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The Antibacterial Effect of Spices against *Streptococcus Pyogenes* and *Klebsiellapneumoniae*

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Abstract:
In order to subvert the resistance being developed by microbes to popular antibiotics new sources of antibacterial agents of plant origins are considered. Ethanol extracts of 10 different spices Sesamum indicum (sesame),Papaver somniferum (poppy), Foeniculum vulgare (fennel), Trigonella foenumgraecum(fenugreek), Coriandrum sativum (coriander), Cuminum cyminum (cumin), Piper nigrum (black pepper), Cinnamomum zeylanicum (cinnamon), Syzygium aromaticum (cloves) and Illicium verum (star anise) were tested using the well diffusion method for the antibacterial effect against a gram-positive bacterium Streptococcus pyogenes anda gram-negative bacterium Klebsiella pneumoniae.
The antibacterial effect of spices was analyzed by measuring the zone of inhibition which indicates the effectiveness of the spices against the bacteria. Positive controls (erythromycin and ciprofloxacin) were used to compare the effect of the spice extracts. Sesame, poppy and fenugreek showed no antibacterial effect against both the bacteria whereas the other spices have antibacterial effects.
The most effective spices in inhibiting the growth of S. pyogenes were cinnamon and cloves which showed a low Minimum Inhibitory Concentration (MIC) of 5% which was 0.045 gmL^{-1} and 0.049 gmL^{-1} respectively. The growth of K. pneumoniae was effectively inhibited by cinnamon and star anise. The MIC for both of the spices was 40%, with a conc. 0.360 g mL^{-1}

1. Introduction

The bactericidal drugs are able to induce cell death whereas bacteriostatic drugs inhibit the cell growth (Kohanski *et al.*, 2010). The first antibiotic principle ever discovered was penicillin during the year 1928 by Fleming. He found that *Staphylococcus aureus* was sensitive to penicillin. Antibiotics hitherto discovered were classified in to several groups based on their antimicrobial spectra (Saga and Yamaguchi, 2009). Antibiotics are able to kill the bacteria or inhibit their growth by inhibiting the DNA replication or suppressing the RNA synthesis, or suppressing the synthesis of cell wall or interfering with the protein synthesis.

Prolonged administration of antibiotics induces the development of resistance mechanisms in bacteria. Seriously enough, many infectious pathogens: *S. aureus* (MRSA), *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* are reported to develop inherent resistance to antibiotics. Antibiotic resistance is caused either by genetic or biochemicalactions. It may also be due to the mutation caused by errors of replication or incorrect repair of damaged DNA (Giedraitiene *et al.*, 2011).

β-lactamase, aminoglycoside-modifying enzymes and chloramphenicol acetyltransferase plays an important role in inactivating the function of antibiotics. β-lactamase cleaves the β-lactam ring of the antibiotic penicillin and cephalosporin in which the β- lactams with ester and amide bonds are hydrolyzed (Giedraitiene *et al.*, 2011).

Streptococcus pyogenes is a gram-positive coccus extracellular bacterium which is often referred to as β-hemolytic Group A streptococci (GAS) (Bessen, 2009). Upper respiratory infections caused by *S.pyogenes* is usually treated with β-lactam drugs such as penicillin. However, due to genetic changes and coexistence of oropharyngeal β-lactamase producing bacteria, penicillin failed to eradicate *S. pyogenes* (Passali *et al.*, 2007). Due to the development of resistance to penicillin, macrolides were introduced to control *S. pyogenes* infections. However, *S. pyogenes* slowly expressed resistance to macrolides by modifying the post-transcriptional target site and macrolide efflux (Jalava *et al.*, 2004).

K. pneumoniae is an encapsulated anaerobic, non-motile, lactose-fermenting gram-negative bacillus which does not react with ornithine decarboxylase (Brisse *et al.*, 2006). Prolonged administration of antibiotics in chronic patients led to the development of multidrug-resistance to *K. pneumoniae*, which naturally produce extended-spectrum-beta-lactamases (ESBLs). ESBLs hydrolyses oxyimino-β lactam antibiotics such as third generation cephalosporins and aztreonam.

