ANTIMICROBIAL EFFECTS OF SPICE'S EXRACTS ON SPOILAGE MICROORGANISMS OF HOMEMADE AKARA

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Abstract: This study examines the antimicrobial effect of six spices on spoilage organisms present on akara (bean cake) prepared from cowpea (*Vigna unguiculata*). Spoiled akara sample diluted up to 10^8 were plated on plate count agar and Sabouraud's dextrose agar. Total viable count of bacteria and fungi in the spoiled akara were 11.21 x 10^4 and 8.09 x $10^{3.}$ Antimicrobial activities of 0.5 g/ml each of water and ethanol extracts of Fenugreek (*Trigonella foenum-graecum*), Coriander (*Coriandrum sativum*), Caraway (*Carum carvi*), Fennel (*Foeniculum vulgare*), Oregnano (*Origanum vulgare*) and Rosemary (*Salvia rosmarinus*) on the isolated spoilage organisms were assessed using the agar well diffusion method. Their activities were compared with standard antibiotic chloramphenicol. Five (5) genera of bacteria isolates (*Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Shigella dysenteriae* and *Pseudomonas spp*) and five (5) fungal genera (*Aspergillus flavus*, *Aspergillus niger*, *Mucor spp*, *Rhizopus spp* and *Saccharomyces spp)* were identified in the spoiled akara samples using staining techniques, biochemical tests and lactophenol blue stain as well as plating on potato dextrose agar and Sabouraud dextrose agar medium respectively.Results showed that ethanol extracts of the spices showed better and strongest activity against the five isolates than the water extract. The ethanol extracts of the spices showed more activity against the gram positive isolates than the water extracts which was only active against the gram negative isolates.

Keyword: Akara, Antimicrobial, Microorganisms, Spices, Spoilage

Introduction

Spoilage is a chemical process that takes place in food causing it to be undesirable for human consumption due to changes in organoleptic and nutritional qualities (Diniz *et al*., 2020). Spices are plant substances which are aromatic in nature with lots of fragrance used to improve the taste of foods (Diniz *et al*., 2020).Spices is often used alone in cooking or in combination with herbs, vegetables and dried fruits. Spices are used in the production of cosmetic, pharmaceutical drugs, and food processes (Puvaca, 2022). In food processing industries, spices have been used as colorants, flavorings and preservatives. It has a long history of use in the production of herbal drugs and supplements in the pharmaceutical companies. Spices are known to have unique bioactive compounds which make them to possess antimicrobial properties against food pathogens and human pathogenic organisms (Puvaca, 2022).

Fenugreek is a member of the *Fabaceae* family (Syed *et al*., 2020). It is widely cultivated in Central Asia and other countries around the world. It is a leguminous plant with great nutritional and antimicrobial properties (Syed *et al*., 2020). Fenugreek is a plant that has multipurpose crop that is used in food and medicinal purposes. The plant contains substantial amount of fatty acids, phytochemicals, vitamins and minerals which play vital role in the cure and management of human diseases (Mahendra and Bisht, 2011). Fenegreek seeds are spices used worldwide to improve the sensory qualities of foods. It is also used in the development of functional foods as well as to prevent the development of food spoilage organisms (Mahendra and Bisht, 2011).

Coriander (*Coriandrum sativum*) is a member of the *Apiaceae* family (Rajeshwari and Andallu, 2011). The plant has green leaves which are lanceolate, borne flowers and erect stems with approximately 120 cm in height. It is mainly cultivated in Sudan and Egypt (Rajeshwari and Andallu, 2011). Its cultivation has mostly been because of its seeds which is highly fragrant. Due to the fragrance and colour of the spice, it is added to food as garnishee to improve their taste (Chawla and Thakur, 2013).

Caraway (*Carum carvi* L.) is a dried fruit otherwise known as *Persian cumin* belonging to the *Apiaceae* family and a native to countries like Asia and Europe (Mahboubi, 2019). The plant has similar appearance to the carrot family. Caraway is one of the oldest aromatic spice plant which have a pungent flavor and aroma that makes it find application in food production (El-Rady *et al*., 2023). Caraway is mainly cultivated for its seeds which are about 2 mm and are crescent shaped. The seeds are used in making rye bread and other food products. Additionally, other by products of this seed is used in food as well as in medicinal industries.

