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Peppermint (*Mentha piperita*) inhibits microbial biofilms *in vitro*

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Abstract

Microbial biofilms have become increasingly problematic in the food processing and medical industries where they cause food and surface contamination. Biofilms have also been implicated as the cause of serious infections in humans as their occurrence makes it difficult to treat common infections and the likelihood of recurrent infections is high. Due to emerging resistance, conventional control methods are fast becoming ineffective. In this study, the use of a selection of commercial plant extracts is investigated. The inhibitory effects of eight herbal extracts on the development of microbial biofilms was investigated against clinical and reference strains of *Pseudomonas aeruginosa* and *Candida albicans*. The antimicrobial activity was investigated on the planktonic forms using the minimum inhibitory concentration assay. The extracts that showed the highest antimicrobial activity against the two test organisms were *Echinacea angustifolia* (cone flower), *Mentha piperita* (peppermint) and *Rosmarinus officinalis* (rosemary) with minimum inhibitory concentration values between 0.38 and 2.5 mg/ml. The crystal violet assay was used to assess the effect of pre-treating a surface with plant extracts on cell attachment and the extent of biofilm development following exposure to extracts (biofilm biomass). Most of the extracts reduced microbial colonization by at least 50%. In contrast, preformed biofilms were less responsive to the majority of extracts, thus growth inhibition was more difficult to achieve. *Mentha piperita* was the only extract that showed some antibiofilm activity against both pathogens.
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1. Introduction

The formation of micro-organisms in layers and their metabolic and decomposition products on various surfaces results in the formation of biofilms (Kumar and Anand, 1998). Advantages associated with biofilm formation include protection from the environment, nutrient availability, metabolic cooperation and acquisition of new genetic material (Jabra-Rizk et al., 2004). The most common problematic biofilms which occur on almost every individual is plaque that harbours oral bacteria on teeth and causes tooth decay (Bauman, 2006). Biofilms have also been implicated as the cause of serious infections although infections are not exclusively a result of biofilm formation (Spoering and Lewis, 2001). In addition, contamination of indwelling medical devices such as artificial joints, prosthetic heart valves, catheters and dentures has also resulted in some serious infections (Hugo and Russell, 2004).

The methods that are currently used for the control of biofilms include physical, chemical and more recently biological methods (Kumar and Anand, 1998; Steinberg et al., 2005). Physical methods commonly used include heat and mechanical scrubbing, brushing, scraping and high pressure spraying (Dreeszen, 2003). The use of chemical biocides such as disinfectants, detergents and preservatives is also common (Kumar and Anand, 1998). Bacteriocins and enzymes are also used on a smaller scale as biocontrol agents in the food industry, however, limitations include availability and cost (Kumar and Anand, 1998). Although a number of control measures are in place for biofilm control, most of these seem to be almost ineffective due to the increased resistance conferred by sessile cells. In an effort to identify alternative biofilm control methods, plants have been identified as potential candidates as many herbal extracts have shown to exhibit antimicrobial properties.

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Literature confirms that plant-based antimicrobial studies have almost exclusively focused on the planktonic micro-organisms while the sessile forms that are more resistant to antimicrobial agents and therefore more difficult to control, remain largely unexplored. This paper reports the antimicrobial activity of planktonic forms, antibiofilm adhesion potential and the prevention of further growth of established biofilms of eight plant extracts using two micro-organisms, *Candida albicans* and *Pseudomonas aeruginosa*.

2. Materials and methods

2.1. Selection and preparation of plant material

Eight herbs of commercial importance were purchased from Warren Chem Specialties, Herbs-A-Plenty and Unilever South Africa. These were selected on the basis of their commercial importance as most of them are consumed due to their health benefits. The herbs used and their commercial importance are presented in Table 1. The fresh herbs were initially air-dried at room temperature and ground to a fine powder in a blender. Dichloromethane/methanol (CH_2Cl_2/MeOH, 1:1) extracts of the powdered herbs were prepared using a method adapted from Sandasi et al. (2008).

