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Antimicrobial activity of the crude extracts of *Houttuynia cordata* and *Centella asiatica* on some human gut microflora

Research Article

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Abstract

C. asiatica and *H. cordata* plants are used as vegetable since time immemorable by the people of Assam. These are also consumed for their medicinal values. Plenty of information on the action of the plants extract on the pathogens are available, but could not retrieve any information on the non-pathogens of human guts. Therefore, an attempt has been made to find action of the extracts on few of the non-pathogens of human guts. The study shows that none of the plant extracts inhibit *Bacillus coagulans, Bacillus mesentricus*, and *Lactobacillus* sp, whereas a minimum inhibition were recorded in case of pathogens *Escherchia coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Shigella dysenteriae.*

Key Words: *Plant Extracts, Inhibition zone, Pathogens and Non-pathogens.*

Introduction

Centella asiatica, and *Houttuynia cordata* are plated as leafy vegetable by the people of North – East India. These plants are quite often used as herbal remedy against stomach infections by the people of Assam, India. Literature suggests that extracts of these plants have anti-microbial, anti-inflamatory, anti-oxidative properties and thus provides remedy not only to the gastrointestinal problems but also to other infections. Rattanachuaikunsopon and Phumkhachorn 2010) reported that the extract of *C. asiatica* among many plants is best against *F. columnare* (1). The study of Cheng and Koo, 2000 reported that *C. asiatica* extract accelerate cure of gastric lesions in rats (2). NgLT et al 2007, showed the protective effect of *H. cordata* extract on bleomycin induced pulmonary fibrosis in rats (3). Experiments also demonstrated the antidiarrheal activity of *H. cordata* (4). Although antimicrobial activity of *C. asiatica* was determined to be poor than antibiotics such as tetracycline, it showed bacteriostatic activity against isolates from wounds of different etiology. During the period of Severe Acute Respiratory Syndrome SARS) outbreak, *H. cordata* was one of the ingredients in the SARS prevention use for prevention. The formulation was recognised by health ministry of China (5). Recently several studies illustrated its anti-SARS (6), anti-allergic (7); anti-inflamatory (8, 9); virucidal (10, 11); anti-Leukemic (12); anti-oxidative (3, 13) and anticancer activities (14).

The microbiome of human gut includes a wide range of both pathogenic and non-pathogenic microorganisms. Existing literatures mostly inferred on the antimicrobial activity of the extracts of *H. cordata* and *C. asiatica* against most of the pathogenic microorganisms. Literature survey on the actions of the extracts of these plants on non-pathogenic gut microorganisms could not provide much information. It is essentially important to know about the action of the plant extracts on the non-pathogenic microorganisms of the gut. It is also well established that the non-pathogens support human health. Therefore, in view to elucidate the antimicrobial activity of the said plants on the non-pathogens with respect to that on pathogens the present study was conceived. If the extract poise any adverse effect on non-pathogens, there lies the question of using pre or probiotic supplements along with the use of these plants as indigenous herbal composition or Ayurvedic composition.

Materials and Methods

Bacterial Sample Isolation and Identification

The pathogenic microorganisms *Escherchia coli, Shigella dysenteriae, Klebsiella pneumoniae, Staphylococcus aureus* were collected from various clinical specimen at Bacteriology laboratory, Microbiology, Department of Gauhati Medical College and Hospital. The non-Pathogenic bacteria *Bacillus mesentricus* and *Bacillus coagulans* are probiotic isolates and Lactobacillus sp. was collected from curd.

The pathogens were cultured in MacConkey and Blood agar media for identification. The identified colonies were then stored in Luria Bertani LB) broth

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and agar. For antimicrobial tests, the sub-cultures were prepared on Muller Hinton agar media and Nutrient Agar Media. LB broth was used for the purpose of analysis of minimum inhibitory concentration MIC).

A suspension of non-pathogens was prepared in peptone water and was streaked on the MRS De Man, Rogosa and Sharpe) agar media and further sub-cultured in Muller Hinton agar media.

Identification of the microorganisms was based on colonial characteristics and using various standard biochemical tests such as IMViC, Catalase test, Hi Assorted TM Biochemical test, NaCl tolerance test specifically for Non-pathogens.

