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Evaluation of Antimicrobial Activity of Some Spices on Fresh Fruit Juices

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ABSTRACT:

Microorganisms are the causative agent for infective diseases, food spoilage and food borne diseases. Drug resistance and use of chemical preservatives have currently reduced the safety of food products in food industry. Therefore, there is an urgent need for the discovery of antimicrobial agents that could overcome this shortage.

Spices have been used as food and flavouring agents since ancient times and traditionally, many spices are also been used to treat throat and stomach infections. However, many of these spices were experimentally proven to possess antimicrobial properties. Therefore, the current study is an attempt to determine the antimicrobial effect of three spices namely cloves, cinnamon and pepper on microorganisms isolated from freshly prepared fruit juices collected from the local market in the city of Hyderabad. A significant concentration dependent antimicrobial effect was observed by the methanol extract of cloves and cinnamon against gram positive and gram negative bacteria.

Keywords: cloves, cinnamon, pepper, antibacterial activity, fruit juices, cup-plate method.

INTRODUCTION:

Spices are grown in tropical climate with minimal good agricultural practices and are therefore subjected to harbour with various soil organisms and other possible pathogenic organisms due to insects, dust and faeces of rodents, animals and birds [1]. The microflora of the spices depends on factors such as nature of each spices, part of the plant collected, harvesting, drying, transport, processing and post-processing storage [1].

According to the ancient medical system of India called Ayurveda, spices were not only used as flavouring agents but also to preserve the food for long periods. In Ayurveda one of the most important uses of spices is to stimulate, increase appetite and maintain digestive strength. These spices are also beneficial for our health as they are rich in various nutrients, minerals and antioxidants [2]. They are also used to prevent diseases for example cardamom garlands are often worn in India even today. Ayurvedic medicines uses spices for weight loss, improve digestion, reduce or eliminate sugar, pain, swelling and inflammation. In light of this a lot of research is underway in further understanding the potential use of spices in treating

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various diseases [3]. Ancient Greeks, Romans and Chinese writing indicates that spices were frequently used for therapeutic purposes [4].

The US Food and Drug Administration (FDA) defined spices as "aromatic vegetable substances, in the whole, broken, or ground form, whose significant function in food is seasoning rather than nutrition. They are true to name and from them no portion of any volatile oil or other flavouring principle has been removed". The three spices used for the study are defined as below by FDA [5].

1. Cloves – These are dried, unopened flower buds of *Syzygium aromaticum*. The dried buds are dark reddish-brown in color, with a strong aromatic odor, and hot pungent taste. The principal active ingredient in the volatile oil is eugenol.
2. Cinnamon (Cassia) - The dried bark of *Cinnamomum zeylanicum*, is brown to reddish-brown in color. The principal active ingredient is cinnamaldehyde (volatile oil), is responsible for the characteristic odor.
3. Pepperblack– These are dried, immature berries of *Piper nigrum* L. The wrinkle berries are dark brown to black, and have a characteristic, penetrating odour, on grounding with hot, pungent taste. Piperine, is the bioactive compound responsible for the pungent taste.

Fifty years of use and misuse of antibiotics have led to the enrichment of ever evolving multidrug resistant microorganisms [6]. Research studies have shown that antibiotic resistant genes are present in man, animals, plants and these genes may be transferred to disease causing organisms by direct contact or indirectly by consuming contaminated food [7]. Therefore, there is a continuous search for new antimicrobial substances to control the crisis posed by drug resistant strains [8]. Many spices such as pepper, cinnamon, thyme, cumin, clove and oregano have been used to treat infectious diseases or preserve food and were experimentally proved to possess antimicrobial activities against pathogenic and spoilage fungi and bacteria [9,10,11]. The Table 1 is the scientific evidence collected towards the antimicrobial activity of the selected spices namely cinnamon, black pepper and clove for the experimental.

METHODOLOGY:

Spices Material:

The spices for the experimental were purchased from the local market, Hyderabad during the month of February, 2022.

Extraction:

The spices materials were pulverized into fine powder and were cold macerated with methanol (96%) solvent for three days. The collected methanol extracts were concentrated

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under vacuum (50^{o}C), dried and weighed. The dried methanol extract was then subjected for qualitative phytochemical analysis and antimicrobial activity.

