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Screening of Antimicrobial Activity of *Murraya koenigii* Leaf Extracts Against Pathogenic Bacterial Strains *Staphylococcus aureus* and *Escherichia coli* Isolated from Contaminated Water

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: *Murraya koenigii* is a widely used plant both as a potential medicinal agent and also for common cooking purposes. Aim of this present study was to determine the antimicrobial activity of *Murraya koenigii* leaf extracts on *Staphylococcus aureus* and *Escherichia coli.*

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Study Design: Screening and isolation of pathogenic bacterial strains from contaminated water. Preparation of *Murraya koenigii* leaf extracts using petroleum ether, acetone and ethyl acetate by using serial extraction method with Soxhlet apparatus.
Place and Duration of Study: Department of microbiology, Agro biotec research centre Ltd, Poovanthuruthu, Kottayam, Kerala, India, between 2014 January to 2014 May.
Methodology: Staphylococcus aureus and Escherichia coli were the bacterial strains used in this study. Morphological and biochemical analysis of microorganisms were conducted to identify the strains. Leaf extracts (petroleum ether, acetone and ethyl acetate) of *Murraya koenigii* were screened using MHA disc diffusion methods.
Results: Various concentration of plant extracts were used to check its activity against isolated pathogens. Acetone extract of curry leaves exhibit maximum zone of inhibition against Staphylococcus aureus and petroleum ether extracts shown maximum inhibition against Escherichia coli.

Keywords: Murraya koenigii; antimicrobial activity; Staphylococcus aureus; Escherichia coli.

1. INTRODUCTION

Murraya koenigii known as curry leaves is widely used as an essential ingredient in Indian traditional cooking system. The special aromatic oil present in the curry leaves make it as a special ingredient in food preparations. Curry leaves have a pungent, bitter and small acidic taste. In its dry form they also retains its flavour and qualities. Traditionally curry leaves are used in Ayurveda and Unani medications [1]. "Phytochemical analysis revealed that the curry leaves contains proteins, carbohydrate, fibre, minerals, carotene, nicotinic acid, Vitamin C, Vitamin A, calcium and oxalic acid. Moreover it having crystalline glycosides, carbazole alkaloids, koenigin, girinimbin, iso-mahanimbin, koenine, koenidine and koenimbine, cyclomahanimbine, tetrahydromahanimbine, Murrayastine, murrayaline and pyrayafoline carbazole alkaloids. Many of these secondary metabolites have been isolated from *Murraya koenigii* leaves" [2].

"Curry leaves have a number of health benefits regardless their use in cooking. Numerous medicinal properties like anti-diabetic, antioxidant, antimicrobial, anti-fungal, anti-inflammatory, anti- carcinogenic and hepato-protective properties can be seen in curry leaves. Many pharmacological characters like its activity on heart, anti-diabetic and cholesterol reducing property, antimicrobial activity, antiulcer activity, antioxidative property, cytotoxic activity, antidiarrheal activity and phagocytic activity make them medically effective. Fresh leaves of *Murraya koenigii* contains volatile oil. Stem bark and roots of *Murraya koenigii* contains carbazole alkaloids and triterpenes which has been isolated from them" [2,3].

Extensive uses of antibiotics are the major reason for the emergence of multi drug resistance in bacteria. With no doubt we can say that there is need for new antibiotics. Widely used antibiotics and other synthetic drugs have many side effects. So as an alternative, herbal medicines are being experimented [4]. Higher plants are a potential source for new anti-microbial agents [5]. 166 selected plant extracts have been evaluated in an effort to find novel medications that effectively treat a variety of illnesses [6]. There have been numerous concerns raised in the literature regarding the antibacterial screening of therapeutic plant extracts [7,8].

Most of the population are vulnerable to diseases caused by pathogenic bacteria present in water. Among the various enteric pathogens, *Escherichia coli* and *Staphylococcus aureus* causes various infections. The main aim of the present study was to screen antimicrobial activity of *Murraya koenigii* leaf extracts against the major pathogenic microorganisms *Staphylococcus aureus* and *Escherichia coli isolated* from contaminated water.

