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Phytochemical composition and antimicrobial activities of three selected spices seed extracts against food spoilage microorganisms

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Abstract. *Persaud C, Daniel R, Hemraj D. 2019. Phytochemical composition and antimicrobial activities of three selected spices seed extracts against food spoilage microorganisms. Bioteknologi 16: 41-47.* The study emphasizes on three selected spice extracts to be efficiently used as antimicrobial agents in food preservation, with the intention of developing antimicrobial agents capable of replacing synthetic chemical agents and natural agents weakened by antimicrobial resistance. The purpose of this study aims at stepping away from the artificial and exploring the extent to which natural substances, such as spices, provide antimicrobial effects in attempt to be formulated as natural food preservative. Antimicrobial screening of ethanolic and hexane extracts of *Cuminum cyminum* (cumin), *Sinapis alba* (mustard) and *Pimenta dioica* (all spice) against food spoilage bacteria *Bacillus subtilis* and *Pseudomonas aeruginosa* and fungi *Candida albicans* was attempted, with references to synthetics drugs Ciprofloxacin, Erythromycin and Fluconazole. Phytochemical analysis on the extracts showed that there were more secondary metabolites present in the ethanolic extracts. It was observed that ethanolic extracts of *C. cyminum* and *P. dioica* were more effective against the three microbes as compared to the hexane extracts of *C. cyminum* and *P. dioica*, while hexane extracts of *S. alba* was more effective against bacterial species. *S. alba* was not an effective antifungal agent. The results indicated that plant extracts possessing antibacterial and antifungal properties can be formulated as an ideal food preservative.

Keywords: All spice, antimicrobial activities, cumin, mustard, phytochemical analysis, spices

INTRODUCTION

Food spoilage and food poisoning is a complex naturally occurring process (Gram et al. 2002) and to understand how food spoil, the causes must first be assessed. The number one cause of food spoilage is because of microorganisms. In developing countries, food poisoning has been recorded as the one of the most common causes of illness and death; most cases are associated to Gram negative bacteria such as *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa*. Other Gram-positive bacteria that are known to be common causative agents in food poisoning and spoilage, includes *Staphylococcus aureus* and *Bacillus subtilis* (Mostafa et al. 2017). Fungal spores also contribute to food spoilage. Whereas, in undeveloped countries, food borne pathogens are the leading cause of illness and death with fungi being the major causative agent (Mohanka and Priyanka 2014). Ensuring microbial safety of food has become an important issue, but despite many technological advances and cultural techniques, food spoilage and food poisoning is still an ongoing phenomenon.

Prevention of food spoilage today is commonly achieved by chemical preservatives that prevent bacterial growth, but these chemicals cannot completely eradicate several pathogenic bacteria that spoil food products or delay the growth of these spoilage organisms (Liu et al. 2017). The effectiveness of most antimicrobial agents, which are currently being utilized today to extend shelf life and increase the safety of food products in the food industry, have been significantly weakened and reduced by microbial resistance. As a result, it is necessary that new antimicrobial agents that can overcome this resistance be discovered (Liu et al. 2017; Mostafa et al. 2017).

Antimicrobial compounds exist in many plants as secondary metabolites, where they provide natural defense mechanisms. Their broad activities against bacteria and fungi have led researchers to suggest that they can be used as natural preservatives in food (Kong et al. 2017). Natural products as substitutes of synthetic chemical preservatives are increasingly accepted because they are better tolerated by the human body and are less detrimental to human health, reducing the occurrences of side effects and eliminates chemical residues in food and feed chains (Liu et al. 2017; Mostafa et al. 2017).

As a result, scientific research began focusing on botanical condiments in attempt to combat food spoilage. In this study, the growth response of two common food spoilage bacteria (*B. subtilis* and *P. aeruginosa*) and fungus (*Candida albicans*) to the ethanolic and hexanic extracts of three spices will be investigated. Specifically, the effects of 25%, 50% and 100% extracts of *Cuminum cyminum* (cumin), *Sinapis alba* (mustard) and *Pimenta dioica* (all spice) on the growth of the microorganisms will be studied on the surface of Mueller-Hinton Agar by Agar disc diffusion method.