Preparation of Plant Extracts and Antimicrobial Property Analysis

Centella asiatica and *Houttuynia cordata* were collected from the Agricultural Research Institute Kahhikushi, Guwahati, Assam India. Leaves of the plants were washed thoroughly before air drying and then powdered. The extracts were prepared in solvents such as chloroform, petroleum ether, benzene, acetone and methanol using soxhlet apparatus and stored in a sterile screw capped bottle at – 20^{0}C. In addition, leave extracts were also prepared by cold maceration method. In this method, the dried powder was kept dissolved in chloroform, benzene, acetone and methanol for 72 hours. Under reduced pressure the extracts were evaporated and dried by using a rotary evaporator.

Antimicrobial activity of the extracts thus obtained were analyzed by standard well-diffusion technique and Minimum Inhibitory Concentration using Resazurin Dye.

Characterization of plant extract

Thin Layer Chromatography TLC) was performed on TLC silica gel 60 F254, aluminium sheet 20x20 cm. After drying the plates, they were exposed to Iodine vapor for obtaining colored spots spot. The R*f* value of the different spots that were recorded. A preparative TLC was performed on glass silica film with the known potent solvent mixture to get the fraction.

GC-MS Analysis for Plant samples:

Shimadzu GC 2010 plus with triple MS TP-8030) fitted with EB-5MS column was used for the purpose. The temperature program for the column was set at 100^{0}C hold for 1 min, 15 ^{0}C/min to 160^{0}C and 5^{0}C/min to 300^{0}C hold for 7 min. The GC injector was held isothermally at 280^{0}C with a split period of 3 min, as the carrier gas Helium was used, at a flow rate of 1 mL/ min by using electronic pressure control. The interface temperature for GC–MS was maintained at 280 ^{0}C. In electron impact EI) ionization mode, the MS was operated with 70 eV electron energy and the scan to determine appropriate masses for selected ion monitoring ranged from 50 to 500 amu atomic mass unit).

Results

The pathogens and nonpathogens

Based on the colonial characteristics followed by standard biochemical tests the microorganisms were identified to be *E.coli, K. pneumoniae, S. dysentriae, S. aureus* Table – 1). Similarly, the non-pathogens were identified to be *B. coagulans, B. messentricus* and were isolated from probiotic Bifalac sachet and *L. acidophillus* was isolated from curd Table – 2).

Table 1. Biochemical Test Results of Pathogenic Bacteria for identification

Sl	Bacteria	Colonial Characteristics	GS	MO	IN	MR	VP	CO	CI	CA	OD	NR	SR
1	E. coli	Moist smooth surface pink in MacConky/ cled, bacillus slender & thin.	-	+	+	+	-	-	-	+	-	+	Glucose+ Lactose+
2	Stephylococus aeurus	Coccus Clustered colony, show b-hemolysis in blood agar plate	+	-	-	+	+	+	+	+	-	+	Glucose+ Lactose+ Mallose+
3	Klebsela pneumonia	Mucoid bluish pink in cled/ MacConky medium, basilus and stout	-	+	-	-	-		+	+	-	+	
4	S dysentreae	Hemolytic colony, colony circular colourless, convex but moderately translucent with smooth surface and entire edges.	-	-	+	+	-		-	+		+	Glucose+ Lactose-

GS – Gram Stain; MO – MobilityIN – Indole Test; MR - Methyl Red Test; VP - Voges- proskaver Tesr;
CO – Coagulate Test; CI – Citrate Reductase Test; CA – Catalase Test; OD – Oxydase Test;
NR – Nitrate reductase Test; SR – Sugar Test

Table 2. Biochemical Test Results of Non-Pathogenic Bacteria for identification

Sl	Bacteria	Colony Characteristics	GS	MO	IN	MR	VP	CO	CI	CA	OD	NR	SR
1	Bacillus coagulans	The organism with convex, entire margined and smooth surfaced colonies, white to cream in colour and did not grow in 7% NaCl containing media was identified to be *Bacillus coagulans*	+	+	-	+	-	+	+	+	-	-	Maltose- Glucose+
2	Bacillus mecentricus	Elipsoidal spores.The colonies are irregular,wrinkled and pointed.They are smooth, opaque and unpigmented,slightly yellowish.non-haemolytic when cultured in sheeep blood-agar media.	+	+	-	+	+	-	+	+	-	-	Glucose+ Fructose+ Lactose+
3	Lactobacillus sp.	The coloniessmall to medium gray colonies that usually exhibit alpha hemolysis on blood agar are, mucoid colony, white in MRS,	+	-	-	-	-	-	-	-	-	-	Maltose + Lactose + Glucose +

