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Antimicrobial properties and phytochemical analysis of different extracts of *Murraya koenigii*

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Abstract
Murraya koenigii (Curry leaf) is an Indian-native green leafy vegetable. Curry leaves have several health advantages and natural taste. Due to its medicinal value and characteristic aroma it is highly valued plant. In addition to having anti-diabetic characteristics, it also has hepato-protective, anti-carcinogenic, anti-inflammatory antioxidant and antibacterial properties. This study set out to look into antibacterial properties of curry leaves. Samples of curry leaves were imported from Mohali, Punjab. To obtain curry leaf powder, the leaves were air dried at room temperature then pulverized. With the help of disc diffusion method, this powder was utilized to prepare the ethanol, methanol and aqueous extracts for testing the antibacterial activities on both Gram (+ve) and Gram (-ve) bacteria. Using discs of antibiotics, the inhibition zones surrounding the disc were assessed and compared. When tested against *S. aureus*, *E. coli* and *B. subtilis* the curry leaf extract showed a distinct zone of inhibition, demonstrating a broad spectrum of very high antibacterial activity. Results obtained showed that *S. aureus* found to be most effective while *E. coli* was least effective. Among various extracts methanolic extract of curry leaf was found to be most effective while aqueous was observed to be least effective.

Keywords: Antimicrobial, phytochemical, curry leaf

Introduction
Murraya koenigii; commonly called Curry leaves occurs throughout India up to an altitude of 1500 metres (Vats, M *et al*., 2011) [1]. The leaves of the plant are used as a natural medicinal as well as flavouring agent. Due to presence of natural antioxidants different extracts of this plant are utilized as; therapeutic drugs (Tamokou, J.D., *et al*., 2013 and Saafi-Ben Salah, E.B., *et al*., 2012) [2, 3], food preservatives (Preethi, R., *et al*., 2010) [4] and pharmaceuticals (Srivastava, A., *et al*., 2006) [5]. Herbal drugs are often used as antimicrobial, but the problem of bacterial resistance is growing rapidly. Recent interest in natural remedies has increased, especially in regard to alternative and natural products. *Murraya koenigii*, one of them, also plays a significant role because it possesses a number of therapeutic and pharmacological qualities that work directly against antibiotics and microbes. *M. koenigii* is a well-known leafy spice that's utilized in Asian-Indian dishes as a preservative. Because of its unique aroma, the smaller quantity of *M. koenigii* is sufficient for usage (Das, A.K., *et al*., 2011) [6]. *M. koenigii* has also been found to have therapeutic potential in addition to its apparent usefulness in diet (Shruthi, S.D. *et al*., 2012).
The results of antimicrobial activities observed for *Murraya koenigii* are presented in this paper. Disc diffusion and broth dilution method were used to evaluate these activities. The disc diffusion method was used to test the microbes for plant susceptibility. Aqueous, ethanol and methanol extracts of *M. koenigii* were applied to the disc of nutrient agar plate at concentrations ranging from 1.562 to 300 mg/disc, and the optical density was checked using broth dilution method.

Methodology
Microorganisms
Microorganisms such as (*E. coli*: MTCC No-2314, *B. subtilis*: MTCC No-2435, *S. aureus*: MTCC No-1144) were obtained from IMTECH (Institute of Microbial Technology) -39, Chandigarh, India. On nutrient agar plates, the organisms were kept at 4 °C. The strains were biochemically tested before use.

Murraya koenigii sample collection and preparation
Collection of *Murraya* leaves
Murraya koenigii leaves were collected in the late afternoon of April from Mohali, Punjab. The plant was healthy and free of disease. Under flowing tap water, the leaves were washed.

Preparation of leaves extracts

Collected plant material was shadow dried; leaves were converted in the form of powder with the help of mortar and pestle. Powder was poured into the conical flask, where it was mixed with ethanol, methanol, and distilled water individually and kept for 1 week. Then Whatman's No. 1 filter paper was used to filter it. The extracts were concentrated in a water bath that was kept at 65°C until it had been reduced to 1/4 of its original volume. According to protocol, extracts were re-suspended in their respective solvents (Sinha, A., *et al*., 2021) [8].

