Suter, CM. (1941), Relationships Between the Structures and Bactericidal Properties of Phenols. *Chemical Reviews*, **28** pp. 269-99.