Screening of antimicrobial activity of *Murrayakoenigii leaf extracts against* pathogenic bacterial strains *Staphylococcus aureus* and *Escherichia coli isolated from contaminated water*.

ABSTRACT

Aim: *Murrayakoenigii* 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 *Murrayakoenigii* leaf extracts on *Staphylococcus aureus* and *Escherichia coli.*

Study Design: Screening and isolation of pathogenic bacterial strains from contaminated water. Preparation of *Murrayakoenigii* 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.

Methedology:*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 *Murrayakoenigii* 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 exhibited maximum zone of inhibition against *Staphylococcus aureus* and petroleum ether extract showed maximum inhibition against *Escherichia coli.*

Key words:*Murrayakoenigii, antimicrobial activity, Staphylococcus aureus, Escherichia coli*

1. INTRODUCTION

*Murrayakoenigii*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 Ayurvedic and Unani medications [1]. Phytochemical analysis revealed that the curry leaves contains proteins, carbohydrate, fiber, 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 pyrayafolinecarbazole alkaloids. Many of these secondary metabolites have been isolated from *Murrayakoenigii*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 *Murrayakoenigii* contain volatile oil. Stem bark and roots of *Murrayakoenigii* contains carbazole alkaloids and triterpenes which has been isolated from them [2] [3].

Extensive use of antibiotics is 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]. For discovering new drugs effective in treating several diseases, 166 plant extracts of choice have been

Comment [f1]: Delete "pathogenic bacterial strains" and modify the title as "**Screening of antimicrobial activity of *Murrayakoenigii leaf extracts againstStaphylococcus aureus* and *Escherichia coli* isolated from contaminated water"**

Comment [f2]: Correct it as "food ingredient".

Comment [f3]: Delete this word

Comment [f4]: Correct it as "Department of Microbiology, Agro Biotec Research centre Ltd."

Comment [f5]: Correct it as "for six months from January to May, 2014.

Comment [f6]: Correct the spelling

Comment [f7]: Correct it as: were isolated from contaminated water using standard bacteriological method.

Comment [f8]: curry leaf plant

Comment [f9]: Correct it as: in addition to

Comment [f10]: properties

Comment [f11]: medicinally

Comment [f12]: Rephrase the sentence

Comment [f13]: Delete this sentence

UNDER PEER REVIEW

screened [6]. Many concerns about the antibacterial screening of medicinal plant extracts have appeared in the literatures [7] [8].

Comment [f14]: Rephrase and correct the grammatical mistakes

The curry leaf extract showed high antibacterial activity against *E. coli* with 15±3mm 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 [9]. From the Gas Chromatography-Mass Spectroscopy (GCMS) 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* [10]. The ethyl acetate extract of *Murrayakoenigii* 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*[11].

Comment [f15]: Rephrase it scientifically

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. In this study, we are going to screen antimicrobial activity of *Murrayakoenigii* leaf extracts against the major pathogenic microorganisms *Staphylococcus aureus* and *Escherichia coli isolated* from contaminated water.

Comment [f16]: Staphylococcus is not an enteric pathogen

Comment [f17]: Rephrase and articulate scientifically to mention the need and objective of the study.

UNDER PEER REVIEW

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.**???**

Comment [f18]: Reference?

Comment [f19]: Method of isolation of bacteria? - Need description.

2.1 Preparation of MHA

Instant medium MHA (Mueller Hinton agar) is used as bacteriological growth medium. Mueller and Hinton developed Mueller Hinton Agar (MHA) in 1941 for the isolation of pathogenic *Neisseria* species. Nowadays, it is more commonly used for the routine susceptibility testing of non-fastidious microorganism by the Kirby-Bauer disk diffusion technique. MHA medium was prepared using 15.2g MHA in 400ml of distilled water [12].

2.2 Preparation of Nutrient broth

Nutrient media are basic culture media used for maintaining microorganisms and cultivating fastidious organisms. Nutrient broth is one of the several non-selective media useful in routine cultivation of microorganisms. 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. A loopful each of *S.aureus* and *E. coli* were transferred to the broth for incubation. After incubation a swab was dipped in the broth with *S.aureus*and it was swabbed on to the MHA medium. Likewise, it was done with *E. coli* and both were again kept for 24hrs incubation.

Comment [f20]: Temperature?

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 [13] 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. It was impregnated with the compound to be tested and was placed on the surface of the medium. If the compound is effective against the bacteria at a certain concentration, no colonies will grow were the concentration in the medium is greater than or equal to the effective concentration. This is the zone of inhibition. Thus the size of the zone of inhibition was measured to know the compounds effectiveness.

Comment [f21]: Correct it as: on the medium of the plate.

Comment [f22]: Needs rephrasing.

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 30sec, treatment with iodine for 30 sec, treatment with 95% alcohol, counter staining with safranin.