GS – Gram Stain; MO – MobilityIN – Indole Test; MR - Methyl Red Test; VP - Voges- proskaver Tesr;
CO – Coagulate Test; CI – Citrate Reductase Test; CA – Catalase Test; OD – Oxydase Test;
NR – Nitrate reductase Test; SR – Sugar Test

Antimicrobial Activity Analysis

The antimicrobial activity analysis of the extracts was performed by well diffusion and Minimum Inhibitory Concentration methods. The resulting zone of inhibition were measured in millimeter. The resampling values at $P<0.05$ is illustrated in Table 3. The results reveal that the extract have inhibitory effect with varied range depending on the microorganism and the solvent used for extraction. The experiment also has shown that crude extracts of both *H. cordata* and *C. asiatica,* irrespective of nature of isolation distinctly inhibit the growth of *E. coli* and *S. dysenteriae*. However, extent of inhibition by the extracts significantly varies. It has been observed that chloroform and methanol extracts of *C. asiatica* were more effective against *E. coli* 11.65 ± 0.00 and 13.67 ± 0.01 respectively. Acetone, chloroform

and methanol extracts of *C. asiatica* seems to be almost equally inhibiting *S. dysenteriae*. However, no inhibition was seen against *K. pneumonia* by the extract of *C. asiatica*. A varying and insignificant inhibition was observed in case of *S. aureus* by the extracts of *C. asiatica*. On the other hand, the extracts of *H. cordata* seems to inhibiting *K. pneumonia* and *S. aureus*. The extracts prepared in solvents chloroform, methanol acetone, benzene, and petroleum ether of *H. cordata* was found to be effective against *E. coli*, *S. dysenteriae* and *K. pneumoniae*. Acetone and chloroform extract of *H. cordata* even showed antimicrobial activity against *S. aureus* with small sized zone.

Table 3: Antimicrobial assay of *C. asiatica* and *H. cordata* in different solvent extracts

Organism	Plant Species	Acetone	Chloroform	Methanol	Benzene	P. Ether
E. coli	*C. asiatica*	5.01±0.00	11.65±0.00	13.67±0.01	-	-
	H. cordata	-	8.64±0.02	6.34±0.01	5.99±0.01	12.33±0.01
K. pneumonia	*C. asiatica*	NO ACTIVITY SEEN				
	H. cordata	7.69±0.01	15.64±0.01	9.66±0.01	8.99±0.02	11.99±0.01
S.aureus	*C. asiatica*	4.67±0.00	9±0.01	7.65±0.01	-	-
	H. cordata	4.99±0.00	3.67±0.01	-	-	-
S. dysenteriae	*C. asiatica*	10.32±0.00	11±0.00	12±0.00	-	-
	H. cordata	12.67±0.01	14.66±0.01	17.66±0.01	12.67±0.01	8.66±0.02

The Minimum inhibitory concentration was determined by calculating the lowest concentration of the plant extract that inhibited visible growth of the test microorganism. The lowest concentration of the plant extract at which the color change occurred was taken as the MIC value. From the triplicate the arithmetic average were considered. The MIC values of *H. cordata* and *C. asiatica* tested against the pathogenic bacteria are presented in Table 4. The MIC value of different extracts fall in the range within 0.36 mg/ml to 2.5 mg/ml as determined by resazurin based microtitre plate assay. Although the inhibitory zone formed by the extracts of *C. asiatica* in case of *S. aureus* was varying, evident from significantly non-uniform results but it has been observed that a very low concentration is required for inhibition i.e ~ 0.83 mg which is contradicting. Better and uniform results however, was observed in case of *S. dysentriae* and *E. coli*. All the extracts of *H. cordata* seems to be insignificantly varying when a particular type of organism is considered at $P < 0.05$. However, the required concentration for inhibition significantly varies from organism to organism P-value 0.95).