In vitro* antimicrobial activity of *Murraya koenigii

The stock solutions for the leaves were prepared at a concentration of 300 mg/ml. Serial dilution was used to obtain concentrations of 300 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, and 1.562 mg/ml. A spreader was used to equally distribute 25 to 50µl of each organism onto nutrient agar plates, which had been prepared. The inoculum was always freshly produced before the experiment began. Each plate was incubated for 24 hours at 37°C after 25µl of each of the aforementioned concentrations of leaves extract were added to each plate. After 24-48 hours, readings were taken of the clear zones of culture growth inhibitions surrounding the discs containing leaf extract of *Murraya*.

Broth Dilution method

Experiments were carried out in sterile conditions under laminar flow. 2ml of nutrient broth was placed in each test tube before they were autoclaved. Different concentrations (3-60 mg/ml) of a 300 mg/ml stock solution of *Murraya* leaves were taken in various tubes along with broth, excluding the control tube that simply contained broth. 200µl of the newly made inoculum (0.5 x 10 6 CFU/ml) was added to a test tube containing extract of the leaves of *Murraya*. Test tubes that had been inoculated were grown in a shaker incubator until late in the log phase of growth at the appropriate temperature for each organism. The optical density at 600 nm was calculated for each test tube.

Susceptibility to antibiotics

All bacteria, including *S. aureus*, *B. subtilis*, and *E. coli*, were tested using commercially available antibiotic discs. On the Petri plate, 100µl of microbial culture was poured and then distributed evenly using a spreader. Then, under sterile conditions, discs of various antibiotics were put on nutrient agar media plates. After that, plates were incubated for 24 hours at 37°C. Antibiotics spread throughout the media during incubation and stop bacteria from multiplying. Zones of inhibition were evaluated using the aid of vernier calliper scale.

Synergistic activity of plant extract and commercially available antibiotic

The bacterial cultures were raised at 37°C in nutrient broth. Each bacterium was grown before being put onto the nutrient agar plates. To determine the synergistic effect between commercially available antibiotics and plant extract. *Murraya* leaf extract and 20µl antibiotics extract were then poured on the surface of each inoculated plate. The plates were incubated for 24 hours at 37°C. Then, using a vernier calliper scale, the zone of inhibitions were quantified.

Log Colony Forming Unit

Log Colony Forming Unit was calculated by following standard protocols of (Rana, A., *et al*. 2021; 2022abc) [9, 24, 25, 26]. In order to do this, the microbial inoculums were prepared by leaving their culture in nutrient broth for an extended period of time. After incubation, bacteria were cultivated at 37°C, cells were extracted by centrifuging for 10mins at 8000g, the pellet was washed, and the supernatant was discarded before being suspended in phosphate buffer saline (PBS). Optical density (OD) at 600 nm was quantified. Viable counts were assessed after incubation for 24 hours at 37°C using spread plating on nutrient agar and serial dilution. CFU was counted after 24hours.

Phytochemical analysis

Using newly prepared extracts, the following phytoconstituents were examined: carbohydrates, proteins, tannins, saponins, glycosides, flavonoids, phenols, alkaloids and steroids.