2.2. Minimum inhibitory concentrations

To confirm the antimicrobial activity of the selected herbs, the extracts were initially tested on planktonic micro-organisms using the modified *p*-iodonitrotetrazolium violet (INT) microplate, minimum inhibitory concentration (MIC) assay (Eloff, 1998). Both clinical and laboratory strains of *P. aeruginosa* and *C. albicans* were used in this assay. *C. albicans* clinical strain CI002 was supplied by the microbiology laboratory, Department of Pharmaceutical Sciences, Faculty of Science, Tshwane University of Technology. *P. aeruginosa* (clinical strain FRD1) was supplied by Research and Development, Onderstepoort Biological Products. Reference strains *P. aeruginosa* (ATCC 9027) and *C. albicans* (ATCC 10231) were obtained from the microbiology laboratory, Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of Witwatersrand. The organisms were selected on the basis of their pathogenicity and their ability to form biofilms.

The extracts were dissolved in sterile water and tested using the same range of concentrations (0.0625–8 mg/ml). The positive control for *P. aeruginosa* was ciprofloxacin and amphotericin B was used for the *C. albicans* strains. The negative control was water. The MIC procedure was carried out by aliquoting one hundred microlitres (100 µl) of sterile distilled water into all the wells of the microtitre plate. The prepared extracts were then pipetted into the first well in each column in triplicate. Serial dilutions were performed in decreasing concentrations down the columns. Following the serial dilutions, 100 µl of the standard culture (1.0×10^6 CFU/ml) was added to all the wells. The plates were sealed with sterile adhesive tape and incubated at 37 °C for 24 h for *P. aeruginosa* and 48 h for *C. albicans*. Following incubation, the MIC assay was determined. To visualise the bacterial growth, 40 µl of INT (0.04 mg/ml) was added to each well and the plates incubated at room temperature for 6 h. For the yeasts, plates were incubated at room temperature for 24 h. The plates were then examined for colour changes and the MIC was indicated by the first clear well (lowest concentration having no microbial growth) in a column.

2.3. Inhibition of biofilm formation

To prevent initial cell attachment, 100 µl of plant extracts were aliquoted into wells of a 96 well microtitre plate. Standardised cultures (1.0×10^6 CFU/ml) of *P. aeruginosa* and *C. albicans* were then added (100 µl) into the wells and incubated for 4 h at 37 °C without shaking (Sandasi et al., 2008). The final concentration of the extracts in the wells was 1 mg/ml while ciprofloxacin at a concentration of 0.0025 mg/ml (MIC value) and amphotericin B at 0.0050 mg/ml (MIC value) were used as the positive controls for *P. aeruginosa* and *C. albicans*, respectively. Water was used as the negative control. Following incubation, the biofilm biomass was assayed using the modified crystal violet (CV) staining assay and percentage inhibition determined using Eq. 1 (Djordjevic et al., 2002).

$$\text{Percentage inhibition} = \frac{OD_{\text{Negative control}} - OD_{\text{Experimental}} \times 100}{OD_{\text{Negative control}}} \quad (1)$$

Table 1
Minimum inhibitory concentrations (mg/ml) of eight herbal extracts against two pathogens.

Plant	Commercial application	*P. aeruginosa*	*P. aeruginosa*	*C. albicans*	*C. albicans*
		ATCC 9027	Clinical FRD1	ATCC 10231	Clinical CI002
Echinacea angustifolia (Echinacea/cone flower)	Medicinal herb	0.75	1.00	1.00	0.38
Mentha piperita (Peppermint)	Spicing agent	0.75	1.00	2.50	1.00
Rosmarinus officinalis (Rosemary)	Spicing agent	1.50	1.00	1.00	0.75
Melaleuca alternifolia (Tea tree)	Medicinal herb	2.00	2.00	1.50	2.00
Hypericum perforatum (St John's Wort)	Herbal tea	3.00	1.00	3.00	1.00
Thymus vulgaris (Thyme)	Spicing agent	1.00	8.00	1.25	1.00
Aspalathus linearis (Rooibos)	Herbal tea	4.00	8.00	3.00	1.00
Camelia sinensis (Green tea)	Herbal tea	8.00	2.00	2.00	1.50
Controls [a]		0.0025	0.0025	0.005	0.005

