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In vitro inhibitory effect of selective plants against clinical strains of *Escherichia coli* isolated from diarrheal samples from malnourished children

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Abstract
A diarrhoeal disease is a major public health problem among children. The emergence of infection caused by multiple drug resistant enteric pathogens has now necessitated the search for alternative parenteral agents and introduction of natural plant products. Ayurvedic literature indicates decoction of processed medicinal plant parts as an anti-diarrhoeal home remedy. Hence systemic study of medicinal plants is very important. This study attempts to screen etiological agent of chronic diarrhoea in malnourished patients and to study *in vitro* effect of plants on the clinical isolates specifically to *Escherichia coli* isolates. The study and control group in the study included 100 patients each. A random collection of stool samples and its inoculation on culture media was done. The minimum battery of tests was performed for identification of isolates obtained. Culture positivity was 100% and 94% in control and study group patients studied. *E.coli* remained the predominant isolate followed by *Klebsiella pnuemoniae*. Antibacterial effect of selective plants was checked on *E.coli* isolates obtained from study group. The extracts were prepared by hot water decoction method of plant parts like roots of sunthi (*Zingiber officinale)*, seeds of Jeera *(Cuminum cyminum)*, roots of musta (*Cyperus rotundus)* and it's *in vitro* antibacterial activity was carried out by agar well diffusion method against standard strain and clinical isolates of *E.coli***.** Sunthi showed a *in vitro* antibacterial activity, while *musta,* jeera, as well as combination of all three-plants failed to show *in vitro* antibacterial activity, when tested against standard strain and clinical isolates of *E.coli*. Though in literature these plants were documented as one of the promising household remedy as antidiarrhoeal agents towards diarrhoea, *in vitro* results of the present study does not show them as effective antidiarrhoeal agent, except Sunthi.

Keywords: *Zingiber officinale, Cuminum cyminum*, *Cyperus rotundus,* diarrhoea, antidiarrhoeal agent, antibacterial activity

1. Introduction
In India, diarrhoeal diseases is a major public health problem among children under the age of five years [1]. In health institution, up to third of total Pediatric admission is due to diarrhoeal diseases and up to 17 per cent of all deaths in indoors pediatrics patients are diarrhoeal related. Acute diarrhoea lasts for less than two weeks, which is nearly always be presumed to be of infective type [2, 3]. Detection of etiological agents of infective diarrhoea is important for therapeutic aspects and for implementation of appropriate control strategies. In developing countries, the bacterial pathogens are most commonly associated with endemic forms of diarrhoea. The emergence and widespread distribution of drug resistant enteric bacteria have imposed serious limitations on successful antibiotics treatment. Spontaneous acquisition of drug resistance among enteric pathogens is due to selective pressure of antibiotic therapy [4]. This problem of antimicrobial resistance in microorganisms causing diarrhoeal diseases in both developed and developing countries continues to be alarming. These multiple drug resistance strains have caused major disease outbreaks with high mortality and morbidity in developing countries [5].
The emergence of infection caused by multiple drug resistant enteric pathogens has now necessitated the search for alternative parenteral agents and the introduction of natural plant products [6]. This is a very important replacement for the resistance. Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic values. Recently the acceptance of traditional medicine as an alternative form of

health care and the development of microbial resistance to the available antibiotics has led us to investigate the antimicrobial activity of medicinal plants [6] Ayurveda describes certain plant drugs, which can be used in treating or reducing severity of diarrhoea [7]. Ayurveda literature search indicates that the decoction of processed Zingiber officinale (Zo) (sunthi), Cyperus rotundus (Cr) (musta), Cuminum cyminum (Cc) (jeera) can be used as an anti-diarrhoeal home remedy [7]. In order to find out their activity, a systemic study of medicinal plants is very important. Scientific study towards various Ayurvedic plant drugs and its effect on various pathogens *in vitro* as well as *in vivo* are not well documented. Hence, it is need of today to establish such documents for its regular use as remedy, which will be useful for the control and cure of the various infectious conditions like diarrhoea. Keeping in mind the above facts, we have made an attempt to screen etiological agent of chronic diarrhoea in malnourished patients and to study *in vitro* effect of selective plant drugs on the clinical isolates specifically to *E coli* isolates, as *E.coli* is probably very common cause of diarrhoea.