The resistant genes are carried by the plasmids to other antibiotics such as aminoglycosides, chloramphenicol and tetracyclines, resulting in the evolution of multidrug-resistant bacteria (Gupta *et al.*, 2003). In order to overcome this issue, new antimicrobial agents are to be identified in controlling the pathogens.

Spices are natural products derived from different parts of plants that are commonly used in food preparations. The essential organic oils and oleoresins present in the spices offer flavor and aroma to the cooked food. The essential oils serve as natural preservatives in foods (Ceylan and Fung, 2004).

Other than the above reasons spices are also considered as a natural antimicrobial agent. The use of spices as antimicrobial agents was first reported in 1880s wherein spores of anthrax bacilli were reported to be controlled by cinnamon oil (Tajkarimi *et al.*, 2010). It is pertinent to observe that early in the Pyramid Age of Egypt (2600 BC), laborers involved in the construction of pyramid were fed with onions as a medicinal herb to preserve their health (Ceylan and Fung, 2004).

Therefore, in this work a fair evaluation was made to assess the antimicrobial impact of ten spices (which are being popularly used in cooking) against two microbial species.

2. Materials & Methods

Ethanol extracts of 10 different spices (Table 1) were tested using well diffusion method to evaluate the antibacterial effect of them against the gram-positive *S. pyogenes* (ATCC 19615) *and* gram-negative *K. pneumoniae* (ATCC 13883). The bacterial cultures were obtained from Institut Jantung Negara, Malaysia. They were purified using selective media. *S. pyogenes* and K. *pneumoniae* were cultured in blood agar and MacConkey agar media respectively.

The colonies which appeared in the petri plates were then stained with Gram's stain to confirm the identification of the bacteria. A few colonies were then taken out using a wire loop and cultured in nutrient broth. The broth was incubated at 37^oC for 24 h. Of the 10 spices tested, only those expressed positive antibacterial effects were further proceeded with MIC assay by well diffusion method.

The extract of the spices was prepared in ethanol using Soxhlet apparatus. The antibacterial effect of spices was evaluated by measuring the zone of inhibition. Positive controls (erythromycin and ciprofloxacin) were parallelly used to compare the effectiveness of the spice extracts.

The zone of inhibition produced by the extracts of each spice was measured in mm. The experiment was repeated 3 times for each spice and the results were calculated as mean ± standard deviation using Microsoft Excel 2007.

The 2-way analysis of variance (ANOVA) was carried out using GraphPad Prism 6 Demo for Windows. Difference was considered to be significant if p value is < 0.05. The botanical names of the spices and the plant- parts from which the antibacterial principles were extracted are given in Table 1.

Spice	Botanical name	Part used
Sesame	*Sesamum indicum*	Seed
Poppy	*Papaver somniferum*	Seed
Fennel	*Foeniculum vulgare*	Seed
Fenugreek	*Trigonella foenumgraecum*	Seed
Coriander	*Coriandrum sativum*	Seed
Cumin	*Cuminum cyminum*	Seed
Black pepper	*Piper nigrum*	Fruit
Cinnamon	*Cinnamomum zeylanicum*	Inner bark
Clove	*Syzygiwn aromaticum*	Flower bud
Star anise	*Illicium verum*	Fruit

Table 1: Botanical names and parts of each spice used in the assay

3. Results & Discussion

The positive control used in *S. pyogenes* is erythromycin which expressed an inhibitory zone of 30.0 mm, whereas in *K. pneumoniae*, ciprofloxacin was used and it resulted in an inhibitory zone of 36.0 mm. It is observed that the extracts of sesame, poppy and fenugreek had no antibacterial effect against both of the bacteria whereas the other spices had shown positive effects.

