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Antibacterial Activity of Polyphenolic Fraction of Kombucha Against Enteric Bacterial Pathogens

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Abstract The emergence of multi-drug-resistant enteric pathogens has prompted the scientist community to explore the therapeutic potentials of traditional foods and beverages. The present study was undertaken to investigate the efficacy of Kombucha, a fermented beverage of sugared black tea, against enterotoxigenic *Escherichia coli*, *Vibrio cholerae, Shigella flexneri* and *Salmonella* Typhimurium followed by the identification of the antibacterial components present in Kombucha. The antibacterial activity was evaluated by determining the inhibition zone diameter, minimal inhibitory concentration and minimal bactericidal concentration. Kombucha fermented for 14 days showed maximum activity against the bacterial strains. Its ethyl acetate extract was found to be the most effective upon sequential solvent extraction of the 14-day Kombucha. This potent ethyl acetate extract was then subjected to thin layer chromatography for further purification of antibacterial ingredients which led to the isolation of an active polyphenolic fraction. Catechin and isorhamnetin were detected as the major antibacterial compounds present in this polyphenolic fraction of Kombucha by High Performance Liquid Chromatography. Catechin, one of the primary antibacterial polyphenols in tea was also found to be present in Kombucha. But isorhamnetin is not reported to be present in tea, which may thereby suggest the role of fermentation process of black tea for its production in Kombucha. To the best of our knowledge, this is the first report on the presence of isorhamnetin in Kombucha. The overall study suggests that Kombucha can be used as a potent antibacterial agent against entero-pathogenic bacterial infections, which mainly is attributed to its polyphenolic content.

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Introduction

Enteric bacteria have been reported to be the causative agent for significant worldwide morbidity and mortality in the form of dysentery, diarrhoea and enteric fever. The pathogenic members include enterotoxigenic *Escherichia coli* (ETEC), *Vibrio* sp., *Campylobacter jejuni*, *Shigella* sp., *Salmonella typhi*, non-typhoidal *Salmonella* sp. which are predominantly associated with acute gastroenteritis in people living in the less-developed nations as well as the developing countries including India [3]. This has had two consequences, firstly investments towards production of antibiotics have increased and secondly, continuous use of such antimicrobial agents has resulted in the emergence of multi-drug-resistant (MDR) strains among these pathogenic microorganisms [3]. Thus, the burden of enteric bacterial infections has acquired new heights due to the emergence of MDR strains. This has resulted in a major shift in global perspective towards battling such infections, and more focus is being made on traditional foods and beverages for their therapeutic potentials [23].

Kombucha, an oriental beverage, is prepared by fermenting sugared black tea by a consortium of acetic acid

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bacteria and yeast [8, 14, 15]. It has acquired significant popularity as a traditional fermented beverage due to its various claimed and a few established pharmacological effects [13, 16, 18, 25]. It is believed that most of these beneficial properties may be attributed to the polyphenols [25, 26], organic acids specifically acetic acid [16] and a host of other ingredients that are inherent to the raw materials or are produced as a result of the microbial fermentation [4, 26]. The health-promoting effects of this ferment include hepatoprotective [5], anti-diabetic [6], antioxidant [6, 7], as well as reduction of arthritis, atherosclerosis, metabolic disorders, inflammatory problems [18] and even cancer [17, 18]. The antimicrobial property of Kombucha has been reported in a number of previous research works [4, 16, 18, 25]. However, as these studies have mainly dealt with the overall properties of the beverage, reports on the quantitative analysis have largely been missing from the scientific discourse. Moreover, no report is available regarding the activity-guided screening of Kombucha against different enteric bacteria as well as identification of its antibacterial constituents by biophysical methods.

In this article, we describe the progression of the antibacterial activity of Kombucha against enteric bacteria at different fermentation time points. We also evaluated the antibacterial activity-guided analysis of different solvent extracts and thin layer chromatography fractions of Kombucha against the enteric bacterial strains. In addition, the active constituents of Kombucha were identified and quantified by High Performance Liquid Chromatography (HPLC) analysis. Therefore, our study might be able to shed some light for future applications of Kombucha as an effective antibacterial agent in the treatment of enteric bacterial infections.

Materials and Methods

Chemicals

Commercially available black tea (Tata tea Gold) was used for the study. Chloroform, ethyl acetate, n-butanol, acetonitrile (HPLC grade), water (HPLC grade), and methanol (HPLC grade) were purchased from Merck Specialities Private Limited (Mumbai, India). Sucrose, Folin-Ciocalteu reagent, gallic acid, quercetin, aluminium chloride, potassium acetate and sodium carbonate were obtained from Sisco Research Laboratories (Mumbai, India). Luria–Bertani broth, Agar powder (Bacteriological), Kanamycin, Dimethyl sulfoxide (DMSO) were purchased from HIMEDIA Laboratories Private Limited (Mumbai, India). Mueller–Hinton Agar was purchased from BD, Difco. Catechin (HPLC grade) and isorhamnetin (HPLC grade) were bought from Sigma Aldrich, USA.

Microorganisms

The test microorganisms enterotoxigenic *E. coli* O157:H7, *Vibrio cholerae* N16961, *Shigella flexneri* 2a 2457T, *Salmonella* Typhimurium NCT 572 and the reference strains *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were obtained from National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India. The microorganisms were maintained in Luria–Bertani medium.