Table 4. Minimum Inhibitory Concentration of various extracts of *C. asiatica* and *H. cordata* on bacteria

Plant	Extracts	Bacteria	Minimum Inhibitory Concentration in mg)				
			1	2	3	Mean	P-Value
C. asiatica	Acetone	*S. dysentriae*	2.5	2.5	2.5	2.5	0.06
		E. coli	0.62	1.25	1.25	1.05	0.20
		S. aureus	1.25	2.5	1.25	1.6	0.13
	Methanol	*S. dysentriae*	1.25	1.25	1.25	1.25	0.17
		E. coli	2.5	2.5	2.5	2.5	0.06
		S. aureus	1.25	0.62	0.62	0.83	0.25
	Chloroform	*S. dysentriae*	1.25	2.5	2.5	2.08	0.09
		E. coli	2.5	2.5	2.5	2.5	0.06
		S. aureus	0.62	1.25	1.25	1.04	0.20
H. cordata	Acetone	*S. dysentriae*	1.25	1.25	1.25	1.25	0.17
		E. coli	2.5	2.5	2.5	2.5	0.06
		S. aureus	2.5	2.5	2.5	2.5	0.06
		K. pneumonia	2.5	2.5	2.5	2.5	0.06
	Methanol	*S. dysenteriae*	0.15	0.15	0.31	0.4	0.36
		E. coli	2.5	2.5	2.5	2.5	0.06
		S. aureus	1.25	1.25	2.5	1.6	0.13
		K. pneumoniae	2.5	2.5	2.5	2.5	0.06
	Chloroform	*S. dysentriae*	0.62	0.31	0.15	0.36	0.38
		E. coli	2.5	2.5	2.5	2.5	0.06
		S. aureus	2.5	2.5	2.5	2.5	0.06
		K. pneumoniae	2.5	2.5	2.5	2.5	0.06

Fractionation of plant extract

Preparative TLC with a thickness of 1mm silica gel as well as TLC silica gel 60 F245 aluminum sheet 20 x 20 cm, was used to separate different components of the crude extracts of the two plants Table 5). The different solvents used for the fractionation were methanol, chloroform, acetic acid and ethanol at varied ratio.

The most prominent fraction spotted on TLC were scrapped and then dissolved in DCM Dichloromethane), filtered and centrifuged and finally given for GC-MS, analysis. The gas chromatography characterized the compounds present in the fractions of

H. cordata and *C. asiatica* by comparison of mass spectra on NIST library. Those peaks matching similarity index greater than 70% in the NIST library were assigned. The library search of the highest peaks revealed five basic compounds Benzenamine, Cyclotetrasiloxane,N-2-Ethylhexyl) trifluoroacetamide11- Methyldodecanol in case of *H. cordata* extracts Figure 1). GCMS chromatogram analysis of *C. asiatica* showed four predominant peaks with high percentage namely bicyclo(7.2.0)undec-4-ene,4,11,11-trimethyl-8-methylene ,(1r.1r*,4z,93*)) ; aryophylene;1,2-benzenedicarboxylic acid, bis2-methylpropyl) ester1h-3a,7-methanoazulene, octahydro-1,4,9,9-tetramethyl Figure 2).

Table 5 TLC fractions of the extracts

Plant	Extracts	Solvent System	No. of Fractions	Rf. Values
H. cordata	Acetone	Chloroform : Ethanol 3:7)	2	0.80; 0.68
	Methanol		2	0.73; 0.65
	Chloroform		2	0.75; 0.61
	P. Ether		2	0.73; 0.61
	Benzene	Choloroform : Benzene 7:3)	5	0.86; 0.30; 0.23; 0.16; 0.11
	P. Ether		5	0.90; 0.60; 0.20; 0.11; 0.09
C. asiatica	Acetone	Chloroform : Methanol 9:1)	3	0.88; 0.82; 0.78
	Chloroform		3	0.86; 0.78; 0.74
	Methanol		3	0.84; 0.78; 0.72
	Acetone	Chloroform : Glacial acetate : Methanol : Water 6:2:1:1)	3	0.95; 0.71; 0.47
	Chloroform		4	0.86; 0.63; 0.52; 0.36
	Methanol		3	0.89; 0.67; 0.47