[a] Ciprofloxacin and amphotericin B were used as controls against *P. aeruginosa* and *C. albicans*, respectively.

2.4. Inhibition of preformed biofilms

To test the ability of the extracts to prevent biofilm development, biofilms were pre-formed in 96 well microtitre plates by aliquoting 100 µl of standardized *P. aeruginosa* and *C. albicans* (1.0×10^6 CFU/ml) into the wells and incubated for 4 h at 37 °C. Following incubation, 100 µl of each of the plant extract that showed some degree of inhibiting cell attachment (six extracts) was added to a final concentration of 1.00 mg/ml in the wells. Ciprofloxacin at a concentration of 0.0025 mg/ml (MIC value) and amphotericin B at 0.0050 mg/ml (MIC value) were used as the positive controls for *P. aeruginosa* and *C. albicans,* respectively. Water was used as the negative control. The plates were further incubated for 24 h at 37 °C (Sandasi et al., 2008). Following incubation with the extracts, the crystal violet assay was performed to assay for biofilm biomass.

2.5. Crystal violet staining assay

The plates were washed three times with distilled water following incubation, then oven-dried at 60 °C for 45 min. Following drying, the wells were stained with 100 µl of 1% crystal violet and incubated at room temperature for 15 min after which the plates were washed three times with sterile distilled water to remove unadsorbed stain. The semi-quantitative assessment of biofilm formation was performed by adding 125 µl of ethanol to destain the wells. One hundred microlitres (100 µl) of the destaining solution was then transferred to a new plate and the absorbance determined at 590 nm using a microplate reader (Universal microplate reader EL_X 800). The mean absorbance ($OD_{590\,nm}$) of the samples was determined and percentage inhibition obtained using Eq.1.

3. Results and discussion

3.1. Minimum inhibitory concentrations

Generally, most extracts used had MIC values in the order of 1.00–3.00 mg/ml against both pathogens (Table 1). Noteworthy activity (MIC 0.38–1.00 mg/ml) was observed for *E. angustifolia* against all the isolates. This was followed to a lesser extent by *M. piperita* (0.75–2.50 mg/ml) and *R. officinalis* (0.75–1.50 mg/ml). For *C. albicans*, the clinical strain showed higher susceptibilities for all plant extracts with the exception of *M. alternifolia*. For *P. aeruginosa,* the clinical and ATCC strains exhibited variable susceptibility which was extract dependant.

The results confirm the antimicrobial activity of these extracts as previously reported (Cowan, 1999; Kuete et al., 2008; Landete et al., 2008; Taylor et al., 1996). Research has shown that various spices exhibit antimicrobial activity against a wide range of micro-organisms; however the efficacy differs depending on the choice of plant material, solvent used for extraction, composition and concentration of secondary metabolites, and choice of test organisms. Phenolic compounds are believed to significantly contribute to the overall antimicrobial activity of extracts (Kim et al., 2002; Shan et al., 2007). The good activity observed thus warranted further investigation for antibiofilm activity.