2. MATERIALS AND METHODS

The plant materials used in this study were collected from local farms. Fresh curry leaves were collected and washed properly. It was dried in shade and finely powdered. Extracts were prepared by serial extraction method with soxhlet apparatus by using petroleum ether, acetone and ethyl acetate. Then the extracts were dried using

rotary evaporator and kept in a cool place for further analysis. Contaminated water sample was collected from drainage outlet of industrial area, Poovanthuruthu, Kottayam, Kerala.

2.1 Preparation of MHA

Instant medium MHA (Mueller Hinton agar) is used as bacteriological growth medium. MHA medium was prepared using 15.2g MHA in 400ml of distilled water [9].

2.2 Preparation of Nutrient broth

Preparation of broth required 0.25g of NaCl, 0.05g of beef extract or 0.05g of yeast extract and peptone. All these were added in 50ml of distilled water and mixed well and transferred to test tube and sterilized. For a 24-hour incubation period, a loopful of S. aureus and E. coli were added to the broth. Following the incubation period, a swab was dipped into the S. aureus broth and then swabbed onto the MHA medium. E. coli was used in a similar manner, and both were once more maintained for incubation.

2.3 *In vitro* Assay by Disc Diffusion Method

Disc diffusion method for antimicrobial susceptibility testing was carried out according to the standard method by Bauer et al. 1966 [10] to assess the presence of antibacterial activities of the plant extracts. The bacteria were swabbed across the plate. Filter paper discs made from Whatmann no. 1 filter paper were sterilized and was impregnated with the ethyl acetate, acetone and petroleum ether extracts of curry leaves respectively. The filter paper discs were placed on the surface of the medium.

2.4 Gram Staining

Gram staining involves six important stages. They are: preparation of smear, fixing of the smear by heating, staining with crystal violet for 30- 60sec, treatment with iodine for 1 min, treatment with 95% alcohol, counter staining with safranin [11].

2.5 Motility Test

Hanging drop technique is used. Here the fluid containing microbes is placed in the centre of a thin cover slip. On each of the four corners of the cover slip a tiny droplet of Vaseline is placed. The cavity slide is placed over the slip with the cavity facing downwards. The slide is quickly turned upside down so that the drop cannot run off to one side.

2.6 Culturing

The organisms were cultured in peptone water prepared by adding 2g peptone in 100ml distilled water taken in conical flask and sterilized. Organisms were inoculated and kept for 24hr incubation. Nutrient agar was used for colony characterization and identification. Mac Conkey's agar- a selective medium was used for the culture and isolation of gram negative lactose fermenting bacteria. Differential media like blood agar, eosin-methylene blue agar (EMBA), and mannitol salt agar (MSA) were used. Gram positive organism *S. aureus* and gram negative *E. coli* were isolated using these media. *S. aureus* was plated on blood agar. Eosin-methylene blue agar (EMBA) was used for *E. coli* culture. Mannitol salt agar (MSA) was used for the selective isolation of presumptive pathogen *Staphylococci* [12].

2.7 Biochemical Analysis

2.7.1 Catalase test

When performing the Catalase test, a drop of hydrogen peroxide was dropped onto the microscope slide with bacterial smear.

2.7.2 Urease test

The surface of urea agar slant was streaked with a portion of well isolated colony. The cap was left slightly loose and the test tube was incubated at 35 in ambient air for 48hrs to 7 days.

2.7.3 Triple Sugar Iron test

"The triple sugar iron or TSI test - a microbiological test roughly named for its ability to test microorganism's ability to ferment sugars and to produce hydrogen sulphide. Using a straight inoculation needle, a well-isolated colony was separated and inoculated into TSI by stabbing through the centre of the medium to the bottom of the tube , then streaked the surface of the agar slant and incubated the tube at 35°-37°C in ambient air for 18 to 24 hours with the cap left loosely on it" [13].

2.7.4 IMViC test

2.7.4.1 Indole test (tryptone broth)

A loopful of bacteria was inoculated into tryptone broth and incubated for 48hrs. After incubation a few drops of indole reagent was added to the broth culture.

2.7.4.2 Methyl red test (MRVP broth)

Inoculated a loopful of bacteria into MRVP broth and incubated for 3 to 5 days. After incubation 1ml of broth was removed and placed into a sterile tube before performing the methyl red as the same broth was used for VP test. 3-4 drops of methyl red was added to original broth.