MATERIALS AND METHOD

Collection and preparation of seeds

Approximately 1360g each of dry seeds of *S. alba, C. cyminum* and *P. dioica* were purchased from N&S Mattai. Seeds were stored in airtight zip lock bags and containers, with suitable labelling after purchase. Seeds were incubated at 45℃ until constant weight was achieved. Seeds were grounded with a stainless-steel manual hand mill until they became fine and powdery. They were then stored in airtight zip lock bags and containers with suitable labelling until further use.

Preparation of crude seed extracts by solvent extraction and rotary evaporation

Ethanol extract

Seed extract was prepared by solvent extraction method. 500g of each powdered seed material was extracted with 1000 mL of ethanol. The seeds and the solvent mixture were frequently agitated for 48 hours at 30°C. The mixtures were strained using a gauze and then filtered with Whatman's no.1 filter paper. The filtrates were evaporated in a rotary evaporator at 45°C. The extraction process continued until all the ethanol was removed and collected in the condensing flask and the extracts became thick and gel like. The extract was then collected and labelled, before being kept in sealed containers and wrapped in aluminum foil. The containers were stored at room temperature to prevent contamination until further use in phytochemical analysis and paper disc diffusion.

Hexane extract

Seed extract was prepared by solvent extraction method. 250g of each powdered seed material was extracted with 500 mL of Hexane. The seeds and the solvent mixture were frequently agitated for 48 hours at 30°C. The mixtures were strained using a gauze and then filtered with Whatman's no.1 filter paper. The filtrates were evaporated in a rotary evaporator at 45°C. The extraction process continued until all the hexane was removed and collected in the condensing flask, with the extracts being thick and gel like. The extract was collected, labelled and kept in sealed containers and wrapped in aluminum foil. The containers were store at room temperature.

Preparation of extract concentration

The extract was broken down into three concentrations, 25%, 50% and 100% which were all tested. For the Ethanolic extracts, ethanol was added to the extract to achieve desired concentrations. 10 mL of each extract was prepared. For 25% extract, 2.5 mL extract was added to 7.5 mL ethanol, 50% extract was prepared with 5 mL extract and 5 mL ethanol and 10 mL extract was used and treated as 100%. For the Hexane extracts, hexane was used in the extract to achieve desired concentrations. 10 mL of each extract was prepared. For 25% extract, 2.5 mL extract was added to 7.5 mL hexane, 50% extract was prepared with 5 mL extract and 5 mL hexane and 10 mL extract was used and treated as 100%. Extracts were stored in accurately labelled sealed glass vials at room temperature.

Preparation of 0.5 McFarland standard

McFarland 0.5 was prepared by mixing 0.05 mL 1% Barium Chloride ($BaCl_2$) and 9.95 mL 1% Sulphuric acid (H_2SO_4) in a test tube to obtain an estimated bacterial density $1.5x10^{8.}$ The McFarland was used to prepare the bacterial suspension.

Preparation of inoculum and bacterial suspension

Each bacterium (*B. subtilis* and *P. aeruginosa*) and fungus (*C. albicans*) were sub-cultured from cultures obtained from the Georgetown Public Hospital in Mueller-Hinton Agar petri dishes. Bacterial strains were incubated for 24 hours 30°C, while the fungus was incubated for 48 hours at 30°C. Bacterial suspension was prepared in test tubes containing 10 mL sterilized water. The microbial growth was harvested from the sub-cultured petri dishes with a sterile loop and inoculated the water. The turbidity of the suspension was compared to the turbidity of 0.5 McFarland standard in front of a Wickerham Card.

