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Research Article

COMPARATIVE ANTIMICROBIAL ACTIVITIES OF NEEM AND CURRY LEAF EXTRACTS AND THEIR SYNERGISTIC EFFECT AGAINST SELECTED PATHOGENIC BACTERIA AND FUNGUS

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ABSTRACT

There is a growing demand today for naturally derived drugs and medicine in place of synthetic drugs that are more commercially available. Such drugs derived from indigenous plants are effective as they contain potent therapeutically active compounds of the plants that show antimicrobial activity against a variety of pathogens that cause common diseases. Such diseases as periodontitis and oral candidiasis are a growing concern these days. This study was undertaken to investigate the antimicrobial activity of two indigenous Indian plants, Azadirachta indica and Murraya koenigii and their synergistic effect, if any, against three pathogenic microorganisms associated with oral diseases and one pathogenic bacteria known to be a nosocomial causative agent. Three bacterial species (Streptococcus mutans, Streptococcus gordonii, Pseudomonas aeruginosa) and one fungal species (Candida albicans) were selected for antimicrobial assays. Both ethanolic and methanolic extracts were prepared from the leaves of the two plant species in addition to an aqueous extract of A. indica and an n-hexane extract of M. koenigii. Antibacterial and antifungal assay was done using agar well diffusion method under standard culture conditions. Inhibitory activity was observed for all extracts against P. aeruginosa while A. indica and M. koenigii showed ineffectiveness in inhibiting growth of C. albicans and S. mutans respectively. C. albicans and S. mutans were selected for study of synergistic effect based on the results of the initial assay, however there was no synergistic activity observed for any combination of extract mixtures.

Key Words: Azadirachta indica, Candida albicans, Murraya koenigii, Pseudomonas aeruginosa, Streptococcus gordonii, Streptococcus mutans, synergistic effect

INTRODUCTION

The medicinal potential of several plant species against a plethora of pathogenic microorganisms is well documented in literature, with an exponential growth of academic research on the antimicrobial activities of such plants. More importantly, the study of these indigenous species is a commendable way of preserving the Indian medicinal and scientific heritage as well as move towards developing indigenously made herbal medicines for a variety of diseases instead of importing foreign manufactured drugs. Often, these plants have been demonstrated to have more potency than standard drugs, for instance, when Pillai and Santhakumari 1981¹ revealed that nimbidin, derived from neem oil, served as a much more potent antiinflammatory drug than the standard drug phenylbutazone. Another concern is the increasing resistance of common bacterial strains to standard antibiotics as Clewell 1981² discusses how isolated strains of Streptococcus sp. have become resistant to erythromycin, chloramphenicol, aminoglycosides and tetracyclines. Indigenous plants with medicinal properties are thus being explored for their potential activity against drug-resistant bacterial strains too, for instance, in the US patent US6599541 B1 (2003) 3 wherein Brindavanam et al disclose a composition for treating resistant bacteria, comprising an extract obtained from Azadirachta indica (neem).

The present study focuses on the antimicrobial activity of *Azadirachta indica* and *Murraya koenigii* against 3 bacterial strains namely: *Streptococcus gordonii* MTCC 2695, *Streptococcus mutans* MTCC 497 and *Pseudomonas aeruginosa* MTCC 1688 and 1 fungal strain, namely *Candida albicans*.

Streptococcus gordonii is a gram-positive, mesophilic cocci that grow in short, bead-like chains. They are non-motile, non-sporing and mainly aerobic.4 It exists as part of the human oral microflora and colonizes the smooth tooth surface, contributing to formation of dental plaque. Upon invading the bloodstream, it may also cause endocarditis.⁵ Entenza et al⁶ demonstrates the tolerance of the tolerant derivative of S. gordonii to penicillin in the penicillin treatment of experimental endocarditis. This demands the need for a potent alternative antibiotic, with A. indica serving as a potential candidate since its bark extracts have been shown to inhibit growth of Streptococcus sanguis⁷, a close relative to S. gordonii. Experimentation with curry leaves (Murraya koenigii) has shown antimicrobial activity, albeit not very substantive, against Streptococcus mutans8, again a close relative of S. gordonii yet no literature was found for antimicrobial activity of M. koenigii against S. gordonii itself. Hence, strains of S. gordonii and S. mutans have been selected for this study as their genetic similarity to S. sanguis may lead to similar inhibition patterns due to presence of A. indica.

Streptococcus mutans is a gram-positive, non-capsulated coccus, about 0.75 m in diameter when grown on a medium with a neutral or alkaline reaction. S. mutans is a major pathogenic agent of dental caries⁹ and like S. gordonii, it has been known to cause infective endocarditis¹⁰ as well as periodontitis and atheroma. Yavier et al (2007) 11 reports the significant activity of A. indica against S. mutans but even though Prabhakar A.R. et al (2009) 8 shows antibacterial activity of M. koenigii against S. mutans, it is reported to show poor substantiality.

