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# *IN VITRO* ANTI-BACTERIAL ACTIVITY OF *ROSMARINUS OFFICINALIS* L. AND *MURRAYA KOENIGII* L. AGAINST MULTI-DRUG RESISTANT *STAPHYLOCOCCUS* SPECIES

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## ABSTRACT

*Staphylococcus* species are the most versatile and adaptive organisms. They are widespread in nature and inhabit the skin, mucosa and nose in humans. Search for medicinally valuable plants with antimicrobial activity is being emphasized due to the increasing antibiotic resistance in bacteria. In the present study, the antibacterial potential of *Rosmarinus officinalis* **(**Rosemary) and *Murraya koenigii* (curry leaves) was evaluated against multi-drug resistant *Staphylococcus* clinical isolates using well diffusion method.

In this study, 60 multi-drug resistant clinical isolates of *S. aureus* (43) and Coagulase Negative *Staphylococci* (CoNS) (17) were screened. Out of these 60 isolates, 43 were sensitive to aqueous infusion of rosemary; 23 to aqueous decoction and 58 to ethanolic extract whereas, 24 isolates were sensitive to the essential oil. In case of the curry leaves, no antibacterial activity was observed in aqueous infusion and decoction while only 14 isolates were sensitive to the ethanolic extract. The aqueous infusion of rosemary exhibited an average zone of inhibition of 21±5.69 mm against CoNS and 17 ± 4.77 mm against *S. aureus*, the zone of inhibition of aqueous decoction of rosemary was also greater against CoNS (17 ± 5.78 mm) then *S. aureus* (13 ± 6.91 mm) and the ethanolic extract showed almost similar zone of inhibition against *S. aureus* (22 ± 3.61 mm) and CoNS (21 ± 7.64 mm). Whereas, the essential oil of rosemary showed greater zone of inhibition against *S. aureus* (16 ± 4.67 mm) as compared to CoNS (15 ± 6.94 mm) strains. Aqueous infusion and decoction of curry leaves revealed no significant antibacterial potential against all tested Staphylococcal isolates and ethanolic extract also showed only a weak response. These results demonstrate that *Rosmarinus officinalis* possesses anti-staphylococcal activity.

**Keywords:** Antibacterial activity, curry leaves, multidrug resistant, rosemary, *Staphylococcus*.

## INTRODUCTION

*Staphylococcus* species are the most versatile and adaptive organism (Romero-Pastrana *et al.*, 2010; Vijayakumaran and Dattani, 2010). They are widespread in nature and found on the skin, mucosa (Romero-Pastrana *et al*., 2010; Coutinho *et al*., 2009) and in the nose of humans (Vijayakumaran and Dattani, 2010) as colonizers and can also cause a variety of infectious diseases. An increasing incidence of resistance against antibiotics has been reported in Staphylococci due to the indiscriminate use of antibiotics (Coutinho *et al*., 2009) resulting in alterations in the human microbial flora (Hardy *et al*., 2004). Natural plant products are considered safer as compared to the synthetic antibiotics (Yogisha *et al*., 2009) and could be used as alternative healing products (Coutinho *et al*., 2009). Phytomedicines have been used in primary health care for centuries (Okigbo *et al*., 2008). The emergence of multiple drug resistant bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA) (Chan *et al*., 2008) and the side effects of antibiotics are the main factor for treatment of infectious diseases with medicinal plants (Galadime *et al*., 2010).

Medicinal plants have enormous potential to be used for different clinical conditions including infectious diseases (Yogisha *et al*., 2009). Basic benefits of using herbal medicine are easy availability and affordability for the poor (Tambekar *et al.*, 2009). Different studies have demonstrated that plants and their chemical compounds have antimicrobial properties (Coutinho *et al*., 2009) which provide protection against variety of diseases (Tambekar *et al.*, 2009). Herbs have traditionally been used in the treatment of dysentery, fever, wound infection, jaundice, chicken pox, snake bite, hook worm infection, diarrhea, tooth ache, gingivitis, cough, bronchitis, skin diseases (Galadime *et al*., 2010) and ulcers (Arote *et al*., 2009). The crude extract of plants can be used as a broad spectrum antimicrobial agent (Liag *et al*., 2006).

Essential oils of plants are known as volatile oil, isolated from different parts of the plant by various methods like fermentation, extraction or steam distillation. They are used for the treatment of cancer and have demonstrated antibacterial, antifungal, antiviral and insecticidal activity as well. They are also used as a food preservative, aromatic agent and in fragrance industries (Prabuseenivasan *et al*., 2006).