Paper disc diffusion

Paper disc diffusion was done in triplicates for each extract, at the three concentrations, with the three microbial species. The petri dishes containing Mueller-Hinton agar was swabbed with a sterilize swab to prepare a lawn of the microorganism. Sterile paper disc were soaked in the different concentrations of the extract for 24 hours, before being placed over the lawn. Four discs were placed to each plate. The petri dishes were sealed with transparent tape and incubated. Petri dishes containing bacteria was incubated for 24 hours at 30°C, while petri dishes containing fungi was incubated for 48 hours at 30°C. The formation of zones of inhibition were observed upon formation, which is a circular field around the paper discs. The zones were measured with a ruler in millimeter (Agabalogun et al. 2012).

Preparation of controls

Ciprofloxacin, Fluconazole and Erythromycin were used as the reference or positive control. 0.01g of each drug was mixed in 100 mL water. Paper discs were infused in the solution and was placed on the surface of the agar streaked with the microbes. Antibiotics, Ciprofloxacin and Erythromycin, were tested against *B. subtilis.* and *P. aeruginosa.* Antifungal drug Fluconazole was tested against *C. albicans.* The solvents Hexane and Ethanol were used as the negative control. Paper discs were infused in the solvents and were placed on the surface of the agar streaked with the microbes. Petri dishes containing bacteria was incubated for 24 hours at 30°C, while petri dishes containing fungi was incubated for 48 hours at 30°C.

Qualitative phytochemical analysis

The extracts were analyzed by the following tests to qualitatively test for phytochemical including steroids, phenols and tannins, alkaloids, saponins, volatile oils and terpenoids.

Test for steroids

5 mL of crude seed extract was placed in a test tube and mixed with 2 mL acetic anhydride. Followed by 2 mL chloroform, concentrated Sulfuric acid (H_2So_4) was added dropwise at the side of the test tube. A red color formation indicates the presence of steroids (Deshpande et al. 2014).

Test for phenols

In a test tube, 5 mL of crude seed extract was mixed with 2 mL of 2% iron tri-chloride ($FeCl_3$). A black-dark green color change indicates the presence of both phenols and tannins (Deshpande et al. 2014).

Test for tannins

In a test tube, 5 mL of crude seed extract was mixed with 2 mL of 2% iron tri-chloride ($FeCl_3$). A green color change indicates the presence of catecholic tannin, while a blue color indicates the presence of gallic tannins (Deshpande et al. 2014).

Test for alkaloids

2 mL of the crude extract was measured in a test tube, with picric acid added drop wise until an orange color persisted (Deshpande et al. 2014).

Test for saponins

0.5 mL of the crude extract was measured in a test tube and 2 mL water was added and shaken; a persistent foam for 10 minutes indicates the presence of saponins (Deshpande et al. 2014).

Test for volatile oils

2 mL of extracts was placed in a test tube, with 0.1 mL dilute NaOH and a small quantity of dilute Hydrochloric acid (HCL) being added. The formation of a white precipitate indicates the presence of Volatile oils (Deshpande et al. 2014).

Test for terpenoids

5 mL of crude seed extract was placed in a test tube and mixed with 2 mL acetic anhydride. Then 2 mL chloroform, concentrated Sulfuric acid (H_2So_4) is added dropwise at the side of the test tube. A red color formation indicates the presence of steroids (Deshpande et al. 2014).

Data analysis

Data recorded was analyzed by Analysis of Variance (ANOVA). Tukey Honest significant difference test was carried out to identify whether there was a significant difference between the different concentrations of spice extracts as well as comparing the concentrations between the different groups of extracts. There was a significant difference if the p-value obtained was below 0.05

RESULTS AND DISCUSSION

The yield of ethanol extracts was higher than hexane extracts, albeit they showed no significant difference when assessed statistically. These results are supported by Widyawati et al. (2014). Ethanol extracts were more effective compared to the hexane extracts, except for *S. alba,* where the hexane extracts were more effective (Table 1-5). These results are supported by Mohanka and Priyanka (2014) and Deshpande et al. (2014).

Microorganisms used in the study were chosen because of their dynamic roles in food poisoning and food spoilage. Bacterial strains *B. subtilis* (locally known as the Fried-Rice spoilage bacteria) and *P. aeruginosa,* are common bacterial species that produce toxins and other metabolites that induce human gastrointestinal diseases, in accordance with Mostafa et al. (2017). The antimicrobial activities generated by the extracts of these spices were more effective against bacterial strains than fungi, like the results of Erturk (2006).

