Research Article

Combined Antimicrobial Activity of Honey and Commercial Green Tea Extract against Some Pathogenic Bacterial Species

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ABSTRACT

Honey is one of the oldest traditional medicines considered important in the treatment of various human diseases. However, differently processed honeys exhibit different antibacterial properties. On the other hand green tea, one of the most-consumed beverages worldwide has a lot of broad spectrum of biological activities. The intention of the proposed work is to investigate the synergic antimicrobial activity of honey and green tea. Present study deals with the anitimicrobial activity of three different honeys (Natural Honey- FH, Dabour Honey-DH and Patanjali Honey-PH), green tea (commercial) and honeys with tea (mixed with various concentrations) were investigated against some enteropathogenic bacteria using agar diffusion technique. Minimum inhibitory and minimum bactericidal concentrations were also determined among the strain. Inhibition zone of mixture of honey and tea extract against all tested organism was significantly ($P \leq 0.05$) greater than honey and tea extract alone. Tea extract and Natural honey-tea mixture (FH+TE) shows higher antibacterial activity especially on *E. coli, Proteus vulgaris* and *Staphylococcus aureus* with the zones of inhibition of 20 to 24mm at the concentrations of 100 % (v/v) which is near standard antibiotic. The results of this study justify the application of honey with green tea extract in treatment of many bacterial diseases.

Keywords: Honey, Tea extract, Antibacterial activity, Zones of inhibition, Minimum Inhibitory Concentration, Minimum Bactericidal Concentration

INTRODUCTION

The utilize of conventional medicine to treat infection has been apply ever since the origin of mankind and honey is one of the oldest traditional medicines considered as traditional therapy for microbial infections[1,2]. It produced by *Appis mellifera* is a sweet food made from the synthesis of nectar from flowers, plant saps, man waste products. There are numerous reports of the antimicrobial activity of honey against a wide range of bacterial and fungal species and it was found to be effective against some clinical isolates and disease causing in man[3,4].

It's also helping in relieve of the night cough and allow proper sleep. External use of honey has been shown to be as helpful in healing wounds. The variety of honey formed by honey bees is the most well-known due to its worldwide commercial production and human consumption.

Nowadays, many people took honey for its antibacterial and anti-inflammatory properties. Holistic practitioners believe it one of nature's best versatile remedies. However, the antimicrobial activity of honey is also associated to its geographical region and flower from which the ultimate product is derived[5,6].

Products derived from plants have been used for medicinal purposes for centuries, it was reported that the antimicrobial agents are synthesized chemotherapeutic substances obtained majorly from microorganisms, plants and some animal products. Though some of these products perform less or higher than synthesized antibiotics, in some cases, they have been found safe and good source of pharmacological effect for man.

Tea has a long history as a treatment for various diseases. The most well known *Camellia sinensis* (green tea) in many parts of the world has medicinal values.

Many scientists have reported antimicrobial properties, anti-tumor, anti-inflammatory and anti-necrotic properties of tea[7,8].

The benefits of green tea are weight loss, hydrates the body, cancer prevention, serves as a stimulating drink, fights inflammation, reduce stress, boosts immunity, provides pain relief, reduces risk of heart disease, inhibits formation of blood clots, reduces risk of high blood pressure, reduces unhealthy blood sugar and prevents wrinkles[9,10].

The importance of green tea and honey cannot be over emphasized as regards their rule in health remedy.

However, differently processed honeys exhibit different antibacterial properties.

Green tea, one of the most-consumed beverages worldwide has a lot of broad spectrum of biological activities, thus this study was aimed at investigating the phytochemical analysis, antioxidant activity and antibacterial activity of three different processed honeys and green tea extract against five enteropathogenic bacteria for possible inhibition.

The minimum inhibitory concentration (MIC) of all samples against bacterial stains also was evaluated.

MATERIALS AND METHODS

Preparation of Sample

Source and Dilution of Honey

A Total of three types of honey - Natural Honey (FH), Dabour Honey (DH) and Patanjali Honey (PH) were used in this study. Natural honey was collected from gope garh forest, west Bengal, India. Commercial Dabour and Patanjali honey was purchased from supermarket, Kharagpur, West Bengal, India. Samples were filtered with a sterile mesh to remove debris. Pure honeys refer to as "neat'. It was then diluted with sterile distilled water to 6 different concentration-100%, 80%, 60%, 40%, 20%, and 10% and kept at room temperature prior (25±2°C) to use[11].