Curry leaf tree (*Murraya koenigii L.*) is one of the most extensively researched medicinal plants, belongs to family *Rutaceae* and famous for its flavourant, aromatic and medicinal properties (Bansode and Chavan, 2014; Ningappa *et al*., 2010). Curry leaf tree is native to Indo-China but commonly grown in the tropical area. Biologically active compounds of curry leaf are carbazole alkaloids (Ningappa *et al*., 2010) i.e., monomeric, binary carbazoles, simple, furo, pyrano-coumarins (Adebajo *et al*., 2006) tocopherol, β-carotene and lutein (Ningappa *et al*., 2010). Antioxidant protein (APC) present in curry leaf has antibacterial activity against human pathogenic bacteria (Ningappa *et al*., 2010). The fresh curry leaves contain volatile essential oils (Prabhakar *et al.*, 2009; Adebajo *et al*., 2006) and their numerous constituents i.e., sesquiterpenes and monoterpenes contain broad antimicrobial properties (Prabhakar *et al.*, 2009).

Rosemary plant (*Rosmarinus officinalis L.*) belongs to the family of *Lamiaceae* (*Labiatae*). Previous researches have shown that essential oil of rosemary contains biologically active compounds which have high antioxidant and antimicrobial activities (Prabuseenivasan *et al*., 2006) and act as anticancer agent (Genena *et al*., 2008). Important compounds isolated from rosemary plant from different regions of the world are flavonoids, volatile oils, diterpenoids and phenolic acids including caffeic acid, crygogenic acid and rosmarinic acid (Moghtader *et al*., 2009). The antimicrobial effect of rosemary can be attributed to rosmarinic acid, rosmaridiphenol, carnosol, epirosmanol, carnosic acid, rosmanol and isorosmanol (Nieto *et al*., 2018). The compounds with pharmacological significance include ∝-pinene, 1,8-cineol, verbenone and camphor (de Oliviera *et al*., 2017; Moghtader *et al*., 2009). Scientists have isolated many important compounds from rosemary i.e. flavones, diterpenes, steroids and triterpenes (Genena *et al*., 2008).

The aim of this study was to determine the effectiveness of different extracts of curry leaves and rosemary against MDR Staphylococcus clinical isolates as they have limited sensitivity to the available antibiotics and are difficult to treat.

## MATERIALS AND METHODS

The aqueous infusion, aqueous decoction, ethanolic extract of curry leaves and rosemary and essential oil of rosemary were tested for their antimicrobial activity by agar well diffusion assay (Mariam and Abu-Al-Basal, 2009).

### Plant material

Plants were purchased from the local grocery stores. The plant components were washed with tap water and rinsed with distilled water, air dried at room temperature for 4 days and ground to coarse powder form in an electric grinder.

### Preparation of aqueous infusion

A weighed amount of 10, 20, 30, 40, and 50g of powder was soaked in 100mL of sterile distilled water in separate flasks for 24h at room temperature and then filtered through Whatman filter paper No.1, yielding 10, 20, 30, 40 and 50% concentrations. All the aqueous extracts were filter sterilized and stored at 4°C in a sterile bottle until used for antibacterial assay.

### Preparation of aqueous decoction

Aqueous decoction was prepared by soaking 10, 20, 30, 40, and 50g of powder in 200mL of sterile distilled water in separate flasks for 24 h at room temperature. The contents of flasks were then boiled over low flame for 15-20 minutes and allowed to cool. The contents of the flasks were filtered through Whatman filter paper No.1, yielding 10, 20, 30, 40 and 50% concentrations. All the decoctions were filter sterilized and stored at 4°C in a sterile bottle until used for antibacterial assay.

### Preparation of ethanol extract

Ethanol extract was prepared by soaking 10, 20, 30, 40, and 50g of finely powdered curry leaves and rosemary in 100 ml of absolute ethanol in separate flasks for 24 h at room temperature and filtered through Whatman filter paper No.1, yielding 10, 20, 30, 40 and 50% concentrations. The contents were filter sterilized and stored in a sterile bottle.

### Essential oil of rosemary

Essential oil of rosemary was purchased from the local market of Karachi, Pakistan. It was considered as 100% in concentration.

**Test organisms**

Sixty strains of multi-drug resistant Staphylococcus species isolated from various clinical samples as described in a previous study (Mushtaq and Naim, 2015) were used for *in vitro* antibacterial assay.

**Antibacterial assay**

**(a) Base medium**

Mueller-Hinton agar (MHA, Merck) and Mueller-Hinton broth (MHB, Oxoid) were used as base media to detect the anti-staphylococcal activity of different plant extracts by agar well diffusion method.

**(b) Preparation and standardization of inoculum**

A loopful of culture was inoculated in 1ml of Mueller-Hinton broth. The tubes were incubated at 37°C for 18-24 hours. Next day, inoculum was standardized by matching its turbidity with McFarland's tube number 0.5 to get $15\times10^8$ CFU/ml. When the turbidity of the culture was inadequate, additional colonies were inoculated and when the turbidity of culture was excessive then broth was added. The standard inoculum suspension was used within 15-20 minutes.

