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INCIDENCE OF BACTERIA AND ANTIBACTERIAL ACTIVITY OF SELECTED TYPES OF TEA

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ABSTRACT

The purpose of this study was to determine *in vitro* antibacterial activity of selected teas (Assam: Indian black tea from *Camellia sinensis*, Pu-erh: darkpu-erh (shu) from *Camellia sinensis*, Sencha: Japanese green tea from *Camellia sinensis*) against five species of pathogenic microorganisms. In our study, we determined the total viable count (TVC), yeasts (Y) and *Enterobacteriaceae* counts (E). MALDI-TOF MS Biotyper was used for identification of colonies after cultivation. Evaluation of the antimicrobial activity was performed by disc diffusion method, well diffusion method and detection of minimum inhibitory concentration (MIC). For antibacterial activity against *Escherichia coli* CCM 2024, *Yersinia enterocolitica* CCM 5671, *Klebsiella pneumonie* CCM 2318, *Staphylococus aureus* CCM 2461 and *Bacillus thurigiensis* CCM19 were detected. The inhibition zones were measured in mm in disc diffusion method and well diffusion method. The MIC of the individual extracts was measured spectrophotometrically. The high number of total viable count was found in Pu-erh tea (2.1 log CFU.g^{-1}) and lowest number was found in Assam tea (0.7 log CFU.g^{-1}). The high number of *Enterobacteriacea* was found in Pu-erh tea (2.03 log CFU.g^{-1}) and lowest in Assam tea (0 log CFU.g^{-1}). The higher number of yeasts was found in Pu-erh tea (1.83 log CFU.g^{-1}) and lowest in Assam tea (0.3 log CFU.g^{-1}). Mass spectrometry revealed the presence of seven Gram positive bacteria *Bacillus cereus, B. mycoides, B. pumilus, Enterococcus durans, Staphylococcus epidermis, S. hominis, S. warneri*, four Gram negative bacteria *Acinetobacter junii, Hafnia alvei, Klebsiella pneumoniae, Sphingomonas* spp. and two yeast - *Candida glabrata, Cryptococcus albidus*. The results show that certain tea extracts are particularly active against various pathogenic bacteria. Tea extracts (Sencha, Rooibos, Mate, Assam) were found to have the strongest antibacterial activity against *Staphylococcus aureus* CCM 2461.

Keywords: bacteria; antibacterial activity; MALDI TOF MS Biotyper; tea

INTRODUCTION

Tea is a popular beverage due to its inherent liquor and flavour characteristics. Two major types of tea available in the market are green tea and black tea. Linnaeus first classified the tea plant as Thea sinensis and later named as (*Camellia sinensis* (L) O. Kuntze). The native place of tea plants is claimed as the area touching Nagaland, Manipur along Assam and Burma frontier in the west, even though China in the east touching southwardly through the hills of Burma, Thailand and Vietnam. India is the second largest producer of black CTC tea with a production of 1135.07 million kilograms in the year 2012. It supplies approximately 26% of global back tea demand. Moreover, Assam tea estates manufacture more than 50% of all Indian black tea production. Taste of the tea liquor and appearance of the made tea are two major characteristics quality parameters of black tea **(Dutta and Baruah, 2014)**.

Pu-erh black tea, which is subjected to a long-time of secondary oxidization and fermentation (post-fermented), is defined as a new type of tea in recent years **(Liang et al., 2005)**. Pu-erh black tea, originally produced in the Yunnan province of China, is used as a health beverage to prevent a variety of diseases. First parching crude green tea leaves (*Camellia sinensis* var. *assamica* (L.) obtain Pu-erh black tea O. Kuntze; *Theaceae*) and then undergoes secondary fermentation with microorganisms such as *Aspergillus niger* (postfermented). During the fermentation process, catechins are oxidized into quinone by polyphenol oxidase and then condensed to form bisflavanol, theaflavin, thearubigen, and other high molecular components **(Wang et al., 2010)**. These are regarded as the biologically important active components of Pu-erh black tea which may be responsible for its acclaimed health benefits. Examples of such health benefits include hypocholesterolemia, anti-obesity **(Fujita and Yamagami, 2008a, 2008b)**, anti-atherosclerosis **(Hou et al., 2009)** and anti-mutagenicity **(Wang et al., 2011a,b)**. It is generally believed that the popularity of Pu-erh black tea is linked to its long history of use, especially in Asia, and its health benefits.

