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Research Article

Antibacterial Activity of Spices against Enterobacteriaceae

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Abstract

Present research work deals with the Study of Antibacterial Activity of Spices against Enterobacteriaceae (E.coli). Aqueous solution of Spices (Bay leaf (Laurus nobilis), Black pepper (Pippali nigrum), Cinnamon (Cinamomum verum), Clove (Syzygium aromaticum) and Star Anise (Illicium verum) was prepared and tested against Enterobacteriaceae (E.coli). Antibacterial activity of Spices was tested on E.coli by Disc diffusion method and antibacterial activity of each spice was observed in the form of zone of inhibition. Black pepper (Pippali nigrum) showed 2cm antibacterial activity on E.coli as compared to Bay leaf (Laurus nobilis) 1.3cm and Star Anise (Illicium verum) 0.9cm. Cinnamon (Cinamomum verum) Clove (Syzygium aromaticum) and does not showed any antibacterial activity at the concentration of 10µl/disc. The results showed that the aqueous decoction of black pepper possesses great antibacterial potential (75%) as compared to aqueous decoction of bay leaf (53.4%) and star anise (18.1%).

Key words: Bacterial culture (E.coli), Spices [Bay leaf (Laurus nobilis), Black pepper (Pippali nigrum), Cinnamon (Cinamomum verum), Clove (Syzygium aromaticum) and Star Anise (Illicium verum)], Agar Disc Diffusion Method

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1. Introduction

There has been a renewed interest in improving health and fitness through the use of more natural products. Spices are an important part of the human diet. They have been used for thousands of years to enhance the flavor, color, and aroma of food. In addition to boosting flavor, spices are also known for their preservative and medicinal value which forms one of the oldest sciences. [3]

Spices word come from a Latin species, meaning a commodity of value and distinction can be defined as “any dried, fragrant, aromatic, or pungent vegetables or plant substance in whole, broken, or ground forms that contributes flavor,

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whose primary function in food is seasoning rather than nutrition and that may contribute piquancy of foods and beverages." Although as natural substances spices are easily absorbed by our bodies and generally do not have any adverse effects, spices as medicine should be used judiciously. This is because substances' being derived from a plant does not mean it is always harmless. The latest finding suggests that the chemicals present in spices can be allergens, carcinogens, and mutagens. Keeping this view, the study was conducted to determine the antibacterial potential of aqueous decoction of (Bay leaf (Laurus nobilis), Black pepper (Pippali nigrum), Cinnamon (Cinamomum verum), Clove (Syzygium aromaticum) and Star Anise (Illicium verum). [3]

Antibacterial: anti means against i.e. against bacteria any chemical substance that inhibits the bacteria to grow or reproduce on a medium. Any substances which show this activity are antibacterial agents.

Enterobacteriaceae: A large family of Gram negative rod shaped bacteria of the order Eubacteriales, whose natural habitat is the intestinal tract of humans and animals. Salmonella, Escherichia coli, Yersinia pestis, Klebsiella, Shigella, Proteus Enterobacter, Serratia, Citrobacter. They are rod shaped and typically 1-5um in length. They are facultative anaerobes, Non- sporing, motility by peritrichous flagella. Ferment sugars and produce lactic acid. Most also reduce nitrate to nitrite some enterobacteria produce endotoxins which resides in cell cytoplasm.

Disc Diffusion Method: is a test which uses antibiotic-impregnated wafers to test whether bacteria are affected by antibiotics. In this test, wafers containing antibiotics are placed on an agar plate where bacteria have been placed, and the plate is left to incubate. If an antibiotic stops the bacteria from growing or kills the bacteria, there will be an area around the wafer where the bacteria have not grown enough to be visible. This is called a zone of inhibition. [13]

2. Materials and Methods

Pure culture of E.coli bacteria, Nutrient Agar Media (NAM), Nutrient Broth (NB), Triple Sugar Iron Agar Media (TSI), Simmon's Citrate Agar Media, Trypticase Soya Agar (TSA), Bromo cresol falkow medium, Endo Agar Media (EA), Hi-Chrome Media.

Method of Preparations

Isolation of E.coli Bacteria: Collection of soil sample

Soil sample has been collected from pot soil in a sterile Beaker from the Mata Gujri College Campus and brought to the Microbiology Laboratory for further processing.

