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POLYPHENOL RICH SUGAR CANE EXTRACT INHIBITS BACTERIAL GROWTH

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ABSTRACT

Plants that are primarily used as a food source commonly have undervalued biological properties beyond the basic supply of nutrients. One important example of this are the antimicrobial properties of plants. Inclusion of natural and food grade antimicrobial ingredients in recipes to prevent food spoilage and disease transmission, or in cosmetic products to prevent transient and pathogenic bacteria would have world-wide public health implications. A patented natural polyphenol rich sugar cane extract (PRSE), is marketed as a high anti-oxidant and polyphenol ingredient, but its anti-microbial activity has not been reported previously. We determined the anti-bacterial properties of PRSE on common human pathogens relating to a range of diseases including food poisoning, tooth decay, acne and severe skin infections using disc/well diffusion experiments. Our findings indicate that PRSE is an efficient antimicrobial, which could be included at differing dosages to target a range of food borne and environmental pathogens.

Keywords: sugar cane extract, anti-bacteria, polyphenol, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Propionibacterium acnes, Escherichia coli, PRSE, phytolin

INTRODUCTION

It has been estimated by the World Health Organization that over 2 billion food borne diseases episodes world-wide are a result of unsafe food practices. As a safety measure, thermal processing or chemical processing is used to eliminate vegetative microorganisms, but this results in diminished nutritional properties [1, 2]. In addition, since the advent of antibiotics in the 1950s, the use of plant derivatives as anti-microbials has been abolished [3]. However, with the rise of antibiotic resistance, the search for new antimicrobial compounds compatible with food products has become a matter of urgency, and universal spending on finding new anti-infective agents is expected to increase dramatically over the next few years. Health practitioners and the public are becoming increasingly aware of problems with over prescription and misuse of traditional antibiotics for their use in the treatment and prevention of bacterial infections [3-5]. New antimicrobial sources, especially plant sources, are increasingly being investigated.

Polyphenols are health protective compounds rich in anti-oxidant activity found in foods and beverages that are of plant origin [6, 7], such as, fruits, vegetables, red wine, grains, chocolate, coffee and tea leaves [8-11]. An increase in some of these plant products in the diet may improve overall health and in recent years, much research has focussed on dietary polyphenols in preventing a range of degenerative and chronic diseases [12]. Indeed, polyphenols have the ability to aid in the treatment and prevention of cardiovascular diseases, cancers, inflammation, obesity, pain, diabetes, osteoporosis, as well as several neurodegenerative diseases [13]. There is evidence that Neanderthals living 60,000 years ago used plants such as hollyhock, a plant with anti-microbial and food preservation abilities which is still used in ethno-medicine around the world today [3, 14, 15]. A number of biochemical and epidemiological studies have shown that polyphenols of various foods and herbs are beneficial to human health, and some extracts of polyphenol rich foods, such as green tea, have anti-bacterial properties [10, 16, 17]. For example, green tea has been shown to prevent tooth decay by exerting its anti-bacterial effects on oral bacteria [18].

Sugar cane (Saccharum officinarum L.) is a rich source of polyphenols with high anti-oxidant activity and a natural anti-microbial agent [19]. It was shown that sugar cane bagasse extract possessed anti-microbial and bacterostatic activity against Salmonella typhimurium, Staphylococcus aureus, Listeria monocytogenes and Escherichia coli causing damaged cellular proteins and bacterial cell wall damage [19]. Furthermore, ethanol, chloroform and ethyl acetate extract of the leaves of sugar cane has been shown to have anti-microbial activity against Escherichia coli, Salmonella typhi and Pseudomonas aeruginosa [20]. Moreover, lignins (complex organic polymers important for tissue support of plants), isolated from sugar cane have also been shown to have anti-oxidant and anti-bacterial properties against Bacillus aryabhattai and Klebsiella sp [21].