2.7.4.3 Voges- proskauer test

A loopful of bacteria was inoculated into MRVP broth and incubated for 3-5 days. After incubation, 0.6 ml Barrit's reagent A (VP A) with 5% α- naphthol and 0.2 ml Barrit's reagent B (VP B) with 40% KOH was added to the culture from the MRVP broth and was shaken well [14].

2.7.4.4 Citrate test (Simmon's citrate slant)

Bacteria was streaked onto citrate agar slant and incubated for 24 to 48 hrs with a loose cap.

2.7.4.5 Carbohydrate utilization test

With a sterile inoculating loop, a well-isolated colony of sample bacteria was separated and inoculated to the phenol red glucose broth. The tubes were incubated at 35±2°C for 18 to 24 hours and were observed for a colour change of the broth and trapped air bubbles in Durham's tube [15].

3. RESULTS AND DISCUSSION

The antimicrobial efficiency of curry leaves extracts against *Staphylococcus aureus* and *Escherichia coli* were screened using disc diffusion method. Then the following results were obtained.

3.1 Screening of Organisms

From the direct microscopic examination and cultural characteristics of isolated organisms it can be seen that Strain 1 was a gram negative, motile bacteria showing greyish white, moist, smooth, opaque colonies on nutrient agar (Table 1). They formed lactose fermenting pink coloured colonies on Mac Conkey's agar. It produced a green colour metallic sheen on EMB agar. There were no golden yellow colonies on MSA plate and no hemolysis was observed on Blood agar. Strain 2 was a gram positive, non-motile bacteria showing small white colour colonies on nutrient agar and they formed pink coloured colonies on Mac Conkey's agar. No metallic sheen was observed on EMB agar. It produced golden yellow colonies on MSA and showed beta hemolysis on blood agar.

"Mac Conkey's agar with an inclusion of crystal violet and bile salts prevent the growth of gram-positive bacteria and fastidious gram-negative bacteria. The tolerance of gram-negative enteric bacteria to bile is partly a result of the relatively bile-resistant outer membrane, which hides the bile-sensitive cytoplasmic membrane" [16]. "Gram-negative bacteria that ferment lactose cause the pH of the media to drop and neutral red is absorbed by the bacteria, which appear as bright pink to red colonies on the agar. Strong lactose fermenters cause precipitation of the bile salts, resulting in a pink halo in the medium surrounding the colonies. Weak lactose fermenters will appear pink to red with no pink halo in the surrounding medium. Non lactose fermenting bacteria appear colourless on the medium and the agar that surrounds the bacteria remains transparent" [17].

"Eosin-methylene blue agar contains peptone, lactose, sucrose, and the dyes eosin and methylene blue. The dye methylene blue in the medium inhibits the growth of gram-positive bacteria. Eosin responds to change in pH, from colourless to black under acidic conditions. Lactose-fermenting gram-negative bacteria acidify the medium, produce a dark purple complex associated with a green metallic sheen which is an indicator of lactose or sucrose fermentation" [18].

"Mannitol salt agar was used in the isolation of staphylococci. It contains 7.5% sodium chloride which helps selecting bacteria which tolerates high salt concentrations. MSA also distinguishes bacteria based on the ability to ferment the sugar mannitol. Staphylococci withstands the osmotic pressure in the medium.

MSA uses phenol red as a pH indicator in the medium. Upon mannitol fermentation, acid is produced. Resulting in the decrease in pH and the formation of a yellow area surrounding bacterial growth. A non-fermenting bacterium that withstands the high salt concentration would display a red to pink area due to peptone breakdown" [19,20].

"Blood Agar, an enriched medium used to grow fastidious organisms and to differentiate bacteria based on their hemolytic properties. To observe the hemolytic reaction, the plate must be held up to a light source with the light coming from behind. Beta hemolysis (β) is the complete lysis of red blood cells. A clear zone surrounds the colony. Many species of bacteria produce toxic by- products that are capable of destroying red blood cells" [21].

