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Research Article

MORPHO-MOLECULAR CHARACTERIZATION OF *ESCHERICHIA COLI* AND ITS REGULATION USING NATURAL SPICES

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ABSTRACT

In present study, the effect of *E. coli* against different natural species were evaluated by using Kirby Bauer and the efficacy of the different natural spices against *Escherichia coli* was performed. The isolated *E. coli* strain from sewage sample which was collected from drain of Pension Mohalla located at second cross, Shivamogga town, Shivamogga district was identified based on its morphological and biochemical characters and confirmed by genomic characterization using 16s rRNA gene sequencing method and deposited to GenBank, NCBI, confirmed as *Escherichia coli* strain KUMBSRNGBT-113, and allocated with accession number PQ197716. Antibiotic susceptibility of different antibiotics was tested against isolated *E. coli* and standard *E. coli* by using Kirby Bauer method. Based on zone of inhibition, the isolated *E. coli* shows highest susceptible to cefpodoxime, (30 mm), Ciprofloxacin (28mm), Streptomycin (26mm) and Chloramphenicol (25 mm) and the isolated culture was resistant to Bacitracin, Amoxicillin, Erythromycin, Vancomycin, Ampicillin and Rifampicin. The antibacterial potency of aqueous extracts of different spices were tested against isolated and standard *E. coli* cultures by Agar well diffusion assay. Chloramphenicol was used as the standard antibiotic. The aqueous extract of some spices has shown antibacterial effects against the isolated *Escherichia coli*, Clove extract has shown highest zone of inhibition which can be described as highly effective. Dried ginger extract, Cinnamon extract, bay leaf extract has shown moderate zone of inhibition whereas Black pepper extract, coriander seeds extract and pandan leaves has shown slightly better zone of inhibition.

Keywords: *Escherichia coli*, Antibiotic susceptibility, Kirby Bauer method, Aqueous extract, Antibacterial effect.

INTRODUCTION

Microorganisms are fundamental to life on earth, playing roles in ecological processes, human health, and environmental sustainability. Their omnipresence in the environment, from the deepest ocean trenches to highest mountain peaks, underscores their adaptivity and significance. This essay explores the diverse habitats, functions, and impacts of microorganism in the environment, emphasizing their indispensable role in maintaining ecological balance. Microorganisms, including bacteria, archaea, fungi, viruses and protozoa are found in virtually every environment. Microorganism play crucial role in sustaining life on earth, impacting various ecological, industrial and medical processes.

Microorganisms play a crucial role in ecosystems, nutrient cycling, and industry by decomposing organic matter and recycling elements like carbon, nitrogen and sulfur. They contribute to soil fertility and are used in the production of antibiotics, enzymes, biofuels, and fermented food. In medicine, microorganisms are pathogens and essential components of human health, contributing to digestion, immune system function, and protection against pathogens. Advances in microbiology have led to the development of vaccines, antibiotics and probiotics, enhancing public health.

German physician Theodor Escherich (1857–1911) first isolated and identified the bacteria *Escherichia coli* in 1885. He isolated it from infants' faeces. Gram-negative, non-sporulating, rod-shaped, facultative anaerobe. *E. coli bacteria* belong to the genus *Escherichia* and are frequently

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found in food, the environment and the lower digestive tracts of warm-blooded animals. It is the most researched prokaryotic model organism in the fields of microbiology and biotechnology. Gram-negative, rod-shaped, straight, non-sporing, non-acid fast *Bacilli*.

Typically, cells have a rod-like form and measure 1-3 μm × 0.4-0.7 μm (micrometre). They have peritrichous flagellar arrangement, hence it is motile and very few strains are not. Since 37 °C is the ideal temperature for *E. coli* growth, but many lab strains may survive at as high as 49°C. When conditions are right, it can replicate in as little as 20 minutes. It includes both motile and non-motile fibriated strains. Negative staining techniques result in a brilliant halo over a dark *Escherichia coli* backdrop, making the *E. coli capsules* visible. They only have one or two peptidoglycan layers in their thin cell wall (Basavaraju *et al*., 2022).