Cuminum cyminum was the most useful spice against bacterial strains *B. subtilis* and *P. aeruginosa,* as compared to *P. dioica* and *S. alba*, to which there was a statistically significant difference. This was in accordance with Mostafa et al. (2017), who found *C. cyminum* to be effective against several strains of Gram-positive and Gram-negative bacteria implicated in food poisoning. *C. cyminum* inhibited the growth of *B. subtilis* at 25%, 50%, and 100% concentrations and there was a statistically significant difference between the *C. cyminum*-ethanol extracts and the *C. cyminum*-hexane extracts at all three levels (Figures 1-3), with *C. cyminum*-ethanol extracts at all three concentrations being the most effective against *B. subtilis*. *C. cyminum* inhibited the growth of *P. aeruginosa* at 50% and 100% concentrations for *C. cyminum*-hexane extract and 25%, 50% and 100% concentrations for ethanol extracts. Ethanol extracts of *C. cyminum* were more effective against *P. aeruginosa.* However, *C. cyminum* was not effective against the fungi *C. albicans.*

Pimenta dioica-hexane was not effective against Gram-negative bacteria *P. aeruginosa,* but *P. dioica*-ethanol was effective against *P. aeruginosa* at all three concentrations. Gram-positive bacteria *B. subtilis* and the fungi *C. albicans* were the most susceptible microorganisms to *P. dioica*. *P. dioica* could inhibit the growth of *B. subtilis* and *C. albicans* at 25%, 50% and 100 % concentrations for both ethanol and hexane extracts. Ethanolic extracts of *P. dioica*, however, were more effective and produced larger inhibitory zones compared to the hexane extracts. There was a statistically significant difference between the *P. dioica* in two solvents at all three concentrations (Figures 4-6). These results supported by Tajkarimi et al. (2010) and Azzouz et al. (1982).

Table 1. ANOVA analysis of the spice extracts in the ethanol versus hexane at the three concentrations.

Extract	P-value	Difference
C. cyminum 25 % (ethanol-hexane)	0.007734	+
C. cyminum 50 % (ethanol-hexane)	0.0000000303874	+
C. cyminum 100 % (ethanol-hexane)	0.0000336138	+
P. dioica 25 % ethanol-hexane)	0.000984	+
P. dioica 50 % (ethanol-hexane)	0.0000288718	+
P. dioica 100 % (ethanol-hexane)	0.000000414	+
S. alba 25 % (ethanol-hexane)	0.063133731	-
S. alba 50 % (ethanol-hexane)	0.02132	+
S. alba 100 % (ethanol-hexane)	0.000000274424	+

Note: +: Statistically significant difference; -: No statistical difference

Table 2. ANOVA analysis of the difference between the three spices used.

Spice	P-value	Difference
C. cyminum-P. dioica	0.0497385	+
S. alba-P. dioica	0.0000023	+
S. alba-C. cyminum	0.0000000	+

Note: +: Statistically significant difference

Table 3. ANOVA analysis of the difference between the three microorganisms.

Microorganisms	P-value	Difference
C. albicans -B. subtilis	0.0052063	+
P. aeruginosa-B. subtilis	0.7824462	-
P. aeruginosa-C. albicans	0.0356384	+

Note: +: Statistically significant difference; -: No statistical difference

Table 4. Phytochemicals detected in the extract of the three spices: *S. alba*, *C. cyminum* and *P. dioica.*