Sugar cane is of great economic importance for food production and processing, including that of food preservation, ethanol and sugar production including syrup, juices and molasses [19]. PRSE, a natural plant extract of sugar cane and a by-product of sugar processing, is high in polyphenols and in general provides health benefits in reducing obesity, aiding in diabetes and controlling blood glucose levels [22] and decreasing the glycemic index of high carbohydrate foods [23]. However, the anti-microbial properties of PRSE on common pathogenic bacteria affecting humans have not been reported. Herein, we present the anti-microbial activity of PRSE against 5 bacterial strains (Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Escherichia coli and Propionibacterium acnes). Escherichia coli is a gram-negative anaerobic bacteria mostly found in the intestine. Most strains of Escherichia coli are harmless and constitute part of the normal gut flora and are involved in vitamin K synthesis [24]. However, some Escherichia coli serotypes are often responsible for food poisoning and disease (i.e. gastroenteritis, hemorrhagic colitis, Crohn's disease, travellers' diarrhea, urinary tract infections, sepsis, pneumonia) [25]. Antibiotics are used to treat Escherichia coli infections and to decrease the course of illness, though, the rate of bacterial resistance to commonly used antibiotics is increasing and antibiotics are more commonly not recommended [26]. Staphylococcus epidermidis is a gram-positive anaerobic bacterium part of the normal skin flora which is non-pathogenic although those with compromised immune systems are at risk of developing infection [27]. In addition, Staphylococcus epidermidis is commonly hospital acquired infection, in addition to drug users and those patients needing catheters and prosthetic heat valves; hand washing has been used in hospitals to reduce Staphylococcus epidermidis contamination and spread. Due to it being part of the normal skin flora, Staphylococcus epidermidis has developed resistance to commonly used antibiotics [28]. Of interest, Staphylococcus epidermidis is often present in affected acne vulgaris pores together with Propionibacterium acnes [29]. Propionibacterium acnes, also a gram-positive anaerobic bacterium is closely linked to acne, blepharitis and atopic dermatitis [30], and is susceptible to a vast number of antibiotics, natural anti-microbials (i.e. tea tree oil, citrus oil, honey) and over the counter anti-bacterial chemicals. However, in recent years there has been an emergence of antibiotic resistant Propionibacterium acnes strains which has resulted in a problem worldwide [31]. Likewise, Staphylococcus aureus (gram-positive bacterium) part of the normal flora of the skin, nose and respiratory tract, is not commonly pathogenic but can cause minor to severe life threatening infections [32]. With the emergence of antibiotic resistant Staphylococcus aureus strains this has become a problem worldwide and accounts up to 50,000 deaths each year in the USA alone [33]. Finally, Streptococcus mutans (gram positive anaerobic bacterium) mostly found in the oral cavity plays a crucial role in tooth decay, oral diseases and certain cardiovascular diseases [34]. With the increased use of fluoride and anti-bacterial

reagents such as triclosan and chlorhexidine containing toothpastes and oral rinses in recent years in order to decrease Streptococcus mutans growth, this has resulted in Streptococcus mutans resistant strains [35]. We therefore, determined the anti-bacterial properties of PRSE, on common human pathogens using disc/well diffusion experiments. Our findings can be extended to a range of other human pathogens as well as in the veterinary sector and could lead to a translational outcome in that PRSE could be ingested, included in skin ointments and/or in soap products.

MATERIAL AND METHODS

Bacterial strains and materials

Bacterial strains, Staphylococcus aureus ATC® 25923, Staphylococcus epidermidis ATC® 14990, Streptococcus mutans ATC® 25175, and Escherichia coli ATC® 25922 were purchased from In vitro technologies (Noble Park, VIC Australia). Propionibacterium acnes ATCC® 6919 was ordered from Thermo Scientific™ Culti-Loops™ (Waltham, MA USA). Tryptone soya agar with 5% horse blood plates, and, tryptone soya broth were purchased from Thermo Scientific™. Petri dishes (90x15mm), loop inoculating PS blue, anaerobic jars (2.5 Litre) and anaerobic Gen Sachets were purchased from Thermo Scientific™. Penicillin-streptomycin was obtained from Sigma Aldrich (Castle Hill, NSW Australia). PRSE, isolated and purified from sugar cane as previously described [36] and was provided by The Product Makers (Australia) Pty Ltd (Keysborough, VIC Australia).

Culturing and growth conditions

All strains used were in freeze dried form and transferred into enrichment sterilized tryptone soya broth (TSB) media. The cultures were propagated 3 times to optimize growth at 37 °C and at different incubation times, depending on the strain type; Escherichia coli was incubated for 24 hours (h), Staphylococcus aureus, Staphylococcus epidermidis and Streptococcus mutans were incubated for 48 h, and Propionibacterium acnes for 72 h. Propionibacterium acnes (loop form) was ready to culture directly into blood agar. There were no differences in the preparation of agar plates for disc and well diffusion assays. Briefly, 100 µL of a bacterial suspension was added into 6 mm wells/discs of tryptone soya agar plates with 5% horse blood (TSAB).

