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ORIGINAL ARTICLE

In vitro activity of a combination of bacteriophages and antimicrobial plant extracts

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Significance and Impact of Study: This preliminary study provides insights into the potential combination of bacteriophages and antimicrobial plant bulk extracts to target bacterial pathogens. It is to our knowledge the first time in which virulent bacteriophages have been combined with antimicrobial plant extracts.

Keywords
antimicrobial compounds, bacteriophages, combination therapy, natural products, phage therapy.

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Abstract

The continuing threat of antimicrobial resistance presents a considerable challenge to researchers to develop novel strategies ensuring that bacterial infections remain treatable. Many plant extracts have been shown to have antibacterial properties and could potentially be combined with other antibacterial agents to create more effective formulations. In this study, the antibacterial activity of three plant extracts and virulent bacteriophages have been assessed as individual components and in combination. When assessed with a modified suspension test, these plant extracts also exhibit antiviral activity at bacterial inhibitory concentrations. Hence, to investigate any potential additive effects between the extracts and virulent phages, the extracts were tested at subantiviral concentrations. Phages alone and in combination with plant extracts significantly reduced ($P < 0{\cdot}05$) the bacterial concentration compared to untreated and extract treated controls up to 6 h (2–3$\log_{10}$), but this reduction did not extend to 24 h. In most cases, the phage and extract combinations did not significantly reduce bacterial content compared to phages alone. Additionally, there was little impact on the ability of the phages to reproduce within their bacterial hosts. To our knowledge, this study represents the first of its kind, in which antimicrobial plant extracts have been combined with virulent phages and has highlighted the necessity for plant extracts to be functionally characterized prior to the design of combinatorial therapies.

Introduction

The potential for a future in which bacterial infections become untreatable is undeniable, with a number of bacterial infections failing to respond to drugs of last resort or antibiotic combination therapy (Fifer *et al.* 2016; Bi *et al.* 2017). While this is due not only to an increase in the level and type of resistance that exists (Zhu *et al.* 2013; Oz *et al.* 2014), the overuse and misuse of antibiotics in both human and veterinary applications cannot be understated (Berendonk *et al.* 2015; Meek *et al.* 2015; Fleming-Dutra *et al.* 2016) and has shifted focus towards new strategies for combatting multi-resistant infections, including reducing antibiotic consumption (Sabuncu *et al.* 2009) and the development of new therapies (Brown and Wright 2016). Both bacteriophages and plant extracts are amongst those being investigated either as individual therapies (Bonifácio *et al.* 2014; Semler *et al.* 2014; Takemura-Uchiyama *et al.* 2014) or as adjuvants to conventional antibiotics (Coulter *et al.* 2014; Cushnie *et al.* 2014; Kamal and Dennis 2015).

A number of strategies are being pursued to reduce antibiotic resistance levels, including the development of new types of therapy and reduction of antibiotic consumption. Although combinatorial therapy between antibiotics and phages has been shown to be successful *in vitro,* and furthermore against resistant phenotypes (Comeau *et al.* 2007; Torres-Barceló *et al.* 2016), it is perhaps counteractive to employ at the same time as trying to reduce overall consumption of antibiotics, particularly in veterinary applications where antibiotic resistance is a major issue (Allen 2014; Van Boeckel *et al.* 2015). As such, combinations of naturally antimicrobial plant extracts with virulent phages could potentially address such issues.

In the current study, the antimicrobial potential of subinhibitory concentrations of three antimicrobial plant extracts in combination with virulent phages has been investigated.

The plant extracts were derived from *Stephania suberosa* roots (SSE, Menispermaceae), *Oroxylum indicum* fruits (OIE, Bignoniaceae) and *Boesenbergia rotunda* rhizomes (BRE, Zingiberaceae), all used in traditional Asian Medicine. The virulent bacteriophages SU16 (Myoviridae) and SU27 (Siphoviridae) were isolated from wastewater in 2010 and have been previously shown to be able to infect 16 and 4 strains of *Eshcherichia coli* at high or moderate efficiency of plating, respectively (Khan Mirzaei and Nilsson 2015).