Collection and Preparation of Tea Extract

Commercial green tea Lipton® was purchased from market, Kharagpur, India. The plant material was identified by the taxonomist of the Botany Department at the Raja N. L. Khan Women's College, Midnapore. Hydroethanol extracts was prepared by addition of 100 gm of tea with a solution of hydroethanol (1:1) in a shaker at 37°C for 24 hours, then filter through Whatman No.1 filtered paper. The filtrate is concentrated to dryness by a rotary evaporator at 37°C followed by freeze drying (CSIR Protocol, 1997). Dry crude extract of tea after rotary evaporation is 6.32 gm. Out of 100 gm tea of 100 mg extract was dissolved in 1 ml sterile distilled water and it considered as 100%. Then 80%, 60%, 40%, 20%, and 10% of extracts were prepared by diluting with appropriate volume of sterile distilled water (w/v) for determination of antimicrobial activity and minimum inhibitory concentration (MIC)[12,13].

The honey-tea mixture was prepared by dissolving 1 gm of the tea extracts in 10 ml of pure honey (each honey samples) to make a concentration of 100 mg/ml and it considered as 100%. Then 80%, 60%, 40%, 20%, and 10% of samples were prepared by diluting with appropriate volume of sterile distilled water (v/v) for determination of antimicrobial activity and minimum inhibitory concentration (MIC)[11].

Preparation of Inoculums

Four gram negative [*Escherichia coli* (MTCC 40); *Proteus vulgaris* (MTCC 426); *Pseudomonas aeruginosa* (MTCC 424) *and Salmonella typhi* (MTCC 3904)] and one Gm positive [*Staphylococcus aureus* (MTCC 3160)] bacteria were collected from Microbial Type Culture Collection (MTCC), Pune, India. The microorganisms were incubated into Muller – Hinton broth for 24 hrs at 37°C. After incubation bacterial broth was kept at 4 °C for further use[14,15].

Phytochemical Screening of Tea Extracts and Honey

The preliminary phytochemical investigation was carried out for the tea extract and honey[16]. The following experimental work has been done for the determination of different phytochemicals which were present in the investigated tea and honey sample by following different methods[15,17].

Alkaloid Test

Five gm of the tea extract and 5 ml each of the honey was stirred with 5 ml of 1% aqueous HCl on a water bath at 60°C for 5 minutes.

The sample was filtered with a 3 layered muslin cloth. One ml of the filtered was treated with few drops of Draggendoff's reagent.

Blue black colour precipitation was obtained immediately that showed the presence of alkaloids.

Saponins Test

Five gm of the tea extract and 5 ml each of the honey were shaken separately with distilled water in a test tube. Frothing which persists on warming was taken as preliminary evidence of the presence of the saponins.

Tannins Test

Five gm of the tea extracts and 5 ml each of the honey was stirred separately with 100 ml distilled water and filtered. One ml ferric chloride reagent was added to the filtrate. A blue-black or blue green precipitate was an indication of the presence of tannins.

Phlobotannins Test

Five ml each of honey mixed with 5 ml of distilled water and also mixed with 1% aqueous HCL. The appearance of red colour precipitation indicates the presence of phlobotannins.

Flavonoids Test

Five ml of diluted ammonia solution was added to aqueous filtrate of the test samples followed by the addition of 1 ml concentrated H_2SO_4. A yellow coloration indicates the presence of flavonoids.

Salkoski Test

Five gm of the tea extract and 5 ml each of honey were dissolved in 20 ml of chloroform. Few drops of sulphuric acid were carefully added that allows forming a layer at the lower portion. Appearance of reddish-brown colour between the interfaces indicates the presence of steroids.

Thin Layer Chromatography Analysis for Antioxidant Constituents

About 2 µg of samples were loaded on a TLC plate (Merck, 20 cm x 20 cm). The plate was developed with methanol: chloroform: hexane (7:2:1, v/v/v) and sprayed with 0.05% DPPH reagent.

The developed plates were dried by hair drier.

The active antioxidant constituents were detected as yellow colour, produced by the reduction of DPPH in the purple background on the TLC plates. Ascorbic acid was used as standard antioxidant compound[18].