Escherichia coli is a gram-negative facultative anaerobe which acts as harmless commensals gastrointestinal system to dangerous strains that cause death in people and animals. In humans, *E. coli is* toxin producing and divided into different pathotypes based on the presence of virulence factors, such as enterotoxigenic, enteroaggregative, Shiga, enteropathogenic, diffusely adherent and enteroinvasive. In 1977, *E. coli* O157:H7 bacteria generating Shiga toxin were isolated from cattle in Argentina and designated as *E. coli* O157:H7. Shiga toxin-producing *E. coli* O157:H7 is responsible for many outbreaks and occasional outbreaks (Hemorheagic Uremic Syndrome). However, non-O157 strains can cause serious sickness in humans. Several cases of bloody diarrhea caused by *E. coli* O157:H7 have been reported in the US, Canada, and Japan. In 2011, Germany and other European countries saw an unusual outbreak caused by an extremely lethal *E. coli strain* containing Shiga toxin acquired through gene transfer. Around 100 *E. coli serotypes* that produce Shiga toxins have been identified (Bano *et al*., 2019, Gugsa *et al*., 2022)

Escherichia coli is responsible for 90% of community-acquired and nearly 50% of nosocomial UTIs in both inpatient and outpatient settings. Treatment involves antimicrobial medications like amoxicillin, cephalexin, clarithromycin, ciprofloxacin, gentamicin, nalidixic acid, nitrofurantoin, norfloxacin, and tetracycline's. However, issues arise when bacteria develop resistance to antibiotics during an infection. This resistance can occur through mutations, plasmid transfer, stress response system stimulation, horizontal gene transfer, transposition, or uptake of external DNA. Resistant microorganisms are becoming more common in hospitals. Treatment failure, which can have serious implications, especially for critically ill patients (Rabbee *et al*., 2016, Tabbasum *et al*., 2024).

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MATERIALS AND METHODS

In the present comparative study, the isolation, identification, biochemical and molecular characterization of *Escherichia coli* and antibiotic susceptibility, antimicrobial activity against selected spices were tested by agar well diffusion and disc diffusion technique.

Collection, isolation and identification of samples

The sewage sample was collected from drain of Pension mohalla second cross of Shivamogga town, Shivamogga district. The sewage water sample was collected and stored in sterile screw capped containers and transported to lab. The collected water samples were serial diluted in physiological saline up to 10^{-9} dilutions and $10^{-5,}$ 10^{-6} and 10^{-7} dilutions were plated on eosin methylene blue agar plates through spread plate method. EMB agar plates were incubated at 37 ºC for 24 hours. The characteristic green metallic sheen growth of colonies is a presumptive identification for *E. coli*. Colony morphology and color on EMB agar plates together with the Gram-stain and KOH test was used as an initial identification for *E. coli* colonies.

Cultural characteristics of E. coli

E. coli can be grown on a solid or in a liquid growth medium under laboratory conditions. A basic media for *E. coli*, requires glucose or other sugars as a carbon and energy source, ammonium salts as a nitrogen source, other salts, and trace elements. As *E. coli* have simple nutritional requirements it was cultured on a different medium, such as Nutrient agar, MacConkey agar, EMB agar, Blood agar, Endo agar, Muller Hinton agar, Voilet red bile agar and in Nutrient broth to study colony morphology on different agar plates.

Biochemical Characterization

The selected isolates were subjected for biochemical tests as mentioned by Bergey's Manual of Systematic bacteriology. *E. coli* isolates can be confirmed biochemically by the use of a traditional method called IMViC tests. This is a set of four tests that are used to differentiate members of the family *Enterobacteriaceae*. IMViC (Indole, Methyl red, Voges-Proskauer and Citrate utilization tests), Catalase test and Oxidase test.

Genomic characterization

20 μg/μL of extracted genomic DNA was used for polymerase chain reaction (PCR) amplification, qualified through gel electrophoresis using the Chromous Biotech gDNA minispin kit and CTAB methodologies, while the 16S rRNA gene was amplified using PCR. The

amplification of the sequence was performed utilizing universal primers, namely 27F (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and 1492R (5′-AAG GAG GTG ATC CAG CCG C-3′). The amplification cycles were modified, with an initial denaturation at 94 °C for 5 min. This was followed by thirty cycles at 94 °C for synthesis, with a final extension step consisting of one minute at 55 °C, one minute at 72 °C and a seven-minute incubation at 72 °C for band validation. The amplified result has undergone analysis using agarose gel electrophoresis with a concentration of 1.2% w/v. Subsequently, staining was carried out using ethidium bromide. The gel documentation equipment was employed to document the bands, while the gel extraction kit was utilized to purify the PCR result. The obtained 16S rRNA sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI). Pairwise alignment was conducted using ClustalW and the resulting alignment was used to construct a phylogenetic tree constructed in RAxML (Randomized Axelerated Maximum Likelihood) using maximum likelihood analysis. The phylogenetic tree was analyzed using FigTree (v1.4.4) software, with a bootstrapping value 100 (Tamura *et al*., 2011).