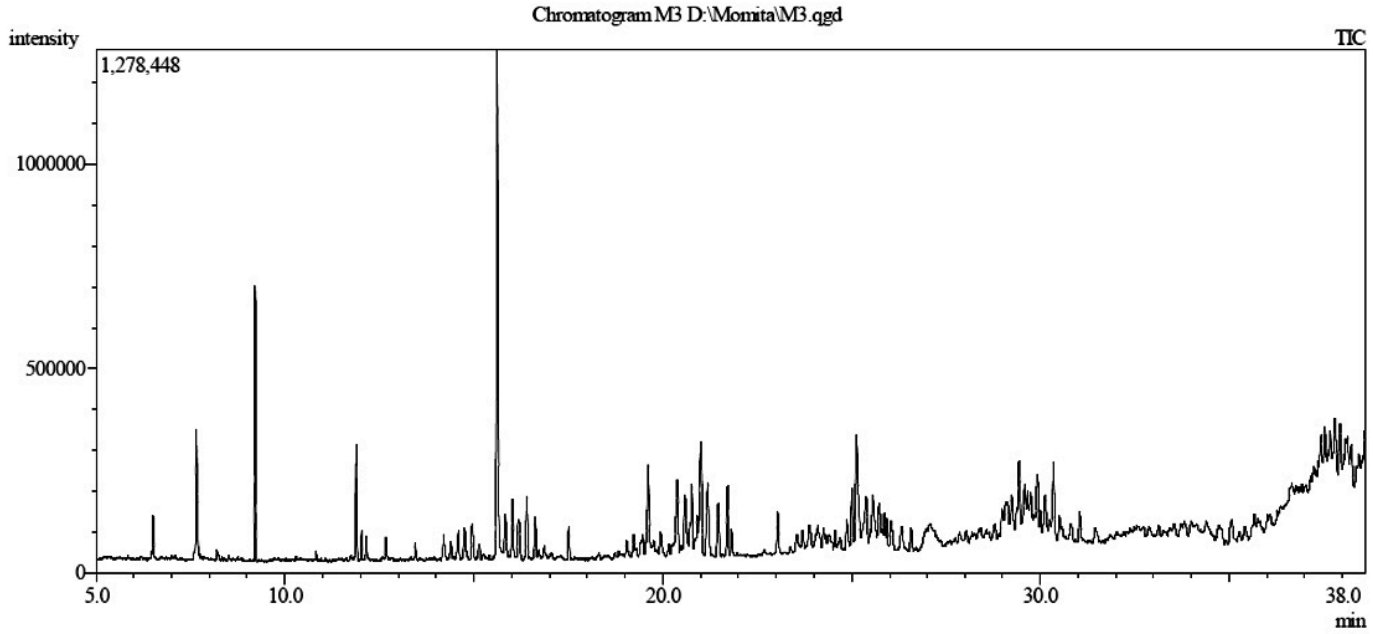

Figure 1: **GC/MS analysis Clarus 680 GC/Clarus 600 C MS was used using Capillary column: Elite-5MS. For H. cordata.**