1. **Test for carbohydrates:** The crude extract was mixed with 2ml of Benedict's reagent and cooked for 5mins in a water bath. Precipitates produced with a reddish-brown appearance, indicating the presence of carbohydrates.
2. **Test for proteins:** Add few drops of concentrated nitric acid and sodium hydroxide to the extracts in test tubes. Appearance of yellow colored indicates the presence of proteins.
3. **Test for tannins:** 3ml of extract was mixed with 2ml of 10% $Fecl_3$. Tannins were present as indicated by the presence of a dark blue-blackish color.
4. **Test foe saponins:** 0.5g of the extract was added to 2ml of water and shaken. The presence of saponins is indicated if the formed foam persists for 10 minutes.
5. **Test for glycosides:** 1-2 ml of NH_4OH was added to the extract and shake. Appearance of cherish color indicates the presence of glycosides.
6. **Test for flavonoids:** Lead acetate was added to the extracts. Presence of yellow colour indicated the presence of flavonoids.
7. **Test for phenols:** The extract was mixed with 3–4 drops of 5%$Fecl_3$. The presence of phenols was indicated by blue-black color.
8. **Test for alkaloids:** To 2ml of extract, add few drops of Wagner's reagent in test tube formation of reddish-brown colored ppt. indicate the presence of alkaloids (Sofowra, 1993; Trease, and Evans, 1989; Harborne, 1973) [10, 11, 12].

Result and discussion

An extensive review of the literature showed that, *Murraya* has a variety of pharmacological properties including; antibacterial, hepatoprotective, nephroprotective, anti-inflammatory, anti-diarrhea, anti-dysenteric and also utilized to prevent vomiting (Vats, M., *et al*. 2011) [1]. Leave and fruits of this plant are also found to be a source of an essential oil which makes it an important fixative for perfumes.

The decreasing effectiveness of antibiotics provided by pharmaceutical firms as well as the rise in medication resistance against bacteria, natural product consumption is on the rise nowadays. Microbes are developing ways for resisting antibiotics, and some are developing multi-drug resistance. In order to determine *Murraya koenigii*'s antibacterial properties against different Gram (+ve) and Gram (-ve) bacteria, the disc diffusion method and broth dilution method were used in this experiment.

***In vitro* antimicrobial activity of *Murraya koenigii* leaves**

Disc diffusion method: *Murraya's* antibacterial activity was examined using three different types of extracts: ethanolic, methanolic and aqueous. Earlier non-pathogenic Gram (+ve) and Gram (-ve) bacteria, such as *Bacillus subtilis* and *Escherichia coli*, were chosen for antibacterial experiments with *Murraya*. Following that, disc diffusion method and the broth dilution method were used to test the inhibitory activity of *Murraya* against pathogenic Gram (+ve) bacteria, specifically *Staphylococcus aureus*. Stock solutions of the leaves of plant extract were made at concentration of 300 mg/ml. The following concentrations were obtained by diluting these in order: 300 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, and 1.562 mg/ml. On nutrient agar plates, each organism was equally spread throughout. Every time, the inoculum was freshly prepared before the test started. Then, 25µl of each product was added to a different agar plate and incubated at 37°C for 24 hours. Bacterial growth was seen to be inhibited in a zone of inhibition around discs containing leaf extracts. The following tables illustrate the results:

Table 1: Antimicrobial activity of aqueous extract of *Murraya* leaf

Aqueous extract of *Murraya*		ZOI (mm)		
S. No.	**Concentration (mg/ml)**	***E. coli***	***B. subtilis***	***S. aureus***
1.	1.562-12.5	NI	NI	NI
2.	25	NI	NI	NI
3.	50	NI	NI	NI
4.	100	NI	NI	2.7
5.	300	NI	1.6	3.5

Table 2: Antimicrobial activity of ethanolic extract of Murraya leaf

Ethanolic extract of *Murraya*		ZOI (mm)		
S. No.	**Concentration (mg/ml)**	***E. coli***	***B. subtilis***	***S. aureus***
1.	1.562-12.5	NI	NI	NI
2.	25	NI	NI	0.9
3.	50	NI	1.5	2.1
4.	100	1.9	2.6	3.6
5.	300	2.7	3.2	5.3