By indole and methyl red confirmatory test it was confirmed that the isolated strain 1 was gram positive bacteria. It showed negative result for Voges - Proskauer, citrate and urease tests (Table 2). It showed acid butt and acid with gas production in TSI test. It shows H_2S production and it is a Catalase negative bacteria. Isolate 2 was MR and VP positive, negative for indole, citrate and urease tests. It does not show H_2S production and it is Catalase positive. Controls were also kept along with the test and the reaction was observed. Table 3 shows that isolate 1 is *Escherichia coli* and isolate 2 is *Staphylococcus aureus.*

Indole Production Test was to determine the ability of microorganisms to decompose the amino acid tryptophan by the enzyme tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. The presence of indole was detected by adding Kovac's reagent composed of paradimethyl aminobenzaldehyde, which produces a cherry red reagent layer.

Methyl Red Test was conducted to determine the ability of microorganism to oxidize glucose with the production and stabilization of high concentrations of acid end products. The pH indicator methyl red detects the presence of large concentrations of acidic products which turns red throughout the tube, indicating of a positive test. A slightly high pH still indicates the presence of acid, but the indicators turn yellow indicating the negative test.

Voges – Proskauer Test determines the ability of bacteria to ferment carbohydrates with the production of non- acidic end products, acetyl methyl carbinol or its reduction products, 2, 3 Butylene glycol from the organic acids. The reagent used in this test, Barrett's reagent, consists of a mixture of alcoholic α-

Table 1. Direct microscopic examination and cultural characteristics of isolated organisms

	Motility	Gram staining	NA	Mac A	EMB	MSA	BA
1	+	-	Grayish white, moist, smooth, opaque colonies	Lactose fermenting pink colored colonies	Green color metallic sheen produced	No golden yellow colony produced	No hemolysis
2	-	+	Small white color colonies	Pink colored colonies	No metallic sheen	Golden yellow color colonies	Beta hemolysis

Table 2. Biochemical tests for isolated pathogens

Isolates	I	MR	VP	C	U	TSI	Sugar fermentation				H_2S	Catalase
							G	L	S	M		
1	+	+	-	-	-	A/AG	AG	AG	V	AG	+	-
2	-	+	+	-	-	A	A	A	A	V	-	+

Table 3. List of microorganisms identified

Sl. No.	Isolates	Organisms
1	Isolate 1	*Escherichia coli*
2	Isolate 2	*Staphylococcus aureus*

naphthol and 40% potassium hydroxide solution. Development of deep rose colour in culture within a minute following the addition of Barrett's reagent indicates the presence of the acetyl methyl carbinol representing a positive result. The absence of rose colouration represents negative result.

Citrate Utilization Test used to determine the ability of a microorganism to utilize citrate as the sole source of carbon and as energy source for the growth and ammonium salt as a sole source of nitrogen. Citrate negative culture will show no growth and the medium will remain green. In the absence of glucose or lactose some microorganisms utilize citrate as a carbon source based on the presence of citrase enzyme that facilitates the transport of citrate in the cell. Citrate gets enzymatically converted to pyruvic acid and carbon dioxide. During this reaction the medium becomes alkaline where CO2 combines with sodium and water to form carbonate. This converts the bromothymol blue indicator in the medium from green to Prussian blue. Bromothymol blue is green when acidic and blue when alkaline. Formation of blue colour constitutes a positive test.

Urease test for determining the ability of microorganism to degrade urea by means of the enzyme urease. The presence of urease is noted when the organism grown in urea agar medium with urea as the substrate and phenol red as the pH indicator. Upon the splitting of urea, the phenol red turns to a deep pink indicating a positive reaction. Failure of deep pink colour indicates negative reaction.

The triple sugar- iron agar test is designed to differentiate carbohydrate fermentation patterns and hydrogen sulphide production by the various intestinal organisms. Carbohydrate fermentation is indicated by gas production and a colour change of the pH indicator, phenol red. Formation of a black precipitate that will blacken the medium in the butt of the tube indicates the production of hydrogen sulphide in the medium.

Catalase test for demonstrating the presence of catalase which mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. Release of bubbles was observed in catalase positive organisms. No bubble formation indicates negative reaction [22].

Table 4 shows the pure extract and the extract eluted in 1ml H_2O, the antimicrobial activity of *Murraya* against *Staphylococcus aureus* is in the order acetone> ethyl acetate> petroleum ether. In the extracts eluted in 2ml and 3ml H_2O, the antimicrobial activity of *Murraya* against *Staphylococcus aureus* is in the order ethyl acetate> acetone> petroleum ether.