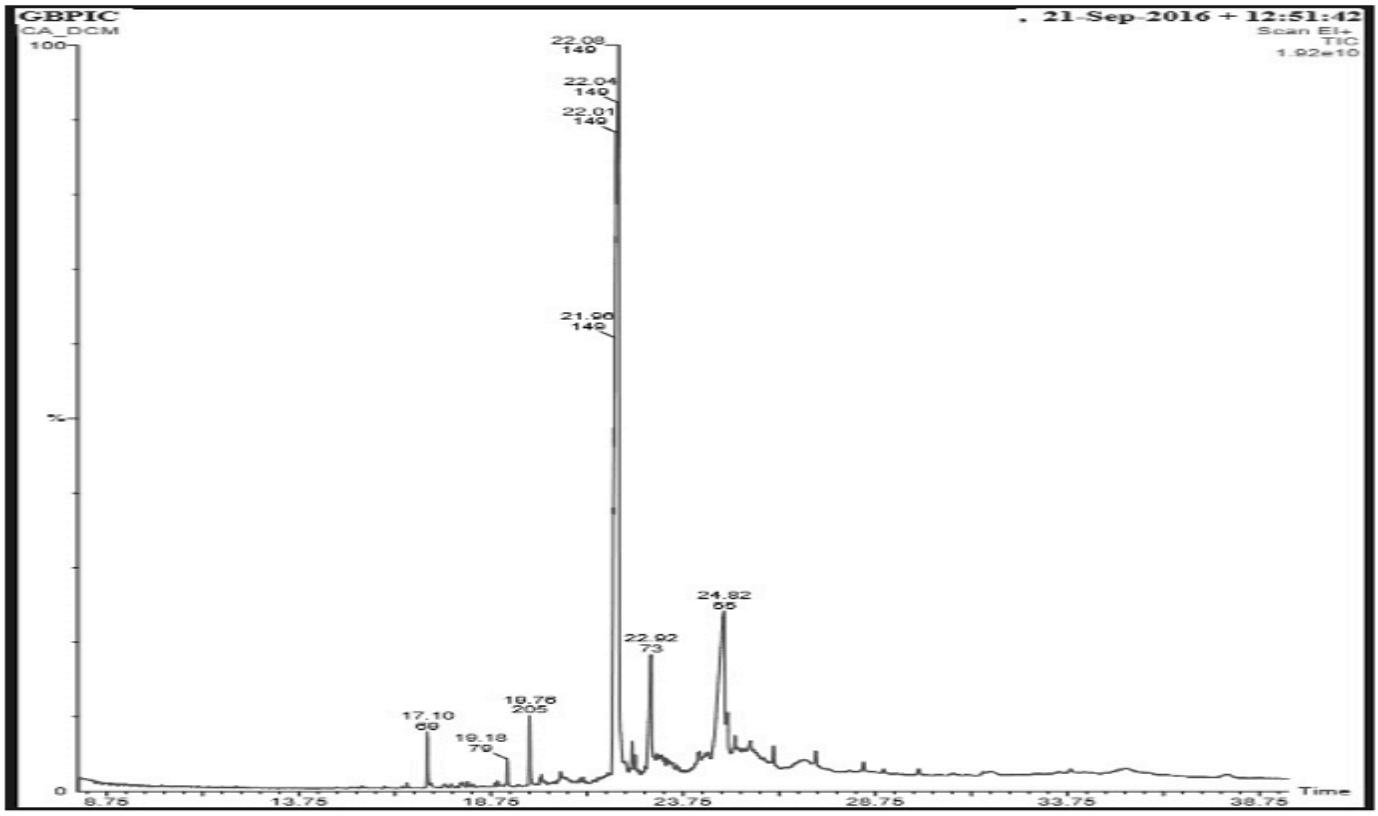

Figure 2: GC/MS analysis Clarus 680 GC/Clarus 600 C MS was used using Capillary column:Elite-5MS. For C. asiatica.

Comparative analysis on the use of antibiotics and the plant extracts to the pathogen as well as non-pathogens.

The comparative analysis was performed based on the formation and size the zone of inhibitions. For the purpose comparation susceptible antibiotic disks were used along with pants extracts in wells on bacterial culture inoculated in MHA media. A comparative analysis was performed using known antibiotic Gentamycin GEN) and the best extract performer Table 6). It has been observed that although the size of inhibition zone is smaller comparative to that of the zone of inhibition former by the antibiotics they inhibit the growth of the pathogenic microorganisms. It is interesting to observe that none of the non-pathogens were affected by the extracts. If the concentration of the extracts increases it may be possible to inhibit the growth of all of the pathogen.

Table 6: Inhibitory activity of known antibiotics and plant extracts a comparison

Organisms	Antibiotic	Zone size mm)	Plant extracts	Zone size
Shigella dysentraie	GEN	22	CA methanol)	12 ± 0.00
			HC methanol)	17.66 ± 0.01
Staphylococcus aureus	GEN	15	CA Chloroform)	9 ± 0.01
			HC Methanol)	4.99 ± 0.00
E. coli	GEN	18	CA Methanol)	13.67 ± 0.01
			HC P. Ether)	12.33 ± 0.01
B. coagulans	GEN	15	PLANT EXTRACTS	NIL
B. mecentricus	GEN	22		
Lactobacillus sp	GEN	24		

GEN Gentamycin; *Centella asiatica* CA Extraction Solvent)); *Huttunia cordata* HC Extraction Solvent))

Discussion

The increase of multidrug resistant microorganism poises a major threat to human population worldwide. Therefore, search for newer drug molecule is becoming a continuous process. The present study is an effort to detect, antimicrobial property that exists if any in *C. asiatica* and *H. cordata*. If it has such property what is the extent it can inhibit harmful and useful bacteria of human gut. The experimental findings suggest that the extracts of both the plants inhibit the pathogens but interestingly do not inhibit the non-pathogens.