Preparation of Kombucha

Kombucha was prepared by adding 0.5 % (w/v) of black tea to single distilled water and boiled for 5 min. It was then steeped for 20 min, filtered through a sterile sieve and poured into a sterile 1 L glass beaker. After cooling, the volume of the tea was adjusted to 500 ml by adding 5 % (w/v) sucrose and 10 % (v/v) previously fermented broth. The tea solution was then inoculated with a portion of freshly grown Kombucha biofilm, the microbial population of which was already characterised in previous works [8, 14, 15]. The beaker was then covered with sterile cheese cloth and secured properly. Separate fermentation systems were maintained at 28 ± 2 °C and terminally harvested after 0, 7, 14 and 21 days. Sugared black tea (SBT) was kept separately as a control. After the specified time periods, the fermented samples were centrifuged at 10,000×*g* for 15 min at 4 °C, and the respective supernatants were collected. After measuring the pH, each of the fermented samples and SBT were lyophilized to powder and weighed to make stock solutions of 200 mg/ml in single distilled water which were filter-sterilized (0.22 µm, Millex-GP filter) and stored at 4 °C until further studies.

Antibacterial Assays of SBT and Kombucha Samples by Determining Inhibition Zone Diameter, Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

Susceptibility test of the bacterial strains was performed by agar-well diffusion method. Each of the strains were incubated in Luria–Bertani (LB) broth at 37 °C under shaking conditions until the final inoculum size reached to 1×10^8 CFU/ml [20]. A volume of 100 µl suspension was spread evenly on Mueller–Hinton Agar (Difco) plates using a sterile glass rod spreader, and the plates were allowed to dry at room temperature. Wells of diameter 6 mm were then bored in the agar plates. A volume of 50 µl of 100 mg/ml of SBT and Kombucha samples at the specified fermentation time periods were added into the wells. Antibiotic Kanamycin (30 µg) was used as the experimental positive control and single distilled water as the

negative control. The plates were first kept at 4 °C for 2 h for pre-diffusion of the samples into the agar plates and then incubated at 37 °C. Inhibition zone diameter (IZD) was measured after 16 h. The experiment for each strain was carried out in triplicate, and the IZD was expressed as mean diameter ± standard deviations [10, 25].

The MICs of SBT and the Kombucha samples at the specified fermentation time periods, were determined by micro-broth dilution assay method using flat-bottomed 96-well microtitre plates [2, 10]. SBT and the Kombucha samples were serially diluted twofold with LB broth (100–1.56 mg/ml). The standard bacterial inoculum was adjusted to 5×10^5 CFU/ml. Kanamycin (30–0.94 µg/ml) was used as the experimental positive control. Other control wells were also prepared in each plate: sterility control (without any bacterial inoculum) and control for inoculum viability (without any sample solution). After incubation at 37 °C for 24 h, the plates were visually examined for turbidity; bacterial growth was further examined by measuring the $O.D._{600}$ in iMark micro-plate reader (Bio-Rad). The lowest concentration of the samples or antibiotic which showed no growth was taken as the MIC.

The MBCs of SBT, the Kombucha samples and Kanamycin were determined by sub-culturing from the wells showing no visible growth (in the micro-broth MIC assay) on fresh Mueller–Hinton Agar plates and incubated at 37 °C for 24 h. The least concentration showing no visible growth on the subculture was taken as the MBC. From the acquired values of MIC and MBC, MIC_{index} (MBC/MIC) values were calculated for all the samples against each of the target strains [10].

Solvent Extraction and Thin Layer Chromatography of Kombucha Under Antibacterial Activity-Guided Screening

Kombucha sample which showed the highest antibacterial activity after the specified fermentation time periods, was subjected to liquid–liquid extraction in a separating funnel. The whole Kombucha broth (500 ml) of three separate batches was successively extracted with different organic solvents, i.e. chloroform, ethyl acetate and n-butanol [17] in a 1:1 v/v ratio. Then chloroform, ethyl acetate and butanol were evaporated under vacuum, and the remaining aqueous phase was lyophilized to obtain 130 ± 26.46, 183.92 ± 20.63, 144.45 ± 25.46 and 112.8 ± 28.46 mg of the respective extracts. The organic solvent extracts were dissolved in 0.5 % DMSO, while the aqueous extract was dissolved in single distilled water to a stock concentration of 200 mg/ml (each). All the extracts were then filter-sterilized and used in antibacterial assays. The most active solvent extract was further fractionated by thin layer chromatography (TLC) on silica gel plates (TLC aluminium sheets; Silica Gel 60 F_{254} UV plates; 20 × 20 cm; Merck, Germany) using the solvent system ethyl acetate: methanol: water (100: 13.5: 10, v/v/v) [29]. After development and drying of the plates, the positions of the spots (or the TLC-fractions) on the TLC plate were ascertained under UV light at 254 and 365 nm. The TLC fractions obtained, were then scraped off separately from the silica plate and eluted with methanol (HPLC grade). The methanol was evaporated under vacuum, and the solidified fractions were dissolved in 0.5 % DMSO, filter-sterilized and evaluated for antibacterial activity.

Estimation of Total Phenolic Compounds and Flavonoids in SBT, Kombucha and Its Solvent Extracts

The total content of the phenolic compounds present in the SBT and the Kombucha sample showing highest spectrum of antibacterial activity and its different solvent extracts were determined by the Folin–Ciocalteu method [6]. A volume of 0.1 ml of the respective samples (50 mg/ml) was added in test tubes and then mixed with Folin–Ciocalteu reagent (0.2 ml), purified water (2 ml) and 15 % sodium carbonate (1 ml). The mixture was then incubated for 2 h at room temperature, and the absorbance was measured at 765 nm by Jasco UV/Vis Spectrophotometer. The standard compound used in the assay was gallic acid, and the total phenolic content of each of the samples was given as gallic acid equivalents (GAE) in milligrams per gram of dried weight of the samples.

The total flavonoid content in the SBT and the most active Kombucha sample and its solvent extracts were determined by the colorimetric method [6] using quercetin as the standard. A volume of 0.5 ml of the respective samples (50 mg/ml) was taken and mixed with methanol (1.5 ml), 10 % aluminium chloride (0.1 ml), 1 M potassium acetate (0.1 ml) and distilled water (2.8 ml). After incubation of the mixture for 30 min at room temperature, the absorbance was measured at 415 nm by Jasco UV/Vis Spectrophotometer. The results for the total flavonoid content were expressed as quercetin equivalents (QE) in milligrams per gram of dried weight of the samples.