Fennel (*Foeniculum vulgare*) is a food spice belonging to the *Apiaceae* family (Noreen *et al*., 2023). It is widely cultivated in both the tropical and temperate areas of the world. Fennel has found application in traditional medicine and in food processing owing to its various antimicrobial activities (Noreen *et al*., 2023). In traditional medicine, fennel plant parts have been used in the treatment of a wide range of human diseases (Saber and Eshra, 2019). The plant is rich in nutrients, vitamins, minerals and other functional components. In food processing, various parts of the plant such as seeds, foliage and bulbs because of their potential source of vital nutrients are used in preparation of different delicacies (Hao *et al*., 2021). The plant is aromatic in nature and used as herbs, vegetable and spice for flavoring foods and as a preservative in beverages and confectionaries (Saber and Eshra, 2019).

Oregano (Origanum vulgare), belongs to the *Lamiaceae* family (Veenstra and Johnson, 2019). It originated in North America and have been used as food and medicinal plant. It is a perennial herb up to about 80 cm in height (Veenstra and Johnson, 2019). It has fragrant, oval shaped leaves which are dark and flowers of varying colours (Veenstra and Johnson, 2019). Oregano has vital constituents that make it useful in pharmaceutical industry and in the treatment of human diseases (Hao and Shi, 2021). This herb has been used for the treatment of health issues such as skin infections, drowsiness, cancer, convulsion and as antidotes for poisoning (Liaqat *et al*., 2020). Essential oil can be obtained from different parts of this herb and the oils have been reported to have antimicrobial properties (Liaqat *et al*., 2020). Because of its antimicrobial properties it is widely used as spices in food production for flavoring the taste of foods and as natural preservatives in processed foods (Boskovic *et al*., 2020). Oregano contains appropriate composition of vitamins and minerals which are needed to support growth and development in humans (Boskovic *et al*., 2020).

Rosemary (*Rosmarinus officinalis* L) is a green shrub and a member of *Lamiaceae* family (Rahbardar and Hosseinzadeh, 2020). Its height is approximately 2.5 m .The shrub has quadrangular stems, leaves without stalks and tiny flowers. It is cultivated in the USA and Mexico. Its optimum growth period is August (Rahbardar and Hosseinzadeh, 2020). Rosemary is a spice herb widely used in the preparation of most foods because of its taste and smell. The plant parts contain appreciable amount of oil which are used in cooking food and preserving it (Pawlowska *et al*., 2020). Rosemary has antimicrobial properties which makes it useful in the treatment of human infections (Gonzalez-Minero *et al*., 2020).

Materials and Methods

Collection of samples

Dried spices including Fenugreek (*Trigonella foenum-graecum)*, Coriander (*Coriandrum sativum)*, Caraway (*Carum carvi)*, Fennel (*Foeniculum vulgare)*, Oregnano (*Origanum vulgare)* and Rosemary (*Salvia rosmarinus*) were purchased from UK. Cowpea (*Vigna unguiculata*), vegetable oil, table salt, pepper and onion were purchased from Sabo market, Yaba, Lagos.

Extraction of spice's extracts

The method of extraction described by Alwhibi and Soliman (2014) with some modifications was used for the preparation of the spice extracts. 320 grams each of dried spices was blended into fine powdery form and soaked separately in 180 ml sterile distilled water and ethanol for 48 h (Figure 1). At intervals of 24 h, each spice mixture was stirred vigorously using a glass rod. Once it was 48 h, each spice extract was filtered through a No 1 Whatman filter paper to obtain a clear filtrate. The filtrates of each spices was air dried in an oven at 40°C to remove the solvents. At the end, dried crude samples of each spice extract was dissolved in 2 ml distilled water and stored in tubes at -18°C.