Spice extracts	P-value	Difference
C. cyminum ethanol-*P. dioica* ethanol	0.3597876	+
C. cyminum hexane -*P. dioica* ethanol	0.0000306	+
S. alba ethanol -*P. dioica* ethanol	0.0000000	+
S. alba hexane -*P. dioica* ethanol	0.0000000	+
C. cyminum ethanol-*P. dioica* hexane	0.0000000	+
C. cyminum hexane -*P. dioica* hexane	0.1879712	-
S. alba ethanol -*P. dioica* hexane	0.1046461	-
S. alba hexane -*P. dioica* hexane	0.9999923	-
S. alba ethanol -*C. cyminum* ethanol	0.00000000	+
S. alba hexane -*C. cyminum* ethanol	0.00000000	+
S. alba ethanol -*C. cyminum* hexane	0.0000896	+
S. alba hexane -*C. cyminum* hexane	0.0956633	-

Note: +: Statistically significant difference; -: No statistical difference

Table 5. ANOVA analysis of the difference between the spice extracts in solvents, ethanol, and hexane.

Phytochemicals	Hexane			Ethanol		
	C. cyminum	*P. dioica*	*S. alba*	*C. cyminum*	*P. dioica*	*S. alba*
Alkaloids	-	-	-	-	-	+
Tannins	-	+	-	+	+	-
Saponins	-	-	+	-	+	-
Volatile oils	-	-	+	-	+	-
Phenols	-	-	-	-	+	-
Steroids	-	-	+	-	+	+
Terpenoids	-	-	+	+	+	+

Sinapis alba was the least effective spice tested in this study. The *S. alba* extracts were not effective and completely susceptible to the fungal species *C. albicans*. However, bacterial strains *B. subtilis* and *P. aeruginosa* were inhibited by the *S. alba* extracts with *B. subtilis* being most sensitive to the *S. alba* extracts, specifically the *S. alba*-hexane extract (Figures 7-9). The Gram-positive *B. subtilis* was inhibited at 50% and 100% concentrations of the *S. alba*-hexane extract. These results are in accordance with Tajkarimi et al. (2010). *P. aeruginosa* was inhibited by *S. alba*-hexane at 25% and 100% and by *S. alba*-ethanol at 25% concentration only. *S. alba* ethanolic extracts were not effective against *B. subtilis.* and *C. albicans*. This result is like Widyawati et al. (2014), ethanol is a polar solvent and chemical compounds of *S. alba* seeds are likely mostly non-polar. Therefore, *S. alba* seeds are insoluble in the polar solvent ethanol hence, there were no strong antimicrobial effects exhibited. There was no statistically significant difference between the 25% *S. alba*-hexane extract and the 25% *S. alba*-ethanol extract in relation to the zone of inhibition produced and effectiveness because they were both only effective against *P. aeruginosa* at that concentration.

Table 6. The mean zone of inhibition of the spice extracts at concentrations 25%, 50% and 100% in solvents hexane and ethanol. Values are the mean ± SD.

Solvent	Spice extract	Concentration (%)	Inhibition zones (mm)		
			Microorganisms		
			B. subtilis	*P. aeruginosa*	*C. albicans*
Hexane	*C. cyminum*	25	10.25 ± 5.34	0 ± 0	0 ± 0
		50	13.75 ± 0	17 ± 1.38	0 ± 0
		100	21.25 ± 2.05	14.25 ± 3.50	0 ± 0
	P. dioica	25	2.83 ± 1.83	0 ± 0	5.16 ± 5.17
		50	8.41 ± 1.83	0 ± 0	11.16 ± 1.18
		100	10.5 ± 2.60	0 ± 0	4.33 ± 5.62
	S. alba	25	0 ± 0	6.5 ± 3.68	0 ± 0
		50	12.58 ± 6.23	0 ± 0	0 ± 0
		100	14 ± 1.94	9.08 ± 1.20	0 ± 0
Ethanol	*C. cyminum*	25	13.83 ± 3.47	27.1	19.8
		50	23.83 ± 1.01	24.6	11.5
		100	20.33 ± 0.73	21.2	15.25
	P. dioica	25	16.16 ± 1.92	18.25	10.3
		50	16.75 ± 2.47	14.66 ± 0.58	15.66 ± 0.36
		100	20.16 ± 0.65	10.16 ± 5.53	14.08 ± 1.37
	S. alba	25	0 ± 0	10.83 ± 5.42	0 ± 0
		50	0 ± 0	0 ± 0	0 ± 0
		100	0 ± 0	0 ± 0	0 ± 0