As seen in fig 1 and in the ANOVA results (Table 2), it is inferred that there is a significant difference in the effects of 10 different spices between the gram-positive and gram-negative bacteria. Fennel, coriander, black pepper, cinnamon and clove, indicated a relatively low $p$value < 0.05.

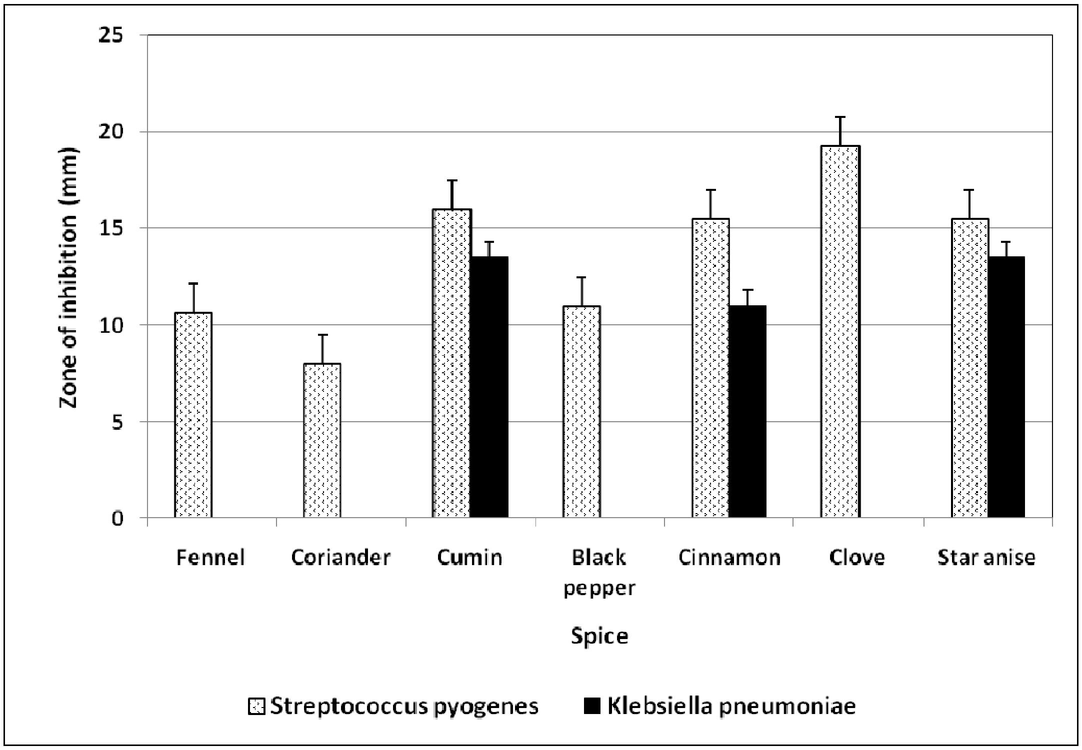

*Figure 1: The comparison of the effectiveness of 10 different spices at 100% concentration against the growth of Streptococcus pyogenes and Klebsiella pneumoniae.(*p-value ≤ 0.05; **p-value ≤ 0.01; ***p-value ≤ 0.001;****p-value ≤ 0.0001)*

Coriander, cumin, black pepper, cinnamon, clove and star anise showed low antibacterial effect against *K. pneumoniae*. This difference in action may be due to the variation in the structure of cell wall between both gram-negative and gram-positive bacteria.
The gram-negative bacterium is more resistant in its antibacterial effect of spices. The cell walls of the Gram-negative bacteria have an outer membrane rich in lipid-protein which has a unique concentration of gel-like matrix referred to as periplasmic space.
The hydrophilic surface of the outer membrane is rich in lip polysaccharides of low permeability since the pore space is narrow. These features control the diffusion rate thereby limiting the penetration rate of antibacterial molecules in to the bacterial cells (Nikaido, 1998).