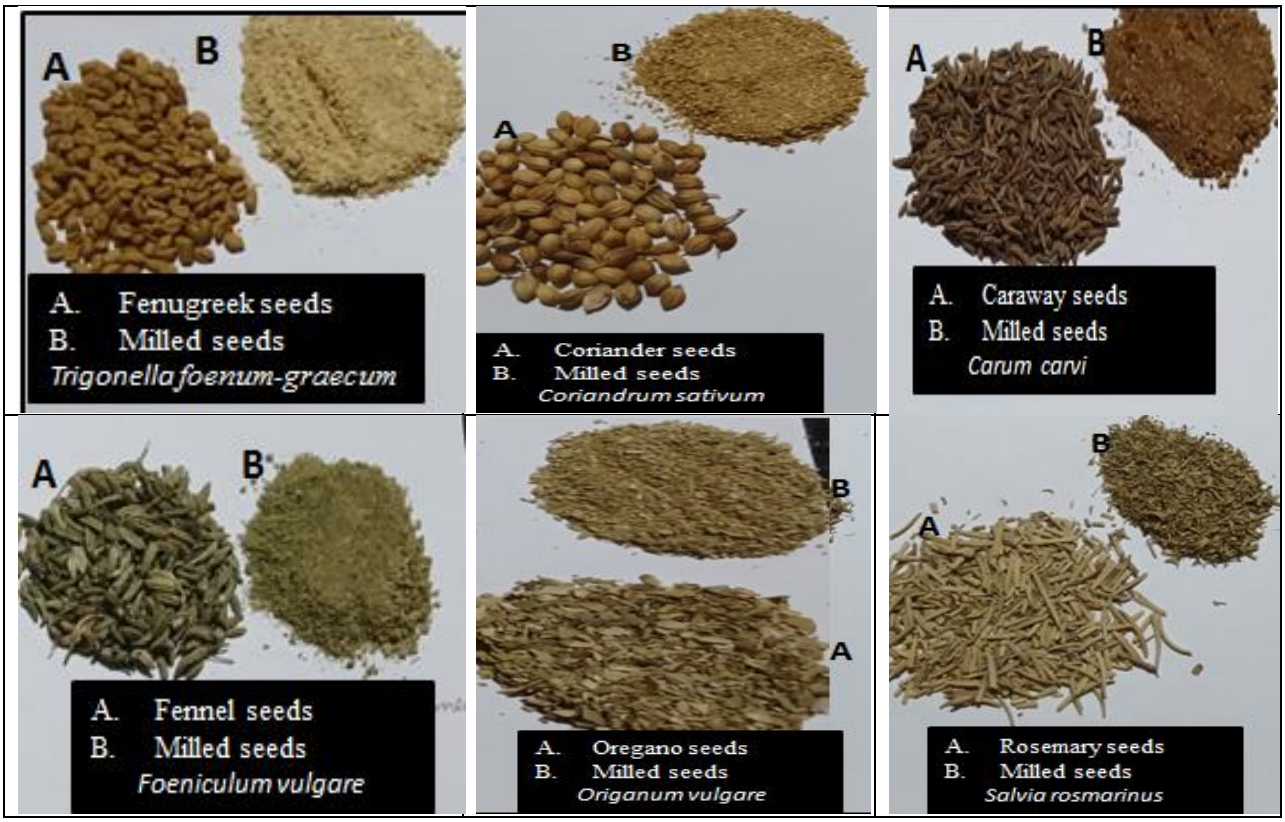

Figure 1. Pictorial representation of the six types of spices used in this study

Preparation of akara (bean cake) and spice's extracts

Cowpea (*Vigna unguiculata*), 500 g was cleaned to remove stones and foreign particles. Thereafter, the beans were soaked for 4 h in distilled water. After soaking, the outer covering of the bean seeds was removed and the water drained. The beans were blended using a blender. After blending, the necessary ingredients were added to taste and the paste was molded into cylindrical shapes and fried. The akara samples were left for five days in white plates kept on open shelf at room temperature to allow spoilage microorganisms to act on them.