Phytochemical Analysis:

1. Test for alkaloids: Three millilitre of each extract was evaporated to dryness and the residue was heated on a boiling water bath with 2N hydrochloric acid (5ml). After cooling the mixture was divided into 2 equal portions. One portion was treated with a few drops of Mayer's reagent and the other with equal amounts of Wagner's reagent [21]. The samples were then observed for the presence of turbidity or precipitate.

2. Test for flavonoids: Five millilitre of each extract was treated with a few drops of concentrated hydrochloric acid and magnesium turnings (0.5g). The presence of flavonoids was indicative if pink or magenta-red color developed within 3 minutes [22].

3. Test for tannins: Ten millilitre of each extract was evaporated and the residue was extracted by 10 millilitres of hot 0.9% sodium chloride solution, filtered and divided into 3 equal portions. Sodium chloride solution was added to one portion of the extract, 1% gelatin solution to a second portion and gelatin-salt reagent to the 3rd portion. Precipitation with the latter regent or with both the 2nd and 3rd reagents is indicative of the presence of tannins.

   Positive tests are confirmed by the addition of ferric chloride ($FeCl_3$) solution to the extract and should result in a characteristic blue, blue-black, green or blue-green color or precipitate [23].

4. Test for saponins: About 2.5g of the dried powdered sample was extracted with boiling water. After cooling, the extract was shaken vigorously to froth and was then allowed to stand for 15-20 minutes and classified for saponin contents as follows: no froth=negative; froth less than 1 cm= weakly positive; froth=1.2cm high= positive; and froth> 2cm high= strongly positive [24]

5. Test for cardiac glycosides: Five millilitre of each extract was treated with two millilitre of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with one millilitre of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout this layer [25].

6. Test for steroids: Dried powdered sample was extracted with chloroform. Two millilitre of acetic anhydride was added to 0.5 millilitre chloroform extract. Then one millilitre of concentrated sulphuric acid was added from the sides of the test tube. A

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reddish brown ring at the junction of two layers indicates positive test for steroids [25].

7. Test for triterpenoids: Dried powdered sample was extracted with chloroform. Two millilitre of acetic anhydride was added to 0.5 millilitre chloroform extract. Then one millilitre of concentrated sulphuric acid was added from the sides of the test tube. A red ring at the junction of two layers indicates positive test for triterpenoids [25].

8. Test for Carbohydrates: i) Molisch's test: To one millilitre of extract 0.4 millilitre of Molisch's reagent was added followed by addition of 1 millilitre of concentrated sulphuric acid along the side of the test tube. A purple colour indicates the presence of carbohydrates (starch). ii) Benedict's test: To one millilitre of methanol extract, 1 millilitre of Benedict's reagent was added and heated for 5 minutes. The presence of carbohydrates (disaccharides) was shown by the formation of an orange precipitate. iii) Fehling's Test: One millilitre of extract was boiled and filtered with two millilitre of purified water. To two millilitre of filtrate two millilitre of Fehling's reagent were added and heated. Reddish brown precipitate indicates the presence of carbohydrate (glucose) [25].

Enumeration and identification of the spices microflora:

a) Preparation of the samples

One gram of each type of spices were added to 10ml of saline solution and left it aside for 30min. A serial dilution was performed to obtain dilutions ranging from 10^{-1} to 10^{-7}.

b) Experiment

The viable count of the spices samples were enumerated by spread plating technique where, 0.1millilitre of the dilutions from 10^{-4} to 10^{-7} were plated onto sterile nutrient agar plate in triplicates. The plates were incubated at 37ºC for 24 hours in a bacteriological incubator. The obtained colonies were counted in a colony counter and calculated the number of colony forming units (CFU) per millilitre of the sample using the formula:

$$\text{No. of CFU/millilitre} = \frac{\text{Number of Colonies (average of triplicate) x Dilution factor}}{\text{Volume}}$$

Based on their colony characteristics the isolated colonies were sub-cultured on to selective media and further identified by biochemical tests [26].

Enumeration and identification of microorganisms from fruit juices:

Three freshly prepared fruit juices namely; apple, banana and pineapple are used for the experimental purchased from the local market, Hyderabad during the month of February, 2022.