Table 3: Antimicrobial activity of methanolic extract of Murraya leaf

Methanolic extract of *Murraya*		ZOI (mm)		
S. No.	**Concentration (mg/ml)**	***E. coli***	***B. subtilis***	***S. aureus***
1.	1.562-12.5	NI	NI	NI
2.	25	NI	1.3	2.3
3.	50	1.2	2.1	3.2
4.	100	2.6	3.5	5.3
5.	300	3.8	4.7	8.5

Leaf of *Murraya*

a. **Aqueous extract: -** for aqueous extract of leaf of *Murraya* the zone of inhibition observed against *S. aureus* varied from 2.7-3.5mm at the range of concentration from 100-300mg/ml. This study is in support with Kumar, A. (2018) [20], where antimicrobial properties of *Murraya* was tested against *S. aureus* with the help of disc diffusion method and showed the inhibition of 8.09mm; for *B. subtilis* it was 1.6mm at the concentration 300mg/ml as corroborated from studies of Selvamani S. (2014) [21], where antimicrobial properties were tested by agar well diffusion method against *B. subtilis*.

The zone of inhibition observed for aqueous extract of leaves of *Murraya* against *B. subtilis* varied from 9-9mm at the range of concentration 25-100mg/ml; for *E. coli* no inhibition was observed.

b. **Ethanolic extract: -** For ethanolic extract of leaf of *Murraya* the zone of inhibition against *S. aureus* varied from 0.9-5.3mm at the range of concentration 25-300mg/ml. This study is in support with Irfan *et al*., (2016), where antimicrobial properties of *Murraya* was tested against *S. aureus* with the help of disc diffusion method and showed inhibition of 2.2-15.0mm at the range of concentration 6.25-100mg/ml; for *B. subtilis* it varied from 1.5-3.2mm at the range of concentration 50-100mg/ml. this study is in agreement with N. Uma Maheswari and N. Cholarani (2013) [23], where antimicrobial properties were tested by agar well diffusion method against *B. subtilis*. The zone of inhibition observed for aqueous extract of leaves of *Murraya* against B. subtilis showed 10.2mm zone of inhibition; for *E. coli* it varied from 1.9-2.7mm at the range of concentration 100-300mg/ml. It is supported from previous studies of Shruthi, S.D. *et al*., (2012), where antimicrobial properties were tested by agar well diffusion method against *E. coli*, where zone of inhibition was observed for aqueous extract of leaves of *Murraya* against *E. coli* was 7.47±0.15 at the range of concentration 0.781-100mg/ml.

c. **Methanolic extract: -** For methanolic extract of leaf of *Murraya* the zone of inhibition observed against *S. aureus* varied from 2.3-8.5mm at the range of concentration 25-300mg/ml. Our study is in agreement with Singh, H. *et al*., (2017) [18], where antimicrobial properties were tested by well diffusion method against *S. aureus* and zone of inhibition observed in methanolic extract of leaves of *Murraya* against *S. aureus* was 10mm; for *B. subtilis* it varied from 1.3-4.7mm at the range of concentration 25-300mg/ml as corroborated from studies of Seniya, C. *et al*., (2011), where antimicrobial properties were tested by disc diffusion method against *B. subtilis*. The zone of inhibition observed for methanolic extract of *Murraya* leaves against *B. subtilis* was 10mm; for *E. coli* it varied from 1.2-3.8mm at the range of concentration 50-300mg/ml. This is supported from previous studies of A. Mounikana *et al*., (2016) [19], where antimicrobial properties were tested by diffusion assay method against *E. coli*, where zone of inhibition was observed for methanolic extract of leaves of *Murraya* against *E. coli* varied from 25-32mm at the range of concentration 250-500mg/ml.

The effectiveness of *Murraya* was also compared with range of antibiotics available commercially.