Antibiotic sensitivity of *Escherichia coli* using Kirby-Bauer method

A broth culture was chosen for testing, diluted to the 0.5 McFarland standard. Before inoculating the culture to a petri plate, the surface and sides were examined to ensure no visible colonies or water. If there were colonies, the plate was discarded. The bacteria were resuspended using a sterile cotton swab, and excess liquid was removed. The Mueller-Hinton agar plate was inoculated in three directions to ensure even spreading. The plate was allowed to absorb the liquid for 2 to 3 minutes, then filter paper antibiotic discs were placed onto the surface. Five different discs fit in a 100-mm petri plate, and the discs were tapped onto the surface using a disinfected forceps. The plate was labeled, incubated at 37 ºC for 24 hours, and the results were observed.

Evaluation of antibacterial efficiency of different spices against *Escherichia coli*

Fresh spices samples were collected from Gandhi bazar of Shivamogga. 10 gram of fine powder of each spices were boiled in 100 mL of water. Allow to boil for 10-15 minutes. After cooling the filtrate was collected. The nutrient broth media was prepared and the test microorganism is grown in a broth medium and incubated overnight at 37°C for 24 hours. The bacterial inoculum is adjusted to a standardized concentration, typically 0.5 McFarland standard. A sterile swab is dipped into the microbial suspension and spread uniformly over the solidified agar plate. A sterile cork borer is used to create wells into the agar surface, spaced apart to prevent overlapping zones of inhibition. A specific volume, typically 50-100 µL, is pipetted into each well, including a positive control (known antibiotic) and a negative control (sterile water or solvent). The plates were allowed to stand for 15-30 minutes to allow the test substance to diffuse into the agar (Yadav *et al*., 2022).

RESULTS AND DISCUSSION

The sewage sample was collected from drainage of pension mohalla 2nd cross Shivamogga. The sample was serially diluted and plated on EMB agar plate and observed for green metallic sheen colonies. 8 green sheen-colored colonies were observed in 10^{-6} dilution. These colonies were further sub cultured in EMB plates to obtain pure culture.

Figure 1. Collection of sewage water sample.

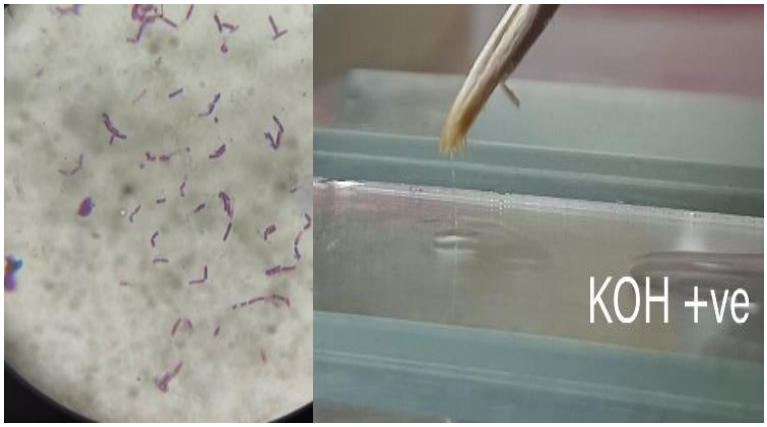


Figure 2. Identification of bacteria using different culture characters. **a.** Grams staining and **b.** KOH test.

Gram's staining

Pure cultures further subjected to morphological identification by gram staining the isolates. On staining isolates were identified as Gram-negative bacilli in 100X magnification.

KOH test

A loopful of isolate was mixed with 3% KOH to observe sticky thread like appearance which confirms the gram-negative cell wall.

Cultural Characteristics of *Escherichia coli*

***E. coli* on Nutrient agar**

E. Coli, on nutrient agar, formed large, thick, greyish white, moist, smooth, opaque, or translucent discs like colonies as shown in. The smooth forms (s) of colonies seen in fresh isolation are easily emulsifiable in saline.

***E.* coli on Blood agar**

E. coli on Blood agar formed grey colony without forming haemolysis. This signifies that the isolated *E. coli* is non-pathogenic in nature.

***E.* coli on MacConkey agar**

E. coli on MacConkey formed pink in color colony due to lactose fermentation, which helps in distinguishing *E. coli* from other non-lactose fermenting bacteria. Non–lactose fermenters and produce colorless colonies on MacConkey agar media.

***E.* coli on Eosin methylene blue agar**

E. coli grown on EMB agar shows a green metallic sheen, which is due to the metachromatic property of dyes and the lactose fermenting property of *E. coli*, which changes the pH of the medium to acidic. Hence, making the medium more selective for *E. coli* makes the identification much easier.

***E.* coli on Violet red bile Agar**

Violet red bile agar (VRBA) is a selective medium used to detect and enumerate lactose-fermenting coliform. Lactose-fermenting microorganisms produce pink to red colonies.

Figure 3. Colony characteristics of *E.coli*.

