

In-Vitro Analysis of *Murraya Koenigii* Root extract with Colistin Against MDR *Pseudomonas Aeruginosa*.

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ABSTRACT

Objectives: To analyze susceptibility of *MDR Pseudomonas aeruginosa* to chloroform root extract of *Murraya koenigii*.

Methodology: For this research *Murraya koenigii* chloroform root extract (*MKCRE*) was obtained from PCSIR, Lahore with total yield of 3.4%. A 14-day study was conducted on blood samples of 12 patients admitted to Sheikh Zayed Hospital (SZH) Lahore over a period of one year, assessed positive for MDR *P. aeruginosa* based on a series of confirmatory bench tests performed in the Microbiology lab of SZH Lahore. Simple Randomized sampling was done by rotary method and the samples were randomly divided into 2 groups, each group having 6 samples. Groups 1 & 2 were tested for susceptibility to *MKCRE* and Colistin respectively using Disk diffusion and Agar well diffusion methods for determination of zone of inhibition and MIC (Minimum Inhibitory Concentration) was determined by broth macro-dilution method. Data was statistically analyzed using SPSS version 25.

Results: Our study concluded that *MKCRE* have significant anti MDR *P. aeruginosa* activity with pharmaceutical potential.

Conclusion: The current study revealed that *Murraya koenigii* chloroform root extract individually carries Anti MDR *Pseudomonas* potential better than Colistin.

Keywords: *Murraya koenigii* root extract, Colistin, *Pseudomonas aeruginosa*

Authors' Contribution:

^{1,2}Conception; Literature research; manuscript design and drafting; ^{3,4}Critical analysis and manuscript review; ^{5,6}Data analysis; Manuscript Editing.

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Introduction

Antibiotic resistance is emerging globally and becoming a major public health threat. Excessive and inappropriate use of antibiotics has eventually led to the development of antibiotic resistance in microbes.¹ *Pseudomonas aeruginosa* is an aerobic gram-negative bacillus that is reported to be the second most frequently isolated pathogen in patients with infections like catheter associated

urinary tract infections, wound sepsis, ventilator associated pneumonia and infections related to stents, grafts, sutures and prosthetic heart valves.² *Pseudomonas aeruginosa* is an opportunistic pathogen that has numerous reservoirs in hospital environment such as equipment, food, taps, toilets, sinks, mops and surfaces. Due to cross resistance to multiple classes and types of antibiotics, these infections are difficult to manage.³ In February 2017 World Health Organization (WHO) published a data

of priority organisms that were resistant to multiple antibiotics, *P. aeruginosa* was included in the first critical priority pathogens.⁴ Recently prevalence of high-risk clones of MDR (Multidrug resistant) and XDR (Extended drug resistant) *P. aeruginosa* is increasing with rates of 15% to 30% in some geographical areas⁵.

Pseudomonas aeruginosa resistance is also being reported in local hospitals of Pakistan. In Pakistan 10,000 patients are admitted annually and mortality rate is up to 20%. In Sindh frequency of MDR *P. aeruginosa* infection is 30% - 55% while in Punjab this frequency is 20% - 50%.⁶

Over the past ten years carbapenems (imipenem and meropenem) belonging to newer class β lactams antibiotics have emerged as front-line treatments for *P. aeruginosa* infections, however, currently bacteria have acquired resistance to these antibiotics.⁷

Now Colistin or Polymyxin E is considered as a last resort against multidrug resistant gram negative infections⁷. The side effects of this group include hypersensitivity reactions, malaise neurotoxicity and nephrotoxicity.⁸ However, the newly found Colistin resistance has emerged as a major threat⁹. New agents having in vitro anti-microbial activity against MDR *P. aeruginosa* includes Tazobactam, Carbapenem, Ceftazidime and Cilastatin. To deal with these deadlier pathogens there is an urgent need for alternative natural treatment strategies to stop further resistance in antibiotics¹⁰. As plants have important bioactive compounds which can boost the effect of other antimicrobials resulting in better outcome¹¹. Plants are being used by mankind for medicinal purposes since ages. In this study chloroform root extract of *Murraya koenigii* was tested for their anti-MDR *P. aeruginosa* activity.