Microbiological examination of spoilage organisms on akara

Preparation of culture media

The four culture media used for the growth and isolation of microorganisms includes Sabouraud dextrose agar (SDA), MacConkey agar (MA), Potato dextrose agar (PDA) and Plate count agar (PCA). The media used were prepared following the directives given by the manufacturers. Different concentrations of media were weighed as follows SDA (68 g), MA (55 g) and PCA (23.5 g) and dissolved in 1000 mL of distilled water. The media were autoclaved at 121 ° C for 15 minutes, left to cool and poured into petri-plates to solidify.

Total viable count of spoilage microorganisms

Ten grams of spoiled akara sample for microbial analysis was weighed and put into 90 mL of 0.1 % sterile peptone water for homogenization and serial dilution of the homogenates was prepared up to 10^6 (Oseghale *et al*. 2020). Then 0.1 mL of the serially diluted homogenate was transferred into the petri-plate. To the dilutions, sterile plate count agar and Sabouraud's dextrose agar was poured and plates were incubated for 37° C for 24 h and 25 ° C for 3 days.

Isolation of spoilage microorganisms

After 24 h, pure cultures of the isolates were obtained by re-streaking them on fresh medium using standard microbiological methods. Then isolates that appear with different morphologies on agar plates were isolated and kept in the freezer at - 20° C.

Fungal isolates were isolated by using Oseghale *et al*. (2020) direct plate method. This method was used to culture the molds observed on the akara. Portions of akara samples to be examined was transferred into petri-plates containing solidified SDA using a sterile forceps and each plate was incubated at 28 ° C for 5 days.

Identification and characterization of spoilage microorganisms

The bacteriological methods of analysis used by Ugwuanyi *et al*., (2019) were used with minor modification to identify the isolated bacterial species. The isolates were evaluated for the presence of glucose, catalase, oxidase, citrate, coagulase as well as gram staining.

The fungal isolates observed on the SDA plates were identified with standard method described by Mailafia *et al*., (2017).Both the morphological and cultural characteristics of each fungi was examined. The colonial growth pattern and morphology of conidial was examined. Lactophenol stain was used for the identification of the fungi species isolated. This was conducted by putting a drop of the stain on a clean slide with a needle and a tiny part of the mycelia from the fungi cultures was placed in the stain, spread and covered with slip. Then the slides were mounted and viewed under the light microscope with x40 objective lenses.

Preparation of spores of spoilage fungi for identification

Fungal suspensions of each isolate were prepared using Al-Garadi *et al*. (2023) method of analysis. For spore identification, the isolated cultures of *Aspergillus niger*, *Aspergillus flavus*, *Mucor*, *Rhizopus* and *Saccharomyces spp* were separately grown on potato dextrose agar at 35 °C to prepare inoculum suspensions. After growth, colonies of each fungal strain observed in the plate were covered with 0.1 % Tween 20 and 1 mL of distilled water. Also the conidia of each fungus was collected, placed into sterile tubes and blended for about 30 minutes after which the conidia of each sample was examined for hyphae.

Antimicrobial analysis

The agar well diffusion method of Yangomodou *et al*., (2020) was used for the analysis. Inoculum of the bacterial isolates were prepared from broth cultures grown overnight at 37 °C for 18 h. A loop full of the cultures was separately diluted with 0.9 % Nacl to obtain a bacterial suspension of 1.5 x 10^8 CFU/ml corresponding to 0.5 McFarland turbidity standards. Thereafter, the suspensions was adjusted and made up to standards by adjusting the density to 1.0 x 10^6 of the bacterial isolates. I mL of the suspension of each bacteria isolate was uniformly spread over the entire surface of the MHA medium plate and plates were allowed to stay on the sterile slabs in the laboratory for 10 mins. On the agar plates, wells of about 6 mm diameter were made in the MHA medium using a sterile cork borer. 10 µL of moulting MHA was used to seal the base of each well. Each of the wells was loaded with 0.5 g/ml of the crude spice extracts. Then 0.5 mL of each concentration was put into each hole on the agar plate. The plates were left to stand on the slabs for about 1 hr to allow the diffusion, and thereafter they were incubated for 37 °C for 24 h. The inhibition zones produced by the bacteria isolates were measured in mm (millimeter). This experiment was carried out in triplicates.

