Original Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

IN VITRO EVALUATION OF SELECTED MEDICINAL PLANTS FOR ANTIMICROBIAL ACTIVITIES AGAINST ANTIBIOTIC RESISTANT CLINICAL BACTERIAL AND FUNGAL PATHOGENS

QASIM TURQI KADHIM[*1] AND HUSSEIN HAMEED ABBAS[2]

[1,2]Department of Biotechnology, Acharya nagar juna University, Guntur, India.

ABSTRACT

Dried leaves of *Rubia cordifolia*, *Psidium guajava* and *Ocimum sanctum* obtained from Amrut Kesari, Bangalore, were extracted using methanol as extraction solvent via percolation method at temperature of 50°C. The leaf extracts were tested for antimicrobial activity against clinical isolates of *Streptococcus mutans*, *Streptococcus salivarius, Escherichia coli*, *Salmonella typhimurium*, *Aspergillus flavus* and *Candida albicans* using disc diffusion and broth dilution techniques. The leaf extracts of *Rubia cordifolia*, *Psidium guajava*, and *Ocimum sanctum* showed antibacterial activity against the Gram-positive *Streptococcus mutans*, *Streptococcus salivarius* and Gram-negative *Escherichia coli*, *Salmonella typhimurium* and a remarkable antifungal activity were shown against *Candida albicans*. *P. guajava* showed the highest antimicrobial activity (10-20 mm inhibition in diameter) for these microorganisms in the disk diffusion and minimum inhibitory concentration (256-1024µg/ml) assays in comparison with standard ciprofloxacin which showed maximum inhibitory activity(22-35mm inhibition in diameter) in disc diffusion and minimum inhibitory concentration(0.5-1.0µg/ml) assay.

KEYWORDS: ***Antimicrobial activity, Plant extract, Clinical bacterial and fungal pathogens, Antibiotic resistant, Disc diffusion method.***

QASIM TURQI KADHIM

Department of Biotechnology, Acharya nagar juna University, Guntur, India.

***corresponding Author**

Received on: 24-05-2017
Revised and Accepted on: 12-07-2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.3.p350-355

INTRODUCTION

It is well known fact that infectious disease, particularly those involving micro-organisms i.e. bacteria and fungi causes serious infection in tropical and subtropical countries of the world and responsible for nearly one half of the death in India.[1] Due to indiscriminate use of commercial antimicrobial drugs, commonly used to the human pathogenic microorganisms have develop multiple drugs resistance which creates enormous health problem.[2] India has a rich plant resources providing valuable medicine, which are conveniently used in Ayurveda, Unani, and other system of medicines for the treatment of various diseases.[3] Plants are rich in a wide variety of secondary metabolite such as tannins, terpenoids, alkaloids, flavonoids, etc.[4] The use of plant extracts and phytochemicals both with known antimicrobial properties can be of great significance in therapeutic treatments.[5] Antimicrobial medicines can be grouped according to the microorganisms they act primarily against. For example, antibacterial (commonly known as antibiotics) are used against bacteria and antifungals are used against fungi. They can also be classed according to their function. Antimicrobials that kill microbes are called microbicidal; those that merely inhibit their growth are called microbiostatic. Disinfectants such as bleach are non-selective antimicrobial.[6] Antibacterials or antibiotics are used to treat bacterial infections. The toxicity to humans and other animals from antibiotics is generally considered low. However, prolonged use of certain antibiotics can decrease the number of gut flora, which may have a negative impact on health.[7] Antifungals are used to kill or prevent further growth of fungi. In medicine, they are used as a treatment for infections such as athlete's foot, ringworm and thrush and work by exploiting differences between mammalian and fungal cells. They kill off the fungal organism without dangerous effects on the host. Unlike bacteria, both fungi and humans are eukaryotes. Thus, fungal and human cells are similar at the molecular level, making it more difficult to find a target for an antifungal drug to attack that does not also exist in the infected organism. Consequently, there are often side effects to some of these drugs. Some of these side effects can be life-threatening if the drug is not used properly.[8] Conditions must be met for the screening of antimicrobial activity:1) there should be intimate contact between the test organisms and substances to be evaluated. 2) Required conditions should be provided for the growth of microorganisms. 3)Conditions should be same through the study. 4) Aseptic / sterile environment should be maintained. Various methods have been used from time to time by several workers to evaluate the antimicrobial activity. The evaluation can be done by the following methods: 1) Turbidimetric method. 2) Agar streak dilution method. 3) Serial dilution method. 4) Agar diffusion method. The plant extracts and the secondary metabolites possess antimicrobial, antifungal and antiviral activity.[9] The various plant products that are regularly used for their therapeutic potential, and plant or plant products that form the part of the food or as dietary components, have been consider attention. Plants extracts and essential oil have been used for many thousands of years.[10] Looking for new antimicrobial agents is a priority, one new/old approach is using medicinal plants. Many antimicrobial studies showed that plant crude extracts have bactericidal and fungicidal effects which could be a solution for the raising problems caused by the antibiotic-resistant bacteria.[11-12] Rubia cordifolia L., is a traditional Indian and Chinese medicinal plant which have been listed at the Chinese pharmacopoeia in 2015.[12] Psidium guajava is a medium sized tree with evergreen ,opposite,aromatic short-petioled leaves. The inflorescence axillary 1-3 flowered trees are used for treatment of various disease conditions especially in developing countries.[13] Different parts of the plant are used in flok medicine for the treatment of various human ailments such as wounds, ulcers, bowels and cholera.[14] Among the medicinal plants, *Ocimum sanctum*, Tulsi, or Holy Basil from the family Lamiaceae has been described as the “Queen of plants” and the “mother medicine of nature” due to its perceived medicinal qualities.[15] The 3 plants have been used locally for their traditional medicinal properties, all of which have documented antimicrobial activities. However, their efficacies against MDR bacteria have not been studied.In the present study, *Rubia cordifolia*, *Psidium guajava* and *Ocimum sanctum* were selected for evaluation antimicrobial activity and minimum inhibitory concentration assay.

