Original Research Paper

Volume-8 | Issue-1 | January-2018 | PRINT ISSN - 2249-555X

Microbiology

ANTIBACTERIAL POTENTIAL OF ALCOHOLIC AND AQUEOUS EXTRACTS OF *GARCINIA INDICA*(DU PETIT-THOU.) CHOISY (KOKUM) AGAINST CLINICAL ISOLATES OF *CLOSTRIDIUM DIFFICILE*.

Dr. Sherin Justin* Assistant Professor, Athena institute of Health Sciences, Falnir Road, Mangalore, Karnataka-575001 *Corresponding Author

Dr. Beena Antony Professor, Department of Microbiology, Father Muller Medical College, Mangalore, Karnataka- 575002

Chaitra C Research Associate, Nitya Genome Technologies Pvt. Ltd. Bangalore-560 003

ABSTRACT

Introduction : Vancomycin and metronidazole have always been the drugs of choice for *Clostridium difficile* infection. Emerging resistant strains of the organism have created the need for alternative drugs. The study was intended to investigate the antibacterial potential of Kokum (*Garcinia indica* (Du Petit-Thou.) Choisy) extracts against clinical isolates of *Clostridium difficile.*

Material and Methods: The ethanolic and aqueous extracts of Kokum were prepared by soxhlation and maceration methods respectively. The screening of the extracts for their antibacterial activity was done by disc diffusion method. Minimum inhibitory concentration of the extracts was also determined by agar dilution method.

Results: All the toxigenic and non toxigenic isolates of *Clostridium difficile* showed sensitivity to both the Kokum extracts by disc diffusion method. The minimum inhibitory concentration range of the Kokum extracts was 2.5µl/ml to 10µl/ml.

Conclusions: Kokum extracts exhibited good *in vitro* antibacterial action against the clinical isolates of *Clostridium difficile*. This justifies the role of Kokum as a gastrointestinal remedy and could serve as an aid in development of new therapeutic options for the organism.

KEYWORDS : Agar dilution method, *Clostridium difficile*, Disc diffusion technique, Minimum inhibitory concentration.

Introduction

Clostridium difficile (*C. difficile*)is an anaerobic Gram positive spore forming bacillus which has been associated with pseudomembranous colitis (PMC) and antibiotic associated diarrhoea (AAD).[1] The organism causes a variety of clinical syndromes ranging from asymptomatic carrier state, diarrhoea to megacolon and may even be fatal.[2] Various antibiotics in clinical practice have been linked to *C. difficile* associated disease (CDAD) [3].The mutant hypervirulent strain, NAP1/BI/027 (North American Pulse-field gel electrophoresis type 1 /restriction endonuclease analysis BI/ribotype 027) which caused outbreaks of CDAD in various parts of the world made the pathogen notorious.[2]

Treatment of *C. difficile* infection (CDI) primarily depends on vancomycin and metronidazole. Emerging strains of *C. difficile* with greater virulence and resistance have created the need for new therapeutic options other than antibiotics. As an initiative towards the development of alternative drugs against *C. difficile*, extracts of Kokum (*Garcinia indica* (Du Petit-Thou.) Choisy) which is normally used for gastrointestinal problems were tried on the pathogen in the present study. Kokum trees grow in the humid tropical areas of Western Ghats of India.[4]In India, it is known by various names such as Bindin, Biran, Katambi, Bhirand, Amsool or Punarpuli.[5] It is known by diverse names like Mangosteen, wild Mangosteen, or Red Mango in English.[5]The seeds, rind and pulp of Kokum have numerous health benefits.[4]Kokum rind has been used for the treatment of piles, colic problems, ulcers, inflammation, diarrhoea and dysentery.[6] A recent study even reported the antidepressant and anxiolytic effects of Kokum fruit rind.[7]

Eventhough it is well recognized that antibiotic usage is the main predisposing factor for CDI, studies on herbal treatment options for the organism are limited like the one by Hammond and colleagues.[8] Our study demonstrated the antimicrobial efficacy of the alcoholic and aqueous extracts of Kokum by disc diffusion method and their Minimum Inhibitory Concentration (MIC) determination by agar dilution method which may prove as a stepping stone in the development of future therapeutics for *C. difficile.*

Materials and Methods

The present cross- sectional study was conducted in a tertiary care teaching hospital of coastal Karnataka, South India for a period of three years starting from January 2012 to December 2014. Stool samples were collected consecutively from 563 inpatients with clinically significant diarrhoea who were admitted in various wards like Medicine, Paediatrics, Surgery, Oncology and Orthopaedics.