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 organisms like *S. aureus* and gram negative *E. coli* were isolated using these media. *S. aureus* was plated on blood agar. Eosin-methylene blue agar (EMBA) is used for *E. coli* culture. Mannitol salt agar (MSA) was used for the selective isolation of presumptive pathogen *Staphylococci.*

2.7 Biochemical analysis

2.7.1 Catalase test

The Catalase test is done by placing a drop of hydrogen peroxide on a microscope slide. Using an applicator stick, touch the colony, and then smear a sample into the hydrogen peroxide drop.

2.7.2 Urease test

Streak the surface of a urea agar slant with a portion of well isolated colony. Leave the cap on loosely and incubate the test tube at 35 in ambient air for 48hrs to 7 days. If organism produces urease enzyme, the color of the slant changes from light orange to magenta. If organism does not produce urease the agar slant and butt remain light orange (no color change).

2.7.3 Triple Sugar Iron test

The triple sugar iron or TSI test is a microbiological test roughly named for its ability to test microorganism's ability to ferment sugars and to produce hydrogen sulphide. The medium contains 1% each of sucrose and lactose and 0.1% glucose. If only glucose is fermented, the butt will turn into yellow. However, if either sucrose or lactose is fermented, both the butt and slant turns yellow. The medium also contains ferrous sulfate. If the bacterium forms H_2S, this chemical will react with the iron to form ferrous sulphide, which is seen as a black precipitate in the butt.

2.7.4 IMViC test

2.7.4.1 Indole test (tryptone broth)

Inoculate a loopful of bacteria into a tryptone broth. Incubate 48hrs. After incubation the broth must be turbid. A clear broth indicates that your organism did not grow and cannot be tested. Add a few drops of indole reagent to the broth culture. Do not shake the tube. A positive result has a red layer at the top. A negative result has a yellow or brown layer.

2.7.4.2 Methyl red test (MRVP broth)

Inoculate a loopful of bacteria into MRVP broth. Incubate 3 to 5 days. After incubation the broth must be turbid. A clear broth indicates that your organism did not grow and cannot be tested. Remove 1ml of broth and place into a sterile tube before performing the methyl red if you are going to use the

Comment [f23]: Merge them and put under one sub-heading "Preliminary tests". Since these are standard methods, description of procedures is not necessary. Mention only the application of the technique.

UNDER PEER REVIEW

same broth for VP test. Add 3-4 drops of methyl red into original broth. Do not shake the tube. A positive result has a distinct red layer at the top of the broth. A negative result has a yellow layer.

2.7.4.3 Voges- Proskauer test

Inoculate a loop full of bacteria into MRVP broth. Incubate 3-5 days. After incubation read the VP test when you have good turbidity. A clear broth indicates that the organism did not grow and cannot be tested. Barrit's reagent A (VP A) contains naphthol and Barrit's B (VP B) contains KOH. Test 1ml of culture from the MRVP broth. Add the entire contents of the VP A reagent (15 drops) and 5 drops of the VP B reagent to the 1ml of broth culture. Shake well. With a positive reaction the medium will change to pink or red. With a negative reaction the broth will not change colour or will be copper coloured.

2.7.4.4 Citrate test (Simmon's citrate slant)

Streak a loop full of bacteria onto a citrate agar slant, do not stab the butt. Incubate 24 to 48 hrs, longer for *Bacillus* species. Incubate with a loose cap. After incubation a positive reaction is indicated by a slant with a Prussian blue colour. A negative slant will have no growth of bacteria and will remain green.

2.7.4.5 Carbohydrate utilization test

Inoculate the phenol red glucose broth with your unknown bacteria. Inoculate the organism into the tub with the disc and incubate at 25 or 37ºC. A change in the phenol red indicator to yellow shows acid (A) production. In addition, if there is a Durham tube in the sugar broth, there may be CO_2 gas. 1ml of the liquid sugar is added to a2ml sterile phenol red solution (having no sugar) and inoculate with organism and incubate. After incubation, look for the typical yellow colour of acid production, a positive test result.

Comment [f24]: Merge them and put under one sub-heading "Biochemical identification of bacteria"
Since these are standard tests, description of procedures is not necessary. Give only the principle and the inference of the tests in brief.

3. RESULTS AND DISCUSSION

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

Comment [f25]: Delete this.

3.1 Screening of organisms

From the direct microscopic examination and cultural characteristics of isolated organisms it can be seen that Strain 1 is 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.

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. Table 3 shows that isolate 1 is *Escherichia coli* and isolate 2 is *Staphylococcus aureus.*

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.

Comment [f26]: Need to rephrase for clear scientific presentation.
Avoid grammatical mistakes.

Comment [f27]: Attach if any photo image showing antibacterial activities of leaf extracts is available.

UNDER PEER REVIEW

In Table 5 the antimicrobial activity of pure extract of *Murraya* against *Escherichia coli* is inthe 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 18mm. The second-high inhibition zone was observed with petroleum ether extract against *Escherichia coli* with a diameter of 15mm.