MATERIALS AND METHODS

Collection and extraction of plant samples

Rubia cordifolia, *Psidium guajava* and *Ocimum sanctum* were collected from Amrut Kesari, Bangalore. And were authenticated and specimen samples deposited at Skanda Life Sciences Private Limited, Bangalore, Karnataka. 10 g of each plant powdered was taken and mixed with 50ml of methanol for keeping it for 50ºc,4 hrs in the water bath after filter the solution supernatant solution has taken and discarded the pellet after the filtration is evaporated for 80ºc in water bath compound were transfer the tubes.

Determination of Antibiotic susceptibility of selected human pathogens (MIC)

The following test cultures were used : bacterial cultures (*Streptococcus mutans*, *Streptococcus salivarius*, *Escherichia coli* and *Salmonella typhimurium*) and fungal organisms (*Candida albicans* and *Aspergillus flavus).* Inoculums were Bacterial cultures were grown on Trypticase soy broth adjusted to 1-2 x 10^5Cell suspension prepared cells/ml. Antibiotics concentrations: 1-64µg/ml of test antibiotics (Two fold dilutions) in Trypticase soy broth.Control was Trypticase soy broth inoculated with culture and without drug. 90ml drug / test compounds of different test concentration were mixed with 10ml inoculum in 96 well plate in triplicate. The control prepared by mix 90ml Trypticase soy broth without drug with 10ml

Inoculum. Treated bacterial cultures are incubated at 22°C and 35°C. The bacterial test plates were observed after 24-48 hrs and O.D at 600 nm is measured in Tecan plate reader.

Evaluation of antimicrobial activity by Agar Disk Diffusion Method

Agar disk-diffusion testing developed in 1940 [16], is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. Nowadays, many accepted and approved standards are published by the Clinical and Laboratory Standards Institute (CLSI) for bacteria and yeasts testing.[17-18]

Screening of test extracts for antimicrobial activity by Agar disk diffusion technique

Test organisms are the bacteria : *Streptococcus mutans*, *Streptococcus salivarius*,

- Gram positive bacteria: *Streptococcus mutans* and *Streptococcus salivarius, Escherichia coli* and *Salmonella typhimurium* and the fungal pathogens : *Candida albicans* and *Aspergillus flavus.* Inoculum was cell suspension prepared from cultures grown on Trypticase soy broth adjusted to 1-2 x 10^5cells/mL. For fungi spore suspension of the cultures grown on Sabouraud Dextrose agar was adjusted to 1-2 x 10^5 cells /mL.
- Test concentrations: drug concentration prepared
- Test compounds: 100 mg/mL in Methanol
- Control: Methanol