The study was approved by the Institutional Ethics Committee (Ref. No FMMC/ IEC/ 816/ 2012). Written informed consent was taken from the patients and from the guardians of the patients in case of minors. The demographic and clinical details of each patient were extracted from the medical records. The stool samples were collected in sterile wide mouthed containers and were processed immediately on receipt to the lab.

1. Isolation and identification of *C. difficile*

The faecal samples were cultured on cycloserine cefoxitin fructose agar (CCFA) and anaerobically incubated for the isolation of *C. difficile*.[9]The colonies on the plate confirmed as *C. difficile* by standard procedures were then subjected to latex agglutination (with Oxoid *C. difficile* Test Kit, DR 1107A, UK.[9,10] Enzyme immunoassay (EIA)was performed using Premier Toxins A & B (*C. difficile*) EIA kit M/S Meridian Bioscience, Europe, for detection of the toxins A and B of *C. difficile*. The colonies confirmed as *C. difficile* were then analyzed by polymerase chain reaction (PCR) using Applied Biosystems SimpliAmp Thermal Cycler by Life technologies for the detection of toxin A and toxin B genes.[11,12]

C .difficile ATCC 43593 was used as the control strain throughout the study.

Out of 113 *C. difficile* isolates obtained in the study, 54 (47.79%) isolates were toxigenic by toxigenic culture.[13] The remaining 59 (52.21%) were non toxigenic isolates.

2. Preparation of Kokum extracts

Kokum rind was purchased from a reputed store in Mangalore and was submitted for authentication to National Ayurvedic Dietetics Research Institute [Central Council for research in Ayurveda& Siddha, Department of AYUSH, Ministry of Health & Family Welfare, Government of India], Bangalore. The authentication number of the herb was RRCBI-11759.

The extraction was done at the Department of Pharmacology, Shree Devi College of Pharmacy, Mangalore.

Figure 1: Kokum rind

i. Preparation of ethanolic extract

The Kokum rind was washed with water and dried. About 100g of Kokum rind was extracted with ethanol (150ml) using Soxhlet Extractor.

Procedure [14]

In this method, the material (Kokum) to be extracted was filled in a thimble made of cellulose or cloth and placed in the extractor part of the Soxhlet extraction unit. The solvent (ethanol) was placed in the lower part of the apparatus (round bottomed flask) and connected to the extractor part. The other end of the extractor part was connected to the condenser. The solvent in the lower container was heated slowly to boil. The vapours of the solvent passed through the side arm into the reflux condenser, liquefied and then dropped into the thimble containing material to be extracted. The warm solution was percolated through the wall of the thimble and extracted gradually and collected in the extractor part. Once the extract height reached the siphon, the entire liquid flowed back into the lower part of the Soxhlet extraction unit containing the solvent. The process was repeated again and again. The extract was then coarse filtered after the removal of solvent and later sterilized with the help of membrane filters of 0.2micrometre (µm) pore size and was stored at 10^0C for further use.

ii. Preparation of aqueous extract

The Kokum rind was washed with water and dried. About 100g of the Kokum rind was extracted with 100ml of chloroform water (1:99) by simple maceration method.[15] Here the coarse drug powder of Kokum was kept in contact with the solvent for at least two days with several daily shakings at room temperature before the marc was pressed. The extract was then coarse filtered after concentration and later sterilized with the help of membrane filters of 0.2µm pore size. It was stored at 10^0C for further use.

3. *In vitro* antibacterial action of alcoholic and aqueous extracts of Kokum

A total of 65 isolates of *C. difficile* (20 toxigenic isolates and 45 non toxigenic isolates) were subjected to disc diffusion technique as a screening test to determine the antibacterial efficacy of the two extracts.

i. Disc diffusion technique

In brief, an overnight culture of *C. difficile* isolates in Viande- Levure (VL) broth, the turbidity of which was adjusted to McFarland 0.5 standard ($1.5x10^8$ CFU/ml) was swabbed onto Brucella blood agar plates. Sterile discs of Whatmann No.1 filter paper (6mm diameter) were saturated with 1ml of the undiluted extracts. The prepared discs were then placed on the inoculated Brucella blood agar plates and the zones of inhibition in millimeters were measured after anaerobic incubation in Hi gas-pak jar at 37^0C for 48 hrs using BD GasPak EZ Anaerobe container system with Indicator. *C. difficile* ATCC 43593 was also employed in parallel. As a negative control, dimethyl sulfoxide (DMSO) incorporated disc was included. The procedure was done in triplicate. The results were graded depending on the diameter of the zones of inhibition.[16]

