Antibacterial activity of the essential oils of *Syzygium aromaticum* (L.) Merr. Perry (Clove), *Myristica fragrans* Houtt. (Nutmeg) and *Zingiber officinale* Roscoe (Ginger) against clinical isolates of *Clostridium difficile*: An *in vitro* study

Sherin Justin[1], Beena Antony[2]

ABSTRACT

Introduction: Treatment options for *Clostridium difficile* other than vancomycin and metronidazole have always been a challenge. Emergence of hypervirulent and resistant strains of the organism demands newer drugs with less side effects. The study was intended to investigate the antibacterial potential of the essential oils of *Syzygium aromaticum*(L.)Merr.Perry (Clove), *Myristica fragrans* Houtt. (Nutmeg) and *Zingiber officinale* Roscoe (Ginger) against clinical isolates of *Clostridium difficile*.

Material and Methods: The essential oils of clove, nutmeg and ginger were prepared by Neo-Clavenger's method. The screening of the essential oils for their antibacterial activity was done by disc diffusion method against both toxigenic and non toxigenic isolates of *Clostridium difficile*. Minimum inhibitory concentration of the oils was also determined against the isolates by agar dilution method.

Results: 100% of both toxigenic and non toxigenic isolates of *Clostridium difficile* showed sensitivity to clove oil by disc diffusion method. 100% of toxigenic isolates, 93.33% of non toxigenic isolates were sensitive to nutmeg oil and 85% of toxigenic isolates, 80% of non toxigenic isolates exhibited sensitivity towards ginger oil by disc diffusion method. The minimum inhibitory concentration range of clove oil was 1.25µl/ml to 2.5µl/ml; both nutmeg oil and ginger oil was 2.5µl/ml to 10µl/ml.

Conclusions: All the three essential oils used in the study exhibited good *in vitro* antibacterial activity towards the clinical isolates of *Clostridium difficile*. This promising finding could be utilized in development of new treatment options for the organism.

Keywords: Agar dilution method,*Clostridium difficile*, essential oils, minimum inhibitory concentration

INTRODUCTION

Clostridium difficile (*C. difficile*) is an anaerobic, Gram positive spore forming bacillus. The organism has been held responsible for 90% of pseudomembranous colitis (PMC) and 20-25% of antibiotic associated diarrhoea (AAD).[1] The epidemiology of the organism has been showing a drastic shift over the last decade from being a nosocomial pathogen to a community- acquired one.[2]

Antibiotics like clindamycin, penicillin, ampicillin, cephalosporins, fluoroquinolones and even vancomycin and metronidazole which are the main treatment options for the organism have been implicated in *C. difficile* associated disease (CDAD).[3] Treatment options for *C. difficile* infection (CDI) other than vancomycin and metronidazole still remain a challenge. The mutant hypervirulent strain, NAP1/BI/027 (North American Pulse-field gel electrophoresis type 1 /restriction endonuclease analysis BI/ribotype 027) which was responsible for the outbreaks of CDAD in many parts of the world has driven much attention towards the pathogen.[4]

Emergence of more resistant and virulent strains of *C. difficile* has created an increasing need for new therapeutic options other than antibiotics with no side effects and easy availability. As a stepping stone in the development of alternative drugs against *C. difficile*, the essential oils of common herbs like clove (*Syzygium aromaticum*(L.) Merr.Perry), nutmeg (*Myristica fragrans* Houtt.) and ginger (*Zingiber officinale* Roscoe) which are routinely used for gastrointestinal problems as grandmother's remedies were tried on the pathogen in the present study. The importance of essential oils has been highlighted in literature by various authors.[5,6] A variety of volatile molecules present in essential oils like terpenes, terpenoids, phenol- derived aromatic and aliphatic compounds could be responsible for their bactericidal, virucidal and fungicidal action.[6]

Though it has been widely accepted that antibiotic usage is the main predisposing factor for CDI, studies analyzing herbal treatment options for *C. difficile* is lacking in literature except the one which proved the antibacterial activity of Manuka honey.[7] Our study which demonstrates the in vitro antibacterial action of the essential oils of clove, nutmeg and ginger by disc diffusion and their Minimum Inhibitory Concentration (MIC) determination by agar dilution may prove as an aid in development of future therapeutics for *C. difficile.*

MATERIAL AND METHODS

Isolation and identification of *C. difficile*

The study was conducted in a tertiary care teaching hospital of coastal Karnataka, South India. Stool samples of 563 pa-

[1]Research Scholar, [2]Professor, Department of Microbiology, Father Muller Medical College, Mangalore, Karnataka- 575002, South India.