PRSE and control solutions

The raw material, sugar cane (Saccharum officinarum), is diluted in water and phytochemicals (PRSE) are extracted using 75 % w/v ethanol. The concentrated supernatant is freeze dried and PRSE powder was used [provided by The Product Makers (Australia) Pty Ltd (Keysborough, VIC Australia]. Briefly, deionized water is added to sugarcane mill molasses with constant stirring until the final Brix is 20. To a beaker containing 1 liter of 20 Brix feedstock under room temperature (20-25 degrees C), 500 g of wet weight pre-treated ion exchange resin and polymeric adsorbents (Dow Chemical, Australia) is added with gentle stirring to ensure effective mixing of the resin particles with the feedstock. The mixture is then filtered under vacuum, and the resin particles are collected and washed by resuspension in 1 liter of deionized water twice. The final washed resins are then suspended in 1 liter of 70% ethanol, stirred for 10 mins, and the filtrate collected by vacuum filtration. This suspension/filtration process was repeated twice more with 1 liter of 70% ethanol with each filtrate collected. Finally, the 3 batches of 70% ethanol filtrates were combined, and the ethanol removed under vacuum. The aqueous extract or spray-dried into a free-flowing brown powder (PRSE, Sugar Cane Extract) with a final moisture level of 2-4% w/w. PRSE consists of polyphenols 200 mg/g as gallic acid equivalents.

Stock solutions of PRSE were prepared as previously described [9] with small modifications. Briefly, PRSE powder was dissolved in sterilized water and filtered through advantec cellulose acetate filters (0.2 mm pore size; 25 mm diameter). The range of concentrations of PRSE solution was serially diluted in 14 stages (0.1, 0.5, 0.7, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mg/ml). The negative control used was sterilized water and the positive control was penicillin-streptomycin (consisting of 100 units/ml penicillin G sodium salt and 10 mg/ml streptomycin sulfate).

Antimicrobial activity of the PRSE extract

Antibacterial tests were carried out using the Oxford cup assay with minor modifications [37]. After autoclaving, TSB media was cooled to 50 °C. Susceptibility of the test organism to the extract was determined by employing the standard disk or well diffusion technique. Wells 6 mm in diameter were made in TSAB plates. The bacterial suspension in TSB was spread and plated on blood agar media. For the disk diffusion test, sterile paper discs (6 mm) were added of the test sample (100 µl) and placed

onto the inoculated blood agar surface. After cultivation at 37 °C for (24, 48 or 72 h) under anaerobic conditions in an anaerobic jar containing AnaeroGen sachets to maintain anaerobic conditions, the resulting inhibition zone diameters were measured. Using the well (6 mm) diffusion technique, 100 µl/well of the sample was added and the size of the inhibition zone of growth was measured after 24 h or 48 h at 37 °C. All experiments were repeated for each bacterial strain at least 3 times.

Statistical analysis

Analysis of data (one-way ANOVA) was used to determine any significant differences ($p < 0.05$) in the diameter of the inhibition zones, using Minitab 17 software [38]. All data are expressed as the mean of triplicate ± standard deviation.

RESULTS

PRSE inhibits growth of Escherichia coli

Growth of Escherichia coli was evaluated in TSAB plates containing a series of wells/discs loaded with a range of concentrations of PRSE at 37 °C following 24 h incubation period. The diameters of the inhibition zone around the wells/discs increased gradually with increasing concentrations of PRSE, starting at 0.7 mg/ml and up to 10 mg/ml ($p < 0.05$, compared to negative control). The lowest growth inhibition zone was 1.5 mm with at 0.7 mg/ml concentration of PRSE whilst the highest inhibition zone was 10.6 mm at concentrations 9 mg/ml and 10 mg/ml (Fig. 1A, Table 1). The inhibition zone of the positive control penicillin-streptomycin reached 35 mm and the negative control showed no inhibition zone.

PRSE inhibits growth of Staphylococcus epidermidis

Staphylococcus epidermidis was grown on TSAB plates containing a series of wells loaded with a fixed amount of PPRSE (100 µl/well or disc) with increasing concentrations of PRSE from 0.1 - 10 mg/ml. After 48 h of incubation at 37 °C, the diameters of the growth inhibition zone were measured. In general, the inhibition zone diameters gradually increased from 5 mm to 10.4 mm between 2 - 5 mg/ml ($p < 0.05$) after which there was a plateau from 5 – 10 mg/ml PRSE concentration (Fig. 1B, Table 1). The inhibition zone of the positive control penicillin-streptomycin reached 30 mm and the negative control and doses of PRSE less than 2 mg/ml showed no inhibition zones.