In the present study, the screening of plant extracts showed good range phytochemical having antimicrobial activity specially, against *E. coli* and *S. dysenteriae*. When compared the inhibitory zone size chloroform and methanol extracts of both *H. cordata* and *C. asiatica* showed most effective antimicrobial

activity then benzene and petroleum ether extracts. However, extracts of both the plants also have shown significant inhibitory zone against *K. pneumonia* and *S. dysenteriae*. The antimicrobial activity was considered when the zone of inhibition is greater than 5 mm (15), this study has found that *S. auerus* although are inhibited but the zone size were less than 5 mm. They also state that *A. sativum* extracts showed better activity against *E. coli* in disc diffusion method rather than well diffusion method. Therefore, it is possible to encounter a different result depending on the methods adopted. *H. cordata* extracts is found to be more effective than *C. asiatica*. *H. cordata* showed most effective result against *S. dysenteriae* and least against *S. aureus*. Jacob et. al. (16) did not find any antimicrobial activity of water and ethanol extracts of *C. asiatica* against *E. coli* and *S. aureus*. This may be because of the concentrations of the extracts. The study of M. Senthikumar (17) showed that higher concentration of ethanol, benzene and chloroform extracts of *C. asiatica* has antimicrobial activity against *K. pneumonia* on the contrary in this study no action has been recorded against *K. pneumonia*. The difference in antimicrobial activity of *H. cordata* and *C. asiatica* in the present study as well as the previous studies may be attributed to the age of the plants, geographical location, climatic conditions, and extraction procedures.

The potency of the plant extracts was quantified by evaluating the minimum concentration at which they inhibited the growth of the pathogens. The present study have shown the range of MIC values ranged from minimum 0.36 mg/ml to maximum 2.5 mg/ml for different extracts of both *H. cordata* and *C. asiatica*. Amongst all extracts methanol extract of *H. cordata* showed highest potency with least MIC value 0.36 mg/ml and 0.40 mg/ml respectively that inhibited the growth of *S. dysenteriae*. On the other hand extracts of *C. asiatica* showed varied range against *S. dysenteriae* minimum range was shown by methanol extract 2.5 mg/ml. Acetone extract and chloroform extract of *C. asiatica* showed almost similar value i.e. 2.5 mg/ml and 2.08 mg/ml respectively. It was also observed that all the extract of *H. cordata* and *C. asiatica* showed similar range of MIC value i.e. 2.5 mg/ml except acetone extract *C. asiatica* with MIC value 1.05 mg/ml.

The MIC tested against *S. aureus* by all the extract of *H. cordata* and *C. asiatica* showed a mix range of values. The MIC value of acetone and chloroform extract of *H. cordata* was similar that is 2.5 mg/ml against *S. aureus* and *K. pneumonia.* But that of methanol extract was 1.6 mg/ml against *S. aureus*. A previous report (16) also showed MIC value of ethyl acetate extract of *H. cordata* to be 2.5 mg/ml. However, no significant results was found against *E. coli* and *S. aureus* by *H. cordata* extracts. In another report (18) the MIC value against *S. aureus* of water extract of fresh and dry *H. cordata* were 12.5 and 100 mg/ml and those of ethanol extract 25 and 100 mg/ml respectively, which were extremely high as compared to the present study (19). The MIC value of methanol, acetone and chloroform extracts of *C. asiatica* ranged in order of 2.05 mg/ml, 1.6 mg/ml and 1.04 mg/ml against *S. aureus*. The results were very low compared to previous work where MIC values of ethanol extracts of leaf powder of *C. asiatica* showed 8 mg/ml and water extracts of leaf powder of *C. asiatica* showed 32 mg/ml against *S. aureus* (20). The present work results are in agreement with Phadet et. al. 2002) (21) which reports MIC values less than 5 mg/ml for ethanol extract 2.5 mg/ml for water extract. The difference in the MIC values can be because of the variations in method of extractions water and solvents) and strains of pathogens or may be because of the area of collection of the plant specimens.

Conclusion

C. asiatica and H. cordata are important herb used as vegetable as well as ethno-medicine for gasteroenteric diseases since time immemorial. The present study reveals that the extracts of both the plant likely to be effective against *S. dysenteriae* and *E. coli* pathogen) without inhibiting non-pathogenic bacteria. The plant extracts have potential compounds that confirms as targeting pathogen rather than host beneficial bacteria. Therefore, *C. asiatica* and *H. cordata,* may be used as an alternative to antibiotics together with antibiotics against *S. dysenteriae* and other pathogens.

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