Comment [f28]: Present the result methodically and discuss by citing comparable data of other research studies of similar kind. Need at least 3-5 citations for better outcome of the discussion.

Table1: Direct microscopic examination and cultural characteristics of isolated organisms

Comment [f29]: Correct as: Bacterial isolates

Strain	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

???

Comment [f30]: Captions for abbreviations? Ex: MSA – Mannitol Salt Agar

Table 2: Biochemical tests for isolated pathogens

Comment [f31]: Correct as: **identification of bacterial isolates**

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

???

Comment [f32]: Captions for abbreviations? Ex: TSI – Triple Sugar Iron test

Table 3: List of microorganisms identified

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

Table 4: Zone of inhibition produced by *Murrayakoenigii* against *Staphylococcus aureus*

Comment [f33]: Correct it as: **Effect of *M. koenigii* leaf extracts 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

UNDER PEER REVIEW

Table 5: Zone of inhibition produced by*Murrayakoenigii*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

Comment [f34]: Correct it as:
Effect of *M. koenigii leaf extracts* against *Escherichia coli*

CONCLUSION

The different solvent extracts of *Murrayakoenigii*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.

Comment [f35]: Do not repeat the results. Give only significant outcomes.

REFERENCE

1. Suman Singh, P. K. Omre and Sandhya Madan Mohan. Curry leaves *(Murrayakoenigii)* a miracle plant. *Ind. J. Sci. Res.* 2014; 4 (1): 46-52.
2. Bhandari PR. Curry leaf (*Murrayakoenigii*) or Cure leaf: Review of its curative properties. *J. Med. Nutr. Nutraceut.* 2012; 1: 92-97.
3. Disegha GC, and OnuegbuIzionworu V. Antifungal Activities of Curry Leaf (*MurrayaKoengii*) Extract on Some Selected Fungi. *Chem. Mat. Research.* 2014; 6(11): 2224-3224.
4. Anita Joshi, Varsha Dattatraya Shahane, Varsh Gore, and Renu Bharadwaj. *Hind Ant Bulletin.* 2009; 47-48(1-4): 7-12.
5. Mitscher, L.A., Drake, S., Goliapudi, S.R., Okwute, S.K. A modern look at folklore use of anti-infective agents. *J. Nat. Products.* 1987; 50: 1025-1040. https://doi.org/10.1021/np50054a003.
6. Dimayuga R, Garcia S. Antimicrobial screening of medicinal plants from Baja California Sur, Mexico. *J. Ethnopharmacology.* 1991; 31: 181–192.
7. Salvat, Antonnacci, Fortunato, Suarez, Godoy. Screening of some plants from Northern Argentina for their antimicrobial activity. *Lette. App. Microbiology.* 2001; 32(5) 293–297.https://doi.org/10.1046/j.1472-765X.2001.00923.x

UNDER PEER REVIEW

8. Aberra Geyid , Dawit Abebe, Asfaw Debella, Zewdneh Makonnen, Frehiwot Aberra, Frehiwot Teka et al. Screening of some medicinal plants of Ethiopia for their anti-microbial properties and chemical profiles. *J. Ethnopharmacol.* 2005; 97(3):421-7. doi: 10.1016/j.jep.2004.08.021.
9. Maha El Amin, Promy Virk, Mai Abdel Rahman Elobeid, Zainab Mohammed Almarhoon, ZeinabKorany Hassan, Sawsan Ali Omer et al. Anti-diabetic effect of Murrayakoenigii (L) and Olea europaea (L) leaf extracts on streptozotocin induced diabetic rats. *Pak. J. Pharm. Sci.* 2013; 26 (2) 359-365.
10. Mini PriyaRajendran, Blessed BeautlinPallaiyan and NijaSelvaraj. Chemical composition, antibacterial and antioxidant profile of essential oil from Murrayakoenigii (L.) leaves. *Avice. J. Phytomed.* 2014; 4(3): 200–214.
11. Ibrahim Abuga , Shaida Fariza Sulaiman , Ridhwan Abdul Wahab , Kheng Leong Ooi , Mohammad SyaifulBahariAbdullRasad. In vitro antibacterial effect of the leaf extract of Murrayakoenigii on cell membrane destruction against pathogenic bacteria and phenolic compounds identification. *Euro. J. Integ. Medicine.* 2020; 33. *https://doi.org/10.1016/j.eujim.2019.101010*
12. Sagar Aryal. Mueller Hinton Agar (MHA) – Composition, Principle, Uses and Preparation. 2022. https://microbiologyinfo.com/mueller-hinton-agar-mha-composition-principle-uses-and-preparation
13. A.W. Bauer, W.M. Kirby, J.C. Sherris, M. Turck. Antibiotic susceptibility testing by a standardized single disk method. *Amer. J. Clin. Pathol.* 1966; 45(4):493-6.

Comment [f36]: Follow standard reference style.
Adopt the Author guidelines of the journal.

UNDER PEER REVIEW