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

How to cite this article: Sherin Justin, Beena Antony. Antibacterial activity of the essential oils of *Syzygium aromaticum* (L.) Merr. Perry (clove), *Myristica fragrans* Houtt. (nutmeg) and *Zingiber officinale* Roscoe (ginger) against clinical isolates of *Clostridium difficile*: an *in vitro* study. International Journal of Contemporary Medical Research 2016;3(4):1085-1089.

tients with diarrhoea who were admitted in various wards like Medicine, Paediatrics, Surgery, Oncology and Orthopaedics were collected from January 2012 to December 2014. The study was approved by the Institutional Ethics Committee (Ref. No FMMC/ IEC/ 816/ 2012).

The patients admitted to the above mentioned wards with diarrhoea were included in the study. The clinical history and drug use of the patients were recorded. Written informed consent was taken from the patients during the study. Consent was taken from the guardians of the patients in case of minors.

Sterile wide mouthed containers were used for the collection of faecal samples and the samples were processed immediately. The stool samples were cultured on cycloserine cefoxitin fructose agar (CCFA) and anaerobically incubated at 37^0 C for 48 hours for the isolation of *C. difficile*.[8] The colonies on the plate were confirmed as *C. difficile* by Gram stain, morphology and characteristic odour and then subjected to latex agglutination (with Oxoid *C. difficile*Test Kit, DR 1107A, UK) and biochemical reactions.[8,9] Enzyme immunoassay (EIA)was performed using Premier Toxins A and B (*C. difficile*) EIA kit M/S Meridian Bioscience, Europe on all the stool samples 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 Simpli Amp Thermal Cycler by Life technologies for the detection of toxin A and toxin B genes. Toxin A gene detection was done using two primer pairs, primers NK3 and NK2; primers NK11 and NK9.[10] Primers NK104 and NK105 were employed to detect toxin B gene.[10]

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

From 563 stool specimens, 113 (20.07%) *C.difficile* isolates were obtained by culture and confirmed by latex agglutination and biochemical reactions. Out of 113 *C.difficile* isolates, 54 (47.79%) isolates were toxigenic by toxigenic culture.[11] The remaining 59 (52.21%) were non toxigenic isolates.

Preparation of essential oils of Clove, Nutmeg and Ginger

Clove, nutmeg and ginger were purchased from a reputed store in Mangalore. Flower buds, fruits and rhizome of clove, nutmeg and ginger respectively were submitted for authentication to National Ayurvedic Dietetics Research Institute [Central Council for research in Ayurveda and Siddha, Department of AYUSH, Ministry of Health and Family Welfare, Government of India], Bangalore. The authentication number of the herbs are given in table 1.

The essential oils of clove, nutmeg and ginger were prepared at the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences,Manipal University, Manipal. The oils were extracted by Neo-Clavenger's method using Clavenger's apparatus. Clove, nutmeg and ginger were shade dried and reduced to coarse powder.

100 g of the coarse powder (clove/nutmeg/ginger) mixed with 60ml of glycerol, 300 ml of distilled water and a few small pieces of porcelain was placed in the distillation flask of the apparatus. The flask was connected to the still head and was heated with frequent agitation, until ebullition started. The distillation was continued till the lower part of the condenser was cold. After a few hours of distillation, heating was discontinued and the volume of oil in the graduated portion of the tube was read off. The distillation was continued again, till the volume of oil did not differ in the two successive readings. The boiling was stopped, the oil was separated and collected in a sterile vial. The total yield of volatile oil content in the herbal product was noted.

In vitro antibacterial action of essential oils of Clove, Nutmeg and Ginger.

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 three essential oils.

i. Disc diffusion technique

A 48 hour old culture of *C. difficile* isolates in Viande-Levure (VL) broth, the turbidity of which was adjusted to McFarland 0.5 standard ($1.5x10^8$ colony forming units/ml) was swabbed onto Brucella blood agar plates. 1 ml from each of the three undiluted essential oils was added to separate sets of 100 sterile discs (Whatmann No.1 filter paper) of 6mm diameter. 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 37^0 C for 48 hours 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.[12] 9 mm – 12 mm → 1+, 13 mm – 16 mm→ 2+, 17 mm – 20 mm→ 3+, >20 mm →4+

ii. Determination of MIC of the essential oils using Agar Dilution method

MIC was determined for the essential oils of Clove, Nutmeg and Ginger 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.[8,13]

a. Preparation of inoculum

One tube with 2ml VL broth was used for the inoculation of each isolate of *C. difficile*. The broths after inoculation were incubated anaerobically for 48 hours and then the turbidity of the broths was adjusted with sterile VL broth to 0.5 McFarland standard. This served as the inoculum.