Microbiological Analysis

Determination of Antibacterial Activity of Honey and Tea Extracts

The antibacterial test of the extract and honeys were tested on the test strains using the agar-gel diffusion inhibition test. For each inoculums 0.5 McFarland standard was prepared by the method of Cooper and the turbidity adjusted to 1.5×10^8 CFU/ml (corresponding to 0.5 McFarland standards).

In the agar gel diffusion test 0.1 ml of 0.5 McFarland standards of the bacterial test organisms was aseptically introduced and evenly spread using sterile 'L' rod on the surface of sterile Mueller Hinton agar[19].

Using a sterile cork borer (6 mm diameter, 4 mm deep) wells were made in the agar medium between peripheral wells and the edge of the petri dish allowing at least 30 mm. Fixed volumes (0.1 ml) of the different concentration (100%, 80%, 60%, 40%, 20% and 10%) of honeys, tea extract and honey-tea mixture were then introduced into the wells in all plates with appropriately distinguished codes (Natural Honey-FH, Dabour Honey-DH, Patanjali Honey-PH, tea extract-TE). A control well was made in the plate with the diluting solvent. The plates were incubated at 37 ^{o}C for 24 hours. Inhibitions indicated by clear halo around the wells were measured[20].

Determination of MIC

The MIC of samples was determined by broth dilution method. Five ml of sterile nutrient broth was taken in sterile test tubes and to this a loopful of test bacterial strains was inoculated. The samples were added to each test tube in increasing concentration (2.5%, 5%, 10%, 20% and 40%). The contents of the tubes are subjected to gentle shaking for proper mixing and incubated at 37˚C for 24 h. A control tube was kept without the test organism. Same test was carried out with a selective standard antibiotic. After 24 hrs incubation the OD values are recorded at 540 nm. The least concentration of the growth causing complete inhibition of the growth was taken as MIC[20].

Determination of MBC

Dilution showing no visible growth for the MIC was subculture into a fresh MH agar plate and incubated at 37 °C for 24 h. The lowest concentration of the samples yielding no growth on MH plate was recorded as MBC.

Data Analysis

The experiment was carried out in triplicates, the diameter zones of inhibition were measure average value of three replicates and standard error (±). Result were subjected to Microsoft excel 2007. $p<0.05$ was statistically significant.

RESULTS AND DISCUSSION

Various honey samples (Natural Honey- FH, Dabour Honey-DH and Patanjali Honey-PH) and green tea extract collected were tested for their phytochemical analysis and antibacterial activity using the five bacterial strains. The minimum inhibitory concentration (MIC) of all samples against five bacterial stains also was evaluated. The phytochemical tests carried out and result showed that honey identified positive test for saponin and flavonoid and tea extract identified positive for the entire said test (Table1). The antioxidant property of TE and FH in terms of DPPH free radical scavenging activity showed TLC band with strong antioxidant activity and another PH and DH with weak antioxidant activity as compared to standard antioxidant compound ascorbic acid (Figure 1).

Table 1: Phytochemical Screening of Honey and Tea Extract

Phytochemical	Inference	
	Honey	Tea Extract
Alkaloid	-	+
Saponin	+	+
Tannin	-	+
Phlobertannin	-	+
Flavonoid	+	+
Salkwoski's Test	-	+

'+' positive; '-'negative

Figure 1: TLC antioxidant activity analysis of *tea extract – TE, Natural Honey- FH, Patanjali Honey-PH and Dabour Honey-DH,* Standard- A: Ascorbic acid

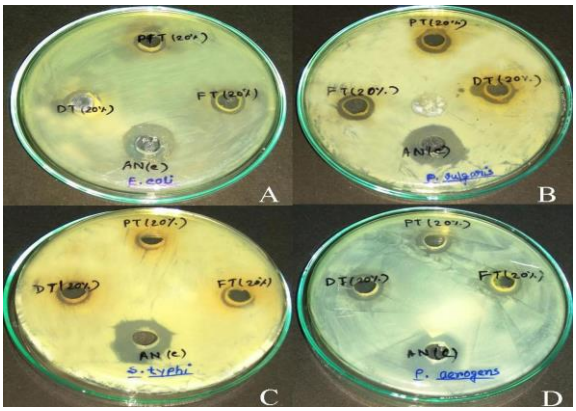

Figure 2: Zone of inhibition against pathogenic bacteria of tea-honey mixture at 20%.