Spice	Mean difference	t	Significance	P value	Summary
Sesame seed	0.0	0.0	No	>0.05	ns
Poppy seed	0.0	0.0	No	>0.05	ns
Fennel	-10.7	7.4	Yes	< 0.0001	****
Fenugreek	0.0	0.0	No	>0.05	ns
Coriander	8,0	5.6	Yes	< 0,0001	****
Cumin	3.0	2.1	No	>0.05	ns
Black pepper	11.0	7.7	Yes	< 0.0001	****
Cinnamon	4.7	3.3	Yes	<0.05	*
Clove	19.3	13.4	Yes	< 0.0001	****
Star anise	2.7	1.9	No	>0.05	ns

Table 2: ANOVA for the comparison of antibacterial effect of 10 different spices at 100% concentration against Gram-positive Streptococcus pyogenes and Gram negative Klebsiella pneumoniae.

It is to be known that gram-negative bacteria possess active efflux pumps which are capable of pumping the antibacterial principles including the commercial antibiotic, β-lactamase out of the cells *via* proton-motive force (Nikaido, 1998).

However, fennel shows a result contrary to the principle that Gram-negative bacteria are more resistant.
This may be due to the structural complexity of the cell walls of Gram-positive bacteria, causing difficulties for the antibacterial principles to penetrate the cell wall. In order to report the broad spectral effects of spices against Gram-positive and Gram-negative bacteria, more bacterial strains are needed to be tested so as to arrive at a clear benefit

Source of variation	% of total variation	P value		
Interaction	6.24	< 0.0001		
Concentration	3.59	< 0.0001		
Spice	86.94	< 0,0001		
Source of Variation	**P value summary**	**Significance**		
Interaction	***	Yes		
Concentration	***	Yes		
Spice	***	Yes		
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	36	550.9	15,30	5,346
Concentration	4	317.1	79.27	27.69
Spice	9	7680	853.4	298.2
Residual	100	286.2	2.862	

Table 3: Two-way ANOVA results on the comparison of antibacterial effect of spices at different concentration against the growth of Streptococcus pyogenes.

Based on the results obtained in this study, it is inferred that gram-negative bacteria are more resistant to antibacterial effect of spices. Based on the results obtained in this study, it is known that the most effective spices in inhibiting the growth of both bacteria are clove and cinnamon

Spice	Difference	t	P value	Summary
Sesame seed	0.0000	0.0000	P > 0.05	ns
Poppy seed	0.0000	0.0000	P > 0.05	ns
Fennel	10.70	44.45	P < 0.001	***
Fenugreek	0,0000	0.0000	P > 0.05	ns
Coriander	0.0000	0.0000	P > 0.05	ns
Cumin	12.70	52.76	P < 0.001	***
Black pepper	0.0000	0.0000	P > 0.05	ns
Cinnamon	11.00	45,70	P < 0.001	***
Clove	0.0000	0.0000	P > 0.05	ns
Star anise	13.00	54.01	P < 0.001	***

Table 4: ANOVA results (Bonferroni post-test) for the comparison of antibacterialeffect of spices (20% concentration vs. 100% concentration) against the growth of Klebsiella pneumoniae.

Spice	Difference	t	P value	Summary
Sesame seed	0.0000	0.0000	P>0.05	ns
Poppy seed	0.0000	0.0000	P>0.05	ns
Fennel	0.0000	0.0000	P>0.05	ns
Fenugreek	0.0000	0.0000	P>0.05	ns
Coriander	12.30	8.904	P<0.001	***
Cumin	3.300	2.389	P>0.05	ns
Black pepper	10.30	7.456	P<0.001	***
Cinnamon	0.3000	0.2172	P>0.05	ns
Clove	3.300	2.389	P>0.05	ns
Star anise	9.000	6.515	P<0.001	***

Table 5: ANOVA results (Bonferroni post-test) for the comparison of antibacterial effect of spices (20% concentration vs. 100% concentration) against the growth of Streptococcus pyogenes.

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