Pseudomonas aeruginosa is a gram-negative, rod-shaped, asporogenous, and monoflagellated bacterium. It is found in a variety of environmental settings including in hospitals, sourced from

respiratory therapy equipment, antiseptics, soap, sinks, mops, medicines, and physiotherapy and hydrotherapy pools. This is a primary reason why its colonization rates exceed 50% in patients upon hospitalization and most of the infections of *P. aeruginosa* are hospital-acquired. These include nosocomial pneumonia, ventilator-associated pneumonia and urinary tract infections. ¹² *P. aeruginosa* has been proved to be resistant to common antibiotics such as kanamycin and ampicillin by Jain et al (2011). ¹³ Mehrotra et al (2010) has shown the effectiveness of ethanolic extract of *A. indica* in inhibiting growth of *P. aeruginosa*. ¹⁴ Mathur et al (2011) has shown activity of *M. koenigii*, especially through its compound 9, 12 octadecadienoic acid, against *P. aeruginosa* although the activity was reported to be only moderate and that too using the ATCC 25619 strain¹⁵, whereas the present study uses the MTCC 1688 strain of *P. aeruginosa*.

Candida albicans is a diploid fungus that grows as both yeast and filamentous cells and is a causal agent of opportunistic oral and genital infection in humans. 16 It is the most common cause of oral candidiasis, an infection that causes white patches on the tongue and other parts of the mouth and the throat. Oral candidiasis occurs due to the overgrowth of the C. albicans colonies existing as part of natural human oral flora, especially when the infected's immunological defences have been undermined.¹⁷ Bohora et al (2010) demonstrates the efficacy of ethanolic extract of neem leaf against C. albicans, showing greater antifungal activity than the standard irrigant sodium hypochlorite. 18 The aqueous extract of A. indica also inhibits growth and colonization of C. albicans although at higher concentrations as shown by Polaquini et al (2006).¹⁹ Doddanna et al (2012) has shown that ethanolic extracts of curry leaf are quite effective in their activity against C. albicans although the aqueous extract has close to no activity. 20 This is further verified by Disegha et al (2014) where alcoholic extract of M. koenigii showed maximum inhibitory effect whereas the cold aqueous extract was the least effective in inhibiting both C. albicans and other fungal species.²¹ There has been no study yet in literature on the synergistic activity of M. koenigii and A. indica on C. albicans.

Neem, or Azadirachta indica, is well known for its numerous pharmacological properties including antibacterial, antifungal, antiulcer, repellent and pesticide among others.²² These activities arise due to the presence of nimbin (C₃₀H₃₆O₉), nimbinene desacetylnimbinase, nimbandial, nimbolide $(C_{27}H_{30}O_7)$ and quercentin (C₁₅H₁₀O₇) in neem leaves.^{23,24,25} Hence, not surprisingly, neem leaf extracts have been used to control leprosy, intestinal helminthiasis and respiratory disorders in children²⁶ and a range of viruses and pathogens including Vaccinia virus, Chikungunya and measles virus²⁶, both Gram-positive (example: Staphylococcus sp.) and Gram-negative (example: Escherichia coli) bacteria as well as a host of other bacterial strains such as Bacillus cerus, Proteus vulgaris, Salmonella typhi, Klebsiella pneumoniae, Shigella dysenterae. Among fungal strains, neem shows antifungal activity against Fusarium oxysporum, Aspergillus flavus, Aspergillus fumigates, Aspergillus niger, Candida albicans, Cladosporium sp., Microsporum canis, Microsporum gypseum, Trichophyton rubrum and Trichophyton mentagrophytes. ²⁶ Thus it would be interesting to study the antimicrobial effect of neem against the selected pathogens too, adding to the repertoire of microbial species that are susceptible to inhibition by neem.

Curry-leaf, or *Murraya koenigii*, is native of India, Sri Lanka and other South Asian countries and is distinguished by its sharp smell. It is well known for its antidysentric, antianemic, antiulcer, antidiarrheal, antidiabetic and analgesic properties in addition to its antibacterial and antifungal activities. The leaves are often applied externally as a traditional remedy for burns and skin itches. Among its antibacterial activity, *M. koenigii* significantly inhibits growth of *Staphylococcus epidermidis*, due to the activity of three carbazole alkaloids namely murrayanol (C₂₀H₂₀O₉), mahanine (C₂₃H₂₅NO₂) and

mahanimbine ($C_{23}H_{25}NO$). ^{27, 28, 29} Common fungal species against which *M. koenigii* shows activity include *Candida albicans*, *Aspergillus niger* and *Trichophyton rubrum*, due to the monoterpenoids and sesquiterpenoids present in the leaves of *M. koenigii*. ³⁰ The plant is also highly valued for its leaves an important ingredient in an Indian cuisine to promote appetite and digestion. ³¹

The present study seeks to study *in vitro* antibacterial activity of *Azadirachta indica* and *Murraya koenigii* against the aforementioned strains of *S. gordonii, S. mutans P. aeruginosa* and *C. albicans* and more importantly, to determine if there exists a synergistic effect upon combining the extracts of the two plants and testing them for antibacterial activity against the 3 bacterial strains and antifungal activity against the fungal strain.