PRSE inhibits growth of Staphylococcus aureus

Staphylococcus aureus was cultured for 48 h on TSAB plates at 37 °C with a series of wells/discs containing different concentrations of PRSE. Antibacterial activity at varying concentrations are shown in Fig. 1C and Table 1. The diameters of bacterial growth inhibition zones around the wells/discs were clearly inhibited between 2 - 10 mg/ml of PRSE, with weak and unclear inhibition between 0.5 - 1 mg/ml concentrations of PRSE. There were no significant differences in zone inhibition between concentration 2 - 8 mg/ml but were significant compared to 0.5 - 1 mg/ml concentrations ($p < 0.05$). The highest diameters of Staphylococcus aureus growth inhibition zones were at concentration 9 - 10 mg/ml of approximately 10.8 mm which was significantly higher than that at < 8 mg/ml ($p < 0.05$) (Fig. 1C, Table 1). The inhibition zone of the positive control penicillin-streptomycin reached 35 mm and the negative control and doses of PRSE less than 0.5 mg/ml showed no inhibition zones.

PRSE inhibits growth of Streptococcus mutans

Streptococcus mutans was added to wells in TSAB plates at 37 °C for 48 h. When inhibition zones were examined in relation to a range of concentrations of PRSE, no Streptococcus mutans bacterial inhibition zones were observed at concentrations less than 6 mg/ml. However, at concentrations between 7 - 10 mg/ml of PRSE, significant inhibition zones were noted ($p < 0.05$) with concentrations 9 - 10 mg/ml being most significant of about 15 mm (Fig. 1D, Table 1). In comparison to positive control penicillin-streptomycin the bacterial inhibition zone was 30 mm and the negative control and doses of PRSE less than 7 mg/ml showed no inhibition zones.

PRSE inhibits growth of Propionibacterium acnes

The anti-bacterial growth of Propionibacterium acnes in the presence of different concentrations of PRSE were examined at 37 °C and 72 h of incubation. Zones of inhibition showed that concentrations of PRSE had good growth inhibition activity (17 - 18 mm) only at concentrations 9 - 10 mg/

ml (Figure 1E, Table 1). The inhibition zone of the positive control penicillin-streptomycin was in the order of 30 mm and the negative control and doses of PRSE less than 9 mg/ml showed no inhibition zones.

Table 1. *Minimum Inhibition Concentrations (MIC) of PRSE against 5 bacterial species*

	S. aureus	*S. epidermidis*	*S. mutans*	*P. acnes*	*E.coli*
Minimum Inhibition Concentration (MIC)	0.5 mg/ml	2 mg/ml	7 mg/ml	9 mg/ml	0.7 mg/ml

E, Escherichia; P, Propionibacterium; S, Staphylococcus

DISCUSSION

Electrodermal response (EDR) is a complex reactNew alternative and effective anti-bacterial agents are required for common bacterial infections linked to acne, oral disease, skin infections and other general infections. As such, resveratrol, honey, pomegranate, garlic, essential oils and cinnamon have been shown to pose anti-bacterial properties [39-45]. In addition, plant based products with high polyphenol content have been shown to prevent the growth of some common bacteria. For example, polyphenols extracted from green tea are effective in preventing the growth of Escherichia coli [46] by intercalating into the phospholipid bilayer making them more susceptible to the antibacterial agents [47].

PRSE, a natural extract from sugar cane is a rich source of polyphenols and is a safe, potential alternative of an anti-bacterial agent. Indeed, we demonstrate herein that PRSE to varying degrees, was able to inhibit the growth of Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans and Propionibacterium acnes using the disc/well diffusion method. PRSE was most potent inhibiting the growth of Escherichia coli compared to the others. Previously it was shown that ethanol, chloroform and ethyl acetate extracts of leaves of sugar cane to have anti-microbial activity against Escherichia coli and Staphylococcus aureus [20], at concentrations of 1 mg/6µl (166 mg/ml) which is significantly higher than that noted with PRSE (inhibition as low as 0.7 mg/ml and 2 mg/ml respectively with the highest inhibition zone at concentrations 9-10 mg/ml). In addition, sugar cane bagasse extract high in polyphenol had bacteriostatic activity against Staphylococcus aureus, Listeria monocytogenes, Eschericia coli and Salmonella typhimurium by disrupting the cell wall morphology and internal structure [19]. The anti-bacterial activity of PRSE to Escherichia coli (0.7 mg/ml) and Staphylococcus aureus (2 mg/ml) compared to sugar cane bagasse against Escherichia coli (2.5 mg/ml) and Staphylococcus aureus (0.625 mg/ml) suggests that PRSE is more potent than sugar cane bagasse against Escherichia coli but requires higher concentration for Staphylococcus aureus. In addition, pure phenolic compounds isolated from sugar cane have anti-bacterial activity against Streptococcus mutans (> 4 mg/ml) and Streptococcus sobrinus (4 mg/ml). Furthermore, maple leaf extract was shown to inhibit the growth of 24 standard bacterial strains with minimal inhibitory concentration of 0.3-8 mg/ml [48], which is in the similar range of the anti-bacterial effects of PRSE for all strains tested.