The antimicrobial activity of pure extract of *Murraya* against *Escherichia coli* shown in Table 5 is in the order petroleum ether > acetone > ethyl acetate. In the extracts eluted in 1ml and 2ml H_2O, the antimicrobial activity of *Murraya* against E*scherichia coli* is in the order ethyl acetate> acetone >petroleum ether. In the extract eluted in 3ml H_2O, the antimicrobial activity of *Murraya* against E*scherichia coli* is in the order ethyl acetate > acetone>petroleum ether. Among all, the highest zone of inhibition was observed with acetone extract against *Staphylococcus aureus* with a diameter of 18 mm. The second-high inhibition zone was observed with petroleum ether extract against *Escherichia coli* with a diameter of 15mm.

Table 4. Zone of inhibition produced by *Murraya koenigii* against *Staphylococcus aureus*

Solvents	Concentration (ml)	Diameter of zone of inhibition (mm)
Ethyl acetate	Pure extract	17
	Extract eluted in 1ml H_2O	14
	Extract eluted in 2ml H_2O	12
	Extract eluted in 3ml H_2O	7
Acetone	Pure extract	18
	Extract eluted in 1ml H_2O	15
	Extract eluted in 2ml H_2O	12
	Extract eluted in 3ml H_2O	7
Petroleum ether	Pure extract	12
	Extract eluted in 1ml H_2O	8
	Extract eluted in 2ml H_2O	0
	Extract eluted in 3ml H_2O	0

Table 5. Zone of inhibition produced by *Murraya koenigii* against *Escherichia coli*

Solvents	Concentration (ml)	Diameter of zone of inhibition (mm)
Ethyl acetate	Pure extract	9
	Extract eluted in 1ml H_2O	8
	Extract eluted in 2ml H_2O	7
	Extract eluted in 3ml H_2O	6
Acetone	Pure extract	13
	Extract eluted in 1ml H_2O	8
	Extract eluted in 2ml H_2O	7
	Extract eluted in 3ml H_2O	0
Petroleum ether	Pure extract	15
	Extract eluted in 1ml H_2O	7
	Extract eluted in 2ml H_2O	0
	Extract eluted in 3ml H_2O	0

"The curry leaf extract showed high antibacterial activity against E. coli with 15±3 mm zone of inhibition. For fungi the leaf extract showed more anti-fungal activity as in Aspergillus niger with a 14±1mm zone of inhibition" [3]. "Aqueous extract of curry leaves and olive leaves has shown a significant decrease in the blood glucose level in STZ-induced diabetic rats when used" [23]. "From the Gas Chromatography-Mass Spectroscopy of curry leaves the chemical contents in the essential oil were obtained and they inhibited antibiotic resistant bacteria such as *Staphylococcus aureus*; *Pseudomonas aeruginosa; Klebsiella pneumonia; Escherichia coli* and *Streptococcus pyogenes"* [24]. "The ethyl acetate extract of *Murraya koenigii* leaves gave the lowest MIC value of 15.63 µg/mL against *Staphylococcus aureus*, *E.coli* 0157:H7.*V.alginolyticus*, *V.parahaemolyticus* and *Y.enterocolitica"* [25].

4. CONCLUSION

Different solvent extracts of *Murraya koenigii* leaves exhibit a very promising antimicrobial effect against *Staphylococcus aureus* and *Escherichia coli* both isolated from contaminated water. The acetone extracts of curry leaves exhibit a better action against *Staphylococcus aureus* within a lower concentration. Effectiveness of petroleum ether extract against *Staphylococcus aureus* was found to be moderate but it acted as a potential antimicrobial agent against *Escherichia coli.* On other hand, ethyl acetate extract of curry leaves doesn't show any antimicrobial effect against *Staphylococcus aureus* and *Escherichia coli.* The results clearly demonstrate that daily usage of curry leaves is an excellent option to keep away water borne pathogenic microorganisms like *Staphylococcus aureus* and *Escherichia coli.* We are planning to extend our research to screen more pathogenic organisms and find the efficiency of curry leaves against them.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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