Determination of Antimicrobial activity

100 µl Inoculum of test cultures was inoculated on Muller Hinton Agar plates (90 mm) for bacterial and Sabouraud Dextrose agar for fungal cultures. Test compounds (10µl, 100 mg/mL), and ciprofloxacin (10 µl, 1 mg/mL) were impregnated on 6mm sterile Whatmann No. 1 Disks for bacterial cultures. Flucanazole (10µl, 10mg/mL) was used as positive control for fungal cultures. Test compounds and standard disks were placed on Agar plates. The plates were Incubated at 35 °C for 24-48 hrs and observe for zone of inhibition around the disk.[19]

MIC determination against test clinical isolates by micro broth dilution technique as per NCCLS method.[20]

The following organisms were tested: the bacterial isolates *Streptococcus mutans*, *Streptococcus salivarius*, *Escherichia coli* and *Salmonella typhimurium*.The fungal islostes were *Candida albicans* and *Aspergillus flavus*. *O. sanctum, R. cordifolia,and P. guajava* were used as a test compounds. The standard antibiotics were for bacterial isolates and crystal violet for fungal isolates. To prepare inoculum suspension prepared from bacterial cultures grown on Tryptone broth adjusted to 1-2 x 10^5cells/ml. Drug concentration prepared as:

i. Ciprofloxacin: 0.25-16µg/ml (Two fold dilutions) in Tryptone Broth
ii. Test compounds

16 - 1024µg/ml (Twofold dilutions) in Tryptone broth for bacterial test cultures

The control was a Tryptone broth inoculated with culture and without test compounds. 100□l drug / test compounds of different test concentration mixed with 10□l Inoculum in 96 well plate in triplicate. Control prepared by mix 100□l Tryptone broth without drug with 10□l Inoculum. Treated bacterial cultures are incubated at 37°C. The bacterial test plates were observed after 24-48 hours and O.D @ 590 nm is measured in Tecan plate reader. Determine MIC as Minimum concentration of drug giving 50% inhibition of OD as compared with control

RESULTS

Antibiotic susceptibility profile of clinical isolates: the antibiotic susceptibility pattern of the clinical bacterial isolates showed among the bacterial isolates tested, all the organisms were sensitive to only Methicillin and ciprofloxacin. All the isolates were resistant to Pencillin , and Amikacin. Gram negative pathogens were resistant to clindamycin, and Oxacillin. Antibiotic susceptibility pattern of clinical fungal isolates showed all the fungal isolates were resistant to common antifungal antibiotics Itracanazole and Ketacanazole. Intermediate resistance was observed for *A. flavus* for posaconazole and Varicanozole.

Evaluation of antimicrobial activity against fungal and bacterial pathogens

Table 1
Inhibitory activity of plant extracts against bacterial pathogens

Test Organisms	Test Extracts	Concentration (10µl, mg/ml)	Zone of inhibition
Escherichia coli	*Rubia cordifolia*	100	12.50 ± 0.50
	Ocimum sanctum	100	9.00 ± 0.00
	Psidium guajava	100	14.00 ± 0.00
	Ciprofloxacin	1	34.50 ± 0.50
Streptococcus salivarius	*Rubia cordifolia*	100	14.50 ± 0.50
	Ocimum sanctum	100	12.50 ± 0.00
	Psidium guajava	100	12.50 ± 0.50
	Ciprofloxacin	1	34.00 ± 0.00
Salmonella typhimurium	*Rubia cordifolia*	100	10.00 ± 0.00
	Ocimum sanctum	100	10.00 ± 0.00
	Psidium guajava	100	13.00 ± 0.00
	Ciprofloxacin	1	34.00 ± 0.00
Streptococcus mutans	*Rubia cordifolia*	100	13.00 ± 0.00
	Ocimum sanctum	100	13.00 ± 0.00
	Psidium guajava	100	16.00 ± 1.00
	Ciprofloxacin	1	36.00 ± 1.00
Candida albicans	*Rubia cordifolia*	100	10.00 ± 0.00
	Ocimum sanctum	100	11.50 ± 0.50
	Psidium guajava	100	13.50 ± 0.50
	Crystal violet	1	22.50 ± 0.50
Aspergillus flavus	*Rubia cordifolia*	100	-
	Ocimum sanctum	100	-
	Psidium guajava	100	11.00 ± 0.00
	Crystal violet	1	24.50 ± 0.50

It is evident from Table 1 that *Psidium guajava* has shown the highest antimicrobial activity (11-16mm inhibition in diameter) against the test pathogens when compared to *Rubia cordifolia* and *Ocimum sanctum*, but less active than the standards ciprofloxacin(34-36mm inhibition in diameter for bacteria) and crystal violet (22-24mm inhibition in diameter for fungal pathogens).