**(c) Well diffusion assay**

The well diffusion method was employed according to the protocol described by National Committee for Clinical Laboratory Standards (NCCLS, 2000a) for the determination of antimicrobial activity of extracts and essential oil. Briefly, the standardized bacterial suspension was spread on the Mueller Hinton agar (MHA) plates using sterile cotton swab. The surface of the agar was allowed to dry for 3-5 minutes. With the help of sterile borer, wells (5mm diameter) were dug out on each inoculated plate at a distance of 25mm from each other. Fifty microliters of plant extracts were loaded in respective wells. Sterile distilled water, absolute ethanol and DMSO were used as negative controls for aqueous preparations, ethanolic extracts and essential oil respectively. The plates were incubated at 37°C for 18-24 hours and the diameters of the zone of inhibitions were measured in millimeter (Cheesbrough, 2000). All tests were performed in duplicates and results were recorded as the mean ± SD of the diameter of zone of inhibition.

## RESULTS

The results pertaining to the antibacterial potential of the tested plant extracts are given in Table 1. The 50% concentrated ethanolic extract of rosemary exhibited highest antibacterial potential with average zone of inhibition of 22 ± 3.61 mm against the MDR isolates of *S. aureus* and 21±7.64 mm for CoNS followed by the 50% concentration of aqueous infusion of rosemary against CoNS (21 ± 5.69 mm) and *S. aureus* (17 ± 4.77 mm) and the 50% aqueous decoction of rosemary demonstrated an average zone of inhibition of 17± 5.78 mm against CoNS and 13± 6.91 mm against *S. aureus*. While the essential oil of rosemary showed an average zone of inhibition of 16 ±4.67mm against *S. aureus* and 15± 6.94mm for CoNS. The zone of inhibition increased with increasing concentration of different plant extracts. The concentration dependent antibacterial activity of rosemary extracts against S. *aureus* and CoNS.

In the present study, the antibacterial activity of aqueous infusion, aqueous decoction and ethanolic extract of curry leaves was much less effective against the tested MDR isolates. These results represent that a weak inhibitory potential is found only in the ethanol extract of curry leaves against the tested MDR clinical isolates of *S. aureus* and CoNS (Table 1).

## DISCUSSION

The growing concern over the increase in antibiotic resistant organisms has prompted scientists to look for alternative treatments. One such alternative is herbal remedies because herbs and their extracts contain antimicrobial properties. Herbal preparations are widely used as self-medication for acute conditions as well as chronic ailments. Therefore, researchers have been studying the activity of herbs against pathogenic bacteria (Mostafa *et al*., 2018; Khan *et al*., 2013). *Staphylococcus* species have the ability to cause life threatening infections and the treatment is difficult due to the high level of antibiotic resistance against multiple antimicrobial drugs, which is an alarming situation. This study focused on proposing an alternate treatment for MDR Staphylococcal infections in the primary care by utilizing common household herbs like rosemary and curry leaves. These herbs are cost effective, easily available throughout the year and have no side effects as compared to antibiotics.

Table 1. Antibacterial activity of *Rosmarinus officinalis L.* and *Murraya koenigii L.* extracts against Staphylococcus species

| Plant material | Extraction | Concentration % | Antibacterial Activity Average Zone of Inhibition ( average ± SD ) | |
|---|---|---|---|---|
| | | | *S. aureus* | CoNS |
| Rosemary | Aqueous infusion | 10 | 7 ± 6.94 | 15 ± 6.92 |
| | | 20 | 12 ± 5.81 | 17 ± 6.19 |
| | | 30 | 13 ± 5.86 | 18 ± 6.60 |
| | | 40 | 14 ± 6.61 | 18 ± 7.98 |
| | | 50 | 17 ± 4.77 | 21 ± 5.69 |
| | Aqueous decoction | 10 | 7 ± 6.25 | 13 ± 5.59 |
| | | 20 | 9 ± 6.53 | 14 ± 6.33 |
| | | 30 | 10 ± 6.95 | 15 ± 6.57 |
| | | 40 | 10 ± 7.02 | 15 ± 6.76 |
| | | 50 | 13 ± 6.91 | 17 ± 5.78 |
| | Ethanolic extract | 10 | 13 ± 2.09 | 13 ± 4.47 |
| | | 20 | 15 ± 1.90 | 15 ± 5.37 |
| | | 30 | 18 ± 2.33 | 18 ± 6.36 |
| | | 40 | 19 ± 2.93 | 20 ± 7.15 |
| | | 50 | 22 ± 3.61 | 21 ± 7.64 |
| | Essential oil | 100 | 16 ± 4.67 | 15 ± 6.94 |
| Curry leaves | Aqueous infusion | 10 | - | - |
| | | 20 | - | - |
| | | 30 | - | - |
| | | 40 | - | - |
| | | 50 | - | - |
| | Aqueous decoction | 10 | - | - |
| | | 20 | - | - |
| | | 30 | - | - |
| | | 40 | - | - |
| | | 50 | - | - |
| | Ethanolic extract | 50 | 15 ± 2.57 | 14 ± 4.42 |