Murraya Koenigii is a small shrub from family Rutaceae. It is native to Asia and used as an important flavoring agent in Indian/Asian cuisine. It is known as "Curry Patta" or Curry leaves. It has been used as an anti-inflammatory, analgesic, antiemetic, antidiabetic,

antioxidant and antimicrobial agent.¹²

In this study antimicrobial effect of *Murraya koenigii* chloroform root extract was determined against MDR *P. aeruginosa* as this part of *Murraya koenigii* plant had antimicrobial susceptibility against regular strains of *P. aeruginosa*.¹³ However, no research was noted against MDR *P. aeruginosa* strains worldwide and significant gap in literature existed. Therefore, in this research, anti- MDR *P. aeruginosa* activity of MKCRE was observed that has not been studied yet.

Methodology

This in vitro experimental study was conducted over a period of one year in the Microbiology Laboratory of Shaikh Zayed Hospital, Lahore, spanning a duration of 14 days. All blood samples collected from patients admitted to the hospital with multi-drug resistant (MDR) *Pseudomonas aeruginosa* were considered for the research. Samples exhibiting multi-drug resistance were included based on the inclusion criteria, while those showing extensive drug resistance or complete sensitivity were excluded. The sample size was calculated by the following formula keeping the power of study equal to 90% and level of significance equal to 5%.

$$n = \frac{(Z_{1-\beta} + Z_{1-\alpha/2})^2 (\sigma_1^2 + \sigma_2^2)}{(\mu_1 - \mu_2)^2}$$

$Z_{1-\beta}$ = Desired Power of study = 90%

$Z_{1-\alpha/2}$ = Desired Level of Significance = 5%

μ_1 = Anticipated mean susceptibility level with Colistin = 12 μ g/ml

μ_2 = Anticipated mean susceptibility level with experimental plants = 13 μ g/ml

σ_1 = Standard Deviation of susceptibility level with Colistin = 0.5

σ_2 = Standard Deviation of susceptibility level with experimental

plants = 0.5

n = calculated sample size for each group = 6

A simple randomized sampling method, specifically the lottery method, was employed for sample selection. This process resulted in the random selection of two groups, each containing six samples:

group 1 comprised isolates numbered 1 through 6, and group 2 comprised isolates numbered 7 through 12.

Group 1- Isolates in this group were tested for susceptibility with *MKCRE*.

Group 2- Isolates in this group were tested for susceptibility with Colistin.

Dimethyl Sulfoxide (DMSO) was used as negative control in disk diffusion and Agar Well diffusion methods. Trypticase soya broth was used as negative control in Broth macro-dilution method. MDR *Pseudomonas aeruginosa* isolate was the test organism. *MKCRE* was used to assess the anti-bacterial activity against MDR *P. aeruginosa*, Colistin sulfate was the drug used for comparison.

Preparation of extract *Murraya Koenigii*

Chloroform Root Extract: The plants of *Murraya Koenigii* were collected from local vendor. Roots were segregated, rinsed and dried under shade and pulverized using blender. The powdered roots (500g) were extracted with chloroform using Soxhlet's apparatus for 12- 14hrs, filtered with What man No 1 paper and desiccated on water bath to obtain semi solid mass of % yield 3.4%. The dried extract stored at 5°C in the refrigerator until further study²³. MDR *P. aeruginosa* samples were stored in Eppendorf tubes containing Trypticase soya broth 1.5 ml and 3 drops of pure glycerol, at 4-8 °C. Stored MDR *Pseudomonas aeruginosa* samples were grown on Mueller Hinton agar for 24 hours to get pure colonies. To store the samples, 4-6 pure colonies were picked and dissolved in previously prepared Eppendorf tubes. These tubes were kept in an incubator at 35°C for 18-24 hours. After confirmation of bacterial growth (sighting haziness in the tubes bottom) tubes were stored in freezer at -80°C in the laboratory. Stored isolates were sub-cultured on the CLED (Cystine-lactose-electrolyte-deficient) agar plates and plates were incubated at 35°C for 24 hours.

Next inoculum was prepared by inoculating fresh colonies to the test tube containing 3 ml of normal saline and mixed thoroughly to make

suspension. Turbidity of inoculum was compared with McFarland (0.5) standard and was adjusted by adding normal saline or more colonies.

Preparation of stock solution:

1. 99.9% DMSO was used as solvent to prepare stock solution of plant extract.