Tea quality is important for its market value and is defined by colour, freshness, strength, and aroma. To date, approximately 600 volatiles have been described in black

tea, with fewer numbers in oolong and green tea, due to the lesser degree of fermentation when producing these teas, and thereby tea quality influences a certain market percentage. Fresh tea leaves of *Camellia sinensis* are steamed immediately after plucking to produce Japanese green tea (Sencha). Endogenous enzymes involved in aroma formation are inactivated by the steam treatment, producing low aroma contents. The commercial value of Sencha is mainly evaluated by umami (taste) and fresh green odor, whereas flowery and fruityodor is essential for black tea or semi-fermented tea (oolong tea). The volatile compounds in green tea, black tea and semifermented tea have been intensively analysed **(Katsuno et al., 2014)**. The potent odorants of Japanese and Chinese green teas and black tea have been investigated based on aroma extract dilution analysis (AEDA) **(Kumazawa and Masuda, 2002; Kumazawa et al., 2006)**. To enrich green tea with more aroma attributes, a selection of raw materials (tea cultivars) can be employed. Amongst several types of Sencha aroma-rich green teas, made from *C. sinensis* cultivars, Kohshun and Shizu 7132 have a sweet odor **(Yang et al., 2009)**, and are becoming popular in Japan.

Despite the significant role of herbal teas in improving nutrition and health, there have been reports of microbial contamination and adverse effects resulting from their consumption. These include neurological, cardiovascular and hematological hazards **(Palmer et al., 2003)**. Toxin-producing microbial contaminants are often the cause of these adverse effects. Therefore, it is important to identify the microbial contaminants of herbal tea products as indicators of safety and quality **(Schweiggert et al., 2005)**. A few reports demonstrating microbial contamination of medicinal herbs from various parts of the world exist in the literature. **Rizzo et al., (2004)** indicated that medicinal plants in Argentina harbored toxigenic fungi such as *A. flavus*, *A. parasiticus* and several members of the Genus *Fusarium*.

In recent years, much attention has been focused on the role of tea flavonoids in the promotion of health, especially of catechins **(Ivanišová et al. 2013; Ivanišová et al., 2015 a,b)**. In plants, these metabolites are involved in their protection against several pathogens including insects, bacteria, fungi, and viruses. In the human organism, these polyphenols may exert health promoting properties, mainly antioxidant, anticancer, anti-inflammatory, antidiabetic and antimicrobial activities **(Dias et al., 2013; Sharangi, 2009; Silva, 2012; Wheeler, 2004)**.

The purpose of this study was to determine *in vitro* the antibacterial activity of selected teas (Assam: Indian black tea from*Camellia sinensis*, Pu-erh: dark pu-erh (shu) from*Camellia sinensis*, Sencha: Japanese green tea from *Camellia sinensis*) against five species of pathogenic microorganisms. In our study, we determined the total viable count, number of yeasts and number of*Enterobacteriaceae* genera.

MATERIAL AND METHODOLOGY

For microbial analysis and antimicrobial activity threeselected teas (Assam: Indian black tea from*Camellia sinensis*, Pu-erh: dark pu-erh (shu) from*Camellia sinensis*, Sencha: Japanese green tea from *Camellia sinensis*) were used.