Antibacterial Assays of Solvent Extracts of the Most Active Kombucha Sample by Determining Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The MICs of the different solvent extracts of the Kombucha sample showing the highest antibacterial activity were determined in the same manner as previously done for SBT and the different Kombucha samples. The solvent extracts were diluted twofold with LB with concentrations

ranging from 100–0.0488 mg/ml. Separate controls were prepared for the inhibitory effects of DMSO. All other conditions were kept same as previously done. The MIC of the samples was determined as the lowest concentration showing no bacterial growth.

The MBCs of the solvent extracts were also determined in same way as done for the Kombucha samples. The MBCs were obtained, and the respective MIC_{index} (MBC/MIC) values were calculated.

Antibacterial Assays of TLC-Fractions of the Most Active Solvent Extract of Kombucha by Determining Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The MICs of the fractions obtained from TLC of the most effective solvent extract of Kombucha were determined by the same manner as described above. All the TLC-fractions were similarly diluted as described above, with concentrations ranging from 12.5–0.006 mg/ml. DMSO controls were also separately taken. Here, also, all other conditions were kept the same. The MIC of each of the TLC-fractions was determined as the lowest concentration showing no bacterial growth in the wells of the microtitre plate.

The MBCs of the TLC-fractions were also detected, and the respective MIC_{index} (MBC/MIC) values were calculated in a similar manner as described above.

HPLC and ESI–MS Analysis of the Active TLC-Fraction of Kombucha

The TLC-fraction showing the highest activity against the enteric bacteria was used for detection of its major components. After TLC, this active fraction was eluted, filter-sterilized by 0.22 μm filter and analysed by HPLC to detect the major compounds present in it. A 20 μl volume of the filtrate was injected to a reverse-phase HPLC system (Shimadzu, Japan) which consisted of a 100 Å LC C-18 column (30 by 2 mm, Phenomenex, USA) and equipped with a photodiode array detector (SPD-M20A). The mobile phase used was acetonitrile: water (40:60, v/v); the flow rate was set at 1 ml/min; running time 50 min; the temperature of the column was maintained at 28 °C. Detection of the peaks was monitored at 280 and 380 nm. The retention times (R_t) of the peaks obtained from the chromatogram were compared with those of the reference standards. Calibration curves were prepared by plotting different concentrations of the reference standards against the peak areas obtained. Then the compounds detected in the sample were quantified from their peak areas against the respective calibration curves. The experiments were carried out in triplicate, and the results were expressed as mean values ± standard deviations. Identification of the compounds was further confirmed by direct infusion of the active TLC-fraction for electrospray ionisation-mass spectrometry (ESI–MS) analysis using the mass spectrometer (Xevo G2 QTof, Waters) in positive ion mode. The conditions used were capillary voltage of 3 kV, sampling cone voltage of 30 V, extraction cone voltage of 3 V, source temperature 130 °C, desolvation temperature 500 °C and the analysis was done as a mass-to-charge ratio (m/z) scan ranging from 0 to 900.

Antibacterial Assays of the Compounds Identified in the Active TLC-Fraction of Kombucha by Determining Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The MICs of the compounds identified in the most active TLC-fraction of Kombucha against the target strains were determined in the same method as described previously for the TLC-fractions. The compounds were dissolved in 0.5 % DMSO and diluted with LB as done previously, with concentrations ranging from 0.2–0.00156 mg/ml. Separate controls of DMSO were also taken. Here, also, all other conditions were kept the same as done previously. The MIC of each of the compound was determined as the lowest concentration which showed no bacterial growth in the wells of the microtitre plate.

The MBC of each of the compounds was also determined, and the respective MIC_{index} (MBC/MIC) values were calculated in a similar method as done for the TLC-fractions.

Statistical Analysis

Statistical analysis was performed using Origin software: Version Pro8. The results are presented as the mean values ± standard deviations (SD) of three independent experimental studies. One-way analysis of variance (ANOVA) along with the Tukey's multiple comparison test was used to determine differences that are significant between all the groups at $P < 0.05$ level.

Results

Antibacterial Assays of SBT and Kombucha Samples

In our study, we first measured the antibacterial activity of Kombucha at different time points (0–21 days) of fermentation against enterotoxigenic *E. coli* O157:H7, *Vibrio cholerae* N16961, *Shigella flexneri* 2a 2457T and *Salmonella*

Typhimurium NCT 572 by the susceptibility test, MIC and MBC assays. Optimum antibacterial activity was observed on the 14th day as well as on the 21st day of fermentation. The antibiotic, Kanamycin, was active against all the test and control bacteria. The SBT and 0-day Kombucha hardly showed any activity against the test and control bacteria. SBT had little antibacterial activity only against *S. aureus*. Likewise, the 0-day Kombucha exerted little activity against *V. cholerae, S. flexneri* and *S. aureus*. The 7-day Kombucha was found to be active against all the test organisms except *S.* Typhimurium. However, the 14-day and 21-day Kombucha samples exerted the highest bacteriostatic and bactericidal activities against all the target organisms (Tables 1, 2). Although the 21-day Kombucha produced greater zone of inhibition in *S.* Typhimurium, no significant difference in the IZD was observed between the 14-day and 21-day Kombucha samples against the other enteric strains (Table 1). Therefore, the 14-day Kombucha sample was selected for successive solvent extraction, thin layer chromatographic analysis and identification of the active components by HPLC and Mass Spectrometry.