9mm – 12mm → 1+, 13mm – 16mm → 2+, 17mm – 20mm → 3+, >20mm → 4+

ii. Determination of MIC of the Kokum extracts using Agar Dilution method

MIC was determined for the ethanolic and aqueous extracts of Kokum using 40 isolates of *C.difficile* (20 toxigenic isolates and 20 non toxigenic isolates) by the agar dilution method according to Wadsworth-KTL anaerobic bacteriology manual sixth edition and Clinical and Laboratory Standards Institute (CLSI), M100-S23 document.[9,17] An overnight broth culture of *C. difficile* in VL broth whose turbidity was adjusted to 0.5 McFarland standard served as the inoculum. Brucella blood agar base supplemented with vitamin K1 and hemin was the medium employed.

Preparation of dilutions of Kokum extracts

Dilutions of the two Kokum extracts were prepared in DMSO.[9,17] The concentration of each of the undiluted extracts was supposed to be 1000 µl. First, each of the undiluted extracts was diluted as 1 in 10 dilution (1ml herbal extract +9ml DMSO) and then proceeded with the dilutions as given in the **Table 1**. Thus the concentration of the respective herbal extract in the agar plates ranged from 10µl/ml of media to 0.3125 µl/ml of media. 2ml from each dilution of the herbal extracts was used for the agar dilution.

Table 1: **Preparation of dilutions of Kokum extracts to be used in agar dilution susceptibility tests (using 1 in 10 dilution of undiluted extracts as the starting concentration).**

Kokum extracts					
Step	**Concentration in microliter (µl)**	**Source**	**Volume in milliliter (ml)**	**Diluent in milliliter (ml)**	**Final concentration at 1:10 dilution in Agar (µl/ml)**
	100	1:10 dilution of undiluted Kokum extract	-	-	10
1	100	1:10 dilution of undiluted Kokum extract	2	2	5
2	100	1:10 dilution of undiluted Kokum extract	1	3	2.5
3	100	1:10 dilution of undiluted Kokum extract	1	7	1.25
4	12.5	Step 3	2	2	0.625
5	12.5	Step 3	1	3	0.3125

Agar dilution method

2ml from respective dilution of the Kokum extract and 1ml of sterile lysed sheep blood were added to 17ml of molten and cooled Brucella blood agar base to obtain 1:10 dilution (Each bottle contained 2ml from respective herbal extract dilution + 17ml Brucella blood agar +1ml of sterile lysed sheep blood).The bottles were mixed thoroughly and poured into petridishes. The broth culture of *C. difficile* adjusted to 0.5 McFarland standard were spot inoculated onto the marked area in the plates which had varying concentrations of the Kokum extract. Two Brucella blood agar plates without Kokum extracts were also inoculated out of which one was growth control (incubated anaerobically) and the other was aerobic contaminant control (incubated aerobically). Then the test plates and anaerobic growth control plate were incubated in anaerobic atmosphere at 37^0C for 48 hours. Aerobic contaminant control plate was aerobically incubated at 37^0C for 48 hours. MIC was interpreted as the lowest concentration of herbal extract yielding no growth.[9]

MIC was determined for both the Kokum extracts against 40 isolates of the organism along with *C. difficile* ATCC 43593.

Statistical Methodology: Data was analyzed by frequency, percentage and Chi-square test.

Results

A total of 65 isolates of *C. difficile* (20 toxigenic isolates and 45 non toxigenic isolates) were subjected to *in vitro* antimicrobial action of alcoholic and aqueous extracts of Kokum by disc diffusion technique. The sensitivity and resistance pattern exhibited by the toxigenic and non toxigenic isolates towards the two herbal extracts are given in **Table 2**. The sensitivity pattern of the isolates towards the two herbal extracts varied between 1+ to 4+ grades as demonstrated in **Table 2**.

Table 2: Antimicrobial action of Kokum extracts against *C. difficile* by Disc diffusion technique

Extracts	Total no. of isolates tested	Total no. of Toxigenic isolates tested	Total no. of Non toxigenic isolates tested	Toxigenic isolates				Non toxigenic isolates			
				Sensitive isolates			Resistant isolates	Sensitive isolates			Resistant isolates
Aqueous extract of Kokum	65	20	45	1+	2	20 (100%)	0 (0%)	1+	2	45 (100%)	0 (0%)
				2+	7			2+	8		
				3+	6			3+	11		
				4+	5			4+	24		
Alcohol extract of Kokum	65	20	45	1+	3	20 (100%)	0 (0%)	1+	3	45 (100%)	0 (0%)
				2+	4			2+	10		
				3+	7			3+	12		
				4+	6			4+	20		
Statistical analysis				p= 1.00, NS				p= 1.00, NS			
Key : 9mm – 12mm → 1+, 13mm – 16mm → 2+, 17mm – 20mm → 3+, >20mm → 4+											

MIC determination was done for alcoholic and aqueous extracts of Kokum against 40 isolates of *C. difficile* (20 toxigenic isolates and 20 non toxigenic isolates).The results are presented in **Table 3**.