Microbiological analysis

Five grams of the tea was sampled using sterile scalpels and forceps, immediately transferred into a sterile stomacher bag, containing 45 mL of 0.1% peptone water (pH 7.0), and homogenized for 60 s in a Stomacher at room temperature. Microbiological analyses were conducted by using standard microbiological methods. Total viable count (TVC) were determined using Plate Count Agar (PCA, Oxoid, UK) after incubation for 2 days at 35°C. For *Enterobacteriaceae*, Violet red bile glucose agar (VRBL, Oxoid, UK) were inoculated with sample suspension and incubated at 37°C for 24 h. Number of yeasts (Y) were determined using Tryptic Glucose Yeast agar (TGYA, Oxoid, UK). Inoculated plates were incubated for 5 days at 25°C. All plates were examined for typical colony types and morphology characteristics associated after the incubation.

We used MALDI-TOF Mass Spectrometer (Bruker Daltonics, Germany) for identification of bacteria and yeasts isolated from tea samples. After incubation of yeasts at 25°C for 5 days, isolated colonies were picked and suspended in 300 µL of sterile distilled water and mixed thoroughly. 900 µL of absolute ethanol was added. The mixture was centrifuged at 13 000 × g for 2 min. After the supernatant was discarded, the pellet was centrifuged again. Residual ethanol was completely removed by pipetting and the pellet was allowed to dry at room temperature. Subsequently 10 µL of formic acid (70%) was added and mixed with the pellet with a sterile toothpick. Next, 10 µL of acetonitrile (100%) was added and mixed thoroughly. The solution was centrifuged at maximum speed for 2 minutes again, and 1 µL of the supernatant was spotted on a polished MALDI target plate (Bruker Daltonics, Germany). Immediately after drying 1 µL of the matrix solution was added to each spot and allowed to air dry. The matrix used was a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) (Bruker Daltonics, Germany) dissolved in 50% acetonitrile with 0.025% trifluoroacetic acid (TFA). The matrix solution preparation (2.5 mg of HCCA) contains 500 µL of acetonitrile, 475 µL of ultra-pure water and 25 µL of trifluoroacetic acid. Next added 250 µL of this solution to the 2.5 mg of HCCA. Samples were then processed in the MALDI-TOF MS (Microflex LT/SH, Bruker Daltonics, Germany) with flex Control software and results obtained with Real-time Classification software (RTC) by used database "Taxonomy" (Bruker Daltonics, Germany).

Antimicrobial activity

The dry materials were crushed, weighed out to 10 g and soaked separately in 100 mL of ethanol p.a. (96%, Sigma, Germany) during two weeks at room temperature in the dark. Exposure to sunlight was avoided to prevent the degradation of active components. Then, ethanolic tea extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove the ethanol (Stuart RE300DB rotary evaporator, Bibby scientific limited, UK, and vacuum pump KNF N838.1.2KT.45.18, KNF, Germany).

For antibacterial activity, bacteria *Escherichia coli* CCM 2024, *Yersinia enterocolitica* CCM 5671, *Klebsiella pneumonie* CCM 2318, *Staphylococus aureus* CCM 2461

and *Bacillus thurigiensis* CCM19 were used. Bacteria were collected from Czech Collection of Microorganisms. The bacterial cultures were cultivated in Muller Hinton broth (Imuna, Slovakia) at 37 °C.

Antimicrobial activity of tea extract was determined using a disc diffusion method and well diffusion method. The MIC of the individual extracts was measured spectrophotometrically. Briefly, a 100 μL of the test bacteria were grown in 10 ml of fresh media until they reached a count of approximately of 10^5 cells.mL^{-1}. An amount of 100 μL of the microbial suspension was spread onto Mueller Hinton agars. Each antimicrobial assay was performed in at least triplicate.

RESULTS AND DISCUSSION

Green tea is produced from tea leaves that have not undergone the process of fermentation. Until recently, the world trade in tea focused almost exclusively on black tea **(Ošťádalová et al., 2014)**.