Total Phenolic Compounds and Flavonoids in SBT, Kombucha and Its Solvent Extracts

The phenolic compounds and flavonoids have been referred to as potent antimicrobial agents against pathogenic bacteria [11, 12]. The total content of phenolic compounds as well as flavonoids of SBT, 14-day fermented Kombucha and its chloroform, ethyl acetate, butanol and remaining aqueous extracts is represented in Fig. 1. The results showed that both the contents of total phenolics and flavonoids increased in Kombucha than SBT. This may possibly be due to the production of several enzymes from the Kombucha microbial population which causes the degradation of the complex polyphenolic compounds into smaller compounds, thereby increasing the total content of the phenolics and flavonoids of Kombucha [6]. Then the 14-day Kombucha was first extracted with chloroform to remove caffeine and other non-polar compounds. The polyphenols and other polar compounds possibly move into the aqueous phase which was subsequently extracted with ethyl acetate and n-butanol [19]. Hence, both phenolic content and flavonoid content were found to be the highest in the ethyl acetate extract of the 14-day fermented Kombucha followed by butanol extract as represented in Fig. 1.

Antibacterial Assays of Solvent Extracts of 14-day Fermented Kombucha

The 14-day Kombucha was sequentially extracted with chloroform, ethyl acetate and n-butanol. All the dried solvent extracts and the remaining lyophilized aqueous extract were screened for antibacterial activity by MIC and MBC. A considerable increase in the antibacterial efficacy was observed with the ethyl acetate extract, as was illustrated by the low values of MIC and MBC against all the strains, when compared with the other solvent extracts (Table 3). Hence, the ethyl acetate extract was found to be the most potent against the enteric bacterial strains followed by chloroform extract, n-butanol extract and aqueous extract. Therefore, the ethyl acetate extract was subjected to thin layer chromatography for further purification of the antibacterial components present in Kombucha.

Antibacterial Assays of the TLC-Fractions of Ethyl Acetate Extract of 14-Day Kombucha

Thin layer chromatographic analysis of the ethyl acetate extract of three separate batches revealed two fractions F1

Table 1 Antibacterial activity of sugared black tea and Kombucha against enterotoxigenic *Escherichia coli* O157:H7, *Vibrio cholerae* N16961, *Shigella flexneri* 2a 2457T, *S.* Typhimurium NCT 572, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 as indicated by inhibition zone diameter (mm)

Bacteria	Inhibition zone diameter (mm)					
	Sugared black tea	Kombucha				Kanamycin[g]
		0-day	7-day	14-day	21-day	
ETEC O157:H7	0^{a}	0^{a}	5.75 ± 0.5^{b}	20.33 ± 0.58^{c}	20.667 ± 0.58^{c}	24.75 ± 0.5^{d}
V. cholerae N16961	0^{a}	7.75 ± 0.96^{b}	11.67 ± 0.58^{c}	19.67 ± 0.68^{d}	20.44 ± 0.58^{d}	25.5 ± 0.62^{e}
S. flexneri 2a 2457T	0^{a}	4.25 ± 0.53^{b}	9.67 ± 0.58^{c}	19.3 ± 0.58^{d}	19.0 ± 0.5^{d}	20.75 ± 0.96^{e}
S. Typhimurium NCT 572	0^{a}	0^{a}	0^{a}	13.75 ± 0.96^{b}	15.5 ± 0.58^{c}	21.67 ± 0.58^{d}
E. coli ATCC 25922	0^{a}	0^{a}	5.25 ± 0.5^{b}	20.5 ± 0.58^{c}	20.67 ± 0.58^{c}	26.5 ± 1.15^{d}
S. aureus ATCC 25923	6.75 ± 0.96^{a}	8.5 ± 1.3^{b}	12.5 ± 0.58^{c}	20.15 ± 0.58^{d}	18.0 ± 0.82^{e}	22.5 ± 0.82^{f}

The data are expressed as mean ± standard deviations of three separate experiments. Rows not sharing a common superscript value $^{(a–f)}$ differ significantly at $P < 0.005$

[g] Antibiotic Kanamycin is taken as a positive control

Table 2 Minimal inhibitory concentration (MIC, mg/ml) and minimal bactericidal concentration (MBC, mg/ml) of sugared black tea and Kombucha against enterotoxigenic *Escherichia coli* O157:H7, *Vibrio cholerae* N16961, *Shigella flexneri* 2a 2457T, *Salmonella* Typhimurium NCT 572, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923

Bacteria	Sugared black tea			Kombucha								
				0-day			7-day			14-day		
	MIC	MBC	MIC_{index}	MIC	MBC	MIC_{index}	MIC	MBC	MIC_{index}	MIC	MBC	MIC_{index}
ETEC O157:H7	>[b]	>	ND[c]	>	>	ND	100	>	ND	3.125	6.25	2.00
V. cholerae N16961	100	>	ND	100	100	1.00	25	50	2.00	6.25	6.25	1.00
S. flexneri 2a 2457T	>	>	ND	100	>	ND	100	100	1.00	3.125	6.25	2.00
S. Typhimurium NCT 572	>	>	ND	>	>	ND	>	>	ND	6.25	12.5	2.00
E. coli ATCC 25922	>	>	ND	>	>	ND	100	>	ND	6.25	6.25	1.00
S. aureus ATCC 25923	100	>	ND	100	100	1.00	25	50	2.00	6.25	6.25	1.00
Spectrum of activity (%)	2/6 (33.33)			3/6 (50.00)			5/6 (83.33)			6/6 (100.00)		

Bacteria	Kombucha			Kanamycin[d]			Strains susceptible (%)[a]
	21-day						
	MIC	MBC	MIC_{index}	MIC × 10^{-3}	MBC × 10^{-3}	MIC_{index}	
ETEC O157:H7	3.125	6.25	2.00	3.75	7.5	2.00	3/5 (60.00)
V. cholerae N16961	3.125	3.125	1.00	3.75	3.75	1.00	5/5 (100.00)
S. flexneri 2a 2457T	6.25	6.25	1.00	15	15	1.00	4/5 (80.00)
S. Typhimurium NCT 572	3.125	12.5	4.00	7.5	15	2.00	2/5 (40.00)
E. coli ATCC 25922	3.125	6.25	2.00	3.75	3.75	1.00	3/5 (60.00)
S. aureus ATCC 25923	12.5	12.5	1.00	7.5	7.5	1.00	5/5 (100.00)
Spectrum of activity (%)	6/6 (100.00)						