SD= Standard deviation; - = No activity

In the present study, the antibacterial activity of different concentrations of aqueous infusion, decoction and ethanolic extract of rosemary and curry leaves and essential oil of rosemary against clinical isolates of Staphylococcus species was investigated. Extracting ingredients from medicinal plants may affect their synergistic activity and thus the therapeutic value. Therefore, the evaluation of crude extracts holds particular significance.

The evaluation of *in vitro* antibacterial effect of rosemary exhibited the highest activity of ethanolic extract against MDR *S. aureus* and CoNS clinical isolates followed by the aqueous infusion. Our results showed that aqueous extracts of the rosemary were able to inhibit the growth of the tested Staphylococcus strains effectively. These results support and further extend former studies on antimicrobial activity of rosemary extracts (Genena *et al*., 2008). They showed that rosemary has antibacterial activity against gram positive as well as gram negative bacteria. Chan *et al*., (2012) also reported stronger antibacterial activity of Rosemary extracts among the eight culinary herbs tested. Results from previous studies have also shown that rosemary extracts have antilisterial activity (Rozman *et al*., 2009). Previous studies have noticed that gram positive bacteria having only peptidoglycan in their cell wall are more sensitive to antimicrobial agents as compared to the gram negative bacteria which have an outer membrane in their cell wall (Marium *et al*., 2009).

The ethanolic extract has potential to inhibit the growth of Staphylococcus species, which provides an evidence for the presence of highly active antibacterial agents in the extract. These *in vitro* results also suggest that the water-soluble compounds present in the aqueous extracts of the rosemary may possess the antibacterial activity. The

results further revealed that preparing an extract with an organic solvent (ethanol) shows a better antibacterial activity while antibacterial activity of aqueous decoction was also affected when extracted in boiling water indicating that the plant material contains thermostable bioactive compounds. Furthermore, in this study, the action of the rosemary extracts on Staphylococcus species at various concentrations showed that the growth of these organisms was decreased by increasing concentration of the extract, indicating a concentration dependent inhibitory effect. The rosemary extract at different concentrations was applied and the results showed that the extract exhibited bactericidal activity even at the lowest concentration tested. Similar results indicating increased inhibitory activity of ethanolic extract with increasing concentration against pathogenic bacteria was reported by Golshani *et al*. (2014).

The essential oil of rosemary inhibited the growth of MDR Staphylococcal strains. Our results are in fair correlation with another study carried out by Abdullah *et al*., (2015) and Prabussenivasan *et al.* (2006).

The preliminary data assessing the *in vitro* antimicrobial effect of curry leaves revealed that aqueous extracts did not exhibit antibacterial activity at the various concentrations used against the MDR strains in the current study and the ethanolic extract also showed weak activity. These findings are in contrast with previous studies on the curry leaves where antifungal, (Adebajo *et al*., 2006), antioxidant, antidiabetic and antimicrobial activity was demonstrated (Ningappa *et al.,* 2010). The information about the antimicrobial activity of the aqueous extract (infusion and decoction) of curry leaves is lacking in the literature. In the present study, aqueous infusion and decoction of curry leaves did not demonstrate any anti-staphylococcal activities while the ethanolic extract exhibited antibacterial activity only to some extent. Ejaz *et al*. (2014) screened 29 Pakistani medicinal plants for antibacterial activity against MDR *S. aureus* strains but reported potent activity in five plant extracts only. The variations in the antibacterial activity of curry leaves reported in different research studies could be due to the differences in the extraction techniques, varying concentrations used, geographical variations in the phytochemical constituents of the plant and the test organisms studied.

This study clearly demonstrates that the different rosemary extracts have antistaphylococcal activity and further investigations are needed to confirm their *in vivo* potential and also evaluate the cytotoxicity of these extracts.

**Conclusion**

To conclude, in the light of these results it can be stated that rosemary can be used as a potential source for the development of a phytomedicine against variety of disease-causing strains of Staphylococcus species. As the global scenario is now changing towards the use of non-toxic plant products having traditional medicinal use, development of modern drugs from *Rosmarinus officinalis* L. should be sort through for the control of Staphylococcal infections.

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