The number of microorganisms identified in tea is shown in Figure 1. The highest number of total viable count was found in Pu-erh tea (2.1 log CFU.g^{-1}) and the lowest in Assam tea (0.7 log CFU.g^{-1}). The high number of *Enterobacteriacea* was found in Pu-erh tea (2.03 log CFU.g^{-1}) and the lowest in Assam tea (0 log CFU.g^{-1}). The higher number of yeasts was found in Pu-erh tea (1.83 log CFU.g^{-1}), while the in Assam tea (0.3 log CFU.g^{-1}). Mass spectrometry revealed the presence of seven Gram positive bacteria *Bacillus cereus, B. mycoides, B. pumilus, Enterococcus durans, Staphylococcus epidermis, S. hominis, S. warneri*, four Gram negative bacteria *Acinetobacter junii, Hafnia alvei, Klebsiella pneumoniae, Sphingomonas* spp. and two yeast *Candida glabrata, Cryptococcus albidus*.

Microflora of tea was studied before. The microorganisms identified in tea wer *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis, Pseudomonas flourescens, Pseudomonas aeruginosa, Klebsiella pneumoniae, Serratia marcencens, Salmonella typhimurium* and *Escherichia coli*, respectively. Fungal isolates were identified as *Aspergillus niger, Aspergillus flavus, Penicillium expansum, Rhizopus stolonifer*, and *Fusarium solanii*, respectively **(Omogbal and Ikenebomeh, 2013)**. This finding indicates that tea could be contaminated with large numbers of different microorganisms.

Mbata et al. 2005 reported that daily consumption of green tea can kill gram positive *Staphylococcus aureus* and other harmful bacteria. Also it has been reported that the green tea contain catechin and polyphenols. These compounds have been found to possess antibacterial and antiviral action as well as anticarcinogenic and antimutagenic properties. This suggests that these compounds could be responsible for the inhibitory of *L. monocytogenes*.

The crude ethanolic extract of white tea exhibited moderate antimicrobial activity against *Sigella sonnei* (11.0 mm), *Pseudomonas aeroginosa* (10.0 mm), *Escherichia coli* (9.0 mm) and *Bacillus cereus* (8.0 mm) at 500 μg.disc^{-1} **(Ur Rashid et al., 2013)**.

The antibacterial activity detected with disc diffusion method is shown in Figure 2. The best antibacterial activity against the tested bacteria with disc diffusion method was found against *Stapylococcus aureus* for Sencha tea (5 mm). The highest antibacterial activity against *Escherichia coli* with disc diffusion method was found for Assam tea (1 mm). The highest antibacterial activity against *Yersinia enterocolitica* was found for Assam and Pu-erh tea (2 mm). The higher antibacterial activity against *Klebsiela pneumonie* was found for Assam and Sencha tea (1 mm). The higher antibacterial activity against *Bacillus thurigiensis* was found for Pu-erh tea (2 mm).

The results of the study showed that the tea extract of *Camellia sinensis* indicates the presence of potent antibacterial activity, which confirms its use against the pathogenic microorganisms. The assessment of antimicrobial activity was based on measurement of inhibition zones formed around the discs. Disk diffusion method did not produce recordable results for all the three type of tea leaves against the pathogens. Among these the methanolic extract of fresh green tea exhibited greater antimicrobial activity. The methanol extracts of the test plant produced larger zones of inhibition against the bacteria. These observations may be attributed to green tea catechin compounds and polyphenols. These compounds have been found to possess antibacterial action **(Saikia et al., 2006)**.

The microorganisms which were found to be sensitive to fresh green tea extracts were *E. coli, Enterococcus faecalis, Staphylococcus aureus, Candida albicans* and *Pseudomonas aeruginosa* **(Archana and Abraham, 2011)**.

It has been documented that green tea contains catechin and polyphenols which are highly sensitive to the oxidation process. The catechin and polyphenols have been found to possess antibacterial and antiviral action as well as anticarcinogenic and antimutagenic properties. These compounds could be responsible for the inhibition of pathogens. The antibacterial effects of tea polyphenols (TPP) extracted from Korean green tea (*Camellia sinensis*) against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) were evaluated in the previous study. The earlier works by **Mabe et al. (1999)** showed that tea catechins have an antibacterial effect against *H. pylori* and may have a therapeutic effect against gastric mucosal injury induced by this organism.