Table 1. Biochemical characterization of *E. coli*.

Biochemical tests	Result
Catalase	Positive
Oxidase	Negative
Indole	Positive
Methyl red	Positive
Voges Proskauer	Negative
Citrate Utilization test	Negative

Biochemical Characterization of *Escherichia coli*

Isolated and Pure cultured microscopically identified *E. coli* was subjected to various biochemical tests. The isolate showed positive results for catalase, indole, methyl red and negative results for oxidase, voges proskauer and Citrate utilization test (Table 1).

Genomic characterization

The isolated *E. coli* was confirmed by 16S rRNA gene sequence analysis. The sequence of 16S rRNA gene of the isolate was amplified using purified genomic DNA and sequenced. The gene sequence was aligned using pair-wise alignment and it shows highest (93%) similarity with *Escherichia coli* strain KUMBSRNGBT-113, the nucleotide sequence was deposited to NCBI database and the sequence was allocated with accession no: PQ197716. The analysis was performed using phylogenetic tree using similar sequence selected from the database by neighbour-joining method is shown in Figure 4 these findings are compared with the earlier results of Clark *et al*., 2016.

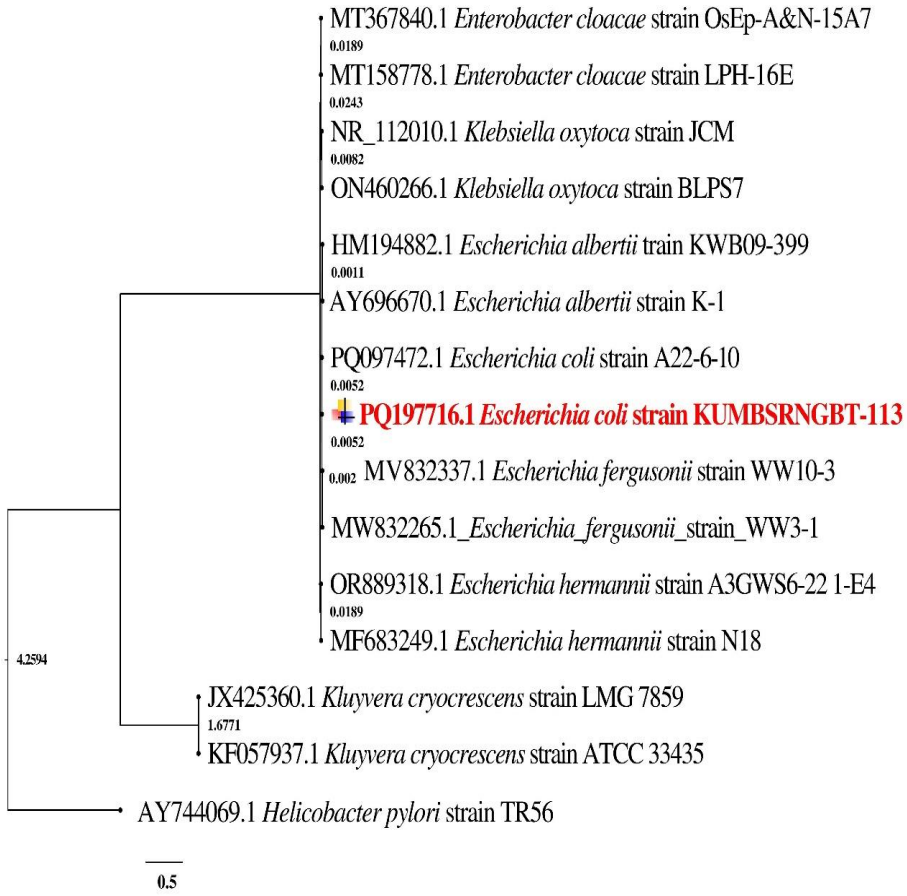


Figure 4. Phylogenetic tree of *E. coli* KUMBSRNGBT-33 isolate with reference strains using neighbour-joining method with bootstrap value of 100.

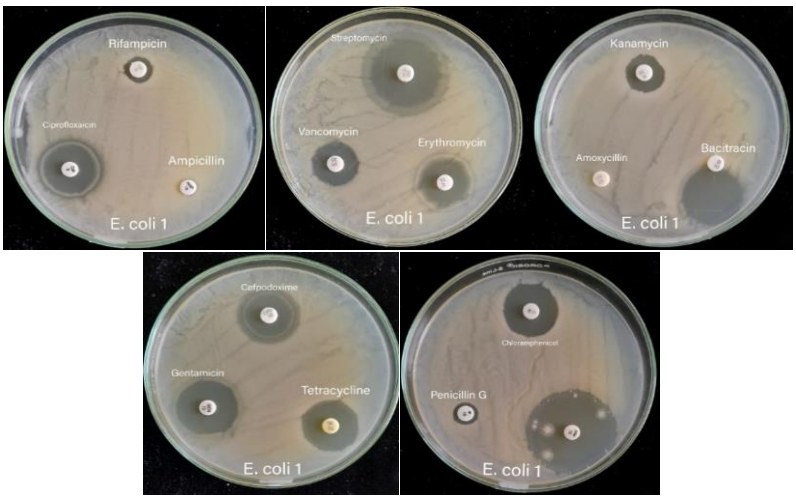
Antibiotic sensitivity of the Escherichia coli using Kirby-Bauer method