Processing of soil sample

One gram of soil sample was weighed and suspension was prepared with 9 ml of distilled water. 10 sterile test tubes were taken and each test tube was filled with 9 ml of distilled water. 10 fold serial dilution of soil suspension was done. Soil suspension was serially diluted from 10^{-1} to 10^{-10} with the help of sterile pipette. Even dilution was taken i.e. 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}. Nutrient Agar Media was prepared and sterilized in Autoclave at 121^{o}C for 15 minutes. Four Nutrient Agar Media Plates were prepared and labeled with 10^{-2}, 10^{-4} ,10^{-6}, 10^{-8} dilution. 0.1 ml of each dilution was taken and spread over their respected plate with the help of sterile spreader. These four plates were

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incubated at 37°C for 24 hours in an incubator. After 24 hours plates were observed for bacterial colonies (Figure 1). Each isolated bacterial colonies were identified by further method. Each isolated colonies were streaked on the Nutrient Agar slant and incubated at 37°C for 24 hours and maintained to obtain a pure culture for further process. [7]

Identification Test

Gram's staining: (Table 1) A heat fixed bacteria smear was covered completely with a few drops of crystal violet solution. After 30- 60 sec the smear was rinsed with water by squirting the slide above the smear and letting the water wash over it until the water runs clear. Several drops of iodine (mordant) was applied to cover the smear and left for 60 sec then rinsed again. A few drops of Isopropanol-acetone mixture was added at a time until it become colorless, then the slide was rinsed again. Aqueous Safranine was applied for 30- 60 sec followed by a rinse. The smear was blotted to remove excess water, using absorbent paper. The slide was then air dried and observed under a microscope. [5]

Carbohydrate Fermentation Test: (Figure 2, Table 2) Performed to find the ability of microorganisms to ferment the given carbohydrate, the ability to degrade amino acids and the ability of microorganism to produce gaseous end products in fermentation. 3 sets of 8 screw tubes and 8 Durham's tubes were made. First set was filled with 5ml of Glucose broth, second with 5ml of sucrose broth and third with 5ml of lactose broth and tube was inserted with inverted Durham's tube filled with corresponded broth. All the sets were sterilized at autoclave. 8 screw tubes inoculated with different bacteria and same process was repeated with rest of other 2 sets. All the sets were incubated and observed for 24 hours and 48 hours for acid and gas production. [15]

Triple Sugar Iron Agar Test: (Figure 3, Table 3) Triple sugar iron (TSI) agar was a tube differential medium used in determining carbohydrate fermentation and H_2S production. Gas from carbohydrate metabolism can also be detected. Bacteria can metabolize carbohydrates aerobically (with oxygen) or fementatively (without oxygen). TSI differentiates bacteria based on their fermentation of lactose, glucose, and sucrose and on the production of hydrogen sulfide. TSI is most frequently used in the identification of the *Enterobacteriaceae*, although it is useful for other gram-negative bacteria. TSI contains three carbohydrates: glucose (0.1%), sucrose (1%), and lactose (1%). Phenol red is the pH indicator, and agar is used to solidify the medium. During preparation, tubes containing molten agar are angled. The slant of the medium is aerobic, while the deep (or butt) in anaerobic. When any of the carbohydrates are fermented, the drop in pH will cause the medium to change from reddish-orange (the original color) to yellow. A deep red color indicates alkalization of the peptones. Sodium thio sulfate in the medium is reduced by some bacteria to hydrogen sulfide (H_2S), a colorless gas. The hydrogen sulfide will react with ferric ions in the medium to produce iron sulfide, a black insoluble precipitate. [14]

Biochemical Test: (Figure 4, Table 4)

A. **Indole test:** Tryptone agar was inoculated and incubated at 37° C for 48 hours and added Kovacs reagent and read immediately. They results were interpreted based on the change of color from yellow to pink. [5]

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B. **Methyl red test (MR):** Buffered glucose broth was inoculated and incubated at 37° C for 48 hours. A few drop of methyl red solution was added to culture and the results were read immediately. The results were interpreted based on the change of color from yellow to red. [5]

C. **Voges Proskauer test (VP):** The organisms were inoculated in buffered glucose broth and were incubated at 37° C for three days and 3 ml of alpha napthol was added followed by 1 ml of 40% KOH. It was mixed well and allowed to stand for 30 min. The results were interpreted based on the change of yellow color to pink. [5]