The antibacterial activity with well diffusion method is shown in Figure 3. The best antibacterial activity against tested bacteria with well diffusion method was found against *Bacillus thurigiensis* for Sencha tea.

The agar-well diffusion method was used for the concentrations of 100, 200, 300, and 400 mg.mL^{-1}, respectively. Results showed that the minimum inhibitory concentration of tea alcohol extract was 400 mg.mL^{-1} with inhibition zone of 20 mm. The extract decreased the bacterial viable count since it showed a visible decrease to $<5\times10^6$ CFU.mL^{-1} after 24 hours of incubation. Black tea extract also had the ability to completely inhibit *Pseudomonas* growth on blood agar and inhibited protease activity and adhesion **(Flayyih et al. 2013)**.

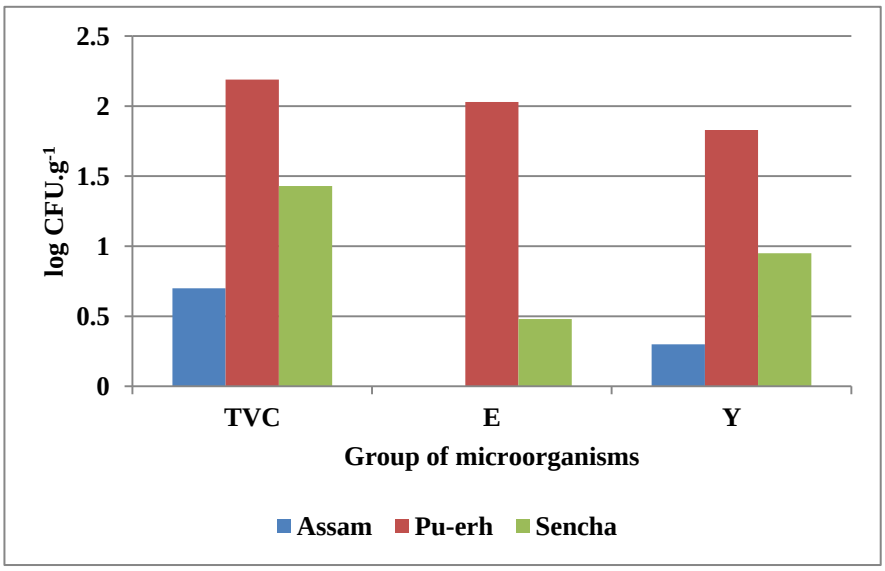

Figure 1 The number of microorganisms in log CFU.g^{-1} in tea samples.

NOTE: TVC - total viable count, E - *Enterocacteriaceae*,Y - yeasts.

