



# Evaluation of phytochemical, antimicrobial and time-killing assay of *Camellia* species

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Received: 27 February 2020 / Revised: 21 July 2020 / Accepted: 2 August 2020 / Published online: 1 September 2020  
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## Abstract

Tea (*Camellia sinensis* and *C. assamica*) possess antimicrobial property due to the presence of bioactive compounds. Qualitative analysis of phytochemicals of both tea species such as alkaloids, flavonoids, carbohydrates, saponins, tannins, steroids, terpenoids and cardiac glycosides was carried out. The antimicrobial activity of both plant species extracts were performed by well and disc agar diffusion methods. The most antimicrobial potential of plant extract was further selected to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against two most susceptible human pathogens. Phytochemicals and antimicrobial activity assays showed different results according to the type of the applied extract solvent. The methanol extract of *C. sinensis* shows more inhibitory effect than the other extracts of both tea species. The lowest concentration of *C. sinensis* was found at 0.048 mg/mL against *Staphylococcus aureus*. The MIC and MBC of *S. aureus* and *Pseudomonas aeruginosa* were ranged from 0.097 to 0.197 mg/mL and 0.19 to 0.39 mg/mL, respectively. Time-killing assay was performed against all tested pathogenic bacteria. After 6 h, *P. aeruginosa* showed no growth followed by *Escherichia coli*, *Candida albicans*, *Klebsiella pneumoniae*, *S. aureus*, *Salmonella typhi*, *Listeria monocytogenes*.

**Keywords** Phytochemicals · Antimicrobial · Agar diffusion · MIC · MBC · Time-killing

## Introduction

A gradual increase in resistance among microorganisms towards the antibiotics is the leading threat in the present world. About 2 million people attained bacterial diseases in the US hospitals, each year and 70% of cases of bacterial strains resistant to one or more drugs (Infectious Diseases Society of America 2004). Among all the plants, tea plant has been found potential against multi-drug resistance pathogens.

Non-fermented tea has a high range of antibacterial activity than fermented and semi-fermented tea (Toda et al. 1989). In recent years, tea acquired scientific attention as an antimicrobial agent against several bacterial species (Singh et al. 2009). *Staphylococcus aureus*, *P. aeruginosa* and *S. typhi* are some of the common human pathogens, generally

causing skin infection, urinary tract infection, and enteric fever respectively.

Tea leaves contain a huge number of polyphenols (flavonoids, catechins, tannins etc.) among which flavonoids are most abundant, possessing the ability to inhibit the spore germination in fungal strains. Besides it also bears several beneficial properties such as anti-inflammatory, antimicrobial, antiallergic, antioxidant, antitumor etc. Therefore, flavonoids are becoming the topic of medicinal research. In contrast, catechins include epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) (Cabrera et al. 2003). Among these, the compounds responsible for the antimicrobial property are EGCG and EGC. EGCG has the potential to decrease the growth of *Escherichia coli* by about 50% (Nazer et al. 2005). Green tea catechins revealed antimicrobial activity against a wide variety of microorganisms including, Gram-positive, Gram-negative bacteria, some viruses, fungi and prions (Reygaert 2018). Many researches proved that tea polyphenols showed positive effects on human health (Yang and Zhang 2019). Tea attributed biological properties to prevent cardiovascular diseases (Sesso et al. 1999) cancer (Mckay and Blumberg 2002), anti-inflammatory, antiarthritic,

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antibacterial, antiangiogenic, antioxidative, antiviral, neuroprotective and cholesterol-lowering effects (Chacko et al. 2010). Due to the high content of flavonoids, it has been attributed to antifungal activity against *Candida* species (Cushnie and Lamb 2005).

Nowadays, the frequency of these pathogens to cause disease has increased. Antibiotics have been used to treat the disease-causing microorganism resulting into side effects on human health. However, plant-based products found to be effective to cure human diseases and for body healthy (Padmini et al. 2010).

## Material and methods

### Collection of *Camellia* species

The species of *Camellia* were collected from Tea Estate, Dehradun, Uttarakhand. The young fresh leaves were washed to remove the dust particle followed by shade-drying and grind to form a powder. Both the plants were identified based on their morphological features and authentic databases by the Botanical Survey of India (BSI). The accession number of *C. sinensis* and *C. assamica* are 19a and 19b respectively.

### Preparation of *Camellia* species extract

The dried plant material of both species was weighed and 100 g of each was kept separately into Soxhlet apparatus for 6 h extraction. Different solvents such as methanol, ethanol and water were used for extraction. The solvents were evaporated by using rotary vacuum evaporator and plant extracts were stored in a refrigerator for further use (Siddhuraju et al. 2002) and their yield percentage were calculated by using the formula (Mostafa et al. 2018):  $\text{Extract yield\%} = \frac{\text{weight of extracted plants residues}}{\text{weight of plant raw material}} \times 100$ .

## Phytochemical analysis of tea extracts

### Tannins

About 5 mL of an extract of the plant was boiled with 20 mL of distilled water and filtered, the boiled filtrate was then treated with 0.1% of ferric chloride. Changing in blue-black colours detects tannins.