D. **Citrate utilization:** The organisms were streaked onto Simmons Citrate agar plate and incubated at 37ºC for 24hours. The results were interpreted based on the change of color from initial green to deep blue if it was positive. [5]

Confirmatory Test for (E.coli): Enzyme test- (Figure 5, Table 5)

A. Catalase test : This test was conducted to detect the presence of the enzyme Catalase. A capillary tube was dipped into 3% H_2O_2 and the colony was touched. [6]

B. De-carboxylase test : 10 ml of Bromo cresol falkow Hi-medium was prepared. 5ml of broth was dispensed in two test tubes and covered with a cotton plug and sterilized by autoclave at 121°C for 15 minutes. Test tubes were labeled as control and sample. The test sample was inoculated with a sterile inoculating loop and incubated at 35°C for 12 hours. Yellow color was observed in first 12 hours due to glucose fermentation and in next 24 hours the broth turns again back to purple colour. Positive result was obtained. [7]

Specific Media for (E.coli): Endo Agar Media, Hi Chrome media**.** (Figure 6)

After the isolation and identification of E.coli bacteria, the bacteria were maintained on Nutrient agar slants and on its specific media. [2]

Preparation of Nutrient Broth culture : 5ml of nutrient broth was taken in a sterile test tube and was sterilized in an autoclave at 121 lbs for 15 minutes and allowed to cool. Broth were inoculated with an E.coli bacterial colony and incubated at 37ºc for 24 hours. [7]

Preparation of Spices Decoction: The aqueous decoction was prepared by boiling 2 g spices in 20 ml distilled water in a flask for 20 minutes. The flask was removed from heat and allowed to cool. The content of flask was filtered to obtain clear decoction. [2]

Screening of Antibacterial Activity of Spices by Agar Disc Diffusion Method (Figure 7, Table 6) **:** 6 Nutrient Agar plates were prepared and labeled as Control, Bay leaf extract, Black pepper, Cinnamon extract, Clove extract and Star anise extract out of which 5 plates were inoculated with bacterial culture with swab culture method and one kept as control. Five wells were made in each plate. Each plate was inoculated with 10µl of extract in their corresponding plate with the help of micropipette and incubated at 37ºc for 24 hours. After incubation the plates were observed for zone of inhibition. [2]

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MACROSCOPIC AND MICROSCOPIC CHARACTERISTICS OF BACTERIA							
NUTRIENT AGAR MEDIA							
CHARACTERISTICS	10^{-2}			10^{-4}	10^{-6}		10^{-8}
COLOUR	Milky White	Creamy yellow	Creamish	Creamish	Buff	Creamish	Creamish
SHAPE	Circular	Circular	Circular	Spreaded	Circular	Spreaded	Spreaded
MARGIN	Entire	Entire	Entire	-	Entire	-	-
ELEVATION	Convex	Raised	Convex	Flat	Convex	Flat	Flat
APPEARANCE	Smooth, Shiny, Glistening.	Smooth, Shiny.	Smooth, Shiny.	Glistening	Smooth, Shiny.	Rough	Rough, Opaque
GRAM STAINING	Gram Positive Bacilli	Gram Negative Bacilli	Gram Negative Bacilli	Gram Positive Bacilli	Gram Negative Bacilli	Gram Negative Bacilli	Gram Negative Bacilli

Table 1: Macroscopic and microscopic Characteristics of Bacteria

CARBOHYDRATE FERMENTATION TEST													
SUGARS													
S.NO.	COLONIES	DEXTROSE				SUCROSE				LACTOSE			
		ACID		GAS		ACID		GAS		ACID		GAS	
		24 HOURS	48 HOURS	24 HOURS	48 HOURS	24 HOURS	48 HOURS	24 HOURS	48 HOURS	24 HOURS	48 HOURS	24 HOURS	48 HOURS
1.	10^{-2}(I)	+++	+++	-	+	++	++	-	+	-	-	-	-
2.	10^{-2}(II)	+++	+++	-	-	++	+++	-	+	-	-	-	-
3.	10^{-2}(III)	+++	+++	+	+	+	+++	++	+++	-	-	+	+
4.	10^{-4}(I)	+++	+++	+	++	+++	+++	-	-	-	-	+	+
5.	10^{-6}(I)	+++	+++	+	+	+	++	+	+	-	-	+	+
6.	10^{-6}(III)	+++	+++	+	+	++	++	-	-	-	-	+	+
7.	10^{-8}(II)	+++	+++	+	++	++	+++	++	++	-	-	+	+