AN: Chloramphenicol (Std), FT: Natural honey + tea, PT: *Patanjali Honey + tea, DT: Dabour Honey + tea.*

Zone of Inhibition of mixture of honeys + tea extract, tea extract and honeys alone against pathogenic bacteria is presented in Table 2. The entire samples have some antimicrobial activity but green tea extract is better in comparism with honey sample and it was more active against *P. vulgaris* and *S. typhi* (≈24 mm and ≈18 mm respectively) and showed lowest activity against *E. coli* and *P. aeruginosa* (≈14 mm).

Among the tested bacteria, *Escherichia coli* was inhibited mostly with the forest honey (21 mm), *S. typhi* was most inhibited with the mixture of forest honey and tea extract (18 mm). Maximum inhibitory activity against *E. coli, P. vulgaris, P. aeruginosa, S. typhi and S. aureus was showed by FH, TE, FH+TE, TE and FH+TE respectively* (≈22 mm, 24 mm, 15 mm, 19 mm and 23 mm).

Some of the standard antibiotics gentamycin, Chloramphenicol and Norfoxacilin as positive control was used in this study. All the test organisms were susceptible to gentamycin with zones of inhibition between 14-23 mm, Chloramphenicol gives 10-29 mm and Norfoxacilin showed 17-24 mm zone of inhibition (Table 3).

As samples were the reasonably effective, the MIC and MBC against the tested organisms were also determined (Table 4). The MIC and MBC values varied from 5 to 20% and 10 to 40% respectively.

Patanjali honey-tea extract (PE-TE) showed lowest MIC value, 5% (mg/ml) for Pseudomonas aeruginos while it was 10 mg/ml for others four organism.

Forest honey with tea extract (FH-TE) showed good MIC value for both gram positive and gram negative bacteria (20% for *P. vulgaris, P. aeruginosa and S. aureus*). MIC of tea extract against *Staphylococcus aureus* is 5 mg/ml (Table 4).

From this study it was showed that all the honey samples and tea extract showed reasonable antibacterial activities on selected pathogenic bacteria.

Though majority of the test organisms were Gram negative bacteria, the Gram positive bacteria were both inclusive in valuable inhibitions with the natural honey and tea extracts.

From the earlier study it was state that honey has three antibacterial properties; like osmotic effect, acidity and hydrogen peroxide[17,21].

The Antimicrobial action of honey depends on the presence of hydrogen peroxide which is produced enzymatically in honey[22].

The glucose oxidase secreted from the hypopharyngeal gland of the bee into the nectar to help the formation of honey from the nectar.

The hydrogen peroxide and acidity formed by two reactions (glucose and oxygen chemically react to produce gluconic acid) has antimicrobial possible[22].

The property made the honey as antimicrobial agent is the osmolarity effect that inhibits bacterial growth.

The high sugar concentration ties-up water molecules, so that bacteria would have lacking water to support their growth[23].

Thereby, the inhibitory action caused by the osmotic effect of honey dilutions clearly depends on the species of bacteria.

In this study various phytochemicals were identified in Table 1. Results of phytochemicals screening were attributed to the effects of present compounds which can play to mitigate different diseases by different mechanisms.

In this study the assumption is that the green tea honey mixture consists of a mixture of active components with other constituents with minor activity that achieves a synergistic effect.

However, this supposition should be confirmed by further analysis. It's also containing saponins and flavonoids which have high antioxidant property to help the reduction of risk of some cancers and heart disease[24].

The positive results provide a scientific indication for the claimed ethenomedical in the treatment of various diseases.