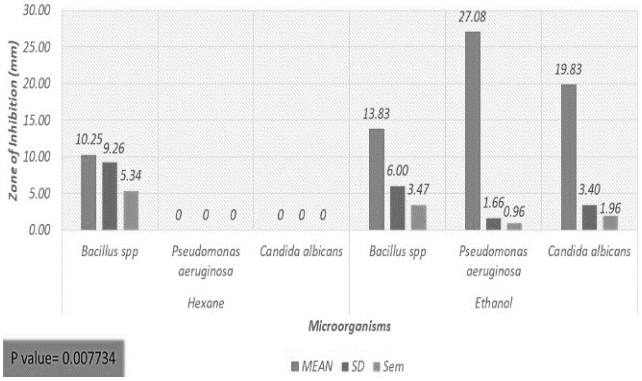

Figure 1. Zone of inhibition of 25% *C. cyminum* against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *C. cyminum* 25% in relation to the zones of inhibition.

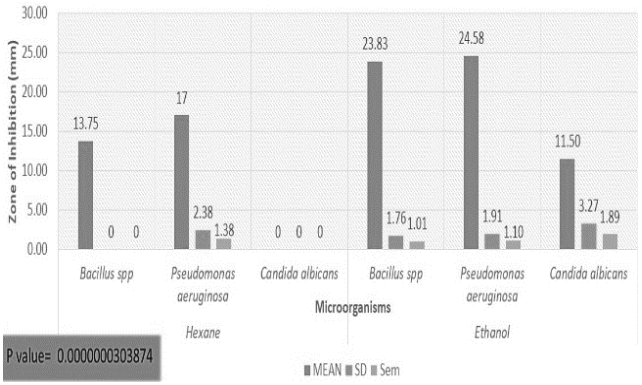

Figure 2. Zone of inhibition of 50% *C. cyminum* against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *C. cyminum* 50% in relation to the zones of inhibition.

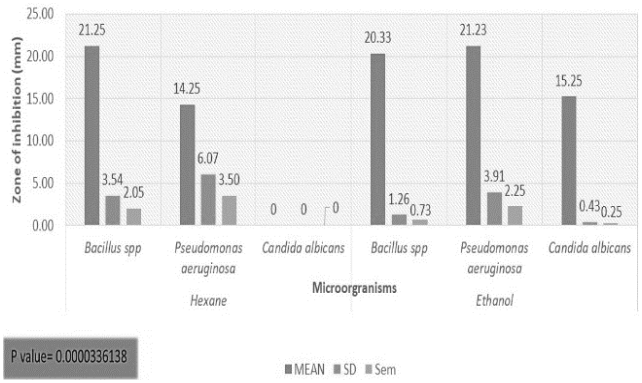

Figure 3. Zone of inhibition of 100% *C. cyminum* against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *C. cyminum* 100% in relation to the zones of inhibition.

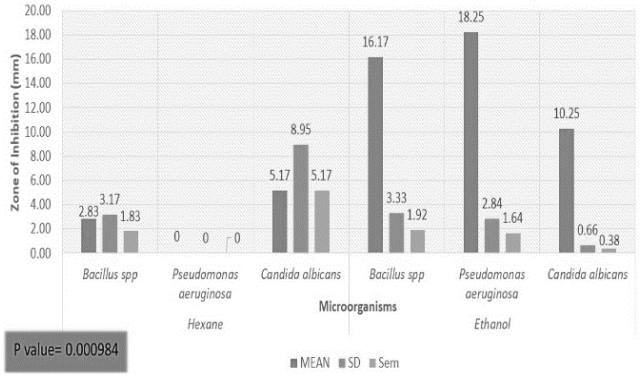

Figure 4. Zone of inhibition of *P. dioica* 25% against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *P. dioica* 25% in relation to the zones of inhibition.