Table 3: Details of the number of isolates of *C.difficile* inhibited at various concentrations of Kokum extracts. (Total number of isolates = 40; Toxigenic isolates = 20, Non toxigenic isolates = 20)

Kokum extracts	Nature of the isolates	Concentrations in microliter/milliliter (µl/ml)					
		10µl/ml	5µl/ml	2.5µl/ml	1.25 µl/ml	0.625µl/ml	0.3125µl/ml
Aqueous extract of Kokum (1 in 10)	No. of Toxigenic isolates inhibited	20	18	18	0	0	0
	No. of Non toxigenic isolates inhibited	20	18	18	0	0	0
Alcohol extract of Kokum (1 in 10)	No. of Toxigenic isolates inhibited	20	19	19	0	0	0
	No. of Non toxigenic isolates inhibited	20	17	17	0	0	0

Discussion

Multidrug resistant organisms are constantly emerging in all parts of the world. Researchers are in search of drugs which have lesser side effects and easy availability. As a stepping stone in the development of alternative treatment options against *C. difficile*, the extracts of Kokum which has been known for its anti diarrhoeal action in traditional medicine were tried on the pathogen. Though antibacterial effect of Kokum extracts have been reported against various bacteria, the studies on *C. difficile* are lacking.

Previous researchers have enumerated the role of Kokum in gastrointestinal problems. A recent review had highlighted the three major bioactive compounds of Kokum namely anthocyanin, hydroxycitric acid, garcinol and their beneficial properties.[18]Baliga *et al* had summarized the various health benefits of Kokum and its phytochemicals like garcinol, isogarcinol and cyanidin-3-glucoside.[6] The methanol extracts of the leaves and fruits of Kokum had shown significant antibacterial activity against the tested isolates in an Indian study.[19]The Kokum leaf extract possessed inhibitory activity against *Salmonella* Typhi*, Salmonella* Paratyphi A*, Salmonella* Typhimurium and the aqueous extract of Kokum rind was reported to have antibacterial activity against *Bacillus subtilis, Escherichia coli, Enterobacter aerogenes* and *Staphylococcus aureus*.[4]Lakshmi and colleagues had reported the antibacterial activity of polyphenols isolated from methanol extract of stem bark of *Garcinia indica* against various aerobes.[20] According to another Indian study, the antimicrobial activity exhibited by Kokum extracts against the bacterial strains employed was principally due to the presence of furfural.[21]

In the present study, the screening of antibacterial activity of the Kokum extracts was done by disc diffusion technique and the MIC of the extracts was determined by agar dilution method.[9, 17] The results of our study indicated that both alcoholic and aqueous extracts of Kokum had significant antibacterial activity against *C. difficile*.There was also no significant difference in their action among toxigenic isolates (p=1.00, NS) or non toxigenic isolates (p=1.00,NS) by disc diffusion method.The MIC range of both the extracts of Kokum was 2.5µl/ml to 10µl/ml. These MIC values highlights the potential of Kokum extracts for development into future therapeutics of *C. difficile*. The only limitation of our study was that analysis of phytochemicals present in the Kokum extracts was not conducted as it was outside the scope of the study. The present study would be an aid in signaling new therapeutics to be tried on the pathogen.

Conclusions

Herbal drugs can complement the action of synthetic medicine since they have fewer side effects. Our study demonstrated that the extracts of Kokum or its purified form could be used in conjunction with antibiotics for *C. difficile*. More extensive research of both *in vivo* and *in vitro* nature are vital before execution of the herb in clinical practice.

Acknowledgement

We acknowledge the valuable advice and help from Dr. I.S.R.Punitha, Department of Pharmacology, Shree Devi College of Pharmacy, Mangalore for the preparation of Kokum extracts. We also acknowledge Dr. Sucharitha Suresh, Assistant Professor, Department of Hospital Administration, Father Muller Medical College, Mangalore for her statistical assistance.