MATERIALS AND METHODS

Collection of plant material

Fresh leaves of *Azadirachta indica* (Family: *Meliaceae*; Common Name: Neem (English)) and *Murraya koenigii* (Family: *Rutaceae*; Common Name: Curry tree (English)) were collected from a local vendor in Matunga (E), Mumbai, India and were authenticated by Dr (Mrs.) A.S. Upadhye at the Agharkar Research Institute, Pune, India.

Preparation of plant extracts

The collected leaves of A. indica and M. koenigii were kept for slow drying in an oven for 48 hours at a temperature of 60°C. After drying, the leaves were powdered using a mixer and collected and stored in a cool, dry place. For extraction, cold extraction procedure was used²³ albeit modified. 1g of powdered plant material of A. indica was added to 10 cm³ of methanol, ethanol and distilled water while 1g of powdered plant material of M. koenigii was added to 10 cm³ of methanol, ethanol and n-hexane. These solvents were chosen in particular as they have been commonly featured in literature for extraction purposes. Polar solvents like ethanol and methanol were chosen to extract bioactive compounds that are usually polar, while n-hexane was used to extract any non-polar compounds that might show desired activity. They were then placed for extraction in a rotary shaker for 27 hours at a rate of 35 rpm. After extraction, the mixtures were filtered through Whatman no. 1 filter paper and the filtrates were kept in a water bath at 80°C for complete evaporation to obtain dry, pure extracts. The dry extracts were then dissolved in 1 cm³ of dimethyl sulfoxide (DMSO) and stored in glass vials.

Preparation of cultures

3 bacterial strains were used, namely – *Streptococcus gordonii* MTCC 2695, *Streptococcus mutans* MTCC 497 and *Pseudomonas aeruginosa* MTCC 1688 along with 1 fungal strain namely *Candida albicans*. The bacterial cultures were grown on Luria Bertani broth (LB) slants for 24 hours at 37°C while fungal culture was grown on Sabouraurd's agar slant for 24 hours at room temperature.³²

Antimicrobial assay

The agar well diffusion technique³³ was used for antibacterial assay of all 4 strains. For the purposes of antibacterial assay, the cultures in actively growing phase were introduced into 20 cm³ molten butts of sterile Mueller Hinton Agar following which they were poured into Petri plates. In each Petri plate, 5 wells were made using a 7mm-diameter cork borer. 50 microlitres of each extract of *A. indica* was poured into one of the wells while the remaining 2 wells contained 50 microlitres of the standard antibiotic, ciprofloxacin 20 ppm (positive control) and 50 microlitres DMSO (negative control) respectively. The same process was repeated for extracts of *M. koenigii*. For study of synergistic activity, in each well, 25 microlitres of each extract of *M. koenigii*

to make a total of 9 combinations. At the end of incubation of 24 hours at 37°C, the diameter of the zones of inhibition was measured. The assay was performed in three independent trials.

RESULTS

The antimicrobial activity of the plant extracts was qualitatively assessed. As seen in Table 1, all 3 extracts of *A. indica* were effective against the pathogenic strains except against *C. albicans* where all 3 extracts failed to inhibit the growth of the fungus. All 3 extracts of *M. koenigii* too were effective in their activities except against *S. mutans*. Across all 4 pathogens and all 3 extracts of both the plant species, the zone of inhibition was similar, falling in the range of 10.0mm – 12.0mm. The exception was activity against *P. aeruginosa* that showed a larger zone of inhibition of 13.0mm – 14.0mm.

In the antibacterial assay, the highest inhibitory effect was shown by the methanolic and ethanolic extract of M. koenigii (zone of inhibition = 14.0 ± 0.5 mm) both against P. aeruginosa, while the least activity was shown by the aqueous extract of A. indica against S. mutans and n-hexane extract of M. koenigii against S. gordonii (zone of inhibition = 10.0 ± 0.5 mm). The aqueous extract of A. indica showed no activity against S. gordonii while none of the extracts of M. koenigii showed activity against S. mutans.