Antibiotic sensitivity of different antibiotics were tested against isolated *E. coli* and standard *E. coli* culture by Kirby Bauer method. The bacterial potency was shown in the form of zone of inhibition. The results are depicted in table 2

Table 2. Antibiotic susceptibility test by using Kirby-Bauer method.

Antibiotics	Zone of inhibition [in mm]			
	E. coli -1	Susceptibility/ Resistance	*E. coli* -2	Susceptibility/ Resistance
Cefpodoxime,	23	Susceptible	30	Susceptible
Gentamycin	24	Susceptible	22	Susceptible
Tetracycline	22	Susceptible	18	Susceptible
Chloramphenicol	21	Susceptible	25	Susceptible
Penicillin G	9	Susceptible	15	Susceptible
Impeniem	35	Susceptible	15	Susceptible
Rifampicin	11	Susceptible	0	Resistant
Ciprofloxacin	45	Susceptible	28	Susceptible
Ampicillin	0	Resistant	0	Resistant
Streptomycin	32	Susceptible	26	Susceptible
Vancomycin	17	Susceptible	0	Resistant
Erythromycin	24	Susceptible	0	Resistant
Kanamycin	15	Susceptible	17	Susceptible
Amoxycillin	0	Resistant	0	Resistant
Bacitracin	23	Susceptible	0	Resistant

The results clearly indicates that isolated *E. coli* culture was highly susceptible to cefpodoxime, (30 mm), Ciprofloxacin (28mm), Streptomycin (26mm) and Chloramphenicol (25 mm). Isolated *E. coli* culture was multiresistant culture, it was resistant to Bacitracin, Amoxicillin, Erythromycin, Vancomycin, Ampicillin and Rifampicin. The isolated culture showed susceptibility to Tetracycline antibiotic. These results were previously reported by Bano *et al*., 2019, according to their study, the antibiotic sensitivity test of *E. coli* showed resistant to Tobramycin, Tetracycline and Streptomycin. The isolated *E. coli* culture was susceptible to streptomycin and ciprofloxacin. The obtained results were compared with previous reports of Jennifer *et al*., 2020, according to their result *E. coli* was moderately resistant to cefixime, ciprofloxacin, and ampicillin and resistant to streptomycin. The isolated *E. coli* showed resistant to Ciprofloxacin and Streptomycin. These results were correlated with previous studies of Praveenkumar reddy *et al*., 2020 according to their result the antibiotic sensitivity test of *E. coli* isolated from sewage treatment plants showed resistant to ciprofloxacin, ampicillin, streptomycin, cefazolin, cefepime. The isolated *E. coli* was susceptible to cefpodoxime, ciprofloxacin, and streptomycin. These results were previously reported by Indira *et al*., 2021 according to study the isolated cultures were less susceptible to Amoxycillin and highly susceptible to ciprofloxacin and cefixime. The Isolated *E. coli* is fairly susceptible to Penicillin. In the previously conducted studies of Kumar *et al*., 2022, the antibiotic sensitivity of *E. coli* strains showed highest level of resistance to amoxicillin and Penicillin.