2. Material and methods

This prospective longitudinal study was undertaken at the Department of Microbiology, T.N. Medical College and B.Y.L. Nair Charitable. Hospital, Mumbai. Selection of subjects for the study and control group was done on the basis of standard classification of malnourished and chronic diarrhoea. The subjects in the present study were of age group of more than 1 month up to 12years old. Study group and Control group included 100 patients each. Random collection of stool sample was done in a sterile wide mouth container before starting any empirical treatment from clinically diagnosed malnourished children with infective chronic diarrhoea. Each stool sample was immediately, within 30 minutes, transported to the Microbiology laboratory [8]. A loopful of stool sample was inoculated and streaked on culture media like MacConkey agar, Blood agar and *E.coli* Hichrome agar and were incubated at 37ºC and growth was observed after 24hrs. The minimum battery of tests was performed for Identification of isolates obtained [9] Antibacterial effect of selective plants were checked on *E.coli* isolates obtained from study group.

Plants parts like roots of sunthi *(Zo))*, seeds of Jeera *(Cc)*, roots of musta *(Cr)* were obtained from authentic sources and their identity was confirmed using standard Pharmacognostic method. The extracts were prepared by Hot water decoction method. Ten grams of dry powder was taken in a clean glass stoppered flask-containing 100ml of distilled water. The solution was boiled in a water bath for 2min. The extract was cooled and filtered to get a clear solution. The filtrate was evaporated on a water bath and dried in a vacuum oven at 100ºC. Dried residue was collected in a mortar and ground to get a fine powder. The powder was then passed through #80 mesh to get a uniform powder. Standardization of *in vitro* antibacterial activity of plant drugs[10] was carried out by agar well diffusion method using standard strain of *E.coli* ATCC 25922 in Muller-Hinton agar. Further In vitro antibacterial activity of plant drugs on clinical isolates of *E.coli*. was carried [11] Result was interpreted by seeing inhibition or by decreased growth, comparing with control. All three plant drugs were tested *in vitro* in different combinations as a, b, c and d against 65 *E.coli* isolates obtained from study group patients using agar dilution method. Details about combination of drug are as follows: (a) Zo + Cc; (b) Zo + Cr; (c) Cc + Cr; (d) Zo + Cc + Cr

3. Results

Table 1: Bacterial is olates from Cases and Controls $n_1 = 100$; $n_2 = 100$

Sr. No.	Bacteria isolated	Groups		Total (%)
		Cases (%)	Controls (%)	
1	*Escherichia coli(E.coli)*	65	77	71.00
2	*Klebsiella pneumoniae (K. pneumonia)*	18	13	15.50
3	*Pseudomonas aeruginosa (P. aeruginosa)*	0	3	1.50
4	*Proteus vulgaris (P. vulgaris)*	1	0	0.50
5	*Staphylococcus aureus (S. aureus)*	0	2	1.00
6	*Shigella flexneri (S. flexneri)*	4	2	3.00
7	*Salmonella paratyphi A (S. paratyphi A)*	3	0	1.50
8	*Salmonella typhi (S. typhi)*	3	0	1.50
9	*Vibrio cholera (V. cholera)*	0	3	1.50
10	No bacterial isolate	6	0	6

Key: n_1 = number of Cases, n_2 = number of controls

Table 1 shows the bacterial isolates obtained from study and control group. Culture positivity was 100% and 94% in control and study group patients studied. *E.coli* remained the predominant isolate among study and control group patients that is 65% and 77%, followed by *Klebsiella pnuemoniae*, as 18% and 13% respectively.

Table 2: *In vitro* effect of plants on *E. coli* ATCC 25922 strain

S. No.	Dilutions	Zone of inhibition in diameter (mm)		
		Zo	Cr	Cc
1	Undiluted (stock-100mcg/ml)	30.33	0	0
2	1:2	26.66	0	0
3	1:4	24	0	0
4	1:8	19	0	0
5	1:10	16	0	0
6	1:12	15	0	0
7	1:14	14	0	0
8	1:16	0	0	0
9	1:32	0	0	0
10	Positive control(Gentamicin)	45	45	45

Sunthi showed a dose dependent *in vitro* antibacterial activity up to 1:14 dilution, when tested against *E. coli* ATCC 25922 strain. Zone diameter was confirmed by taking averages of 3 tests. Musta, Jeera as well as combination of all three-plant drugs failed to show *in vitro* antibacterial activity, when tested against *E.* ATCC 25922 Strain.