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a. Preparation of the samples: One millilitre of each type of fruit juice were added to 10 millilitre of saline solution and left it aside for 10min. A serial dilution was performed to obtain dilutions ranging from 10^{-1} to 10^{-7}.

b. Experiment: Spread plating technique was performed where 0.1 millilitre of the dilutions from 10^{-4} to 10^{-7} were plated onto sterile nutrient agar plate in triplicates. The plates were incubated at 37ºC for 24 hours in a bacteriological incubator. The obtained colonies were counted in a colony counter and calculated the number of colony forming units (CFU) per millilitre of the sample using the formula:

$$\text{No. of CFU/millilitre} = \frac{\text{Number of Colonies (average of triplicate) x Dilution factor}}{\text{Volume}}$$

Based on their colony characteristics the isolated colonies were sub-cultured on to selective media and further identified by biochemical tests [26].

Antimicrobial activity:

The antimicrobial activity of the methanol extracts of clove, cinnamon and pepper was determined by agar well diffusion method against pure cultures procured from IMTECH, Chandigarh and the isolates from fresh fruit juices.

a. Preparation of Samples: All the extracts and the standard antibiotic were suspended in dimethylsulphoxide to yield the concentrations of 15, 30 and 60 µg/ml.

b. Test organisms: Pure cultures from IMTECH, Chandigarh-*Staphylococcus epidermidis, Streptococcus pneumonia, Bacillus cereus, Enterobacter aerogenes, Echerichia coli* and *Pseudomonas aerogenosa*. Isolates from fruit juices-*Staphylococcus aureus, Bacillus subtilis, Bacillus cereus*, *Enterobacter aerogenes* and *Enterococcus* sp.

c. Preparation of Inoculum: The test bacteria were inoculated into liquid media (nutrient broth) and incubated at 37 ^{0}C for 8-10 hr for bacteria. The suspensions were checked to provide approximately10^5 to 10^7 CFU/ml.

d. Experimental: Bacterial cultures each of 20µl was poured over the basal plates containing 25ml of nutrient agar in sterile 9 cm petri plates and spread using L-shaped glass rod. Each of the extracts with 15 and 30µl/ml concentrations was poured into the wells (4mm in size) bored with a sterile metal borer. Each extract was tested in triplicate. Nutrient agar plates were incubated at 37^{0}C for 24hr. Simultaneously, the positive (chloramphenicol) and the negative (dimethylsulphoxide) controls were also tested for the activity and the zones of inhibition were recorded by measuring the diameter of zone of inhibition by the following formula:

Zone of Inhibition (mm) = D-d

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Where,

D = diameter of zone of inhibition

d = diameter of the well (4mm)

RESULTS:

The various phytochemical components present in methanol extracts of clove, cinnamon and pepper are tabulated in Table-2.

The spices were found to be contaminated by microorganisms *Enterobacter aerogenes. Acinetobacter* sp. *Staphylococcus epidermidis, Bacillus cereus* and *Streptococcus pneumoniae* (Table 4). The isolates from clove namely CL1 and CL2 were identified as *Enterobacter aerogenes* and *Acinetobacter* sp.; the isolates from cinnamon namely CN1, CN2 and CN3 were identified as *Staphylococcus epidermidis, Bacillus cereus* and *Streptococcus pneumonia;* and the isolates from black pepper namely P1, P2 were identified as *Enterobacter aerogenes* and *Streptococcus pneumonia*. The contaminating microorganisms isolates coded as A1, A2, B1, B2, PA1 and PA2 from the fruit juices of apple, banana and pine apple are *Enterobacter aerogenes Bacillus subtilis, B.cereus, Staphylococcus aureus,* and *Enterococcus* sp. respectively. (Table 6)

The clove methanol extracts have exhibited concentration dependent inhibition against all the test organisms except *Staphylococcus aureus*. Similarly, cinnamon methanol extracts were effective against all the test organisms except *Streptococcus pneumoniae.* On the other hand, pepper methanol extracts were effective against only some test organisms such as *Staphylococcus epidermidis, Bacillus subtilis, Bacillus cereus* and *Enterococcus* sp. (Table 7 and Table 8).

DISCUSSION:

Amongst the three test methanol extracts clove and cinnamon were found to be more effective against all the test organisms except *Staphylococcus aureus* and *Streptococcus pneumoniae* respectively. Among the test organisms *Staphylococcus epidermidis, Bacillus subtilis, Entrococcus* sp. and *Enterobacter aerogenes* were sensitive to all the three extracts.