### Flavonoids

5 mL of ammonia solution was added to a 3 mL of plant extract followed by careful addition of sulphuric acid. The presence of flavonoids will be observed if the extracted

colour is turned into yellow, but on standing, the yellow colour will be disappeared, subsequently, few drops of aluminium solution (1%) should be added in each plant extract.

### Terpenoids

About 5 mL of each plant extract was mixed with 2 mL of chloroform, then 3 mL concentrated sulphuric acid was added carefully. The presence of terpenoids is proved by the formation of a reddish-brown coloured interface.

### Saponins

2 mL of each plant extract was boiled with 20 mL distilled water in a water bath and filtered then. 10 mL of the filtrate was mixed with 5 mL of distilled water and shaken vigorously. If a froth is observed, olive oil will be added to froth followed by vigorously shaking; formation of a foam appearance confirms the presence of saponins.

### Steroids

Acetic anhydride (2 mL) was added to each plant extract with 2 mL of sulphuric acid. The presence of steroids will be detected as the violet colour is changed into blue or green.

### Cardiac glycoside

Each plant extract was separately treated with 2 mL of glacial acetic anhydride with one drop of ferric chloride solution followed by mixing of 1 mL of concentrated sulphuric acid. The formation of interface with brown colour indicates deoxysugar characteristic of cardenolides. Violet colour ring may appear below the brown ring while the acetic acid is added, a greenish colour ring may form moderately throughout the thin layer.

### Alkaloids

2 mL of each plant extracts was treated with 3 mL of hexane followed by proper shaking and filtration. 5 mL of hydrochloric acid (2%) was poured in a test tube containing plant-extract-hexane mixture, shaken then filtered. A few drops of picric acid were added in the mixture. The formation of yellow precipitate indicates the presence of alkaloids.

### Carbohydrates

2 mL of Benedict's reagent was mixed with each plant extract and boiled; formation of reddish-brown precipitate documents the presence of carbohydrates.

## Bacteria

Six pathogenic bacterial strains viz., *P. aeruginosa* (MTCC 424), *S. aureus* (MTCC 7443), *E. coli* (MTCC 118), *Listeria monocytogenes* (MTCC 657), *Salmonella typhi* (MTCC 733), *Klebsiella pneumoniae* (MTCC 432), *Candida albicans* (MTCC 227) were collected from Microbial Type Culture Collection (MTCC), Chandigarh, India. These cultures were maintained by sub-culturing on nutrient and Sabouraud dextrose agar slants for bacteria at 37 °C and fungi species at 28 °C, respectively.

## Antimicrobial activity of plant extract

### Agar well diffusion method

Tea leaves extracts of both species were investigated for their antimicrobial activities. 100 µL of microbial cultures were swabbed on Mueller Hinton agar plate (MHA) for bacterial strains and Sabouraud dextrose agar (SDA) for fungal strain. Wells (6 mm in diameter) were punched using a sterile cork borer. 80 µL of each plant extract was dispensed in each well. All plates of bacteria were incubated at  $37 \pm 1$  °C in bio-oxygen demand incubator (BOD) for 24 h and fungal plates were incubated at  $25 \pm 1$  °C for 24–48 h to measure the zone of inhibition (Mehrotra et al. 2010).

### Disc diffusion method

Antimicrobial activity of the plant extracts was also evaluated by disc diffusion method. 100 µL of each bacterial culture ( $1 \times 10^8$  CFU/mL) were dispensed in Mueller Hinton Agar medium and Sabouraud dextrose agar medium plates to prepare a lawn. Whatmann filter paper (disc 5 mm) was impregnated with 10 µL of the plant extract then placed on the bacterial and fungal lawn.

Formed inhibition zones were measured in mm and the most potent antimicrobial plant extract was selected for further studies.

### Determination of minimum inhibitory concentration, minimum bactericidal concentration

MIC of the selected plant extract was determined by a double fold serial dilution method against all the pathogenic of both the *Camellia* species. A stock solution of crude plant extract was prepared (25 mg/mL of the corresponding solvent); then it was diluted in test tubes containing sterile Luria Bertani broth using twofold serial dilution method to obtain concentrations ranging from 25 to 0.048 mg/mL. 100 µL of bacterial inoculum ( $1 \times 10^8$  CFU/mL)/100 µL extract dilution was used for each test tube. The mixtures were incubated at 37 °C for bacterial growth. After the

incubation, the lowest concentration that inhibits the growth of bacteria was considered as the MIC. To determine the plant extract MBC, tested bacterial strains were sub-cultured together with the plant extract on agar plates using Nutrient Agar Medium (NAM) and Sabouraud dextrose agar medium (SDA) for the dilution process. MBC was considered as the concentration that ceased the bacterial growth (Mostafa et al. 2018).