Table 2: Carbohydrate Fermentation Test

+	Less	Acid/Gas production
++	Medium	Acid/Gas production
+++	High	Acid/Gas production
-	Negative	Acid/Gas production

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TRIPLE SUGAR IRON AGAR TEST						
S.NO.	TUBE NO.	ACIDIC		ALKALINE		H_2S
		BUTT	SLANT	BUTT	SLANT	
1.	10^{-2}(I)	+	-	-	+++	-
2.	10^{-2}(II)	+++	+	-	+	-
3.	10^{-2}(III)	++	++	-	+	-
4.	10^{-4}(I)	+++	-	-	+++	-
5.	10^{-6}(I)	-	-	+++	+++	-
6.	10^{-6}(III)	-	-	+++	+++	-
7.	10^{-8}(II)	+++	-	-	+++	-

Table 3: Triple Sugar Iron Agar Test (TSI)

Acidic butt	Alkaline butt	Acidic Slant	Alkaline Slant	H_2S Production
Yellow colour	Pink colour	Yellow colour	Pink colour	Black line in the butt

IMViC TEST					
S.NO.	COLONIES	INDOLE TEST	MR TEST	VP TEST	CITRATE TEST
1.	10^{-2}(I)	-	-	-	+
2.	10^{-2}(II)	+	+	-	+
3.	10^{-2}(III)	-	-	-	-
4.	10^{-4}(I)	+	+	-	+
5.	10^{-6}(I)	+	+	-	+
6.	10^{-6}(III)	-	-	-	-
7.	10^{-8}(II)	+	+	-	-

Table 4: Biochemical Test

ENZYME TEST			
S.NO.	COLONIES	CATALASE TEST	DECARBOXYLASE TEST
1.	10^{-2}(II)	+	+
2.	10^{-4}(I)	+	+
3.	10^{-6}(I)	+	+

Table 5: Enzyme Test

\+ POSITIVE

\- NEGATIVE

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ANTIBACTERIAL ACTIVITY OF AQUEOUS DECOCTION OF SPICES AGAINST E.coli				
S.NO.	AQUEOUS DECOCTION OF SPICES	100% CONCENTRATION OF SPICES	DIAMETER OF ZONE OF INHIBITION	
			STANDARD (IN MM)	IN VITRO (IN CM)
1.	Bay leaf	10µl	12 mm	1.3cm
2.	Black pepper	10µl	10 mm	2 cm
3.	Cinnamon	10µl	-	1.2 cm
4.	Clove	10µl	9.07 mm	-
5.	Star anise	10µl	-	-

Table 6: Screening of Crude extract of Spices by Disc Diffusion Method

Figure 1: Bacterial Colonies on Nutrient Agar Plates

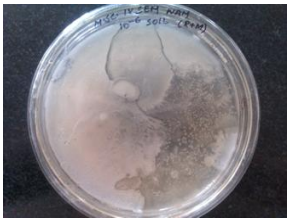
10^{-2} Dilution

No. of colonies: 03

10^{-4} Dilution

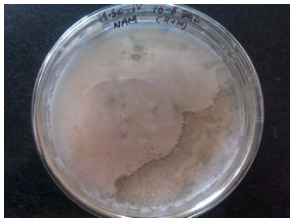
No. of colonies: 01

10^{-6} Dilution



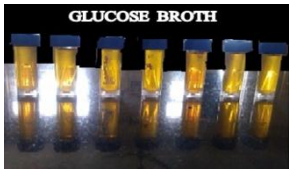
No. of colonies: 02

10^{-8} Dilution



No. of colonies: 01

Figure2: Carbohydrate Fermentation Test



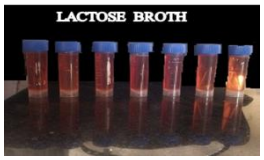


Figure3: Triple Sugar Iron Agar Test (TSI)