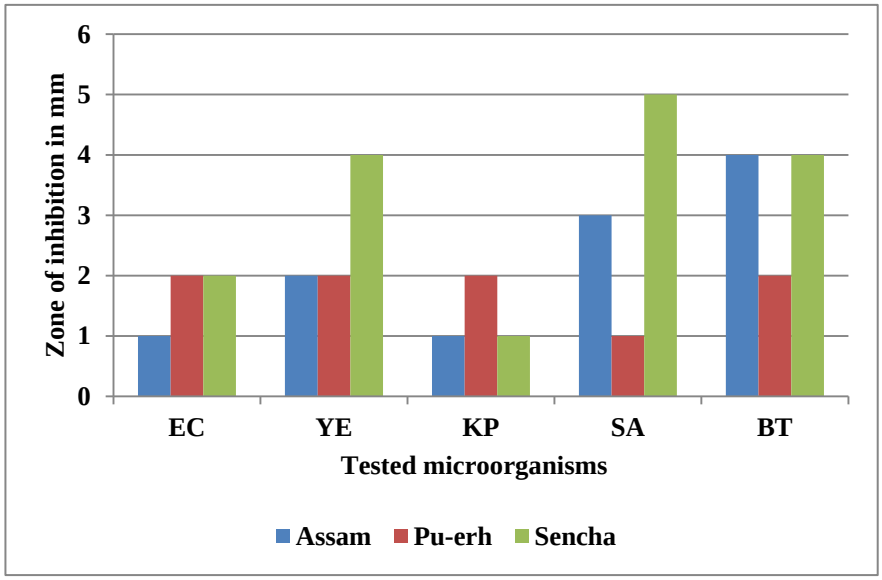

Figure 2 Antimicrobial activity of selected tea against bacteria by disc diffusion method.

NOTE: EC - *Escherichia coli*, YE - *Yersinia enterocolitica*, KP – *Klebsiella pneumonie*, SA – *Staphylococus aureus*, BT - *Bacillus thurigiensis.*

In the study of **Radji et al. 2013** the MIC of green tea extract against MRSA was 400 µg.mL^{-1}, while the MIC for MDR– *P. Aeruginosa* was 800 µg.mL^{-1}. The anti-bacterial activity of green tea extract is comparable to standard antibiotic. The activity of 16 µg of green tea extract against the laboratory strain of *S. Aureus* ATCC 25923 was comparable to that of commercially available oxacillin (1 µg), whereas the activity of 16 µg green tea extract was comparable to that of commercially available gentamicin (10 µg) against the laboratory strain *P. Aeruginosa* ATCC 27853, even though green tea extract was slightly less effective. Green tea extract showed good antimicrobial activity against MRSA and MDR – *P. aeruginosa*, although both of these bacteria have been resistant to multiple classes of antibiotics.

The polyphenol contents of green tea have been reported to inhibit the varieties of pathogenic bacterial growth such as *Helicobacter pylori*, methicillin-resistant *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Salmonella typhi*, *Shigella dysenteriae*, *Shigella flexneri* and *Vibrio cholera*. Polyphenols in green tea were also found to be effective against human immunodeficiency virus, hepatitis, and influenza viruses. Dental caries and periodontal diseases are two the most prevalent plaques associated with oral infectious diseases produced by endogenous oral flora. *S. mutans* and *S. sobrinus* are known as the main etiological

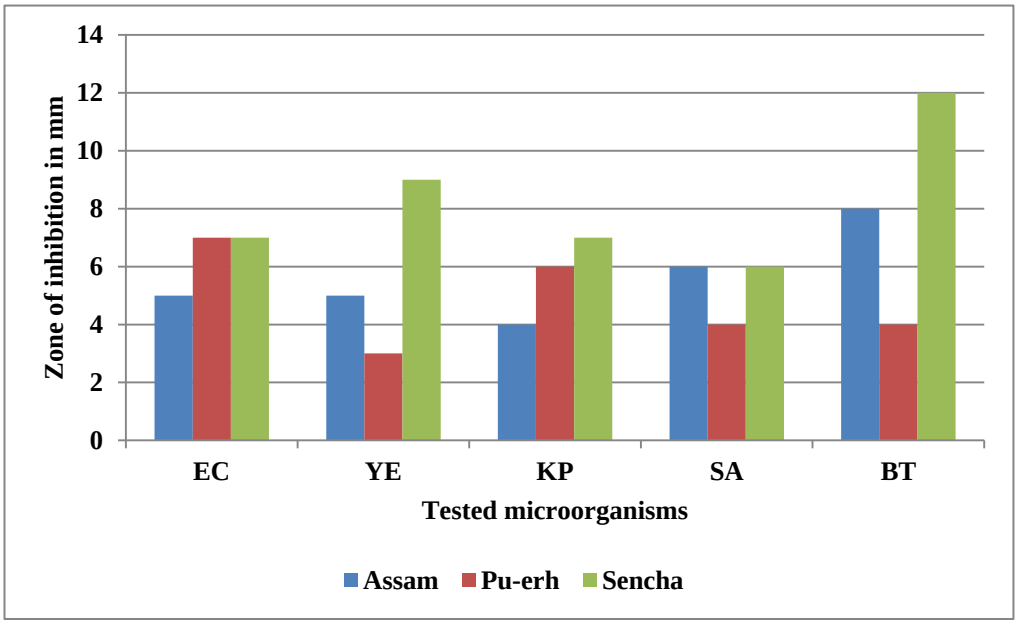

Figure 3 Antimicrobial activity of selected tea against bacteria in mm.