3.2. Prevention of cell attachment

Six of the eight extracts showed some degree of antiadhesion activity however, only 3 extracts showed good activity against the ATCC isolate with a percentage inhibition > 50% (Fig. 1). These were *E. angustifolia* (62%), *M. piperita* (57%), and *R. officinalis* (56%) with activities comparable to ciprofloxacin (60%). None of

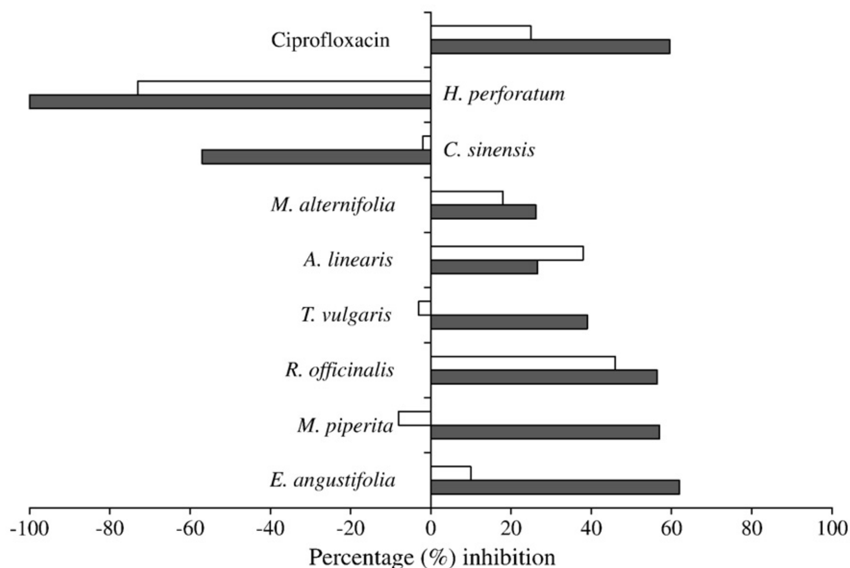

Fig. 1. The effect of herbal extracts on *P. aeruginosa* ATCC 9027 (■) and clinical FRD1 (□) biofilm formation expressed as percentage inhibition. (The extracts that inhibited biofilm formation have positive values while extracts that enhanced formation show negative inhibition).

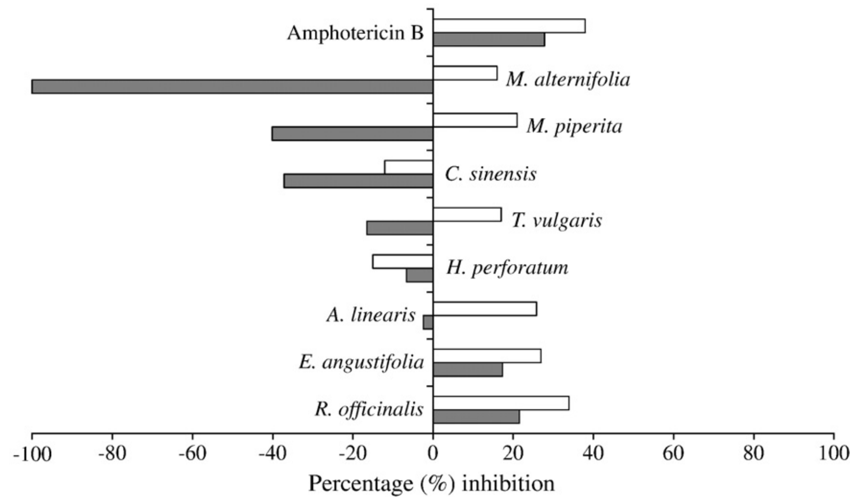

Fig. 2. The effect of herbal extracts on *C. albicans* ATCC 10231 (■) and clinical CI002 (□) biofilm formation expressed as percentage inhibition.

the extracts showed good activity against the clinical isolate as evidenced by the low percentage inhibition values of less than 50% (Fig. 1). This shows that the ATCC isolate was more susceptible to the extracts than the clinical isolate. Fig. 1 also shows 2 extracts (*C. sinensis* and *H. perforatum*) that enhanced cell attachment (negative percentage inhibition) for both the ATCC and clinical isolates. The presence of the extracts seemed to enhance biofilm formation of *P. aeruginosa*.