The effectiveness of the extracts was not due to one phytochemical compound but due to a combination of various compounds present in it [27]. The phytochemical components like steroids, terpenoids, alkaloids, tannins, flavonoids and glycosides are classified as compounds with antimicrobial properties [28]. The preliminary phytochemical screening has shown the presence of, triterpenes, saponins, alkaloids, tannins and glycosides. Therefore, the effectiveness of the extracts on the microorganisms really shows the presence of significant bioactive compounds with antimicrobial properties.

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CONCLUSIONS:

Antimicrobial activity of spices are of great interest due to the fact that they are Generally Recognized As Safe (GRAS), by Food and Drug Administration, US. Spices not only increase the taste and aroma of the food but also enhance the shelf life of the food. It is evident for the study that spices inhibit some types of microorganism contaminating spices and fruit juices as well. A combination of various spices in food can reliably reduce the use of chemical preservatives to a great extent. However, advanced evaluation methods for toxicity, microbial quality, and economic feasibility may give more insight in the usage and bioactive properties of spices.

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TABLES AND FIGURES:

Table 1: Review of literature on the antimicrobial activity of clove, cinnamon, and black pepper against several microorganisms.

Spices	Extract	Microorganisms
Clove	Essential oil	*E. coli*, *S. typhimurium*, *B. cereus*, *S. aureus*, and *L. monocytogenes* [12]
		Enterobacteriaceae, *S. aureus*, and *Pseudomonas* sp.,[13]
Cinnamon	Aqueous	*A.niger*, *Fusarium sambucinum*, *Pythium sulcatum* and *Rhizopus stolonifera* [14]
		S. aureus, *Lactobacillus* sp., *B. thermosphacta*, *Pseudomonas* spp., and *E. coli* [15]
	hydrosols	*S. aureus*, *E. coli*, *S. typhimurium*, *P. aeruginosa*(15% *v/v* hydrosol), and *C. albicans*(no inhibition) [16]
	diethyl ether	*S. aureus*, *K. pneumonia*e, *P. aeruginosa*, *E. coli*, *Enterococcus faecalis* (*E. faecalis*), *M. smegmatis*, *Micrococcus luteus*, and *C. albicans* [17]
Black pepper	Essential oil	*F. graminearum* [18]
	acetone	*Penicillium viridcatum* and *A. ochraceus* *S. aureus*, *B. cereus*, and *B. subtilis* [19]
	ethanol	*S.aureus*, *B. subtilus*, *E. coli* and *K. pneumonia* [20]

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Table 2: Preliminary Phytochemical screening of methanol extracts of Cloves, Cinnamon and Pepper

Photochemical	**Clove**	**Cinnamon**	**Pepper**
Alkaloids	+	-	+
Flavonoids	+	-	-
Tannins	+	-	+
Saponins	-	+	-
Cardiac Glycosides	-	+	+
Steroids	+	-	-
Triterpenoids	+	+	-
Carbohydrates	+	-	+

Table 3: Enumeration of microorganisms in Spices

Dilution	Average count of colonies (triplicates)		
	Clove	Cinnamon	Pepper
10^{-4}	TNTC	TNTC	TNTC
10^{-5}	190	198	195
10^{-6}	157	162	164
10^{-7}	56	69	70

*TNTC: too numerous to be counted

No. of CFU/g of clove extract = $190x10^5$ CFU/g; No. of CFU/g of cinnamon extract = $198x10^5$ CFU/g; No. of CFU/g of pepper extract = $195x10^5$ CFU/g