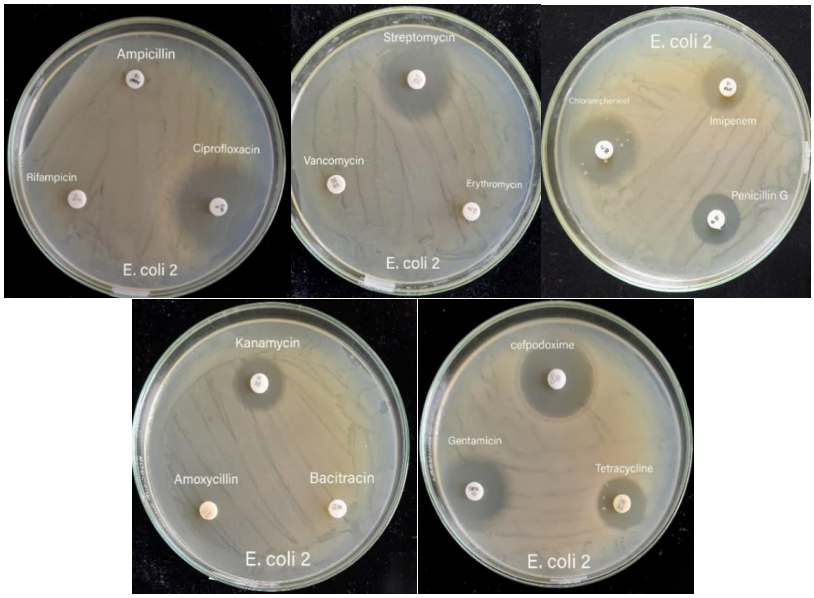


Figure 5 .Antibiotic susceptibility of *E. coli* 1 and *E. coli* 2 isolates by using Kirby-Bauer method.

Evaluation of antibacterial efficiency of different spices against *Escherichia coli*

The antibacterial potency of aqueous extracts of different spices was tested against isolated and standard *E. coli* cultures through Agar well diffusion assay. Chloramphenicol was used as the standard antibiotic. The antibacterial efficiency was shown in the form of zone of inhibition surrounding the well. Lists of spices used and results are depicted in Table 3.

Table 3. List of spices .

Name of the spice	Vernacular name	Family	Scientific name
Cinnamon	Daalcheene,	*Lauraceae*	*Cinnamomum verum*
Cloves	Lavanga, Laung	Myrtaceae	*Syzygium aromaticum*
Black pepper	Kalu menasu,	Piperaceae	*Piper nigrum*
Star anise	Chakra moggu,	Schisandraceae	*Illicum verum*
Fennel	Saunf,	Apiaceae	*Foeniculum vulgare*
Black stone flower	Kallu huvvu, Dagar phool	Parmeliaceae	*Parmotrema perlatum*
Bay leaf	Pallav ele,	Lauraceae	*Laurus nobilis*
Cumin	Jeera, Jeerige	Apiaceae	*Cuminum cyminum*
Coriander seeds	Dhaniya ,	Apiaceae	*Coriandrum sativum*
Cardamom	Elaaichi,	Zingiberaceae	*Elettaria cardamomum*
Nut meg	Jayi kai,	Myristicaceae	*Myristica fragrans*
Asafoetida	Hing , Ingu	Apiaceae	*Ferula asafoetida*
Mint	Pudina	Lamiaceae	*Mentha.piperita*
Ginger	Shunti , Adrag	Zingiberaceae	*Zingiber officinale*
Garlic	Lassan, Belulli	Amaryllidaceae	*Allium sativum*
Mace	Jaivitri Jaipatre	Myristicaceae	*Myristica fragrans*
Long pepper	Hipalli, Pipalli	*Piperaceae*	*Piper longum*
Dried red chilli	Ona menasinakai,	Solanaceae	*Capsicum annuum*
Caraway seeds	Shajeerige,	*Apiaceae*	*Carum carvi*
Sweet flag	Ghorbach, Baje,	Acoraceae	*Acorus calamus*
Turmeric	Arishina, haldi	Zingiberaceae	*Curcuma longa*
Mustard	Savive , sarso	Brassicaceae	*Brassica juncea*
Fenugreek	Menthya, methi	Fabaceae	*Trigonella foenum-graecum*

Purple Flebane	Kali jeera	Asteraceae	*Centratherum Anthelminticum*
Carom seed	Ajwain, Ajowan	Apiaceae	*Trachyspermum ammi*
Dried ginger	Onna shunti	Zingiberaceae	*Zingiber officinale*
Kapok buds	Semul,	Malvaceae	*Ceiba pentandra*
Black Cardamom	Badi ealichi,	Zingiberaceae	*Elettaria cardamomum*
Pandan Leaves	Rambha, Rampe	Pandanaceae	*Pandanus amaryllifolius*

The antimicrobial potency of different spices against different bacteria including *E. coli* was done by using agar well diffusion assay, the turmeric and ginger showed 11mm & 13mm zone of inhibition respectively.

The water extract of ginger and black pepper showed 13mm and 11mm zone of inhibition. The obtained results were compared with previous studies of Chaturvedi *et al*., 2021 according to their study, the ethanolic extract of ginger and black pepper showed no zone of inhibition which was done by using agar well diffusion assay.

The water extract of Clove showed better zone of inhibition than methanolic extract (18mm). These results were correlated with earlier findings of Jadhav *et al*., 2022, according to their study the methanolic extract of clove showed less zone of inhibition (17mm).