Common name	Scientific name	Family	Part of the herb used	Authentication No.
Clove	*Syzygium aromaticum*(L.)Merr.Perry	Myrtaceae	Flower buds	RRCBI-MUS/108
Ginger	*Zingiber officinale* Roscoe	Zingiberaceae	Rhizome	RRCBI-AP/5020
Nutmeg	*Myristica fragrans* Houtt.	Myristicaceae	Fruit	RRCBI-MUS/02

Table-1: Details of the herbs used for extraction

b. Preparation of media

Brucella blood agar base supplemented with vitamin K1 and hemin was prepared in sterile bottles such that each bottle contained 17 ml of the agar. The autoclaved, molten agar was cooled to 48^{0}C in a water bath.

c. Preparation of dilutions of essential oils

Dilutions of the three essential oils were prepared in DMSO.[8,13] The concentration of each of the undiluted essential oils was supposed to be 1000 µl. First, each of the undiluted essential oils was diluted as 1 in 10 dilution (1ml essential oil+9ml DMSO) and then proceeded with the dilutions as given in the table 2. Thus the concentration of the respective oil in the first agar plate was 10µl/ml of media, in the second plate was 5 µl/ml of media and in the last plate was 0.3125 µl/ml of media. 2ml from each dilution of the essential oils was used for the agar dilution.

d. Agar dilution method

2ml from respective dilution of the essential oils 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 essential oil dilution + 17ml Brucella blood agar +1ml of sterile lysed sheep blood). The bottles were mixed thoroughly and poured into petridishes. After the plates were set, they were placed in 37^{0}C incubator for 30 minutes for the evaporation of excess moisture. The organisms (inoculum prepared as above)were spot inoculated onto the marked area in the plates which had varying concentrations of the essential oils. Two Brucella blood agar plates without essential oils were also inoculated out of which one was growth control (incubated anaerobically) and the other was aerobic contaminant control (incubated aerobically). All the plates remained at room temperature for 10 minutes for the inoculum to be absorbed. Then the test plates and anaerobic growth control plate were incubated in anaerobic atmosphere at 37^{0}C for 48 hours. Aerobic contaminant control plate was aerobically incubated at 37^{0}C for 48 hours. MIC was interpreted as the lowest concentration of essential oil yielding no growth.[8]

The procedure was carried out for each of the three essential oils against 40 isolates of *C. difficile*. MIC of the essential oils was also determined with *C. difficile* ATCC 43593.

STATISTICAL ANALYSIS

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 essential oils of clove, nutmeg and ginger by disc diffusion technique. The sensitivity and resistance pattern exhibited by the toxigenic and non toxigenic isolates towards the three essential oils are given in table 3. The sensitivity pattern of the isolates towards the three essential oils varied between 1+ to 4+ grades as demonstrated in table 3.

MIC determination was done for clove oil, nutmeg oil and ginger oil against 40 isolates of *C. difficile* (20 toxigenic isolates and 20 non toxigenic isolates). The results are presented in table 4.

DISCUSSION

Newer antibiotics and antibiotic resistant organisms are in a continuous evolution process. Modern era is in search of drugs which have lesser side effects and easy availability. As a preliminary study in the development of alternative treatment options against *C. difficile*, the essential oils of clove, nutmeg and ginger which are known to be antidiarrhoeal remedies in traditional medicine were tried on the pathogen. Though many investigators have reported the antibacterial effect of the above three essential oils against various bacteria, the studies on *C. difficile* are lacking.

Prabuseenivasan and colleagues had demonstrated strong antibacterial activity of clove essential oil using disc diffusion method.[14] They also determined the MIC using agar dilution method.[14] Another study showed that the essential oil of Clove was more effective compared to their aqueous extracts at different concentrations.[15] Nearly 89% of the clove essential oil is composed of eugenol, its main bioactive compound.[16]

The antibacterial effect of essential oil of Ginger was determined in a study from Sudan by disc diffusion method.[17] Nutmeg has been used for mouth- sores, diarrhoea, intestinal weakness and as anti-inflammatory drug.[18] The effectiveness of the Nutmeg oil against both Gram positive and Gram negative bacteria was proved in a study.[19] It was found that the oil of nutmeg had the highest antibacterial activity when compared to its ethanolic and aqueous extracts.[19] Thanoon and colleagues demonstrated the antibacterial action of Nutmeg oil against *Staphylococcus aureus*.[18] In the study, the antibacterial activity of Nutmeg oil was determined by disc diffusion method.[18]

In our study, the screening of antibacterial activity of the

Essential oils					
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 essential oil	-	-	10
1	100	1:10 dilution of undiluted essential oil	2	2	5
2	100	1:10 dilution of undiluted essential oil	1	3	2.5
3	100	1:10 dilution of undiluted essential oil	1	7	1.25
4	12.5	Step 3	2	2	0.625
5	12.5	Step 3	1	3	0.3125

Table-2: Preparation of dilutions of essential oils to be used in agar dilution susceptibility tests (using 1 in 10 dilution of undiluted oils as the starting concentration).