Results and discussion

MIC/MBC determination and anti-phage activity of plant extracts

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination showed that both host bacterial strains (ECOR16 and ECOR27 from the *E. coli* standard reference collection; ECOR; Ochman and Selander 1984) were inhibited by the bulk plant extracts at the same inhibitory concentrations (Table 1). When the bulk extracts were tested for antiviral properties, all three extracts produced a statistically significant reduction (approx. 0·5–2 $\log_{10}$ PFU per ml; $P < 0{\cdot}05$; Fig. 1) of phage SU16 at MIC concentrations. There was a significant difference in the susceptibility of the two phages, SU16 and SU27, when tested against at multiple concentrations of BRE and OIE (100-1% MIC; $P < 0{\cdot}05$; Fig. 1), while SSE showed no significant difference between the two phages at concentrations of 10% MIC or lower ($P > 0{\cdot}05$; Fig. 1). Due to the differences in anti-phage activity exhibited by the different compounds, the concentration of the plant extracts was optimized on a phage+compound basis for time kill analyses.

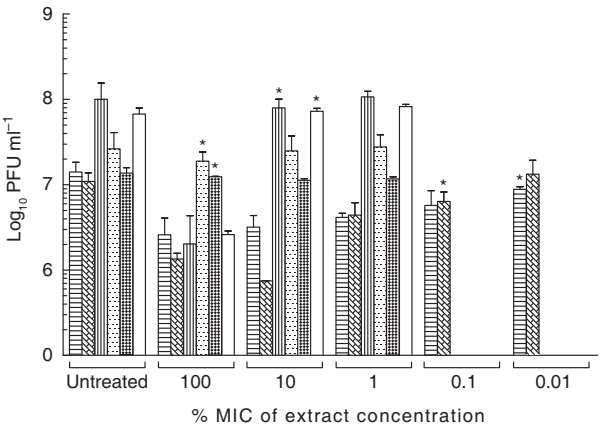

Figure 1 Antiviral activity of plant extracts against bacteriophages SU16 and SU27 after 24 h exposure. (▤) SU16 + *Boesenbergia rotunda* rhizomes (BRE); (▨) SU16 + *Oroxylum indicum* fruits (OIE); (▥) SU16 + *Stephania suberosa* roots (SSE); (▧) SU27 + BRE; (▦) SU27 + OIE; (□) SU27 + SSE. Data are the mean of three replicates ±SD. The minimum inhibitory concentration (MIC) of extract concentrations were 16 mg ml^{-1} for BRE and OIE and 4 mg ml^{-1} for SSE against both bacterial host strains. *Indicates selected concentration that shows nonsignificant difference to the untreated control ($P > 0{\cdot}05$).

Table 1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of extracts (mg ml^{-1}) used in the current investigation to bacterial host strains

	Bacterial strain			
	ECOR16		ECOR27	
Compound/Extract	MIC	MBC	MIC	MBC
SSE	4 ± 0	16 ± 0	4 ± 0	16 ± 0
OIE	13·33 ± 4·62	42·67 ± 18·48	13·33 ± 4·62	42·67 ± 18·48
BRE	16 ± 0	32 ± 0	16 ± 0	32 ± 0

Data are the mean of three replicates ± SD. *Stephania suberosa* root (SSE), *Oroxylum indicum* fruit (OIE), and *Boesenbergia rotunda* rhizome (BRE) extracts.

Combined activity of bacteriophages and bulk plant extracts

vTwo bacteriophages (one *Myoviridae,* SU16 and one *Siphoviridae,* SU27) which infect *E. coli* were investigated for their antibacterial activity when combined with bulk plant extracts with known antimicrobial properties. The phages were only tested against the strains from which they were originally isolated, but have been previously shown to be active against several *E. coli* strains (Khan Mirzaei and Nilsson 2015). Overall, the combination of bulk plant extracts and bacteriophages exhibited no significant difference in antibacterial activity at 24 h when compared to the untreated control or the individual components ($P > 0{\cdot}05$; Figs 2 and 3). However, both phage only and phage with extract significantly reduced bacterial content in all cases for up to 6 h compared to the untreated and extract only controls ($P < 0{\cdot}05$; Figs 2 and 3), which suggests that the reduction was primarily driven by the phage component. No phage SU16 + extract-treated samples showed significant differences compared to the phage only samples ($P > 0{\cdot}05$; Fig. 2). In the case of phage SU27 and extract treated samples, a significant additive effect could be seen when BRE (up to 4 h) and OIE (up to 8 h) extracts were added (Fig. 3a,b). Interestingly, a noticeable shift in the bacterial growth could be observed in the BRE and OIE extracts only compared to control which may be responsible for this additive effect. In addition, a significant reduction ($P < 0{\cdot}05$) in phage titre for the OIE-treated sample was observed at 2 to 8 h (Fig. 3b).