Fig. 2 shows that most extracts had minimal or no inhibitory effect on the attachment of *C. albicans*. Instead most extracts promoted cell attachment. *E. angustifolia* and *R. officinalis* had an inhibitory effect against both ATCC and clinical isolates; however none of these was effective enough to give a 50% reduction in cell attachment (Fig. 2). It is noteworthy that the same extracts also gave significant anti-adhesion activities against *P. aeruginosa* thus the two are promising candidates for further investigation as anti-adhesion agents.

3.3. Prevention of biofilm development

The two extracts (*C. sinensis* and *H. perforatum*) that did not show anti-adhesion properties were not further assessed for biofilm development, thus only six extracts were evaluated further. Fig. 3 shows that most of the extracts were unable to inhibit the growth and development of a pre-formed biofilm. Instead, enhanced growth and development of the biofilm was observed for most of the extracts against both isolates. Only *M. piperita* showed antibiofilm development (38% inhibition).

The results for *C. albicans* (Fig. 4) showed a similar trend to that of *P. aeruginosa*. Minimal activity was exhibited by only *A. linearis* (8%) and *M. piperita* (28%) extracts against both isolates. The majority of the extracts enhanced the development of the biofilm with *T. vulgaris* and *M. alternifolia* showing at least a two-fold increase against both isolates. These results indicate that the extracts either had minimal or no antibiofilm

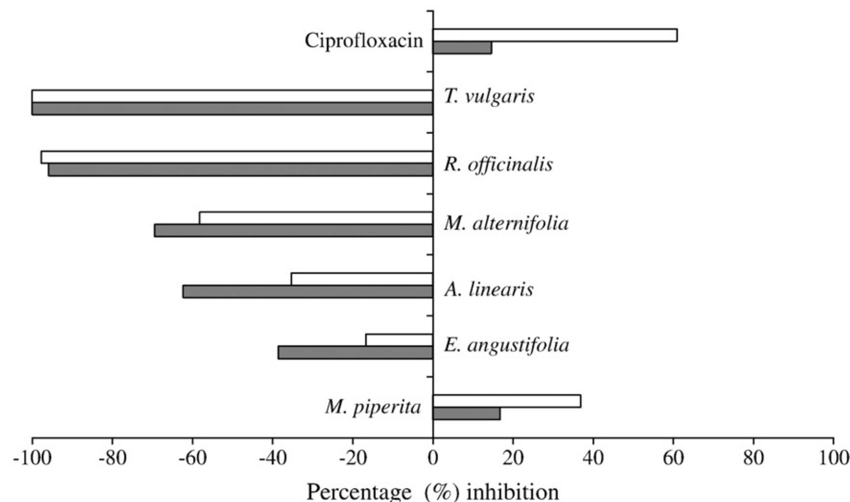

Fig. 3. The effect of herbal extracts on *P. aeruginosa* ATCC 9027 (■) and clinical FDR1 (□) biofilm development expressed as percentage inhibition.