Table 2: Antibacterial Susceptibility of the Five Bacterial Pathogens on Various Honey Samples and with Tea Extract

Organisms Different	Name of Samples**	Diameter zone of inhibition (mm)* produced by Concentration of Samples % (v/v)					
		10	20	40	60	80	100
Escherichia coli	FH	8.3 ± 0.33a	10.6 ± 0.60a	13 ± 0.57a	17.16 ± 0.44a	19.16 ± 0.72a	21.83 ± 0.8a
	DH	8.83 ± 0.16a	9.5 ± 0.28a	10.83 ± 0.44b	11.5 ± 0.76b	13.83 ± 0.44b	14.96 ± 0.26b
	PH	8.3 ± 0.33a	8.83 ± 0.60a	10 ± 0.57b	11.93 ± 0.29b	12.83 ± 0.60b	16.4 ± 0.20c
	TE	9 ± 0.28a	9.16 ± 0.16a	11.26 ± 0.39b	11.63 ± 0.29b	14.4 ± 0.01b	14.66 ± 0.35b
	FH+TE	12.43 ± 0.23b	13.53 ± 0.29b	18.36 ± 0.08c	19.83 ± 0.44c	20.63 ± 0.18a	20.73 ± 0.37a
	DH+TE	9.16 ± 0.16a	12.93 ± 0.56b	14.23 ± 0.14d	14.36 ± 0.13d	15.33 ± 0.35c	15.46 ± 0.24b
	PH+TE	9.33 ± 0.16a	14.6 ± 0.05c	14.23 ± 0.14d	17.5 ± 0.28a	17.6 ± 0.30d	20.16 ± 0.44a
Proteus vulgaris	FH	8.6 ± 0.33a	10.3 ± 0.88a	10.3 ± 0.8a	10.6 ± 0.89a	11.6 ± 1.45a	13.6 ± 0.88a
	DH	7.5 ± 0.28a	8 ± 0.57b	9.1 ± 0.16a	11.6 ± 1.45a	12.3 ± 1.76a	13 ± 2.08a
	PH	6.2 ± 0.26b	7.8 ± 0.2b	8.6 ± 0.6b	10.3 ± 0.89a	11.3 ± 1.45a	11.8 ± 1.01b
	TE	12 ± 1.15c	16.5 ± 0.28c	17.1 ± 0.59c	20.6 ± 0.8b	23.1 ± 0.44b	24 ± 0.57c
	FH+TE	9.1 ± 0.17a	12.2 ± 0.54d	15.5 ± 0.29d	17.4 ± 0.36c	18.1 ± 0.13c	19.3 ± 0.15d
	DH+TE	8.6 ± 0.17a	11.5 ± 0.23d	13.2 ± 0.12e	13.5 ± 0.28d	16.6 ± 0.2d	16.6 ± 0.18e
	PH+TE	12.7 ± 0.15c	16.8 ± 0.11c	16.9 ± 0.58c	18.3 ± 0.15c	20.6 ± 0.2e	21.3 ± 0.18f
Pseudomonas aeruginosa	FH	R	R	R	6.2 ± 0.27e	7.5 ± 0.29a	10.5 ± 0.5a
	DH	R	R	R	R	6.1 ± 0.16a	7.4 ± 0.24b
	PH	R	R	6.8 ± 0.84a	7.6 ± 0.70a	8.0 ± 0.06b	8.4 ± 0.24b
	TE	6.6 ± 0.30a	7.4 ± 0.29a	7.4 ± 0.24a	8 ± 0.06a	11.4 ± 0.29c	13.2 ± 0.61c
	FH+TE	6.4 ± 0.03a	8.63 ± 0.14b	10.83 ± 0.6b	12.6 ± 0.6b	13.3 ± 0.8d	14.5 ± 0.74c
	DH+TE	6 ± 0.03a	8.16 ± 0.16b	8.8 ± 0.08c	10.2 ± 0.41c	11.3 ± 0.17c	13.2 ± 0.11c
	PH+TE	6.3 ± 0.15a	6.2 ± 0.11a	9.1 ± 0.43c	10.3 ± 0.31c	12.4 ± 0.30d	13.3 ± 0.17c
Salmonella typhi	FH	6.36 ± 0.08a	7.6 ± 0.34a	9.16 ± 0.44a	10.66 ± 0.89a	12.3 ± 1.76a	12.66 ± 0.67a
	DH	R	R	7.33 ± 0.33b	10 ± 0.57a	10.6 ± 0.8b	12.8 ± 0.92a
	PH	R	7.2 ± 0.41a	9.16 ± 0.45a	9.8 ± 0.83a	10.1 ± 0.94b	10.83 ± 0.44b
	TE	10.8 ± 0.41b	11.16 ± 0.92b	16.6 ± 0.8c	17.2 ± 0.13b	17.3 ± 0.15c	18.6 ± 0.2c
	FH+TE	8.2 ± 0.11c	8.26 ± 0.13a	13.2 ± 0.41d	14.86 ± 0.13c	17.5 ± 0.28c	18.13 ± 0.13c