Table 3: In vitro effect of combination of plants on *E. coli* ATCC 25922 strain

No.	Dilutions	Zone of inhibition in diameter			
		ZO + Cc	ZO + Cr	Cc + Cr	ZO + Cr + Cc
1	Undiluted Stock (100mcg/ml)	0	0	0	0
2	1:2	0	0	0	0
3	1:4	0	0	0	0
4	1:8	0	0	0	0
5	1:10	0	0	0	0
6	1:12	0	0	0	0
7	1:14	0	0	0	0
8	1:16	0	0	0	0
9	1:32	0	0	0	0
10	Positive control (Gentamicin)	45	45	45	45

None of the combination of plants showed *in vitro* antibacterial activity against *E. coli* ATCC 25922 strain.

Table 4: In vitro effect of plants on clinical isolates of *E. coli*. n = 65

No.	Dilutions	No of *E.coli* isolates		
		Zo	Cr	Cc
1	1:10	17	-	-
2	1:12	17	-	-
3	1:14	17	-	-
4	1:16	17	-	-
5	1:32	17	-	-
6	Control	+	+	+

Key: +: growth; - : no growth; n = no of clinical isolates of *E. coli*

Sunthi showed *in vitro* antibacterial activity by agar dilution method against 17 (26.13%.) clinical isolates of *E.coli*. Musta and Jeera did not show inhibition to any of the *E.coli* isolates from study group patient.

Table 5: *In vitro* effect of plants in various combinations against clinical isolates of *E.coli* n = 65

No.	Dilutions	No of *E.coli* isolates			
		Zo+ Cc	Zo+ Cr	Cc + Cr	Zo+Cr+Cc
1	1:10	-	-	-	-
2	1:12	-	-	-	-
3	1:14	-	-	-	-
4	1:16	-	-	-	-
5	1:32		-	-	-
6	Control plate	+	+	+	+

Key: +: growth; - : no growth; n = no of clinical isolates of *E. coli*

None of the combination of plants showed *in vitro* antibacterial activity against clinical isolates of *E.coli* from study group by agar dilution method

4. Discussion

In India, traditional uses of many household remedies are seen to be useful in diarrhoea and are found to be effective without any side effects. But there is no scientific support and documentation to use these remedies as a routine treatment. Hence we have made an attempt to screen etiological agent of chronic diarrhoea in malnourished patients and to study *in vitro* effect of selective plants like *Sunthi, Musta, Jeera* individually and in various combination against clinical isolates specifically to *E. Coli* isolates which are predominant. *E.coli* constitutes a diverse group of organisms, including non-pathogenic strains, which are among the most common bacteria in the normal flora of the human intestine and pathogenic strains. These Diarrhoeaogenic *E.coli* have been studied extensively and are classified on the basis of serogrouping or pathogenic mechanisms into five major groups. Enter pathogenic *E.coli (EPEC)*, an important cause of diarrhoea in infants in developing countries. Enter otoxigenic *E.coli*, a cause of diarrhoea in infants in developing areas of the world and a cause of traveler's diarrhoea in adults. Enter oinvesive *E.coli*, which cause either a watery Enterotoxigenic like illness or less commonly, a dysentery-like illness. Enter ohemorrhegic *E.coli* (EHEC), which cause hemorrhagic colitis and Hemolytic Uremic Syndrome. Enter oaggregative *E.coli* and diffuse-adherent *E.coli*, which along with EPEC have been implicated as cause of persistent diarrhoea[12].