2. For Broth macro dilution method, TSB was used as solvent.²² To prepare 200mg/ml of *Murraya koenigii* chloroform roots stock solution, 0.2g of extract was dissolved per ml of 99.9 % DMSO. For Colistin stock solution distilled water was used as solvent. 16µg of Colistin sulfate was Dissolved per ml of distilled water. This stock solutions were stored at -60°C for at least 6 months.

Disk Diffusion testing: To prepare working solution of plant extract 8 to 12 dilutions (200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.1mg/ml, 1.5mg/ml, 0.75mg/ml, 0.3mg/ml, 0.1mg/ml) were prepared from stock solution using two fold serial dilutions until a lowest concentration of 0.1mg/ml was achieved while Colistin standard disk of 10µg was used.

Agar-well diffusion testing: 8 to 12 dilutions of *MKCRE* (200mg/ml – 0.1mg/ml) and Colistin (16µg/ml - 0.1µg/ml) were prepared from stock solution using Two-fold serial dilutions.

Broth-macro dilution method: For MIC determination 8 to 12 dilutions of *MKCRE* (200mg/ml-0.1mg/ml) and Colistin (16µg/ml-0.1µg/ml) were prepared in TSB using stock solution by two-fold serial dilution.

Anti-Pseudomonal activity of plant extracts was determined by Kirby-Bauer disk diffusion and Agar well diffusion methods.

Kirby-Bauer disk diffusion method: Filter paper discs of (6mm) were impregnated with 75µl of different concentrations of extracts to make a disk of one particular concentration, allowed it to absorb and dry. The Mueller Hinton agar plates were inoculated by freshly prepared inoculum and were labeled according to inoculum number. Discs impregnated with plant extracts, Colistin and

negative control (99.9 % DMSO) were placed on plates, incubated at 35°C, were examined after 24hrs and the zone of inhibition was measured (area of clearance around the disk). Zone of inhibition >8mm was considered significant.³

Agar well diffusion method: The Mueller Hinton agar plates were inoculated in the same way as done for disk diffusion testing. In each plate wells were prepared with the help of sterilized micropipette tips of 6mm diameter. Then the 100µl of plant extract of different concentrations and drug was poured into each well using micropipette, placed in incubator at 35°C - 37°C and zone of inhibition was observed after 24 hours.

Determination of MIC by broth macro-dilution method: Test tubes were supplemented with 1ml of different concentrations of *MKCRE*. Bacterial suspension (0.1ml) was transferred to a tube containing 9.9ml of TSB. From this suspension 1ml was mixed with plant extract to give the final density of 5x10⁵ CFU/ml. Indicator (Resazurin) was added to check the change in color and measure absorbance. These test tubes were then incubated at 37°C, examined after 24 hours for growth and compared to control. A tube containing only TSB was taken as negative control growth. The lowest concentration where there is no visible growth was taken as MIC.³ The data was analyzed using SPSS version 25. Zone of inhibition was presented in mean ± SD. The normality of data was assessed using the Shapiro Wilk test. One-way ANOVA was used to compare the mean Zone of inhibition among groups. Post hoc Tukey test was used for pair wise comparison. A p-value of ≤ 0.05 was considered statistically significant.

Ethical approval was taken from the institutional review board of Sheikh Zayed Hospital, Lahore (Ref. SZMC/IRB/Internal/0064/2021) on 25-02-2021.

Results

In our study the *MKCRE* (GP1) showed maximum ZI of 19.2mm ± 1.0 at 200mg/ml (P value: < 0.001*) by disc diffusion method, while the *MKCRE* showed

increased zone of inhibition in comparison to Colistin by Agar well diffusion method at all concentrations (Maximum ZI of 23.3 ± 1.0 at 200mg/ml) as compared to Disk diffusion method.

Table I: Comparison of zone of inhibition among groups (*MKCRE* 200mg/ml, 100mg/ml) (Colistin 10µg) using Disk Diffusion Method

Groups	Mean ± SD	Min.	Max.	P-Value.
Group 1 (<i>MKCRE</i>) 50mg/ml	14.7 ± 1.2	13.0	16.0	< 0.001*
Group 1 (<i>MKCRE</i>) 100mg/ml	16.7 ± 0.8	16.0	18.0	< 0.001*
Group 1 (<i>MKCRE</i>) 200mg/ml	19.2 ± 1.0	18.0	20.0	< 0.001*
Group 2 Colistin (10µg)	13.3 ± 0.8	12.0	14.0	< 0.001*

One-way ANOVA, * Significant

Table II: Comparison of zone of inhibition(mm) among groups (*MKCRE* 200mg/ml, 100mg/ml, 50mg/ml & 25mg/ml) (Colistin 16µg, 8mg/ml, 4mg/ml & 2ml) using Agar Well Diffusion Method.