The concentrations of extracts tested in the current investigation were chosen to be subinhibitory to bacterial growth and also of limited toxicity to phages. While this appears to be the case with phage SU16 and ECOR16 (Fig. 2), with modest and often statistically insignificant differences ($P > 0{\cdot}05$) in phage and BRE or OIE extract titre compared to nonextract containing controls (Fig. 2a,b), there are differences in susceptibilities when used in concentration with SU27, most notably for OIE and SSE phage titres.

The combination of phages with adjuvant components is a currently understudied topic, with great focus being placed on the combination of phages and conventional antibiotics (Chaudhry *et al.* 2017; Oechslin *et al.* 2017; Scanlan *et al.* 2017) and purified phage cocktails (Gundogdu *et al.* 2016; Regeimbal *et al.* 2016; Yen *et al.* 2017). However, in order to further develop phages as antimicrobial agents, it is necessary to investigate the impact of more complex formulations which include additional active and excipient components (Abedon 2017). The current proof of concept study has highlighted the variation which can be introduced by including such additional components, particularly when they are poorly characterized bulk compounds derived from natural products.

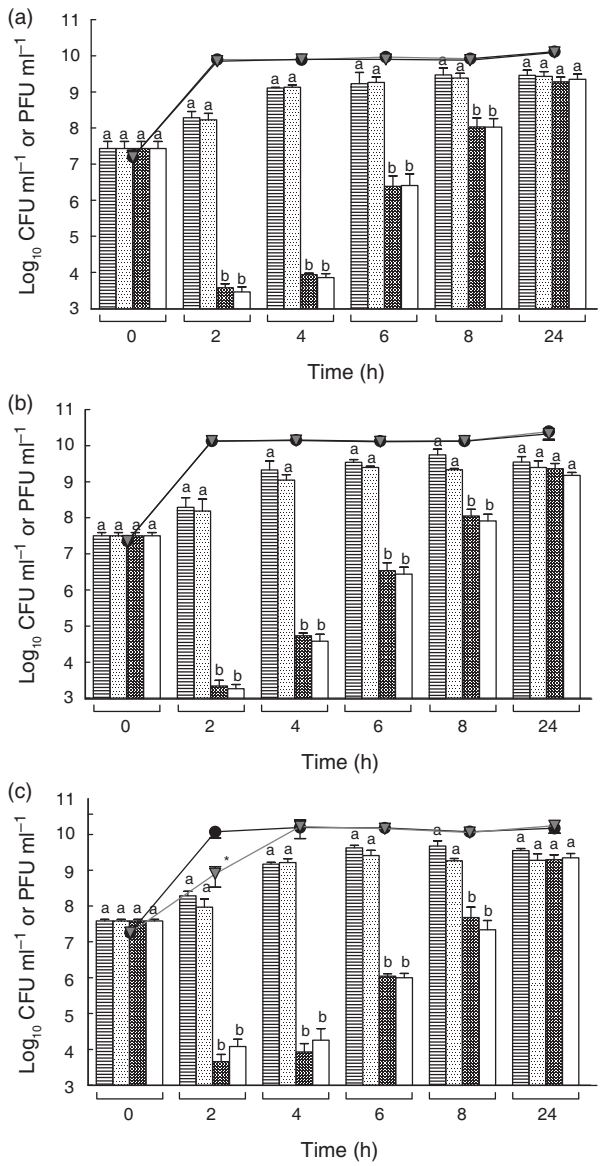

Figure 2 Activity of phage SU16 in combination with different plant extracts against ECOR16. (a) *Boesenbergia rotunda* rhizomes (BRE) (1·6 μg ml^{-1}); (b) *Oroxylum indicum* fruits (OIE) (16 μg ml^{-1}); (c) *Stephania suberosa* roots (SSE) (400 μg ml^{-1}). (▤) Untreated control; (▥) Extract Only; (▦) Phage Only; (□) Extract and phage. The mean ± SD for three replicates are illustrated. Means sharing the same superscript at the same time are not significantly different from each other (Tukey's HSD test, $P < 0{\cdot}05$). Lines indicate phage counts. (–●–) phage only; (···▽···) Extract and phage. *Indicates significant difference to the phage at the same time ($P < 0{\cdot}05$).