Statistical analysis

The mean and standard deviation were calculated using Analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) was used to separate the means.

Results and Discussion

The total bacterial and fungal counts present in the akara sample are shown in Table 1.

Table 1: Total viable count of spoilage microorganisms in akara

S/N	Microorganism	Viable counts (CFU/ml)
1	Bacteria	11.21 x 10^4
2	Fungi	8.09 x 10^3

Microbial examination of spoiled akara showed that it harbored five different types of bacterial isolates including *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus and Shigella dysenteriae* (Table 2) and five food spoilage fungal isolates namely *Aspergillus flavus*, *Mucor sp. Aspergillus niger*,

Rhizopus sp. and *Saccharomyces sp.* (Table 3). Identification of the fungal isolates showed that they possess varying biochemical characteristics (Table 3).

Table 2: Morphological and Biochemical Characteristics of bacterial isolate from spoiled akara

S/N	Morphology of bacterial species	Gram reactions	Lactose	Glucose	Catalase	Citrate	Oxidase	Coagulase	Suspected foodborne patogens
1	Rod shape *bacilli*	Gram negetive	+	+	+	-	-	Not detected	*Escherichia coli*
2	Rod shape *bacilli*	Gram positive	-	+	+	+	-	Not detected	*Bacillus cereus*
3	Rod shape *bacilli*	Gram negetive	-	+		+	+	Not detected	*Pseudomonas spp.*
4	Cocci shape	Gram positive	-	+	+	+	-	+	*Staphylococcus aureus*
5	Rod shape *bacilli*	Gram negetive	-	+	+	-	-	Not detected	*Shigella dysenteriae*

Key:
Positive = +
Negative = -

Table 3: Identification and Characteristics of Fungal isolates from spoiled akara

S/N	Morphology appearance of fungi on spoiled akara	Microscopic examination with lactophenol stain	Identified fungi
1	Front view was black in colour and reverse view cream in colour	Aseptate hyphae with pigments	*Aspergillus flavus*
2	Appears as white candy cotton which became dark in colour with time	Aseptate hyphae with sporandium present at the tips	*Mucor spp.*
3	Colonies were yellow in colour with loose mycelim	Vesicles appear brown in colour with phialides growing along its periphery.	*Aspergillus niger*
4	Appear as dark grey brown in colour	Has sporangiospores with sub globes with irregular shapes and stolones	*Rhizopus spp*
5	Appear as smooth, flat and have glistening colour. Has mucous texture.	Cells are large with globules	*Saccharomyces spp*

The antimicrobial activity of the water and ethanol extracts of plant spices against spoilage organisms found in spoiled akara is presented in Table 4. The activity of the spice extracts was measured using the agar well diffusion method. The antimicrobial activity results showed that the water and ethanol extracts of *T. foenum-graecum* showed the strongest activity against the five bacterial isolates tested. The water extract of *T. foenum-graecum* showed strong activity against *E. coli* (29.04 mm) and *P. aeruginosa* (27.11 mm) whereas the ethanol extract showed stronger activity against *S. aureus, (35.22 mm) and B. cereus* (33.18 mm). The antimicrobial activities of extracts from other spices against these bacterial isolates were also observed. The water extracts of spices like *C. sativum, C. carvi, F. vulgare, O. vulgare* and *S. rosmarinus* showed strong activity against *E. coli* with 27.06 mm, 28.88 mm, 27.11 mm, 28.56 mm and 24.32 mm diameter zones of inhibition. Whereas only the ethanol extract of *F. vulgare* was found to active against *E. coli* with 24.66 mm. The water extracts of the spices seem to be moderately active against *B. cereus*. Whereas the ethanol extracts of *C. sativum, C. carvi, F. vulgare and O. vulgare* showed strong activity against *B. cereus* with 25.23 mm, 30.12 mm, 27.13 mm and 32.34 mm zones of inhibition. Both the water and ethanolic extracts of *T. foenum-graecum, C. sativum, C. carvi, F. vulgare and O. vulgare* were strongly active against *P. aeruginosa.* The ethanol extracts of *C. sativum, C. carvi, F. vulgare and O. vulgare* were strongly active against *S. aureus with* 34.12 mm, 33.43 mm, 32.11 mm and 35.56 mm diameter zones. The ethanol extract was more active than the water extracts of the spices. The water extract of three of the spices, *T. foenum-graecum, C. carvi, O. vulgare* and *S. rosmarinus* were active against *S. dysenteriae* whereas the ethanol extract showed low activity against the isolate.