On comparison to aqueous extract of Bay leaf (14mm) and turmeric (11mm) showed better zone of inhibition when compared to methonolic extract (7mm, 8mm respectively). In the previously conducted study by Yadav *et al*., 2022, Bay leaf and turmeric showed less zone of inhibition by using agar well diffusion assay.

The water extract showed better zone of inhibition than the essential oils. On comparison to our study water extract of Clove (18mm) and bay leaf (14mm) showed better zone of inhibition and highly susceptible. In the earlier findings of Raut *et al*., 2023, the Clove and bay leaf showed less zone of inhibition and less susceptible (14mm, 6mm respectively) was shown in Table 4a, b & c and figure 6a, b & c respectively.

Table 4a. Antibacterial activity of water extract of Spices against *Escherichia coli.*

Spices	Zone of inhibition in millimeter			
	E. coli -1	Susceptible/Resistant	*E. coli*-2	Susceptible/ Resistant
Cinnamon	14	Susceptible	13	Susceptible
Cloves	19	Susceptible	18	Susceptible
Black pepper	12	Susceptible	11	Susceptible
Star anise	10	Susceptible	10	Susceptible
Fennel	13	Susceptible	11	Susceptible
Black stone flower	08	Susceptible	0	Resistant
Bay leaf	14	Susceptible	14	Susceptible
Cumin	9	Susceptible	7	Susceptible
Coriander seeds	9	Susceptible	11	Susceptible
Cardamom	7	Susceptible	0	Resistant
Control	25	susceptible	29	Susceptible

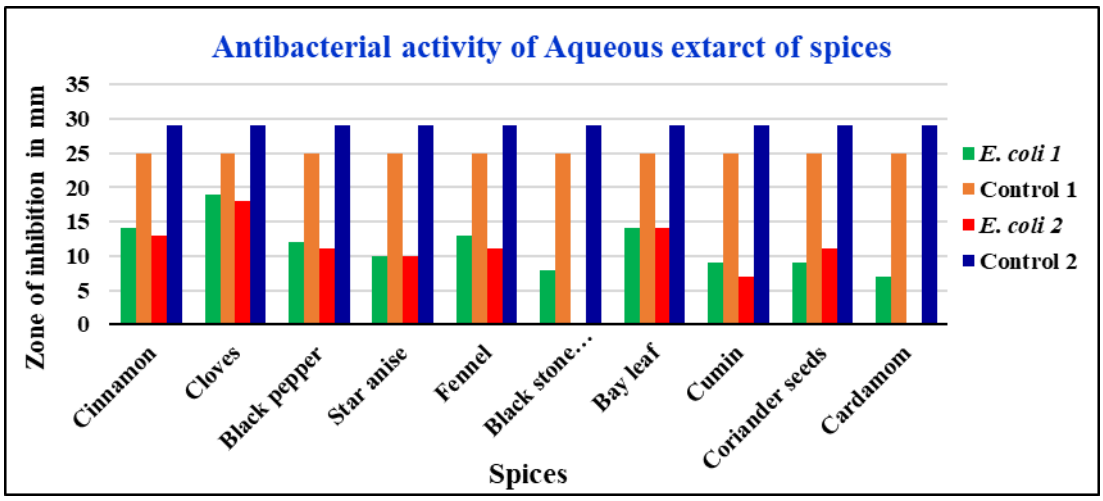


Figure 6a. Antibacterial activity of aqueous extract of spices.

Table 4b. Antibacterial activity of water extract of Spices against *Escherichia coli*.

Nut meg	7	Susceptible	0	Resistant
Asafoetida	12	Susceptible	9	Susceptible
Mint	10	Susceptible	8	Susceptible
Ginger	11	Susceptible	13	Susceptible
Garlic	9	Susceptible	9	Susceptible
Mace	10	Susceptible	0	Resistant
Long pepper	10	Susceptible	9	Susceptible
Byadagi Dried red chilli	7	Susceptible	8	Susceptible
Guntur red Chilli	9	Susceptible	9	Susceptible
Caraway seeds	12	Susceptible	11	Susceptible
Control	25	susceptible	29	Susceptible