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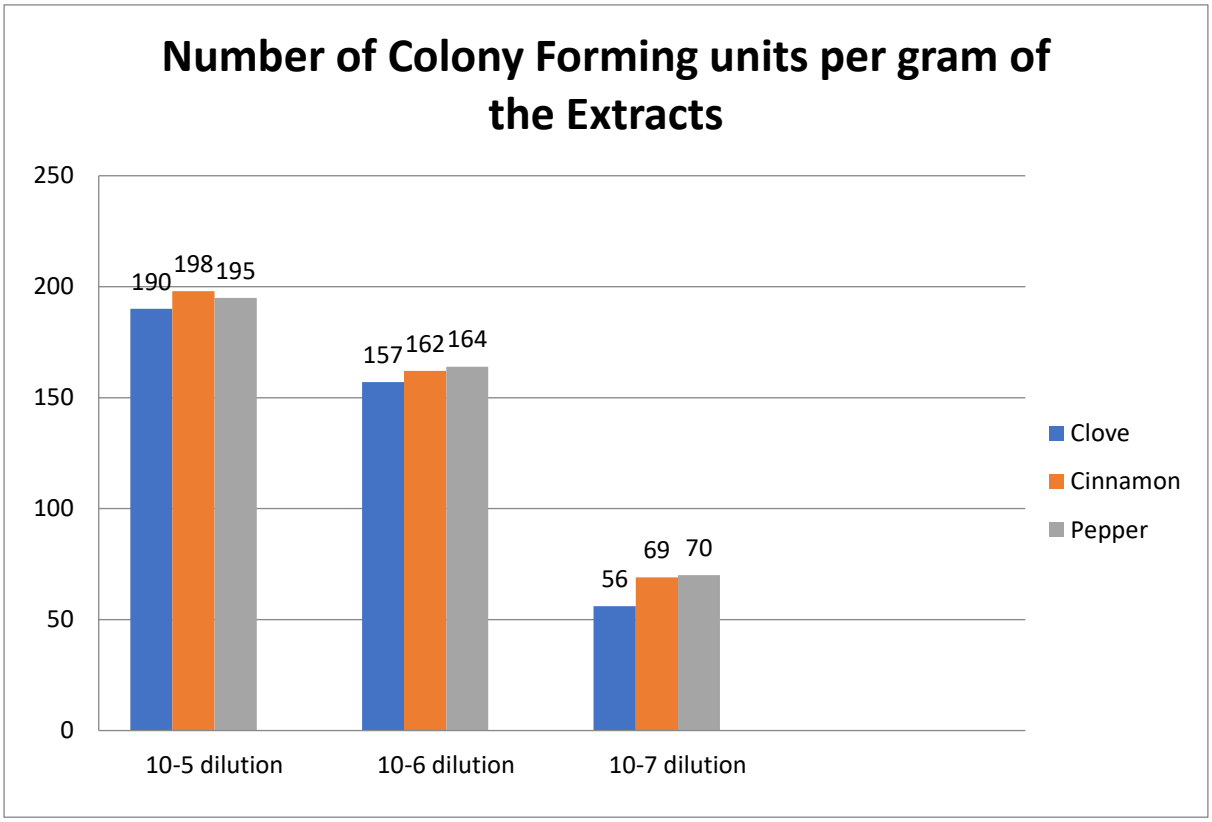

Graph 1: Graphical representation of number of bacteria present at dilutions ranging from 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} in the methanol extracts of cloves, cinnamon and pepper respectively.

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Table 4: Microorganisms isolated and identified from Spices

Spices	Isolates-Code	Grams staining	I	MR	VP	C	CT	O
Clove	CL1	-ve rods	-	-	+	+	+	-
	CL2	-ve coccibacilli	-			+	+	+
Cinnamon	CN1	+ve cocci	-	+	-	-	+	-
	CN2	+ve rods	-	-	+	-	-	+
	CN3	+ve cocci	-	+	-	-	-	-
Pepper	P1	-ve cocci	-	-	+	+	+	-
	P2	+ve cocci	-	+	-	-	-	-
Spices	Isolates-Code	MCA	MSA	EMB	SA	BA	Identification	
Clove	CL1	+	-	+	-	NA	*Enterobacter sp.*	
	CL2			-	-	NA	*Acinetobacter sp.*	
Cinnamon	CN1	-	*	-	-	NA	*Staphylococcus sp.*	
	CN2	-	-	-	+	NA	*Bacillus cereus*	
	CN3	-	-	-	-	β	*Streptococcus sp.*	
Pepper	P1	+	-	+	-	NA	*Enterobacter sp.*	
	P2	-	-	-	-	β	*Streptococcus sp.*	

I-Indole test, MR- methyl red test, VP-Voges Proskauer's test, C-Citrate test, CT-Catalase test, O-Oxidase test, MCA- MacConkey agar, MSA-Mannitol salt agar, EMB- Eosin Methylene Blue agar, SA-Starch agar, BA- Blood agar, "-" no growth, "+" growth or positive for the test, * growth without fermentation of mannitol, β- beta hydrolysis of blood agar.