Table 4: Antimicrobial activity of spice extracts against bacterial isolates from spoiled akara

Plant spices 320 g	Solvent	Inhibition Zone Diameter (mm)				
		Microorganisms				
		Escherichia coli	***Bacillus cereus***	***Pseudomonas aeruginosa***	***Staphylococcus aureus***	***Shigella dysenteriae***
Fenugreek ***(Trigonella foenum-graecum)***	Water	29.04±0.8 [a]	20.12±0.5 [d]	27.11± 0.8 [b]	23.56±0.8 [c]	25.09±0.7 [c]
	Ethanol	26.24±1.0 [c]	33.18±1.1 [a]	28.14±0.9 [b]	35.22±1.1 [a]	20.12±1.3 [d]
Coriander ***(Coriandrum sativum)***	Water	27.06±0.8 [a]	14.56±0.6 [d]	15.23±0.9 [d]	16.43±0.6 [c]	17.32±0.8 [b]
	Ethanol	19.87±0.8 [e]	25.23±0.9 [c]	27.55±0.9 [b]	34.12±1.1 [a]	21.22±1.3 [d]
Caraway ***(Carum carvi)***	Water	28.88±1.0 [a]	13.55±0.7 [e]	25.04±0.5 [b]	21.23±0.8 [d]	23.21±0.7 [c]
	Ethanol	22.23±0.9 [e]	30.12±0.9 [b]	26.29±0.8 [c]	33.43±1.1 [a]	20.65±1.3 [d]
Fennel ***(Foeniculum vulgare)***	Water	27.11±0.8 [a]	16.23±0.7 [d]	24.23±0.9 [b]	20.43±0.6 [c]	15.21±0.7 [e]
	Ethanol	24.66±0.9 [d]	27.13±1.0 [b]	26.21±0.8 [c]	32.11±1.1 [a]	21.45±1.0 [e]
Oregnano ***(Origanum vulgare)***	Water	28.56±0.8 [a]	14.34±0.6 [e]	25.99±0.9 [b]	15.34±0.6 [d]	24.67±0.8 [c]
	Ethanol	23.77±1.0 [d]	32.34±1.0 [b]	24.44±1.0 [c]	35.56±1.1 [a]	19.56±0.7 [e]
Rosemary ***(Rosmarinus officinalis)***	Water	24.32±0.8 [b]	14.34±0.7 [d]	15.22±0.4 [c]	11.66±0.5 [e]	29.32±0.7 [a]
	Ethanol	16.12±1.0 [d]	19.98±1.0 [b]	23.55±1.0 [a]	18.47±1.1 [c]	18.11±0.7 [c]
Control	Water	6.21±0.2 [a]	6.21±0.2 [a]	6.21±0.2 [a]	6.21±0.2 [a]	6.21±0.2 [a]
	Ethanol	7.02±0.4 [a]	7.02±0.4 [a]	7.02±0.4 [a]	7.02±0.4 [a]	7.02±0.4 [a]
	Chloramphenicol	-	36.09±0.8 [a]	-	36.67±0.7 [a]	25.17±0.5 [b]

Values are means ± standard deviation of triplicate determinations. Means bearing different alphabet superscripts within the same row are significantly different ($p<0.05$).