[a] Susceptibility to sugared black tea and Kombucha only

[b] No inhibition with the highest concentration in the test conditions

[c] Not determined

[d] Antibiotic Kanamycin is taken as a positive control

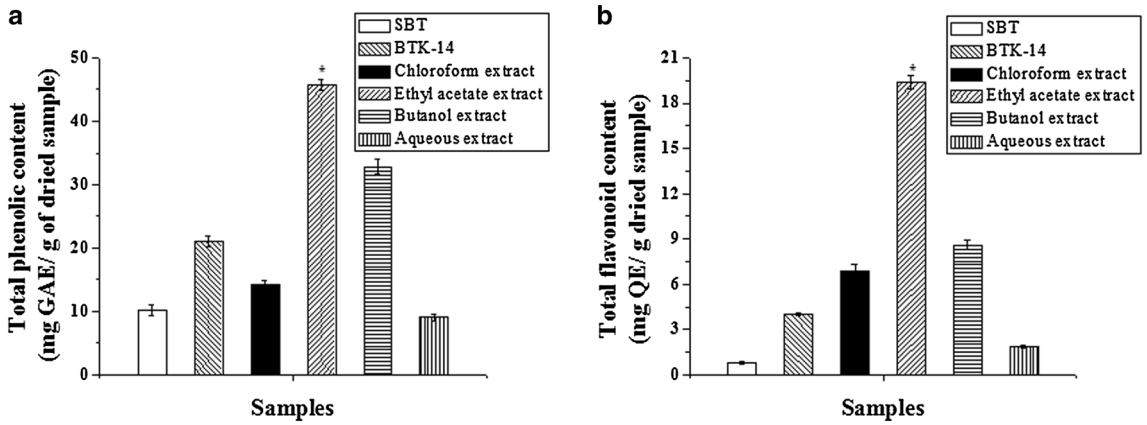

Fig. 1 Total content of phenolic compounds (**a**) and flavonoids (**b**) in sugared black tea (SBT), black tea Kombucha fermented for 14 days (BTK-14) and its different solvent extracts. Results are represented as mean ± standard deviations of three independent experiments. The sign *asterisk* indicates the sample with the maximum amount of phenolic compounds and flavonoids, respectively

(60.5 ± 5.132 mg, R_f 0.78) and F2 (28.6 ± 7.625 mg, R_f 0.63). The fraction F1 was found to be the most potent among the two fractions obtained. *V. cholerae* was observed as the most susceptible organism with each of MIC and MBC of 0.0244 mg/ml, whereas *S.* Typhimurium with each of MIC and MBC of 0.195 mg/ml was the most resistant organism (Table 4). These findings suggest that the antibacterial property of the 14th day fermented

Table 3 Minimal inhibitory concentration (MIC, mg/ml) and minimal bactericidal concentration (MBC, mg/ml) of chloroform, ethyl acetate, n-butanol extracts and remaining aqueous phase of 14-day Kombucha against enterotoxigenic *Escherichia coli* O157:H7, *Vibrio cholerae* N16961, *Shigella flexneri* 2a 2457T, *Salmonella* Typhimurium NCT 572, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923

Bacteria	Solvent extracts of 14-day Kombucha[a]												Strains susceptible (%)
	Chloroform extract			Ethyl acetate extract			n-Butanol extract			Remaining aqueous phase			
	MIC	MBC	MIC_{index}	MIC	MBC	MIC_{index}	MIC	MBC	MIC_{index}	MIC	MBC	MIC_{index}	
ETEC O157:H7	12.5	25	2.00	0.195	0.195	1.00	25	25	1.00	50	100	2.00	4/4 (100.00)
V. cholerae N16961	6.25	12.5	2.00	0.0975	0.195	2.00	12.5	25	2.00	25	100	4.00	4/4 (100.00)
S. flexneri 2a 2457T	12.5	12.5	1.00	0.195	0.195	1.00	25	25	1.00	50	100	2.00	4/4 (100.00)
S. Typhimurium NCT 572	25	25	1.00	3.125	3.125	1.00	25	50	2.00	50	>[b]	ND[c]	4/4 (100.00)
E. coli ATCC 25922	6.25	6.25	1.00	0.0975	0.195	2.00	12.5	25	2.00	50	100	2.00	4/4 (100.00)
S. aureus ATCC 25923	3.125	6.25	2.00	0.0975	0.0975	1.00	6.25	25	4.00	25	100	4.00	4/4 (100.00)
Spectrum of activity (%)	6/6 (100.00)			6/6 (100.00)			6/6 (100.00)			6/6 (100.00)			

[a] Results of the activity of Kanamycin was shown in Table 2

[b] No inhibition with the highest concentration in the test conditions

[c] Not determined

Table 4 Minimal inhibitory concentration (MIC, mg/ml) and minimal bactericidal concentration (MBC, mg/ml) of TLC-fractions F1 and F2 with respective R_f values 0.78 and 0.63 isolated from ethyl acetate extract of 14-day Kombucha against enterotoxigenic *Escherichia coli* O157:H7, *Vibrio cholerae* N16961, *Shigella flexneri* 2a 2457T, *Salmonella* Typhimurium NCT 572 *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923

Bacteria	TLC-fractions[a]						Strains susceptible (%)
	F1 R_f 0.78			F2 R_f 0.63			
	MIC	MBC	MIC_{index}	MIC	MBC	MIC_{index}	
ETEC O157:H7	0.0488	0.195	4.00	>[b]	>	ND[c]	1/2 (50.00)
V. cholerae N16961	0.0244	0.0244	1.00	3.125	6.25	2.00	2/2 (100.00)
S. flexneri 2a 2457T	0.0488	0.0975	2.00	12.5	>	ND	2/2 (100.00)
S. Typhimurium NCT 572	0.195	0.195	1.00	>	>	ND	1/2 (50.00)
E. coli ATCC 25922	0.0488	0.0975	2.00	6.25	6.25	1.00	2/2 (100.00)
S. aureus ATCC 25923	0.0122	0.0244	2.00	3.125	3.125	1.00	2/2 (100.00)
Spectrum of activity (%)	6/6 (100.00)			4/6 (66.67)			

[a] Results of the activity of Kanamycin was shown in Table 2

[b] No inhibition with the highest concentration in the test conditions

[c] Not determined

Kombucha might be due to the fraction F1 derived from TLC of its ethyl acetate extract.