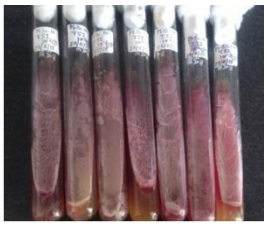


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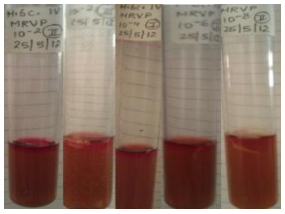
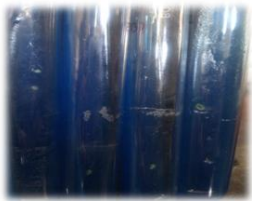
Figure 4: IMViC Test

Indole Test

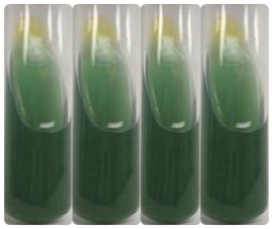


Methyl Red Test

Voges Proskauer Test



Citrate test- positive (blue colour)

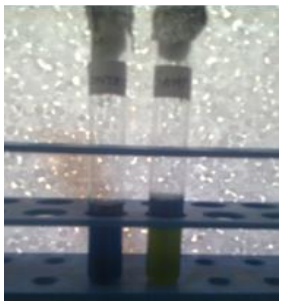


Citrate Test- Negative

Figure 5 : Enzyme test

E.coli colonies on TSA media

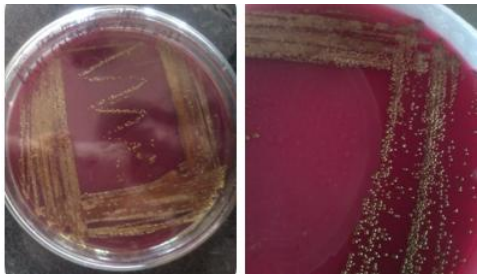
Catalase Test- Positive



Decarboxylase Test-Positive

Figure 6: E.coli Culture maintained on Nutrient Agar Media, Endo Agar Media, and Hi- Chrome Media

E.coli Culture on Nutrient Agar Media Slants



E.coli Culture on Endo Agar Media plates

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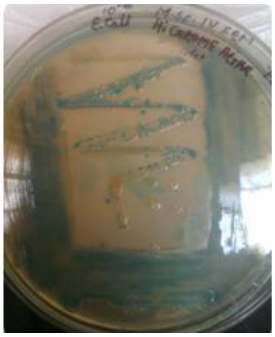
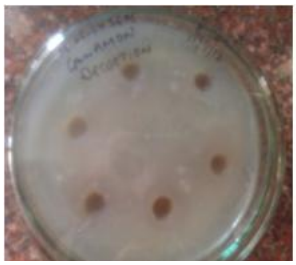


E.coli Culture on Hi-Chrome Media Plates

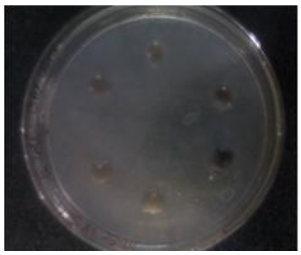
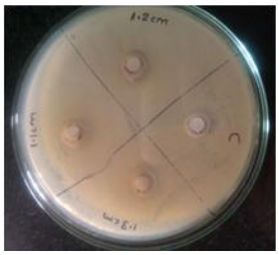
E.coli Culture on Hi-Chrome Media and Endo Agar Media Slants

Figure 7: Agar Disc Diffusion Method

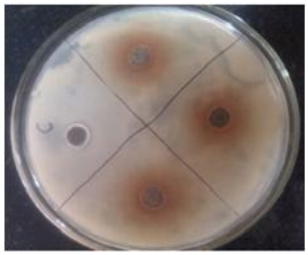


Zone of Inhibition was Observed on Cinnamon extract plate

Zone of Inhibition was Observed on Black pepper extract plate

Zone of Inhibition was Observed on Bay leaf extract plate



No zone of inhibition was Observed on Clove extract plate

No zone of inhibition was Observed on Star Anise extract plate

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3. Results

The isolated E.coli bacteria were highly sensitive at 100% concentration towards Bay leaf, Black pepper, and Cinnamon and resistance towards clove and star anise. The result obtained by in vitro Study of Antibacterial Activity of Spices was different from the standard result.

4. Conclusion

The most promising spice was black pepper. The results showed that the aqueous decoction of black pepper possesses great antibacterial potential (75%) as compared to aqueous decoction of bay leaf (53.4%) and star anise (18.1%).

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