Groups	Mean ± SD	Min.	Max.	P-Value.
Group 1 (<i>MKCRE</i>) 25mg/ml	17.5 ± 1.8	15.0	20.0	< 0.001*
Group 1 (<i>MKCRE</i>) 50mg/ml	20.2 ± 1.6	18.0	22.0	< 0.001*
Group 1 (<i>MKCRE</i>) 100mg/ml	20.7 ± 1.5	18.0	22.0	< 0.001*
Group 1 (<i>MKCRE</i>) 200mg/ml	23.3 ± 1.0	22.0	24.0	< 0.001*
Group 2 Colistin (2µg)	8.8 ± 1.0	8.0	10.0	< 0.001*
Group 2 Colistin (4µg)	10.8 ± 1.0	10.0	12.0	< 0.001*
Group 2 Colistin (8µg)	13.3 ± 0.8	12.0	14.0	< 0.001*
Group 2 Colistin (16µg)	14.0 ± 1.1	13.0	16.0	< 0.001*

MKCRE (200mg/ml - 0.1mg/ml) and Colistin (16µg/ml - 0.1µg/ml) were used to determine their

MIC. Using Broth macro dilution method, the lowest MIC determined for *MKCRE* and Colistin was 50mg/ml and 2µg/ml respectively.

Discussion

Emergence of multidrug resistant bacteria is an ever-growing concern globally. Gram negative bacteria specifically acquire and spread intrinsic resistance very quickly. Treatment of such multidrug resistant strains is a great challenge not only for medical practitioners globally but also a question mark on the cost of care.¹⁵ As medicinal plants are blessed with diverse bioactive constituents; they could be a safe and cost-effective alternative treatment option for an estimated 80% of population that is dependent on these herbal medicinal products as a primary source of health care.¹⁶

Murraya koenigii is an important medicinal herb of Indian origin, its various parts (leaves, roots, bark) are rich source of carbazole alkaloids, terpenoids, flavonoids, phenolics, carotenoids, nicotinic acid and vitamins that produce potent pharmacological and antimicrobial effects (against gram positive and gram-negative bacteria).¹⁷ Unfortunately, limited studies have been conducted in Pakistan and other areas of the world for evaluating their antimicrobial potential against multidrug resistant super bugs specifically MDR *P. aeruginosa*.

Murraya koenigii roots were selected for the research as no research has been conducted to explore its beneficial effects against MDR strains of *P. aeruginosa*. Chloroform was used for extraction of flavonoids, carbazole alkaloids, glycosides and sterols present in roots. *MKCRE* showed significant antimicrobial activity against *P. aeruginosa* even at a very low concentration as compared to petroleum, ether, methanol and hexane.¹⁸ However, its effect on the newly emergent MDR *P. aeruginosa* strains was not studied which was the subject of our research.

A 14-day study was conducted on the 12 confirmed blood samples of MDR *P. aeruginosa*, randomly

taken and divided into two groups. Group 1 and 2 were tested for sensitivity to *MKCRE* and Colistin respectively. The data was analyzed using SPSS version 25. Zone of Inhibition was presented in mean ± SD.

The anti MDR *P. aeruginosa* activity of *MKCRE* was determined against standard drug Colistin which is routinely administered for the treatment of MDR *P. aeruginosa* cases in hospital setup.

In our study following findings were observed, The *MKCRE* (GP1) showed maximum ZI of 19.2mm ± 1.0 at 200mg/ml to minimum ZI of 8.2mm ± 1.6 at 12.5mg/ml (P value: < 0.05). No zone of inhibition was observed by disk diffusion method by *MKCRE* below the concentration of 12.5mg/ml. No previous study related to antibacterial effect of *MKCRE* against MDR *P. aeruginosa* was available for comparison however published data on *MKCRE* showed significant antibacterial activity with maximum ZI of 29mm against sensitive *P. aeruginosa* strains and 9mm ± 0.31 and 10mm ± 0.50 against *E. coli* & *S. Aureus* respectively.