The results obtained in this study showed that akara is a food that is highly susceptible to spoilage organisms. Therefore, the presence of spoilage bacterial and fungal isolates in akara indicated that it is prone to develop unpleasant odour and awful taste after a period of time if not properly refrigerated. This result correlates to earlier findings for the occurrence and identification of these microorganisms in a variety of foods where they are capable of causing foodborne diseases in humans (Agaodlu *et al*., 2007; Joe *et al*., 2009; Zhang and Wu, 2016). Previous results have shown that plant essential oils and extracts from spices have more antimicrobial activity against gram positive than gram negative bacteria isolates (Zhang and Wu, 2016; Okmen *et al*., 2021). This study showed that spice extracts showed different degrees of activity against the five spoilage bacterial isolates tested as observed in the diameter of their zones of inhibition. Both the water and ethanol extracts of spices were active against the tested bacterial isolates. However, the ethanol extract of spices proved to be more active than the water extract of spices because the gram positive bacterial isolates were more susceptible to it than the gram negative isolates. This result supports the earlier reports of Zhang and Wu, (2016). For example in this study, *B. cereus* and *S. aureus* which are both gram-positive organisms were more susceptible to the ethanol extract of spices than the gram – negative bacterial isolates. This sought of difference in sensitivity between the gram negative and gram positive bacterial isolates to spice extracts could be due to the alterations that occur in the outer membrane of gram negative bacteria which inhibit the diffusion of hydrophobic compounds such as antibiotics (Okmen *et al*., 2021). In addition, the stronger activity of the ethanol extract of spices against the five spoilage bacterial isolates may probably be due to the efficiency of the solvent in extracting the entire active compounds available from the spices. This seem to agree with the report of Manandhar, (2002) and his colleagues who reported that garlic powder extracted with solvent had enhanced effect against the tested strains compared to other spice extracts. The activity of water extract of spices against gram negative bacterial isolates agrees with the findings of other researchers. In a study, the inhibitory effects of water extracts of six different spices were analyzed and it was observed that they prevented the growth of microorganisms at a ratio of 0.26-13 mg/ml and is more effective against gram negative bacteria than gram

positive bacteria (Okmen *et al*., 2021). This results shows similarity to the result obtained in this study. Besides, a study on the activity of extracts of seven spices against spoilage bacterial isolates showed that two of the spices exhibited strong activity against *E. coli* than other varieties of spices (Oussalah *et al*., 2007). Out of all the water extract of spices tested, *T. foenum-graecum* exhibited strong activity against *E. coli* than other bacterial isolates. Alwhibi and Soliman (2014) reported that *T. foenum-graecum* seed extract had antibacterial activity against pathogenic *E. coli* than other isolates tested. Apart from *T. foenum-graecum*, other spices such as *C. carvi* and *O. vulgare* showed antibacterial activity against *E. coli*. Seidler-Łożykowska *et al*., (2013) reported that *C. carvi* oil obtained from different cultivars showed antibacterial activity against *E. coli.* This supports the findings obtained in this result. Oregnano (*O. vulgare)* showed antibacterial activity against *P. aeruginosa*. This does not correlate with the report of Gonclaves *et al*., (2013) who found that *O. vulgare* showed antimicrobial activity against all bacterial isolates tested except *P. aeruginosa*. Ethanol extracts of *C. sativum* showed stronger activity against *S. aureus* than other bacterial isolates. However, this result does not agree to that of Okmen *et al*., (2021) who reported no antimicrobial activity for *C. sativum* against the test organisms.

Conclusion

The degree of antimicrobial activity of the different spices examined in this study can be placed in this order fenugreek > oregano > caraway > fennel > coriander > rosemary. These spices may be used as natural antimicrobial agents and suggests the possibility of using them in the preparation of akara especially if spoilage is caused by the activity of microbes. These spices can be used alone or combination with other chemical preservatives or other food preservation systems.

Conflict of Interest

The authors declare that there is no conflict of interest reported in this work.

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