In the present study, comparison between acute and chronic diarrhoea cases revealed that *E.coli* is the commonest etiology in acute as well as chronic diarrhoea cases. Though, exact demarcation between types of *E.coli* was not observed, but finding of this study suggest that multiple acute diarrhoeal episodes in children leads to develop malnourished condition which decreases the immunity in children and may support to develop normal communal flora as etiological agents for repeated prolonged diarrhoeal condition in pediatric cases. For confirmation of EHEC *E.coli* Hicrome agar was used in this study, while EPEC serotyping was carried out using 'O' type standard antisera. Children less than 1 yr were found to be predominant group associated with infective chronic diarrhoea more prone to severe malnutrition

Sixty five clinical isolates of *E.coli* from study group were tested for *in vitro* antibacterial activity of Sunthi, Jeera and Musta by agar dilution method. Among selected plants Sunthi was found to be effective. It showed antibacterial activity against 26% of clinical isolates of *E. coli* obtained from study group cases. Jeera and Musta did not show any *in vitro* antibacterial activity against any clinical isolate of *E. coli*. Also the combination of all three plants did not show any activity against clinical isolates.

Ballal M *et al*. in 2005[5] from India reported that Sunthi extract found to be effective as antifungal at 7 mg/ml concentration, while the study in 2010 [13], focuses the significant antibacterial activity of garlic extract on streptomycin-resistant strains solely and in synergism with streptomycin. Statistical comparison of sole extract and streptomycin synergism with streptomycin control had proved it significant. The study in 2016 [14], showed that standard *S. aureus* strain and *E. coli* strain was completely inhibited by 10 mg/ml and 15 mg/ml of garlic in agar media respectively and their clinical isolates were completely inhibited by 25 mg/ml, indicating that standard isolates were most sensitive and clinical isolates were least sensitive. The study recommended that garlic could be used as effective antibacterial agent for these pathogens.

Jeera was found to be ineffective against clinical isolates of *E.coli* in our study. Study in 2013 [15] showed antimicrobial properties of methanolic extract of cumin seeds on four enteropathogenic and food-spoiler bacterial strains. Minimum concentrations of cumin extract effective against *E,coli, P.aeruginosa*, *S.aureus* and *B.pumilus* were found to be 12.5, 6.25, 25.0 and 6.25 mg dry weight per ml respectively.

Musta was found to be ineffective against clinical isolates of *E.coli* in our study. Daswani PG *et al* [16] carried out study with Musta on EPEC using HEP-2 cells by tissue culture technique to check the production of both toxin and found to be effective only at very high concentrations.Activity of that plant was not necessarily dose dependent. In this study, effect on adherence of enteropathogenic *E. coli* and invasion of enteroinvasive *E. coli* and *Shigella flexneri* to HEp-2 cells was evaluated as a measure of effect on colonization. Effect on enterotoxins such as enterotoxigenic *E. coli* heat labile toxin (LT), heat stable toxin (ST) and cholera toxin (CT) was also assessed. The decoction showed reduced bacterial adherence to and invasion of HEp-2 cells and affected production of CT and action of LT. The decoction of Musta does not have marked antimicrobial activity and exerts its antidiarrheal action by mechanisms other than direct killing of the pathogen. In another study [17], the attempt was made to evaluate the antimicrobial activity of various solvent extracts of contents of Balchaturbhadra Yoga against different gram positive and gram negative bacteria. In the present antimicrobial study of ingredients of Balchaturbhadra Yoga, the Pippali and Karkatshringi showed promising antimicrobial activity against *E. coli, E. faecalis*, and *V. cholera* but antimicrobial activity of Musta and Ativisha were not found.

Difference in the activities of these plants in various studies indicate that the extraction method and in vitro testing method needs to be validated against standard protocol.

5. Conclusion

The study can be thus concluded as follows. Bacterial infection is more common with chronic diarrhoea in malnourished children. *E.coli* is the predominant etiological agent for chronic infective diarrhea in malnourished children. As per suggestive mechanisms of chronic infective diarrhoea in malnourished children, normal commensal *E.coli* can be one of the predominant etiological agent for chronic infective diarrhoea in malnourished children. *In vitro* antibacterial activity of Sunthi was observed but Jeera and Musta did not show any antibacterial activity towards *E.coli* isolates from study group. None of the combination of three drugs showed any *in vitro* antibacterial effect on *E.coli* isolates from study group. I*n vitro* results of these plants did not show them as effective anibacterial agent, except Sunth. But in literature these plants were documented as one of the promising household remedy as antidiarrhoeal agents. Hence the mode of action of these plants needs to be evaluated further as antidiarrhoeal agent.

6. Recommendations

More research work on these plants to combat chronic and acute diarrhoea in malnourished children with respect to its mode of action and decoction methods are needed.

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