CONCLUSION

Our data suggests that PRSE has the potential as a natural bioactive material for antimicrobial applications in the food and beverage industry. PRSE could have a role as a replacement or adjunct for harsh chemical and thermal treatments that decrease food quality and nutritional density, or for medical antibiotics which accelerate the process of microbial resistance. PRSEs are a cost-effective product to manufacture, and are derived from an abundant resource in sugar cane, giving the potential for wide-spread and rapid utilisation. Future studies should evaluate the compatibility of PRSE treatment with food items that are common vectors for bacterial contamination.

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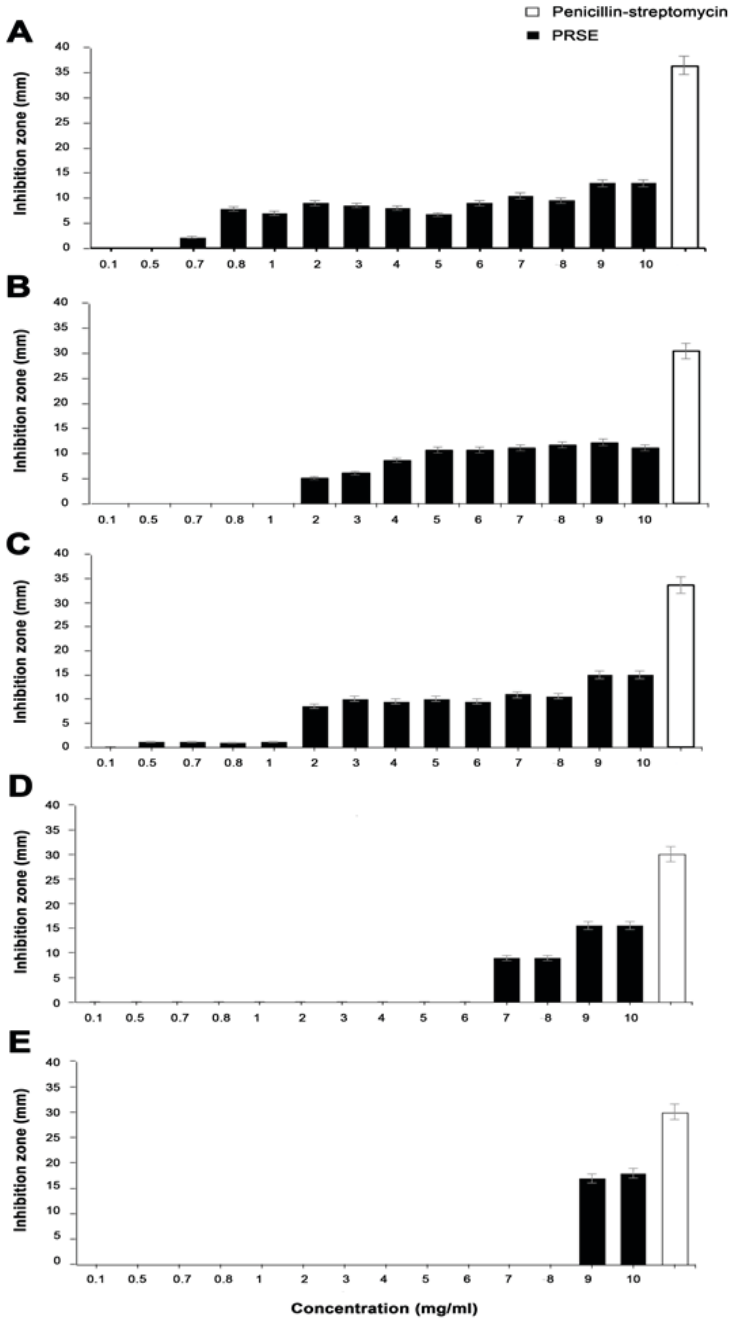

Figure 1. *Anti-bacterial efficacy testing of different concentration (0.1 - 10 mg/ml) of PRSE (black bars) against the growth of (A) Escherichia coli, (B) Staphylococcus epidermidis, (C) Staphylococcus aureus, (D) Staphylococcus mutans and (E) Propionibacterium acnes, in blood agar following 24-72 h incubation at 37°C. Positive control penicillin-streptomycin is shown as white bars. Error bars represent standard deviation of the means, using well and disc methods.*

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