In the current study standard drug Colistin (GP2) showed a ZI of 13.3mm ± 0.8 at 10µg (standard disk) against MDR *P. aeruginosa*. Therefore, in comparison with Colistin the *MKCRE* (ZI of 19.2mm ± 1.0) showed better anti MDR *Pseudomonal* activity by disk diffusion method even without pharmaceutical purification, so anti MDR *P. aeruginosa* activity was significant in both groups whether *MKCRE* and Colistin with the best shown by *MKCRE* by disk diffusion method.

Agar well diffusion was the second method used for susceptibility analysis of MDR *P. aeruginosa* to *MKCRE* and Colistin. *MKCRE* showed increased zone of inhibition

in comparison to Colistin by Agar well diffusion method at all concentrations as compared to Disk diffusion method. Tested over concentration ranges of 200mg/ml- 0.1mg/ml for *MKCRE* against MDR *P. aeruginosa*, sensitivity of *MKCRE* was maximally 23.3mm ± 1.0 at a concentration of 200mg/ml and minimally was 17.5 ± 1.8 at a concentration of

25mg/ml. In comparison with plant extract (*MKCRE*) our standard drug Colistin (GR2) gave a maximum zone of inhibition of 14.0mm \pm 1.1 at a concentration of 16 μ g/ml against MDR *P. aeruginosa*.

As stated above the MIC of *MKCRE* was 50mg/ml. ZI shown by *MKCRE* against MDR *P. aeruginosa* was 20.2 \pm 1.6 with Agar well diffusion method at MIC of 50mg/ml in comparison with a previous study where *MKCRE* had shown ZI of 29mm, 15mm and 8mm against nonresistant *P. aeruginosa*, *Staph. aureus* and *E. coli* respectively with MIC of 0.625mg/ml.¹⁸ However the study has shown that chloroform root extract of *Murraya koenigii* has significant antimicrobial potential against MDR *Pseudomonas aeruginosa* with a relatively high MIC as the bacterial strains are multidrug resistant. In current study MIC of *MKCRE* and Colistin was also determined by a third method (broth macro dilution method). As MIC is a more sensitive parameter to assess the antimicrobial potential of a drug, the lowest MIC and highest efficacy against MDR *P. aeruginosa* was shown by *MKCRE* (50mg/ml). In comparison MDR *P. aeruginosa* showed continued in vitro sensitivity to the standard drug Colistin through a concentration range of 2 μ g/ml to 16 μ g/ml. So, the MIC of Colistin obtained by broth macro dilution method was 2 μ g/ml. The plausible reason for high MIC for *MKCRE* in our study against MDR *P. aeruginosa* is the nature of the strain and its ability to resist by multiple mechanisms through biofilm development.¹⁹ secondly root extract was not pharmaceutically purified as compared to standard drug Colistin. However, the larger MIC of *MKCRE* (ZI =20.2 \pm 1.6 at MIC of 50mg/ml) was more efficacious as compared to Colistin (ZI = 8.8 \pm 1.0 at MIC of 2 μ g) individually. This unusual antimicrobial ability of *MKCRE* against MDR *P. aeruginosa* probably rests in its unique set of common carbazole alkaloids Apigenin, Quercetin, Rutin (flavonoids), saponins, tannins, terpenoids, glycosides and indole alkaloids.²⁰ carbazole alkaloids (mahanine, murrayanole and mahanimbin) of *MKCRE* are responsible for inhibition of bacterial

protein synthesis and the *MKCRE* glycosides lead to bacterial cell death by inhibiting bacterial enzyme activity and metabolism.²¹

These results open a new horizon of opportunity with new treatment options for deadlier MDR *P. aeruginosa* infections that may be cost effective as compared to standard antibiotics like Colistin, possibly reducing the antibiotic related toxic effects and complications,

Conclusion

Our research plant *MKCRE* showed in vitro antibacterial activity against MDR *Pseudomonas aeruginosa*. *MKCRE* showed lowest MIC of 50mg/ml. Therefore, *MKCRE* holds greater anti MDR *P. aeruginosa* potential. With only 3.4% yield, it gave larger ZI than *Colistin*. This also reflects its higher efficacy as anti MDR *Pseudomonas aeruginosa* drug if purified pharmaceutically.

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