HPLC and ESI–MS Analysis of the Active TLC-Fraction of Kombucha

We then further extended our work by subjecting the active TLC-fraction F1 derived from the ethyl acetate extract of Kombucha into HPLC and ESI–MS analyses to get a tentative idea about the antibacterial components of Kombucha. The major compounds detected were catechin ($R_t = 4.481$ min, 68.173 ± 7.86 μg/ml) and isorhamnetin ($R_t = 8.858$ min, 156.384 ± 11.32 μg/ml) in the TLC-fraction F1 by comparing with the corresponding standards at 280 and 380 nm, respectively (Fig. 2).

The molecular masses of the compounds have also been confirmed by direct infusion of the TLC-fraction F1 into ESI–MS analysis (Fig. 3). The fragmentation patterns of catechin and isorhamnetin also matched with that reported in the previous work [24]. The fragment ion (*m*/*z* 246.0791) was due to the loss of $–CH_2CHOH–$ group from catechin

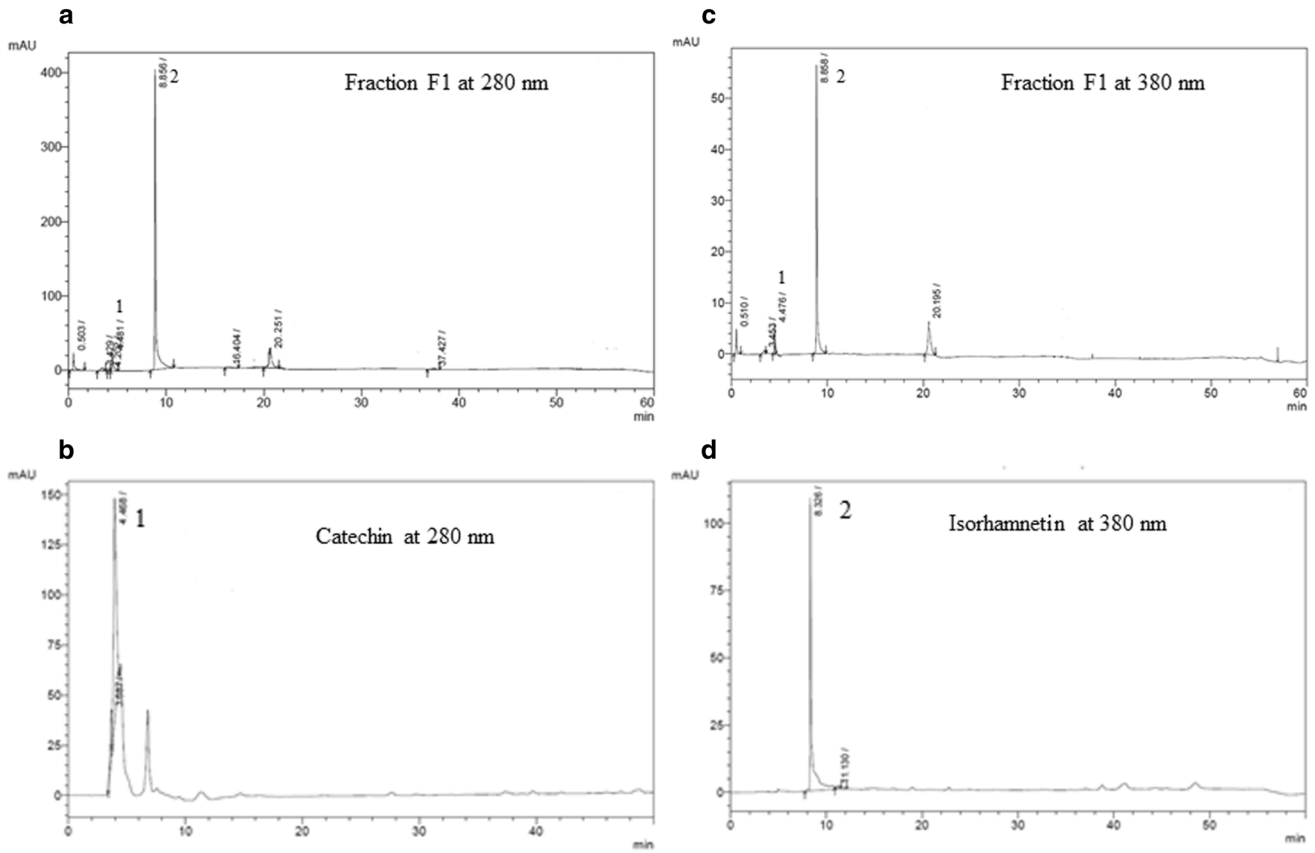

Fig. 2 Reverse-phase HPLC profile of TLC-fraction F1 of Kombucha at 280 nm (**a**) and 380 nm (**c**). The polyphenols are catechin (peak 1) detected at 280 nm and isorhamnetin (peak 2) detected at 380 nm as was identified by comparing the retention times of the respective standards. The chromatograms of the standard compounds are also provided as catechin (**b**), isorhamnetin (**d**)