In the antifungal assay of *C. albicans*, the highest inhibition was demonstrated by the methanolic extract of *M. koenigii* (zone of

inhibition = 12.0 ± 0.5 mm) and the least by the aqueous extract of the same plant species (zone of inhibition = 10.0 ± 0.5 mm). None of the extracts of *A. indica* were effective against the fungal strain of the present study.

The measured zones of inhibition of the plant extracts were lesser than those of the standard inhibitor ciprofloxacin. However, the presence of a zone of inhibition in the cultures inoculated with the plant extracts indicates the potent antimicrobial activity of these extracts and their potential to be used in herbal antibiotics that are not artificially synthesized.

For synergistic effect, nine combinations were selected from extracts of the two plants. These combinations, as shown in Table 1, were methanol (neem) – hexane (curry leaf), methanol (neem) – methanol (curry leaf), ethanol (neem) – hexane (curry leaf), ethanol (neem) – hexane (curry leaf), ethanol (neem) – methanol (curry leaf), ethanol (neem) – ethanol (curry leaf), distilled water (neem) – hexane (curry leaf), distilled water (neem) – methanol (curry leaf) and distilled water (neem) – ethanol (curry leaf). These combinations were chosen since for the two selected pathogens, *S. mutans* and *C. albicans*, no extract of curry leaf and neem was effective respectively. Hence, it was important to see if any of one plant's extracts would have a synergistic effect with any of the other plant's extract for some activity against the selected pathogens.

Table 1.	Antimicrobial	activity of	f various	extracts of A	indica AN	D M koenigii

Plant species	Extract solvent	Zone of inhibition/mm (± 0.5mm)				
		S. mutans	S. gordonii	P. aeruginosa	C. albicans	
A. indica	Methanol	11.0	11.0	13.0	0.0	
	Ethanol	10.5	12.0	12.0	0.0	
	Distilled water	10.0	0.0	12.0	0.0	
M. koenigii	Methanol	0.0	12.0	14.0	12.0	
	Ethanol	0.0	11.0	14.0	11.0	
	n-Hexane	0.0	10.0	13.0	10.0	
Positive control		15.0	24.0	25.0	24.0	
(Ciprofloxacin)						



Figure 1: Antibacterial assay of ethanolic, methanolic and hexane extract of *M. koenigii* against *S. gordonii* (C1 = hexane extract; C5 = ethanolic extract; C7 = methanolic extract)

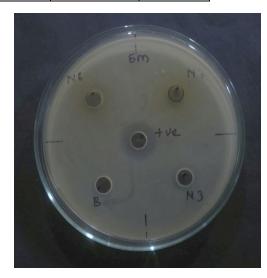


Figure 2: Antibacterial assay of ethanolic, methanolic and aqueous extract of *A. indica* against *S. mutans* (N3 = aqueous extract; N6 = methanolic extract; N7 = ethanolic extract)

DISCUSSION

It has been shown in literature that the antimicrobial activity of both A. indica and M. koenigii are due to certain pharmacologically active phytochemicals sourced from various parts of the plant. Aslam et al (2009) has confirmed the presence of alkaloids, flavonoids, crude and cardiac glycosides, steroids, triterpenoids, tannic acid and saponins in significant concentrations in the leaf extract of A. indica. These are thought to be the active compounds that inhibit bacterial growth, especially against strains of Streptococcus genus.34 The antifungal activity of A. indica can be explained from the findings of Pant et al (1986) that show the leaf extract to contain cyclic trisulphide and cyclic tetrasulphide³⁵ that inhibit fungal growth. As for *M. koenigii*, Mathur et al (2011) not only showed the compound 9,12 octadecadienoic acid to be a potent antimicrobial agent but also confirmed the presence of other active compounds including alkaloids, tannins, saponins and glycosides and with steroids being present prominently. 15

Further studies need to be done to understand why no synergistic activity exists between the 2 plant extracts. One possible reason could be that the compounds extracted via the selected procedure may have had antagonistic effect instead of synergistic. Another reason could be that the phytochemicals of the 2 plant extracts may have not interacted at all. However, the mixing of extracts meant a smaller quantity of extract dissolved in a larger volume of solvent and thus due to decreased concentration, the individual activities of the phytochemicals may have reduced too.

CONCLUSION

All 3 extracts of *A. indica* and *M. koenigii* showed that they were effective in inhibiting the *in vitro* growth of the pathogenic bacteria *S. gordonii* and *P. aeruginosa* with the exception of aqueous extract of *A. indica* for the former. For the pathogen, *S. mutans*, all 3 extracts of *M. koenigii* were ineffective in antibacterial activity. For the fungal pathogen, *C. albicans*, all 3 extracts of *A. indica* were ineffective. On an average, the highest activity was shown against *P. aeruginosa*. No synergistic effect was observed for any of the different mixtures of the plant extracts against *S. mutans* and *C. albicans*.

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