Determination of MIC

Table 2
Minimum inhibitory concentration of standard antibiotic against bacterial pathogens.

Ciprofloxacin Conc mg/ml	% Inhibition			
	E.coli	**S.*typhi***	***S. mutans***	***S.salivaricus***
0.00	0.00	0.00	0.00	0.00
0.50	24.03	37.14	52.71	58.17
1.00	51.76	50.66	62.46	68.21
2.00	67.03	53.04	71.11	76.19
4.00	73.70	60.32	82.16	78.97
8.00	75.73	75.81	86.83	89.30
16.00	80.99	83.40	87.63	91.36
32.00	81.97	84.58	92.72	92.55
MIC (µg/mL)	**1.00**	**1.00**	**0.5**	**0.5**

The minimum inhibitory concentration (MIC) of selected bacterial pathogens for standard ciproflaxacin is depicted in table 2. The minimum inhibitory concentration of *E.coli, S.typhi, S.mutans* and *S.salivaricus* are 1.0, 1.0, 0.5 and 0.5µg/ml respectively for standard ciproflaxacin

Table 3
Minimum inhibitory concentration of standard crystal violet against fungal isolates.

Test Compound	Conc mg/ml	% Inhibition	
		C.albicans	A.flavus
Standard(Crystal violet)	0.00	0.00	0.00
	16.00	20.70	40.31
	32.00	32.67	45.86
	64.00	41.71	56.69
	128.00	59.11	62.52
	256.00	64.30	66.96
	512.00	70.85	69.74
	1024.00	74.69	72.79
MIC (µg/mL)		128	64

The minimum inhibitory concentration(MIC) of selected fungal pathogens for standard crystal violet is depicted in table 3. The minimum inhibitory concentration of *C.albicans* and *A.flavus* are 128 and 64µg/ml respectively for standard crystal violet.

DISCUSSION

The current problem associated with emerging MDR bacteria presents a serious global medical crisis, requiring constant surveillance, which continuously challenges the scientific community The diminishing efficacy and increasing toxicity of synthetic drugs further aggravate this problem, thus scientists are directed to seek more natural or organic materials for solutions. Traditional medicine has been practiced worldwide for centuries, particularly the application of herbal plants for therapeutic purposes. The 3 plants have been used locally for their traditional medicinal properties, all of which have documented antimicrobial activities. However, their efficacies against MDR bacteria have not been studied. In developing countries, it is imperative that effective but less expensive antibacterials should be developed to accommodate all patients, regardless of financial status, in order to eliminate some of the human factors that can cause MDR. The result of susceptibility of clinical isolates to *R.cordifolia, P. guajava* and *O.sanctum* extracts is presented in Table 1. Results of sensitivity test using disc diffusion method indicated that the methanolic extracts presented higher activity in terms of the number of isolates inhibited and the width of the zone of inhibition formed. However, the activity is much lower than that exhibited by standard antibiotic (ciprofloxacin) used as control (Table 2). The antimicrobial assay using disc diffusion test showed that methanolic extract had a remarkable activity on the test isolates except for *Aspergillus flavus* which was slightly sensitive. However the extracts showed a remarkable activity against the clinical isolates, but less active than the standard antibiotic disc used at equal concentration. The results obtained in this study showed that *P. guajava* present a potential for production of drugs used in the treatment of infections caused by *Salmonella* spp. Further research should be carried out on this plant (*P. guajava)* to determine the level of anti-nutrient constituents and toxicity/safety of the plant extracts as well as the active compound responsible for the activity.

CONCLUSION

The results obtained in this study showed that *P. guajava* can be a source of potential drug candidate for the treatment of infections caused by drug resistant *Salmonella* sp., *E.coli Streptococcus* sp. and fungal pathogens. Further research should be carried out on *P. guajava* to determine the level of anti-nutrient constituents and toxicity/safety of the plant extracts as well as the active compound responsible for the activity.