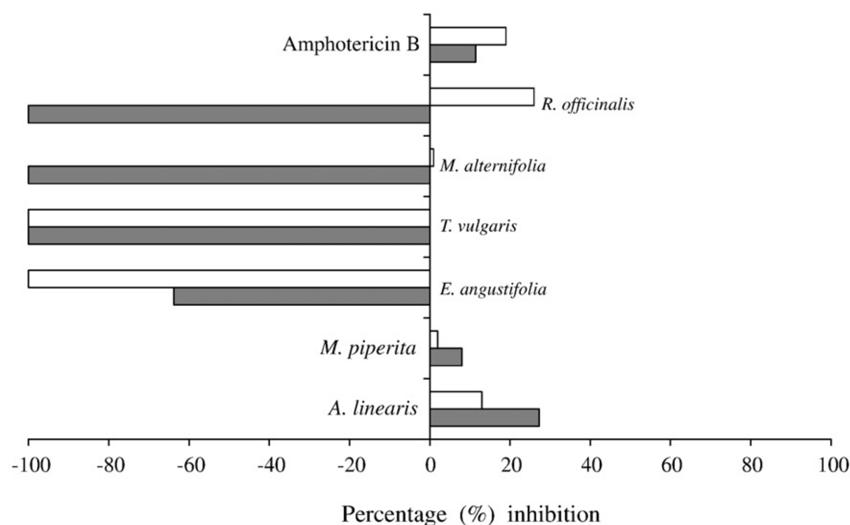

Fig. 4. The effect of herbal extracts on *C. albicans* ATCC 10231 (■) and clinical CI002 (□) biofilm development expressed as percentage inhibition.

activity against *C. albicans*. Overall, *M. piperita* was the only extract that exhibited antibiofilm activity against both pathogens although in both cases activity was less than 50%. The enhanced biofilm development observed for *T. vulgaris*, *R. officinalis*, *M. alternifolia* and *E. angustifolia* is consistent with both pathogens studied. These results show that inhibition of preformed biofilm development proved to be more difficult to achieve than cell attachment.

The absence of antibiofilm activity for most of the plant extracts is evidence that cells in a biofilm are more resistant to antimicrobial agents compared to free-floating or planktonic cells (Frank and Koffi, 1990; Krysinski et al., 1992). The ease with which the plant extracts inhibited cell attachment is confirmation of previous reports where it was found that inhibition of cell attachment to a substrate is easier to achieve than inhibiting the growth of an already established biofilm (Cerca et al., 2005). The success of selected plant extracts in inhibiting cell attachment as shown in this study is a promising tool for reducing microbial colonisation of surfaces and epithelial mucosa which subsequently leads to infections. Regular consumption of selected commercially popular herbs may thus play a role in the prevention of biofilms. Some researchers have demonstrated the success of coating medical devices with biocides such as silver to reduce microbial adhesion and the subsequent disease pathogenesis has been demonstrated (Hashimoto, 2001; Klueh et al., 2000). Milk constituents and chitosan are other non-herbal natural products that have been reported to inhibit cell attachment (Cai et al., 1994; Sharon and Ofek, 2001).

Literature on the antibiofilm activity of plant extracts is currently minimal. The results obtained for *M. piperita* warrant further investigation as a promising candidate for antibiofilm activity and to validate its activity. Previous reports have shown that *M. piperita* exhibits good antibiofilm activity against the Gram-positive pathogen, *L. monocytogenes* (Sandasi et al., 2008). Bupesh et al. (2007) showed that organic leaf extracts of *M. piperita* possess broad spectrum antibacterial activity against a wide range of bacteria. This activity has been attributed to the presence of potent compounds that include menthol, menthone, menthyl acetate and menthofuran. Menthol on its own has been shown to be inhibitory against bacteria, fungi and viruses, which contribute to the overall antimicrobial activity of the *M. piperita* extract (Bupesh et al., 2007).

The enhanced biofilm development observed for some extracts in this study is not fully understood. Literature however reports that the presence of some substances plays an important role in promoting microbial adhesion. Mention has been made of albumin, gelatin, fibrinogen and casein for promoting microbial adhesions (Meadows, 1971). The enhancement of cell attachment observed for some extracts in this study may be due to the presence of certain compounds within the extracts that also promote microbial adhesion. Further investigations on the mechanisms involved in this growth enhancement need to be undertaken to better understand the behaviour of cells in a biofilm.

The results also indicate that good antimicrobial activity against planktonic micro-organisms does not imply good antibiofilm activity. This was observed where most extracts that showed good MIC values had minimal or no antibiofilm activity against the same pathogens. It is therefore imperative to carry out investigations on both sessile and planktonic cells to ensure that extracts that provide a broader inhibitory activity are identified.

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