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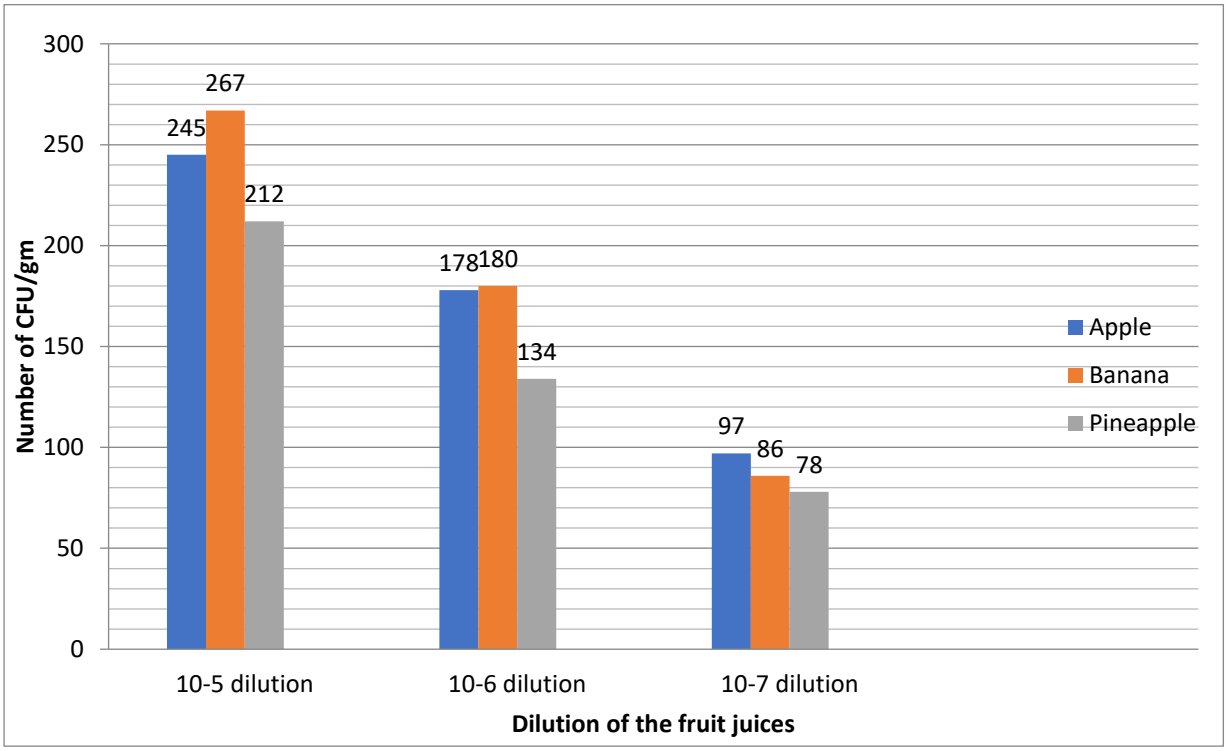
Table 5: Enumeration of microorganisms in Fresh Juice Samples

Dilution	Average count of colonies (triplicates)		
	Apple	Banana	Pineapple
10^{-4}	TNTC	TNTC	TNTC
10^{-5}	245	267	212
10^{-6}	178	180	134
10^{-7}	97	86	78

*TNTC: too numerous to be counted

No. of CFU/g of apple juice= 245 CFU/g; No. of CFU/g of banana juice= 267 CFU/g; No. of CFU/g of pineapple juice= 212 CFU/g



Graph 2: Graphical representation of number of bacteria present at dilutions 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} in the fresh fruit juices of apple, banana and pineapple respectively.

Table 6: Microorganisms isolated and identified from Fruit juices.

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Spices	Isolates-Code	Grams staining	I	MR	VP	C	CT	O
Apple	A1	-ve rods	-	-	+	+	+	-
	A2	+ve rods	-	-	+	-	-	+
Banana	B1	+ve rods	-	-	+	-	-	+
	B2	+ve rods	-	-	+	-	-	+
Pineapple	PA1	+ve cocci	-	+	-	-	+	-
	PA2	-ve cocci	-	-	+	+	+	-
Spices	Isolates-Code	MCA	MSA	EMB	SA	BA	Identification	
Apple	A1	+	-	+	-	NA	*Enterobacter aerogenes*	
	A2	-	+	-	+	NA	*Bacillus subtilis*	
Banana	B1	-	-	-	+	NA	*Bacillus cereus*	
	B2	-	+	-	+	NA	*Bacillus subtilis*	
Pineapple	PA1	-	+	-	-	NA	*Staphylococcus aureus*	
	PA2	+	-	+	-	NA	*Enterococcus sp.*	