Table 4: The zone of inhibition showed by antibiotics against various bacteria

S. No.	Antibiotics (30µg/ml)	ZOI (mm)		
		E. coli	***B. subtilis***	***S. aureus***
1.	Amoxicillin	22.1	23.6	24.8
2.	Chloramphenicol	17.1	18.0	18.9
3.	Vancomycin	15.3	16.2	16.7
4.	Oxacillin	9.4	10.7	11.3
5.	Ampicillin	19.7	20.1	21.4
6.	Erythromycin	12.1	13.3	15.4
7.	Ofloxacin	11.2	12.4	13.9

*All the values are expressed as mean ± S.D (n=5), NI-no inhibition, ZOI-zone of inhibition. mm-millimetre

In this experiment, a variety of antibiotics were used to assess how well antibiotics prevented the growth of bacteria. The

antibiotics were used as positive control included ofloxacin, amoxicillin, chloramphenicol, vancomycin, oxacillin, erythromycin, and ampicillin. By spreading each bacteria on nutrient agar media in a different plate, *E. coli*, *S. aureus*, and *B. subtilis*, various experiments were carried out. This enables us to distinguish between an antibiotic's antibacterial properties when applied against various microbes. *S. aureus* had the largest zone of inhibition, followed by *B. subtilis* and *E. coli*.

Table 5: - Synergistic effect of leaf extracts of *Murraya* against various bacteria.

Plant extract	Antibiotics (30µg/ml)	ZOI (mm)			Comparative effect on ZOI
		E. coli	*B. subtilis*	*S. aureus*	
Aqueous extract of leaf of *Murraya*	Amoxicillin	22.1	23.9	25.7	Activity increases
	Chloramphenicol	17.1	18.4	20.0	Activity increases
	Vancomycin	15.3	16.7	18.1	Activity increases
	Oxacillin	9.4	11.2	12.5	Activity increases
	Ampicillin	19.7	20.7	22.9	Activity increases
	Erythromycin	12.1	13.9	16.7	Activity increases
	Ofloxacin	11.2	13.1	15.0	Activity increases
Ethanolic extract of leaf of *Murraya*	Amoxicillin	22.9	24.6	26.0	Activity increases
	Chloramphenicol	18.4	19.0	20.4	Activity increases
	Vancomycin	16.1	17.3	18.8	Activity increases
	Oxacillin	11.3	12.0	12.9	Activity increases
	Ampicillin	21.4	21.4	23.8	Activity increases
	Erythromycin	13.7	15.2	16.0	Activity increases
	Ofloxacin	12.5	13.6	15.7	Activity increases
Methanolic extract of leaf of *Murraya*	Amoxicillin	24.0	24.8	27.7	Activity increases
	Chloramphenicol	19.2	19.6	21.9	Activity increases
	Vancomycin	17.2	18.1	20.2	Activity increases
	Oxacillin	11.7	13.2	14.7	Activity increases
	Ampicillin	22.9	23.4	24.7	Activity increases
	Erythromycin	14.5	16.1	17.9	Activity increases
	Ofloxacin	14.1	14.7	17.2	Activity increases

Combining a variety of antibiotics with various leaf extracts of *Murraya* has shown synergistic effects on the organisms mentioned above. The strongest additive/synergistic impact was seen when amoxicillin and a methanolic extract of *Murraya* leaf were combined. Amoxicillin had the strongest synergistic effect against *S. aureus*, followed by ampicillin, chloramphenicol, vancomycin, erythromycin, ofloxacin, and oxacillin.

Broth dilution method**: -** The inhibitory concentrations of *Murraya* against the previously mentioned organisms were ascertained by experiments applying the broth dilution method, as well as the effects of a variety of concentrations of various extracts on an organism's growth. Organisms were cultured using a variety of Murraya extracts at concentrations ranging from 3 mg/ml to 60 mg/ml. *Escherichia coli* and *Bacillus subtilis*, two non-pathogenic Gram (+ve) and Gram (-ve) bacteria, had their growth rates monitored at the late log phase. Then, using a broth dilution experiment, it was determined whether the pathogenic Gram (+ve) bacteria *Staphylococcus aureus* was susceptible to *Murraya*'s inhibitory effects. By taking an O.D. at 600nm during the late log phase, each organism's growth was evaluated.