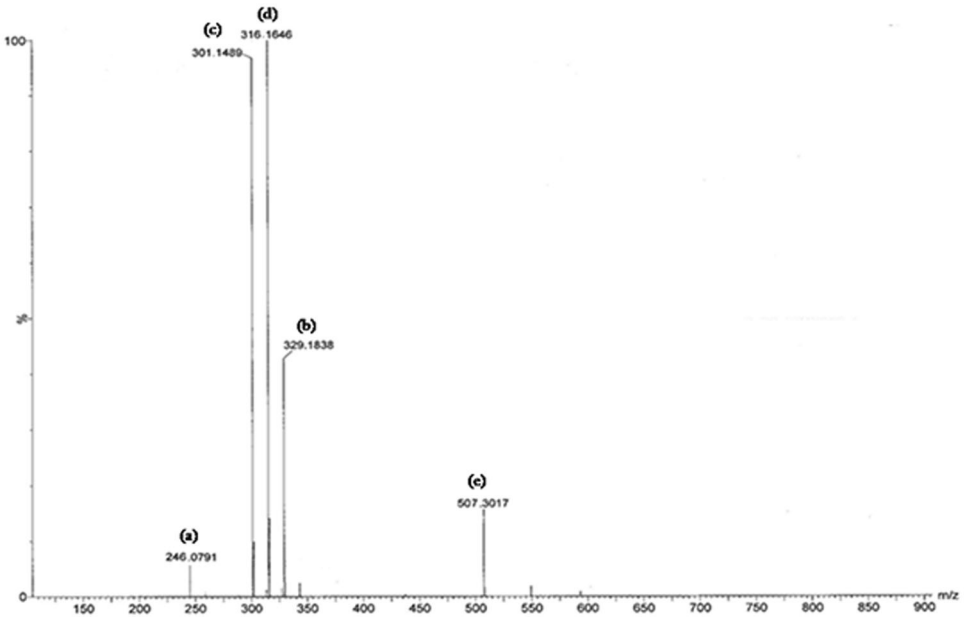

Fig. 3 ESI–MS spectrum (in positive ion mode) of the TLC-fraction F1 of Kombucha. The fragment ions of the compounds are marked as *a* and *b* for catechin, *c* and *d* for isorhamnetin and *e* for an unidentified compound

Table 5 Profile of polyphenols detected in the TLC-fraction F1 obtained from Kombucha

Peak number in HPLC	Retention time (min)	Detection wavelength (nm) in HPLC	Identified Compounds	Molecular mass	Molecular formula (M)	Fragment ions in ESI-MS (*m/z*) in positive mode	Concentration (μg/ml)
1	4.481	280	Catechin	290.27	$C_{15}H_{14}O_6$	(a) 246.0791 $[M\text{-}CH_2CHOH]^+$	68.173 ± 7.86
						(b) 329.1838 $[M + K]^+$	
2	8.858	380	Isorhamnetin	316.26	$C_{16}H_{12}O_7$	(c) 301.1489 $[M - {}^{\cdot}CH_3]^+$	156.384 ± 11.32
						(d) 316.1646 $[M]^+$	
			Unidentified			(e) 507.3017	

The concentrations of the compounds are expressed as the mean values ± standard deviations of three independent experiments

(molecular mass 290). Also the fragment ion (*m/z* 301.1489) was due the loss of ${}^{\cdot}CH_3$ radical from isorhamnetin (molecular mass 316) as described by Sánchez-Rabaneda et al. [24].

The profile of the compounds detected in the polyphenolic fraction of Kombucha has been represented in Table 5. However, the fragment ion (*m/z* 507.3017) could not be identified and has been designated as an unidentified compound.

Antibacterial Assays of the Compounds Identified in the Active TLC-Fraction of Kombucha

From the HPLC and ESI–MS studies, catechin and isorhamnetin were detected as the major compounds present in the TLC-fraction F1 which showed the highest activity against the bacterial strains. The antibacterial activities of catechin and isorhamnetin against the target strains were also performed and have been represented in Table 6. It was observed that both catechin and isorhamnetin exerted bacteriostatic as well as bactericidal activities against all the organisms. The MIC and MBC values for catechin ranged between 0.025–0.1 and 0.05–0.2 mg/ml, respectively, and that for isorhamnetin ranged between 0.025–0.05 and 0.025–0.1 mg/ml, respectively, against all the bacterial strains. Thus, our results also showed that both the compounds catechin and isorhamnetin detected in Kombucha possess potent antibacterial activity against the tested enteric bacteria.

Discussion

Enteric bacterial infections remain a major disease burden in human population in developing countries. Earlier these infections could be treated with low-priced antibiotics. However, recently the treatment has become more expensive and less successful due to the emergence of multi-drug resistance among the enteric strains such as *E. coli*, *C. jejuni*, *Salmonella* spp., *Vibrio cholerae* and *Shigella* spp. [3]. Thus, these drug-resistant bacteria pose a major challenge to human health and the pharmaceutical industry. Hence, it has become imperative that the problem of multi-drug resistance is battled at the earnest by exploring traditional foods and medicines or products from natural

Table 6 Minimal inhibitory concentration (MIC, mg/ml) and minimal bactericidal concentration (MBC, mg/ml) of catechin and isorhamnetin identified from the TLC-fraction F1 isolated from the ethyl acetate extract of 14-day Kombucha against enterotoxigenic *Escherichia coli* O157:H7, *Vibrio cholerae* N16961, *Shigella flexneri* 2a 2457T, *Salmonella* Typhimurium NCT 572 *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923

Bacteria	Identified compounds from TLC-fraction F1[a]						Strains susceptible (%)
	Catechin			Isorhamnetin			
	MIC	MBC	MIC_{index}	MIC	MBC	MIC_{index}	
ETEC O157:H7	0.05	0.1	2.00	0.025	0.05	2.00	2/2 (100.00)
V. cholerae N16961	0.05	0.05	1.00	0.025	0.025	1.00	2/2 (100.00)
S. flexneri 2a 2457T	0.1	0.1	1.00	0.05	0.05	1.00	2/2 (100.00)
S. Typhimurium NCT 572	0.1	0.2	2.00	0.05	0.1	2.00	2/2 (100.00)
E. coli ATCC 25922	0.1	0.1	1.00	0.025	0.05	2.00	2/2 (100.00)
S. aureus ATCC 25923	0.025	0.05	2.00	0.025	0.025	1.00	2/2 (100.00)
Spectrum of activity (%)	6/6 (100.00)			6/6 (100.00)			

[a] Results of the activity of Kanamycin was shown in Table 2

sources as a potential source of novel antimicrobial agents against the resistant organisms [23].