ACKNOWLEDGEMENT

We acknowledge the resources and financial support for the study was provided by the Department of Biotechnology, Aharyana Nagarjuna university, INDIA.

CONFLICT OF INTEREST

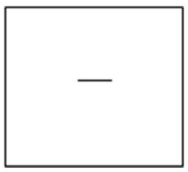
Conflict of interest declared none

REFERENCES

1. Gutmann L, Billot-Klein D, Williamson R, Goldstein FW, Mounier J, Acar JF, Collatz E. Mutation of Salmonella paratyphi A conferring cross-resistance to several groups of antibiotics by decreased permeability and loss of invasiveness. Antimicrob Agents Chemother. 1988 Feb 1;32(2):195-201.
2. Cohen ML. Epidemiology of drug resistance: implications for a post-antimicrobial era. Science (Washington). 1992 Aug 21;257(5073):1050-5.
3. Chopra RN, Nayar SL, Chopra IC. Glossary of Indian medicinal plants. New Delhi.: C SIR. 1956.
4. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev. 1999 Oct 1;12(4):564-82.

5. Tyagi RI, Sharma GA, Jasuja ND, Menghan E. Indian medicinal plants as an effective antimicrobial agent. J Crit Rev. 2016;3(2):69-71.
6. Wainwright M. Moulds in ancient and more recent medicine. Mycologist. 1989 Jan 1;3(1):21-3.
7. Sfetcu N. Health & Drugs: Disease, Prescription & Medication. Nicolae Sfetcu; 2014 May 2.
8. Ahmad I, Mehmood Z, Mohammad F, Ahmad S. Antimicrobial potency and synergistic activity of five traditionally used Indian medicinal plants. Antimicrobial potency and synergistic activity of five traditionally used Indian medicinal plants. 2000;22(4a):173-6.
9. Ghoshal S, Prasad BK, Lakshmi V. Antiamoebic activity of Piper longum fruits against Entamoeba histolytica in vitro and in vivo. J Ethnopharmacol. 1996 Mar 1;50(3):167-70.
10. Jones FA. Herbs-useful plants. Their role in history and today. Eur J Gastroenterol Hepatol. 1996 Dec 1;8(12):1227-31.
11. Malik A, Bai S, Teneja R, Dalal S. *In vitro* screening of some indian medicinal plants for their activity against extended spectrum β lactamases. Br Biotechnol J. 2015 Jan 8 (3): 1-10.
12. Ganie SA, Yadav SS. FT-IR Spectroscopic Analysis of Holoptelea integrifolia (Roxb.) Planch Seed Extracts and their Antibacterial Activity. Res J Med Plant. 2015;9(8):417-26.
13. Geidam YA, Ambali AG, Onyeyili PA. Preliminary phytochemical and antibacterial evaluation of crude aqueous extract of Psidium guajava leaf. J App Sci. 2007 Apr;7:511-4.
14. Begum S, Hassan SI, Siddiqui BS, Shaheen F, Ghayur MN, Gilani AH. Triterpenoids from the leaves of Psidium guajava. Phytochemistry. 2002 Oct 31;61(4):399-403.
15. Singh V, Verma O. Ocimum sanctum (tulsi): Bio-pharmacological activities.
16. Heatley NG. A method for the assay of penicillin. Biochem. J. 1944;38(1):61.
17. CLSI, Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved Standard, 7th ed., CLSI document M02-A11. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA, 2012.
18. CLSI, Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts, Approved Guideline. CLSI document M44-A. CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA, 2004.
19. Ncube NS, Afolayan AJ, Okoh AI. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. Afr J biotechnol. 2008;7(12).
20. Watts JL, Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: approved standard. National Committee for Clinical Laboratory Standards; 2008.

Reviewers of this article



Dr. P.T. Devika Ph.D

Asst. Professor,
Mohamed Sathak College of Arts and Science,
Solinganallur, Chennai-600119

Prof. Y. Prapurna Chandra Rao

Assistant Proffessor,
KLE University, Belgaum, Karnataka

Prof.Dr.K.Suriaprabha

Asst. Editor , International Journal of Pharma and Bio sciences.

Prof.P.Muthuprasanna

Managing Editor , International Journal of Pharma and Bio sciences.

We sincerely thank the above reviewers for peer reviewing the manuscript