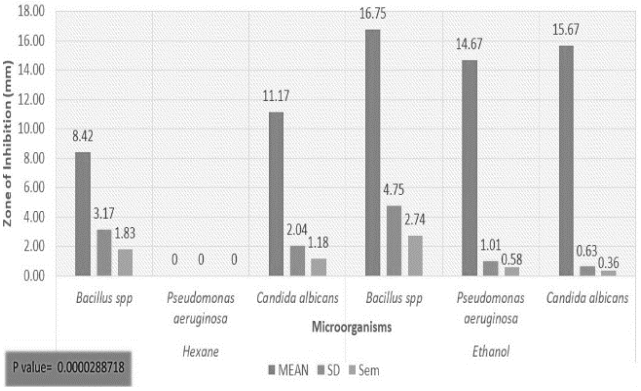

Figure 5. Zone of inhibition of *P. dioica* 50% against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *P. dioica* 50% in relation to the zones of inhibition.

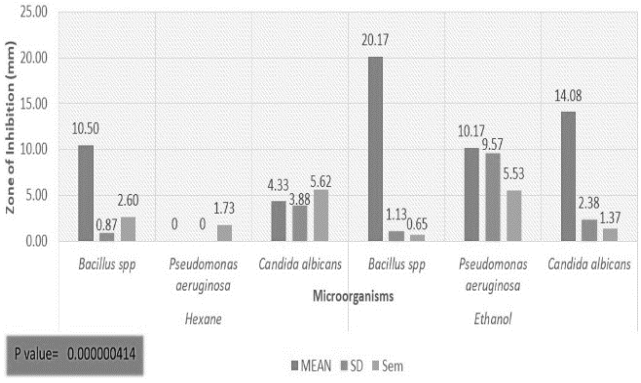

Figure 6. Zone of inhibition of *P. dioica* 100% against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *P. dioica* 100% in relation to the zones of inhibition.

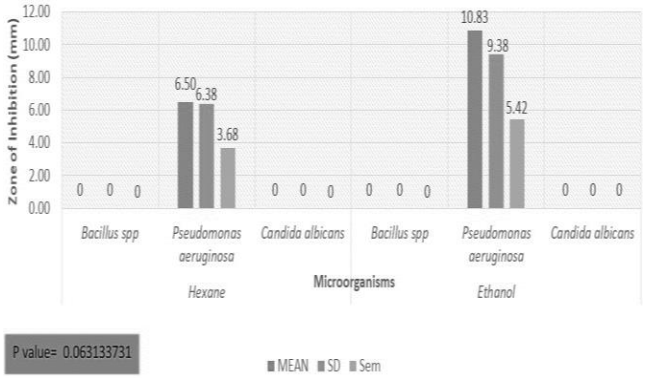

Figure 7. Zone of inhibition of *S. alba* 25% against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was no significant difference between the ethanol and hexane extracts of *S. alba* 25% in relation to the zones of inhibition.

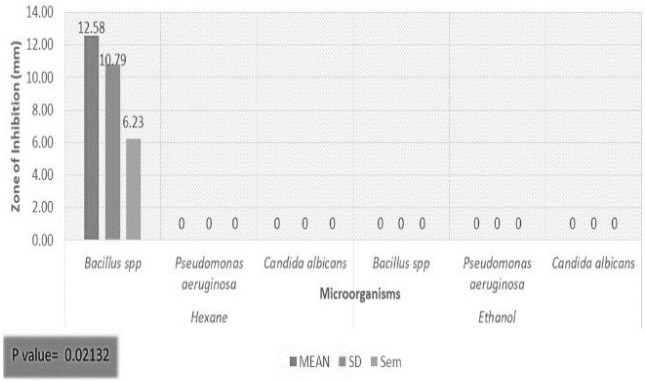

Figure 8. Zone of inhibition of *S. alba* 50% against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *S. alba* 50% in relation to the zones of inhibition.