I- Indole test, MR- methyl red test, VP-Voges Proskauer's test, C-Citrate test, CT-Catalase test, O-Oxidase test, MCA- MacConkey agar, MSA-Mannitol salt agar, EMB- Eosin Methylene Blue agar, SA-Starch agar, BA- Blood agar, "-" no growth, "+" growth or positive for the test, * growth without fermentation of mannitol, β- beta hydrolysis on blood agar.

Table 7: Antimicrobial activity of the methanol extracts of spices on Gram positive and Gram negative bacteria.

	STD	DMSO	Clove			Cinnamon			Pepper		
Concentration (µg/ml)	10	30	15	30	60	15	30	60	15	30	60
Microorganisms	Diameter of zone of inhibition (mm)										
Gram positive bacteria											
Staphylococcus epidermidis	14	0	5	8	16	4	9	17	4	9	16

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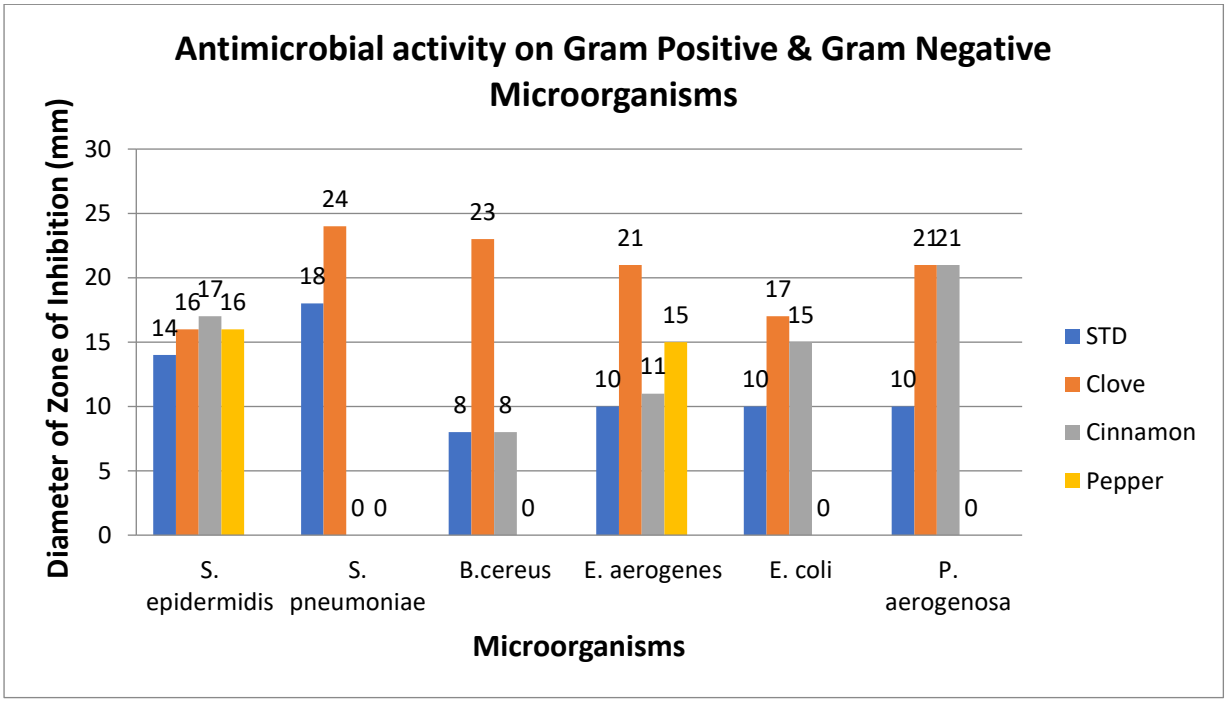
Streptococcus pneumoniae	18	0	6	12	24	0	0	0	0	0	0
Bacillus cereus	8	0	6	12	23	0	4	8	0	0	0
Gram negative bacteria											
Enterobacter aerogenes	10	0	5	11	21	0	5	11	4	9	15
Echerichia coli	10	0	4	8	17	4	8	15	0	0	0
Pseudomonas aerogenosa	10	0	6	12	21	5	10	21	0	0	0

STD: Chloramphenicol, positive control; DMSO: Dimethylsulfoxide, negative control



Graph 3: Graphical representation of the diameter of zone of inhibition obtained by the methanol extracts (at concentration 60 µg/ml) of clove, cinnamon and pepper against the test pure cultures.