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
Ginger oil	65	20	45	1+	3	17(85%)	3(15%)	1+	6	36(80%)	9(20%)
				2+	3			2+	9		
				3+	8			3+	16		
				4+	3			4+	5		
Nutmeg oil	65	20	45	1+	5	20(100%)	0(0%)	1+	6	42(93.33%)	3(6.67%)
				2+	4			2+	6		
				3+	9			3+	12		
				4+	2			4+	18		
Clove oil	65	20	45	1+	1	20(100%)	0(0%)	1+	7	45(100%)	0(0%)
				2+	7			2+	11		
				3+	3			3+	8		
				4+	9			4+	19		
				χ^2= .316, *P*=.854, NS				χ^2= 1.024, *P*=.599, NS			
Key: 9mm – 12mm → 1+, 13mm – 16mm → 2+, 17mm – 20mm → 3+, >20mm → 4+											

Table-3: In vitro antimicrobial action of essential oils against *C.difficile* by Disc diffusion

Essential oils	Nature of the isolates	Concentrations in microliter/milliliter (µl/ml)					
		10 µl/ml	5 µl/ml	2.5 µl/ml	1.25 µl/ml	0.625 µl/ml	0.3125 µl/ml
Nutmeg oil (1 in 10)	No. of Toxigenic isolates inhibited	20	16	9	0	0	0
	No. of Non toxigenic isolates inhibited	20	16	7	0	0	0
Ginger oil (1 in 10)	No. of Toxigenic isolates inhibited	20	8	8	0	0	0
	No. of Non toxigenic isolates inhibited	20	8	8	0	0	0
Clove oil (1 in 10)	No. of Toxigenic isolates inhibited	20	20	20	5	0	0
	No. of Non toxigenic isolates inhibited	20	20	20	3	0	0

Essential oils	MIC
Nutmeg oil (1 in 10)	2.5µl/ml - 10µl/ml
Ginger oil (1 in 10)	2.5µl/ml - 10µl/ml
Clove oil (1 in 10)	1.25µl/ml - 2.5µl/ml

Table-4: Details of the number of isolates of *C.difficile* inhibited at various concentrations of essential oils. (Total number of isolates = 40; Toxigenic isolates = 20, Non toxigenic isolates = 20)

essential oils was done by disc diffusion technique and the MIC of the oils was determined by agar dilution method.[8,13] All the three essential oils employed had exhibited good in vitro antibacterial activity towards the clinical isolates of *C. difficile* and there was no significant difference in their action among toxigenic isolates (χ^2= .316, *P*=.854, NS) or non toxigenic isolates (χ^2= 1.024, *P*=.599, NS) by disc diffusion method. The MIC range of clove oil was 1.25µl/ml to 2.5µl/ml; both nutmeg oil and ginger oil was 2.5µl/ml to 10µl/ml. The MIC values of the present study are in fact promising in development of therapeutics for *C. difficile*.

The only limitation of our study was that we did not do a compositional analysis of the essential oils because it was beyond the scope of the study. But our study is novel in its concept since studies analyzing herbal remedies against *C. difficile* is sparse in literature. The present study would signal new therapeutic options to be tried on the pathogen by future researchers.

CONCLUSION

Herbal drugs can serve as alternative to synthetic medicine. Our study demonstrated that essential oils of clove(*Syzygium aromaticum*(L.)Merr.Perry), nutmeg (*Myristica fragran*s Houtt.) and ginger (*Zingiber officinale* Roscoe) have the potential for progression into future therapeutics of *C. difficile*. Further in vivo and in vitro studies are warranted before implementation of these herbs in clinical use.

ACKNOWLEDGEMENT

We acknowledge the valuable advice and help from Dr. CS Shreedhara, Professor, Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences,Manipal University, Manipal in the preparation of essential oils. We also acknowledge Mrs. Sucharitha Suresh, Assistant Professor,Department of Hospital Administration, Father Muller Medical College, Mangalore for her statistical assistance.

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Source of Support: Nil; **Conflict of Interest:** None

Submitted: 24-02-2016; **Published online**: 20-03-2016