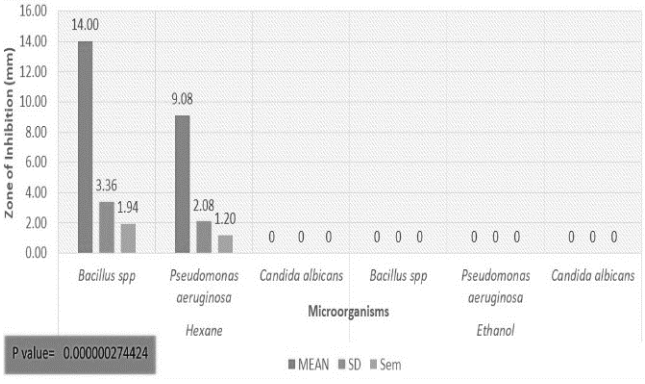

Figure 9. Zone of inhibition of *S. alba* 100% against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *S. alba* 100% in relation to the zones of inhibition.

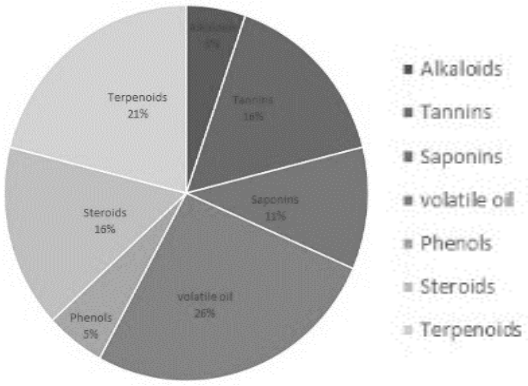

Figure 10. Total percent of phytochemicals found in the extracts of *S. alba*, *P. dioica* and *C. cyminum* in solvents hexane and ethanol.

Ethanol was able to extract most chemical compounds from *C. cyminum*, *P. dioica*, and *S. alba;* including alkaloids, tannins, saponins, volatile oils, phenols, steroids and terpenoids than the hexane extracts where alkaloids and phenols were not extracted (Figure 10). In *P. dioica*-ethanol all phytochemicals tested were detected, except for alkaloids. While *C. cyminum*-ethanol tested positive for tannins, volatile oils and terpenoids. *S. alba*-ethanol tested positive for the presence of alkaloids, volatile oils, steroids, and terpenoids. *C. cyminum*-hexane and *P. dioica*-hexane tested positively only for volatile oils and tannins, respectively. *S. alba*-hexane has the most chemical compounds present for hexane extracts, which tested positively for saponins, volatile oils, steroids and terpenoids. These results are in accordance with that of Widyawati et al. (2014). Hexane, a non-polar compound was ineffective in extracting phenols and alkaloids because they are semi-polar compounds that are easily dissolved in polar solvents such as ethanol. Compounds present in seeds of *C. cyminum*, *P. dioica*, and *S. alba* were mostly polar than non-polar.

All factors were tested and observed against three references ciprofloxacin, erythromycin, and fluconazole; however, the zones of inhibition produced by these drugs were higher than the zones produced by the spices. The three spices tested in this study all exhibited some range of antimicrobial effectiveness. *C. cyminum* was the most effective spice and inhibited *B. subtilis*, *P. aeruginosa*, and *C. albicans*. *P. dioica* was the second most effective spice and inhibited *B. subtilis*, *P. aeruginosa*, and *C. albicans*. *S. alba* was the least effective spice, it slightly inhibited the growth of *B. subtilis*, and *P. aeruginosa* at varying concentrations but was ineffective in inhibiting the mycelial growth on *C. albicans.* Therefore, *C. cyminum* and *P. dioica* can effectively be used as a natural food preservative to control food poisoning and spoilage. The effectiveness of these spices was because of the antimicrobial compounds present in the seeds of each spice, which were detected by phytochemical analysis. It was suggested that these chemical compounds present in the seeds of each spice interact with enzymes and proteins

of the microbial cell membrane causing disruption and dispersal of protons inducing cell death or inhibited enzymes necessary for amino-acids biosynthesis (Mostafa et al. 2017). This study indicated that spice extracts which proved to be potentially effective could be used as natural preservatives to control food poisoning and food spoilage, avoiding application of hazardous chemical preservatives.

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