Table 8: Antimicrobial activity of the methanol extracts of spices on isolates from fruit juices.

	STD	DMSO	Clove			Cinnamon			Pepper		
Concentration (µg/ml)	10	30	15	30	60	15	30	60	15	30	60
Microorganisms	Diameter of zone of inhibition (mm)										

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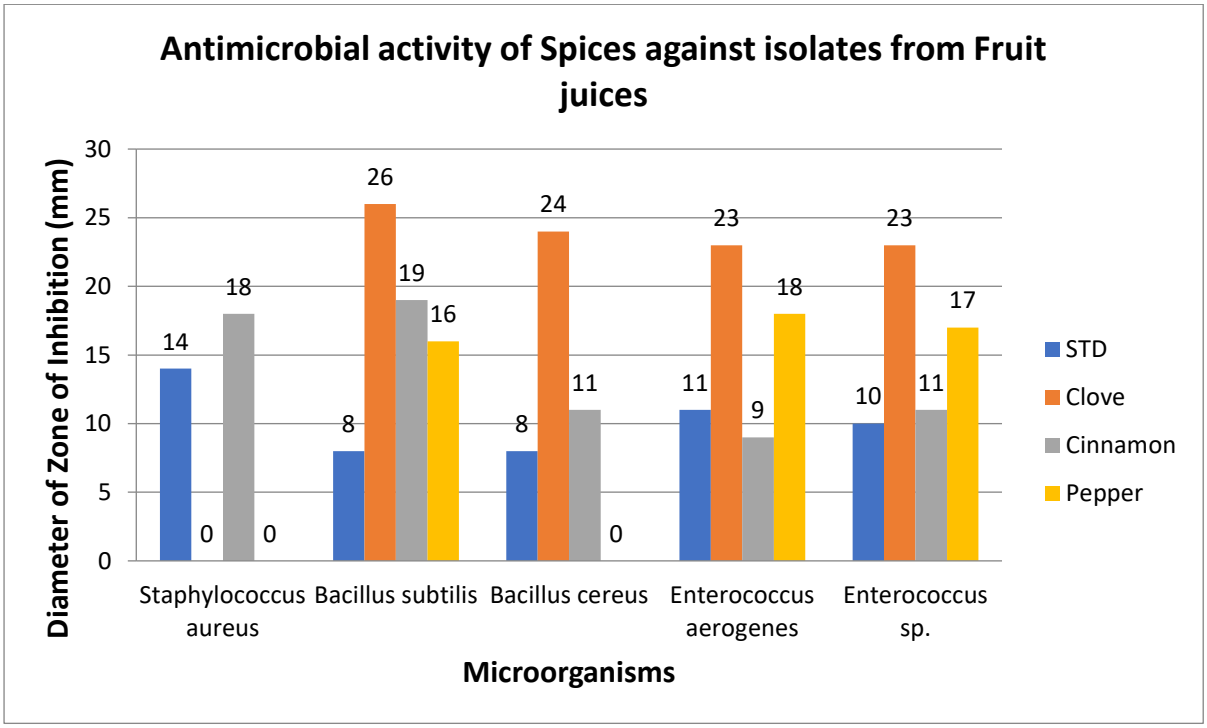
Gram positive bacteria											
Staphylococcus aureus	14	0	0	0	0	4	9	18	0	0	0
Bacillus subtilis	18	0	6	13	26	4	9	19	3	8	16
Bacillus cereus	8	0	6	12	24	0	5	11	-	-	0
Gram negative bacteria											
Enterobacter aerogenes	10	0	5	11	23	0	4	9	4	9	18
Enterococcus sp.	10	0	5	12	23	0	5	11	4	9	17

STD: Chloramphenicol, positive control; DMSO: Dimethysulfoxide, negative control



Graph 4: Graphical representation of the diameter of zone of inhibition obtained by the methanol extracts (at concentration 60 µg/ml) of clove, cinnamon and pepper against the microorganisms isolated from the fruit juices