REFERENCES

1. Ayyagari A, Agarwal J, Garg A. Antibiotic associated diarrhoea: infectious causes. Indian J Med Microbiol 2003; 21: 6-11.
2. Vaishnavi C. Clinical spectrum & pathogenesis of Clostridium difficile associated diseases. Indian J Med Res 2010; 131:487-99.
3. Vaishnavi C. Established and potential risk factors for Clostridium difficile infection. Indian J Med Microbiol 2009; 27: 289-300.
4. Milind P, Isha D. Golden benefits of drinking kokum-cola. Int Res J Pharm 2013; 4: 5-9.
5. Padhye S, Ahmad A, Oswal N, Sarkar FH. Emerging role of garcinol, the antioxidant chalcone from Garcinia indica Choisy and its synthetic analogs. J Hematol Oncol 2009; 2:38. doi:10.1186/1756-8722-2-38.
6. Baliga MS, Bhat HP, Pai RJ, Boloor R, Palatty PL. The chemistry and medicinal uses of the underutilized Indian fruit tree Garcinia indica Chiosy (Kokum): a review. Food Res Int 2011; 44: 1790-9.3 Biotech 2017; 7:131.doi: 10.1007/s13205-017-0766-x.
7. Dhamija I, Parle M, Kumar S. Antidepressant and anxiolytic effects of Garcinia indica fruit rind via monoaminergic pathway. 3 Biotech. 2017; 7: 131. doi: 10.1007/s13205-017-0766-x.
8. Hammond EN, Donkor ES, Brown CA. Biofilm formation of Clostridium difficile and susceptibility to manuka honey. BMC Complement Altern Med 2014; 14: 329.doi: 10.1186/1472-6882-14-329.
9. Jousimies-Somer HR, Summanen P, Citron DM, Baron EJ, Wexler HM, Finegold SM. Wadsworth-KTL Anaerobic Bacteriology Manual. 6th ed. Belmont: Star Publishing Company; 2002.
10. Willis AT. Anaerobic Bacteriology: Clinical and Laboratory Practice. 3rd ed. London: Butterworth and Co (Publishers) Ltd; 1977.
11. Kato H, Kato N, Watanabe K, Iwai N, Nakamura H, Yamamoto T, et al. Identification of toxin A- negative, toxin B- positive Clostridium difficile by PCR. J Clin Microbiol 1998; 36: 2178-82.
12. Justin S, Antony B. Polymerase chain reaction for the detection of toxin A (tcd A) and toxin B (tcd B) genes of Clostridium difficile isolated from diarrhoeal cases and analysis of the clinical spectrum. Journal of Evolution of Medical and Dental Sciences 2015; 4: 4938- 47. doi: 10.14260/jemds/2015/721.
13. Crobach MJT, Dekkers OM, Wilcox MH, Kuijper EJ. European society of clinical microbiology and infectious diseases (ESCMID): data review and recommendations for diagnosing Clostridium difficile- infection (CDI). Clin Microbiol Infect 2009; 15: 1053-66.
14. Redfern J, Kinninmonth M, Burdass D, Verran J. Using Soxhlet ethanol extraction to produce and test plant material (essential oils) for their antimicrobial properties. J Microbiol Biol Educ 2014; 15: 45- 6.
15. Pandey A, Tripathi S. Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. J Pharmacogn Phytochem 2014; 2: 115- 9.
16. Alves TMA, Silva AF, Brandao M, Grandi TSM, Smania EFA, Junior AS, et al. Biological screening of Brazilian medicinal plants. Mem Inst Oswaldo Cruz, Rio de Janeiro 2000; 95: 367-73.
17. CLSI. Performance standards for antimicrobial susceptibility testing; twenty- third informational supplement. CLSI document M100- S23. Wayne, PA: Clinical and Laboratory Standards Institute; 2013.
18. Ranveer RC, Sahoo AK. Bioactive constituents of Kokum and its potential health benefits. Nutrition and Food Toxicology 2017; 1: 236- 44.
19. Tharachand C, Selvaraj CI, Abraham Z. Comparative evaluation of antihelminthic and

antibacterial activities in leaves and fruits of Garcinia cambogia (Gaertn.) Desr. and Garcinia indica (Dupetit-Thouars) Choisy. Braz Arch Biol Technol 2015; 58: 379-86.
20. Lakshmi C, Kumar KA, Dennis TJ, Kumar TSSPNSS. Antibacterial activity of polyphenols of Garcinia indica. Indian J Pharm Sci 2011; 73: 470- 3.
21. Sutar RL, Mane SP, Ghosh JS. Antimicrobial activity of extracts of dried kokum (Garcinia indica C). International Food Research Journal 2012; 19: 1207- 10.