Table 6: OD of aqueous extract of Murraya leaf

Aqueous extract of *Murraya* leaf		OD		
S. No.	Concentration (mg/ml)	*E. coli*	*B. subtilis*	*S. aureus*
1.	3	1.99	1.96	1.92
2.	7.5	1.97	1.92	1.89
3.	15	1.93	1.85	1.75
4.	30	1.89	1.79	1.67
5.	60	1.85	1.71	1.58

Table 7: OD of ethanolic extract of *Murraya* leaf

Ethanolic extract of *Murraya* leaf		OD		
S. No.	Concentration (mg/ml)	*E. coli*	*B. subtilis*	*S. aureus*
1.	3	1.97	1.94	1.89
2.	7.5	1.93	1.90	1.81
3.	15	1.90	1.84	1.69
4.	30	1.84	1.73	1.54
5.	60	1.78	1.67	1.45

Table 8: OD of methanolic extract of *Murraya* leaf

Methanolic extract of *Murraya* leaf		OD		
S. No.	Concentration (mg/ml)	*E. coli*	*B. subtilis*	*S. aureus*
1.	3	1.95	1.93	1.87
2.	7.5	1.91	1.86	1.78
3.	15	1.82	1.80	1.65
4.	30	1.71	1.68	1.49
5.	60	1.62	1.50	1.34

S. aureus, *B. subtilis*, and *E. coli* were tested during the late log phase to determine the inhibitory concentration of various *Murraya* extracts, and viable counts were also measured. Then, using the broth dilution method, Gram (+ve) and Gram (-ve) bacteria were individually tested for inhibitory activity of several *Murraya* extracts. By taking O.D. during late log phase, the growth of *S. aureus*, *B. subtilis* and *E. coli* was assessed at 600 nm. According to tests of antimicrobial effects of several *Murraya* leaf extracts at different concentrations,

bacterial growth slows down as extract concentration rises. The antibacterial activity of various *Murraya* extracts is thus concluded.

Table 9: Phytochemical screening of leaf of *Murraya* in different solvents

S. No.	Phytoconstituents	Extracts		
		Ethanol	Methanol	Aqueous
1.	Carbohydrates	+	+	+
2.	Proteins	_	+	_
3.	Tannins	+	_	_
4.	Saponins	+	_	+
5.	Glycosides	+	+	+
6.	Flavonoids	+	_	+
7.	Phenols	+	_	+
8.	Alkaloids	+	+	+

In methanolic extract of *Murraya* leaf there is presence of alkaloids, carbohydrates, proteins and glycosides; and absence of flavonoids, saponins, phenols and tannins. This study is in agreement with Patil, N.B. (2019) [13]. In aqueous extract of *Murraya* leaf there is presence of carbohydrates, alkaloids, flavonoids, saponins, glycosides and phenols; and absence of proteins and tannins. This study is in agreement with Khatun, (2019) [14]. In ethanolic extract of *Murraya* leaf there is presence of alkaloids, carbohydrates, phenols, glycosides, flavonoids and tannins; and absence of proteins and saponins. This study is in agreement with Dhamane, S.P. (2019) [15].

Conclusion

From the results observed, *Staphylococcus aureus* was found to be most susceptible to the methanolic extract of stem of *T. cordifolia*, followed by *Bacillus subtilis* and *Escherichia coli* was the least sensitive. Among the antibiotics used, Amoxicillin was observed to be most effective followed by Ampicillin, Chloramphenicol, Vancomycin, Erythromycin, Ofloxacin and Oxacillin. Synergistic effect of amoxicillin + methanolic extract of *Murraya'*s leaf showed max. inhibition against *Staphylococcus aureus.* In broth dilution method, as concentration of extract increases there is a decline in growth of microorganisms. Leaves of *Murraya* contain various bioactive constituents such as steroids, carbohydrates, saponins, glycosides, flavonoids, phenols, alkaloids.

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