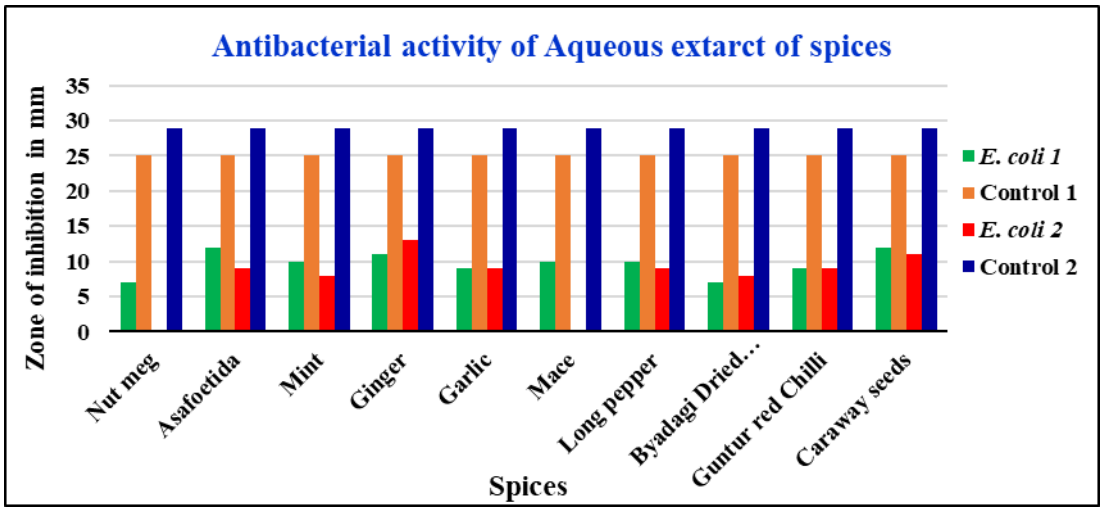


Figure 6b. Antibacterial activity of aqueous extract of spices.

Table 4c. Antibacterial activity of water extract of Spices against *Escherichia coli.*

Sweet flag	7	Susceptible	8	Susceptible
Turmeric	13	Susceptible	11	Susceptible
Mustard	9	Susceptible	9	Susceptible
Fenugreek	10	Susceptible	8	Susceptible
Purple Flebane	9	Susceptible	7	Susceptible
Carom seed	8	Susceptible	10	Susceptible
Dried ginger	8	Susceptible	8	Susceptible
Kapok buds	11	Susceptible	12	Susceptible
Black Cardamom	8	Susceptible	8	Susceptible
Pandan Leaves	8	Susceptible	11	Susceptible
Control	25	susceptible	29	Susceptible

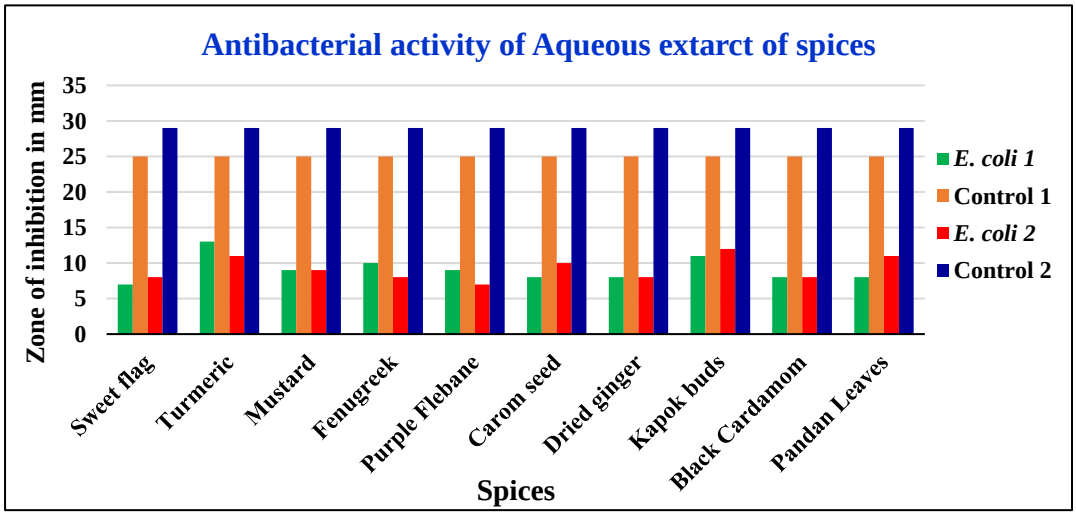


Figure 6c. Antibacterial activity of aqueous extract of spices.

CONCLUSION

In the present study we aimed to isolate *Escherichia coli* from sewage sample. Susceptibility of different antibiotics was measured by using standard method of Kirby Bauer and the susceptibility was measured to find the isolated *E. coli* was multi drug resistant. Susceptibility of water extract of different spices was also measured to find the alternative against highly increasing antibiotic resistance. Result was instrumental to find that spices were effective against multidrug resistance *E. coli.* Further research on the antibacterial activities of extracts of Cloves, Dried ginger, Cinnamon using different extraction methods against multidrug resistance *E. coli.* This is important to establish whether the extract has broad spectrum activity. Research needs to be done on isolation and formulation of active compounds present in the spices so it can be used as antimicrobial drugs when required.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICS APPROVAL

Not applicable

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