NOTE: *EC - Escherichia coli, YE - Yersinia enterocolitica, KP - Klebsiella pneumonie, SA - Staphylococcus aureus, BT - Bacillus thurigiensis.*

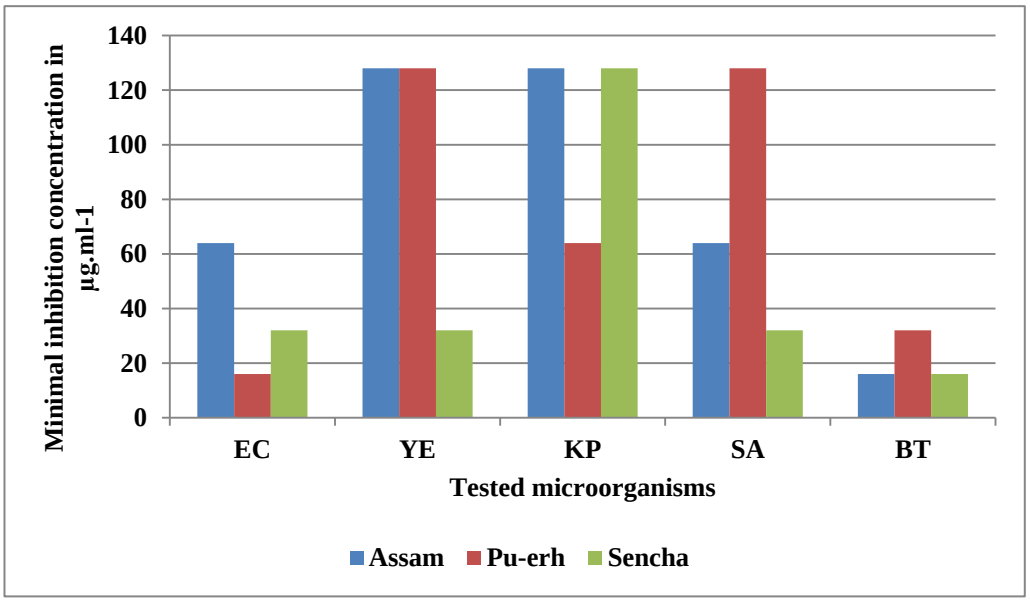

Figure 4 Minimal inhibition concentration of selected tea against bacteria in µg.mL^{-1}

NOTE: *EC - Escherichia coli, YE - Yersinia enterocolitica, KP - Klebsiella pneumonie, SA - Staphylococcus aureus, BT - Bacillus thurigiensis.*

agents of dental caries. These cariogenic bacteria adhere to the tooth surface and produce a sticky glycocalyx film composed of glucan resulting from the action of glucosyltransferase on dietary sucrose. Accumulation of bacteria causes dental plaque formation within which there is continuing acid production by the bacterial plaque **(Araghizadehet al., 2013)**. Tea extract exhibited the inhibitory effect also to those microorganisms.

The minimum inhibitory concentration (MIC) of the ethanol extract is shown in Figure 4. The lowest antibacterial activity was typical for Pu-erh against *Escherichia coli*.

CONCLUSION

The mass spectrometry for identification of tea microflora was used and altogether the presence of seven Gram-positive and four Gram-negative bacteria species were revealed. Tea extracts exhibited the antimicrobial activity, thus have a potential antimicrobial activity against microorganisms even agains the pathogens.

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