Materials and methods

All chemicals and reagents were obtained from either Sigma Aldrich (Stockholm, Sweden) or VWR (Stockholm, Sweden) and of general laboratory grade unless otherwise stated in the text.

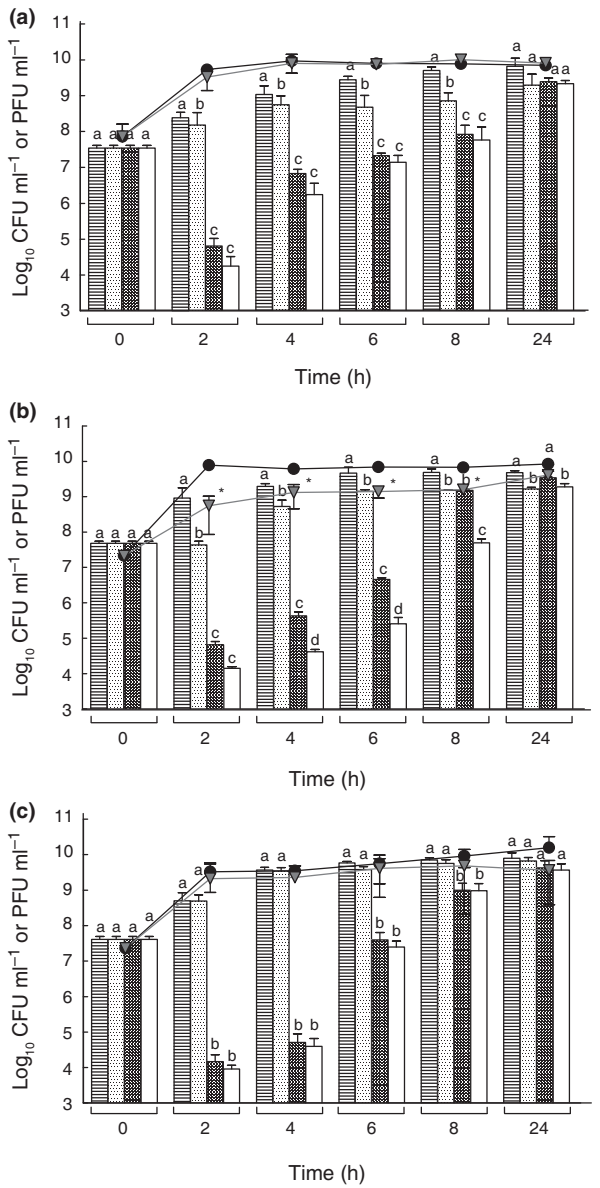

Figure 3 Activity of phage SU27 in combination with different plant extracts against ECOR27. (a) *Boesenbergia rotunda* rhizomes (BRE) (16 mg ml^{-1}); (b) *Oroxylum indicum* fruits (OIE) (16 mg ml^{-1}); (c) *Stephania suberosa* roots (SSE) (400 μg ml^{-1}). (▤) Untreated control; (▨) Extract Only; (▨) Phage Only; (□) Extract and phage. The mean ± SD for three replicates are illustrated. Means sharing the same superscript at the same time are not significantly different from each other (Tukey's HSD test, $P < 0{\cdot}05$). Lines indicate phage counts. (–●–) phage only; (–▽–) Extract and phage. *Indicates significant difference to the phage at the same time ($P < 0{\cdot}05$).

Plant extracts

Fresh samples of SSE, OIE and BRE were obtained locally from Nakhon Ratchasima province, Thailand. Samples were cut into small pieces, dried for 2 days at 40°C and ground into a coarse powder with a mechanical grinder. Crude antimicrobial mixtures were extracted from 500 g of powdered samples with soxhlation extraction in 2 l of ethanol for 8 h. Extracts were passed through Whatman number 1 filter paper to remove large debris and the solvent removed by rotatory evaporation under reduced pressure at 50°C. The remaining sample was lyophilized to obtain dried extracts. Extracts were stored at −20°C until required and suspended in PBS (for SSE) or PBS with 5% DMSO (for BRE and OIE).