Kombucha, owing to its various positive effects on the bio-regularity functions, has been regarded as one of the potential functional foods, enhancing human health. Since Kombucha can be easily prepared at home, it has become quite popular as a traditional health-promoting beverage around the world. However, little scientific evidence is available on the beneficial effects of this fermented beverage until recent past [26]. In the present study, we have evaluated the efficacy of Kombucha against entero-pathogenic bacteria and also detected isorhamnetin, for the first time as an active compound, in the 14-day fermented Kombucha.

Kombucha has been known for its potent antimicrobial properties against a number of pathogenic organisms. Many components such as organic acids (acetic acid, gluconic acid, glucuronic acid etc.), bacteriocins, proteins, enzymes, tea polyphenols and their derivatives which are produced during Kombucha fermentation have been said to contribute to its antimicrobial activity as well as other pharmacological effects [4, 16, 18, 25]. However, very little evidence is there in the literature about the effect of Kombucha on enteric pathogens. Hence, we evaluated the growth inhibitory activity of Kombucha against enteric bacteria. As the 14-day fermented Kombucha exhibited the strongest antibacterial property, it was subjected to successive solvent extraction. The ethyl acetate extract emerged as having the maximum antibacterial activity among the solvent extracts. This result suggests that the phenolic compounds and the flavonoids present in the ethyl acetate extract may be responsible for the antibacterial activity of Kombucha as was reported by previous researchers [4, 18, 25, 26]. Further chromatographic analysis of the ethyl acetate extract of Kombucha led to the isolation of an antibacteriologically active polyphenolic fraction containing catechin and isorhamnetin as the major compounds. To the best of our knowledge, this is the first report on the presence of isorhamnetin in Kombucha.

However, Greenwalt et al. [16] addressed that the antimicrobial efficacy of Kombucha prepared from both black tea and green tea was due to the presence of acetic acid which was considered as a major antimicrobial component of Kombucha. Later, Sreeramulu et al. [25] and Battikh et al. [4] have demonstrated that apart from acetic acid or other organic acids, other biologically active compounds such as polyphenols, proteins, bacteriocins, enzymes may also contribute to the antimicrobial activity of Kombucha. From our study, the polyphenols catechin and isorhamnetin were detected as the major antibacterial compounds of Kombucha which belong to the flavan-3-ol and flavonol classes of flavonoids, respectively [11]. Catechin is one of the predominant polyphenols found in tea [13], which exhibits potent antimicrobial activity [1, 30]. In our study, we also detected catechin as one of the compounds in the active polyphenolic fraction of Kombucha. However, isorhamnetin is an *O*-methylated flavonol and one of the constituents of *Gingko biloba* [9], cocoa [24] and *Hippophae rhamnoides* [28] which exerts potent antimicrobial activity [21]. We also detected isorhamnetin as one of the antibacterial constituents present in the Kombucha polyphenolic fraction. Since isorhamnetin is not reported to be present in tea, we can suggest that the fermentation of black tea by the microbial population of Kombucha may play some role in the production of isorhamnetin in Kombucha. Moreover, the structure–activity relationship for the antibacterial activity of the phenolic compounds including flavonoids has been proposed by many researchers. Based on previous reports by Cushnie and Lamb [11] and Rojas et al. [22], we can hypothesise that the presence of hydroxylation at the positions 5 and 7 of the A ring and position 3 of the C ring contribute to the antibacterial activity of these polyphenolic compounds. The free hydroxyl group(s) in the B ring of the flavonoids are also thought to be responsible for their antibacterial activity [11]. Thus, we can conclude that the combined contribution of these two polyphenols or presence of other additional compounds might be responsible for the antibacterial activity of Kombucha harvested after 14 days of fermentation. Further work also determined that each of the identified constituents catechin and isorhamnetin has potent activity against the enteric bacterial pathogens, thus implying their role in the antibacterial activity of Kombucha.

There are many conflicting reports about the antibacterial spectrum of polyphenols. Many researchers have reported that Gram-positive bacteria are more susceptible to the polyphenols than Gram-negative bacteria [1], but in some cases, this conclusion tends to vary and Gram-negative bacteria were found to be more vulnerable to the polyphenols [27]. In our study also, we found little difference in susceptibility of Gram-negative enteric bacteria and Gram-positive *S. aureus* (reference strain) towards 14-day Kombucha, its solvent extracts and the polyphenolic fraction. Furthermore, apart from being antimicrobial agents, the polyphenols also possess antivirulence property against pathogenesis of different microorganisms [31]. This may pave the way to investigate whether Kombucha can act as an anti-virulent agent, inhibiting the production of different virulence factors in enteric bacteria.

It can be concluded from our results that Kombucha exhibits potent bactericidal activity against the enteropathogenic bacteria due to the combined interaction of its polyphenolic content. Hence our results suggest the fact that Kombucha could be considered as an effective, cheap and easily available functional food as a whole or could be

exploited as an alternative source of various polyphenols and their derivatives for the treatment of enteric bacterial infections. Investigation of the mechanistic principle underlying the antibacterial activity of Kombucha against the enteric bacteria is also under progress. However, further work is required to capitalise on the health-promoting effects of Kombucha in animal models in order to promote it as a potential functional food with characteristic bactericidal and antivirulence properties against enteric bacterial infections in humans.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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