	DH+TE	7.16 ± 0.6a	7.23 ± 0.31a	10.8 ± 0.6a	11.93 ± 1.03a	14.36 ± 0.06d	15.9 ± 0.46d
	PH+TE	6.13 ± 0.13a	9.13 ± 0.46c	10.5 ± 0.28a	12.13 ± 0.13a	12.43 ± 0.80a	13.2 ± 0.11a
Staphylococcus aureus	FH	8 ± 0.57a	10 ± 1.15a	12.16 ± 0.44a	12.66 ± 0.66a	15.5 ± 0.5a	16.83 ± 0.60a
	DH	R	R	6.3 ± 0.15b	7.0 ± 0.17b	8.5 ± 0.28b	9.8 ± 0.41b
	PH	8.1 ± 0.16a	8.4 ± 0.74b	10.3 ± 0.88a	11.3 ± 1.2a	13.66 ± 0.88c	14.16 ± 0.72c
	TE	11.5 ± 0.76b	14.2 ± 0.15c	16.6 ± 0.72c	16.93 ± 1.03c	17.9 ± 0.45d	20.06 ± 0.56d
	FH+TE	13.1 ± 0.59c	18.3 ± 1.2d	19.66 ± 0.8d	21.5 ± 0.86d	22.26 ± 0.81e	22.73 ± 0.81e
	DH+TE	9.1 ± 0.16a	12.8 ± 0.63e	13.8 ± 0.30a	13.8 ± 0.6a	15.3 ± 0.35a	15.8 ± 0.11a
	PH+TE	8.83 ± 0.44a	11.8 ± 0.61e	14.3 ± 0.24a	17.5 ± 0.28c	17.63 ± 0.27d	19.16 ± 0.6d

**mm-millimeter; **Natural Honey- FH, Dabour Honey-DH, Patanjali Honey-PH and tea extract- TE.; R=Resistant (no Zone of Inhibition).; ***Zone of inhibition was expressed as mean ± SE. Letters (a, b, c, d, e, f) in a specific vertical Column are differ from each other significantly (p<0.05).*

Table 3: Antibacterial Activity of Standard Antibiotic Disc

Test organisms	Diameter zone of inhibition (mm)*		
	Gentamycin	Chloramphenicol	Norfloxacin
Gram Negative Bacteria			
Escherichia coli	17.9 ± 0.20a	10.3 ± 0.34a	18.7 ± 0.26a
Proteus vulgaris	15.3 ± 0.8b	17.1 ± 0.60b	20.3 ± 0.88b
Pseudomonas aeruginosa	18.5 ± 0.28a	13.8 ± 0.60c	23.6 ± 0.6c
Salmonella typhi	17.9 ± 0.26a	15.8 ± 0.41d	22.3 ± 0.3c
Gram Positive Bacteria			
Staphylococcus aureus	22.6 ± 1.45c	28.1 ± 0.44e	17.1 ± 0.16a

*mm-millimeter; Zone of inhibition was expressed as mean ± SE. Letters (a, b, c, d, e) in a specific vertical Colum are differ from each other significantly (p<0.05).

Table 4: MIC & MBC Determination of Different Concentrations of Honey, Tea Extract and Mixture of Honey-Tea Extract.

Test Organisms	MIC and MBC (% Of Dilution)													
	FH		DH		PH		TE		FH+TE		DH+TE		PH+TE	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	20	20	20	40	10	20	20	20	20	20	20	40	10	20
P. vulgaris	20	20	20	20	20	20	10	20	10	20	20	40	10	20
P. aeruginosa	10	20	10	20	10	20	10	10	10	10	10	20	05	20
S. typhi	20	40	20	20	20	40	20	20	20	20	10	20	10	10
S. aureus	10	10	20	10	20	20	05	10	10	20	20	40	10	20

*Natural Honey- FH, Dabour Honey-DH, Patanjali Honey-PH and tea extract- TE.

CONCLUSION

It is hoped that this may help to avoid the side effects of antibiotics. In future, the combined use of honey and tea could be also useful in fighting emerging drug-resistant problem especially among enteropathogens.

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