Bacteriophage and bacterial strains

Escherichia coli strains ECOR16 and ECOR27 were obtained from the *E. coli* standard reference collection (ECOR; Ochman and Selander 1984). These strains are the hosts on which phages SU16 and SU27 were originally isolated. Working stocks were produced from −80°C glycerol stocks and maintained on lysogeny agar (LA) plates stored at 4°C. ECOR16 and ECOR27 were routinely cultured in lysogeny broth (LB) at 37°C overnight with shaking at 150 rev min^{-1}. Prior to use, suspensions were centrifuged at 3864***g*** for 15 min, the supernatant discarded and the pellet suspended in 10 ml phosphate buffered saline (PBS) and further diluted in PBS to contain 1–5 × 10^8 CFU per ml.

Previously characterized phages SU16 and SU27 (Khan Mirzaei and Nilsson 2015) were obtained from Stockholm University stocks stored at −80°C in glycerol. Phages were cultured in 10 ml lysogeny broth (LB) using the appropriate bacterial strain (100 μl inoculum of overnight culture) as the regular host at 37°C overnight with shaking. Following incubation, suspensions were centrifuged at 3864***g*** for 15 min at 4°C and passed through 0·45 and 0·2 μm syringe filters. Phage suspensions were enumerated, using the agar overlay method (Adams 1959) and stored at 4°C until required. Prior to use, phages were diluted in PBS to contain 1–3 × 10^8 PFU per ml.

MIC/MBC determination and activity of extracts against phage

The minimum inhibitory concentration and MBC of plant extracts were determined against ECOR16 and ECOR27 using a modified broth method (Clinical and Laboratory Standards Institute 2014). In brief, standardized bacterial suspensions were diluted 1 : 100 in double strength LB (dsLB) and the bacterial content determined by the drop count method (Miles *et al.* 1938). To triplicate wells of the first row of a 96-well microtitre plate, 200 μl of plant extract was added. A 100 μl aliquot was removed and serially diluted 1 : 2 in PBS over the

remainder of the plate. To each well, 100 μl of the diluted bacterial suspension was added and the plate incubated at 37°C overnight. The MIC value was determined to be the lowest concentration where no visible growth could be observed. MBC was determined by spreading 100 μl of suspension from wells showing no visible growth over the surface of pre-prepared LA plates which were then incubated at 37°C overnight. The MBC value was determined to be the lowest concentration where no visible growth could be observed.

The activity of plant extracts against phages SU16 and SU27 was assessed using a modified suspension test assay (Pinto *et al.* 2010). In brief, 1 ml of standardized phage suspension was added to 9 ml plant extract at different concentrations (100, 10, and 1% of MIC) and incubated for 24 h at 37°C). Extracts which maintained significant antiviral activity at 1% MIC were further diluted as necessary. After 24 h incubation, 100 μl aliquots were removed and added to 9·9 ml PBS. Suspensions were then serially diluted in PBS and the phage content determined, using the agar overlay method (Adams 1959).

Combined antibacterial activity of phage+extract combinations

Twenty-five milliliter of dsLB was added to 5 ml of extracts in PBS (or PBS with 5% (v/v) DMSO where appropriate), 5 ml of standardised phage suspension and 5 ml standardised bacterial suspension. The total volume was then increased with PBS to a final volume of 50 ml and flasks incubated at 37°C with shaking at 150 rpm min^{-1}. At defined time points (t = 2, 4, 6, 8 and 24 h) 1 ml aliquots were removed from each flask, and 1 ml of fresh LB was added to each flask to maintain volume. For phage containing samples, a 500 μl aliquot was added to 50 μl chloroform, vortexed and phage content determined, using the agar overlay method (Adams 1959). One hundred μl of the original sample was then serially diluted 1 : 10 in PBS and bacterial content determined by spreading 100 μl of each dilution over the surface of duplicate LA plates. For nonphage containing samples, the bacterial content was determined using the drop count method (Miles *et al.* 1938). Plates were incubated overnight at 37°C and the CFU per ml or PFU per ml content determined. Controls containing PBS/PBS+DMSO (untreated) or phage only (no extracts) were also performed.

Statistical analysis

All experiments were performed in triplicate unless stated in the text. Data were expressed as the mean ± standard deviation. Significant differences in the bacterial viable count among each of treated groups at the same time were analysed by one-way ANOVA followed by Tukey's HSD *post hoc* test. The $P < 0{\cdot}05$ was considered as the statistically significant difference. Also, the significant differences between each tested group and the control were determined, using independent T-tests at $P < 0{\cdot}05$.

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Conflict of Interest

No conflict of interest to declare.

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