## Time-kill efficacy assay/Survival of microbial species

The survival of bacteria, time kill efficacy assay, in the methanolic extract of *C. sinensis* was carried out with slight modification. Different time intervals were applied in the assay at 0, 2, 4 and 6 h. 50 mg of methanolic extract *C. sinensis* was dissolved in 1 mL of methanol to achieve 50 mg/mL concentration. Test bacteria were separately inoculated into sterile Luria Bertani broth and incubated at 37 °C for 24 h. The standard tube dilution method was opted to evaluate the time-kill efficacy of bacteria. Bacterial inoculum (1 mL) was diluted by adding 9 mL sterile saline solution and serially diluted upto  $10^{-3}$ . On testing the bacterial dilution ( $10^{-3}$ ), bacterial colonies were observed in a discrete form which was easily countable. One mL of  $10^{-3}$  bacterial dilution was incubated with an equal amount of 50 mg/mL plant extract at 37 °C for bacteria and 25 °C for fungi for a different time interval (0, 2, 4 and 6 h). 100 µL incubated suspension was transferred on the agar plates and spread through the spreader. Nutrient agar medium was used for bacteria and for fungi Sabouraud dextrose agar was used. Results were expressed in terms of  $\log_{10}$  CFU/mL (Hassan et al. 2014).

## Statistical analysis

The experimental data were performed in triplicate and expressed as mean  $\pm$  standard error. The data were analyzed following Analysis of Variance (ANOVA) using Microsoft Excel 2016. The significance value of data was determined at  $p \leq 0.05$ .

## Results

### Plants extraction yield

The highest yield was found to be of methanol (4.58%) followed by ethanol (3.27%) in the case of *C. sinensis* and ethanol (2.44%) followed by water (2.40%), in the case of *C. assamica* (Table 1).

**Table 1** *Camellia* species and their extract yield percentage

Plant species	Plant part used	Extract	Extract yield (%)
<i>C. sinensis</i>	Young fresh leaves	Methanol	4.58
		Ethanol	3.27
		Water	1.47
<i>C. assamica</i>	Young fresh leaves	Methanol	2.33
		Ethanol	2.44
		Water	2.40

**Table 2** Phytochemical analysis of *C. sinensis*

Phytochemical test	Methanol	Ethanol	Water
Alkaloids	+	+	+
Flavonoids	+	+	–
Carbohydrates	+	–	+
Saponins	–	+	+
Tannins	+	+	–
Steroids	+	+	–
Terpenoids	+	+	+
Cardiac glycoside	–	–	–

“+” present, “–” absent

**Table 3** Phytochemical analysis of *C. assamica*

Phytochemical test	Methanol	Ethanol	Water
Alkaloids	+	+	+
Flavonoids	+	+	–
Carbohydrates	+	–	+
Saponins	–	+	+
Tannins	+	+	–
Steroids	+	+	–
Terpenoids	–	–	+
Cardiac glycoside	–	–	–

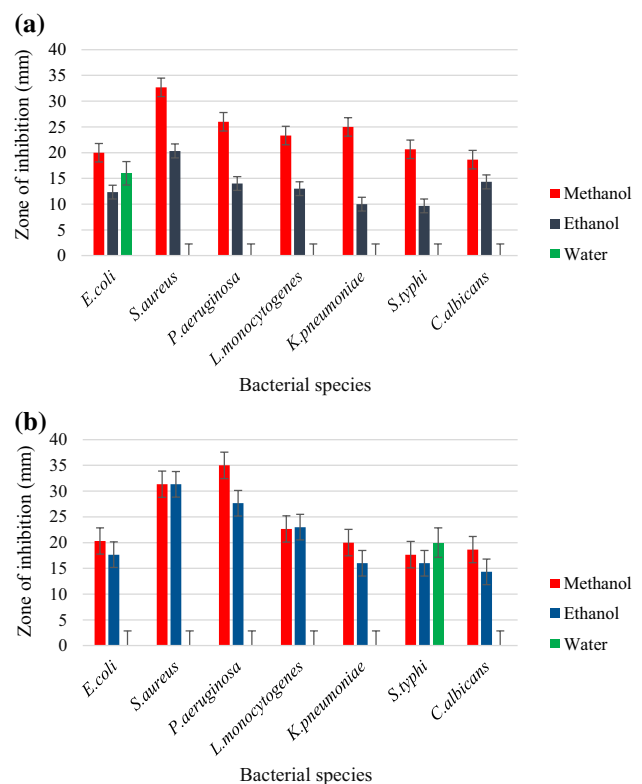
“+” present, “–” absent

## Phytochemical analysis

The different extract of *C. sinensis* and *C. assamica* showed the presence of alkaloids, flavonoids, carbohydrates, terpenoids, saponins, tannins, steroids (Tables 2 and 3).

## Antimicrobial activity assay of plant extract

Results of the antimicrobial activity evaluation of *Camellia* two species by agar-well and disc-diffusion methods were demonstrated in Figs. 1 and 2, respectively. It showed that the *C. sinensis* extract is more effective against *S. aureus* followed by *P. aeruginosa*; whereas, *C. assamica* extract is more effective against *P. aeruginosa* followed by *S. aureus*. The methanolic extract of *C. sinensis* was found more

**Fig. 1** Antimicrobial activity by agar-well diffusion method: **a** *C. sinensis*; **b** *C. assamica*

potential as compared to methanolic extract of *C. assamica* against the pathogenic bacteria. Hence, it was proved that plant extract is very effective against both bacterial species.

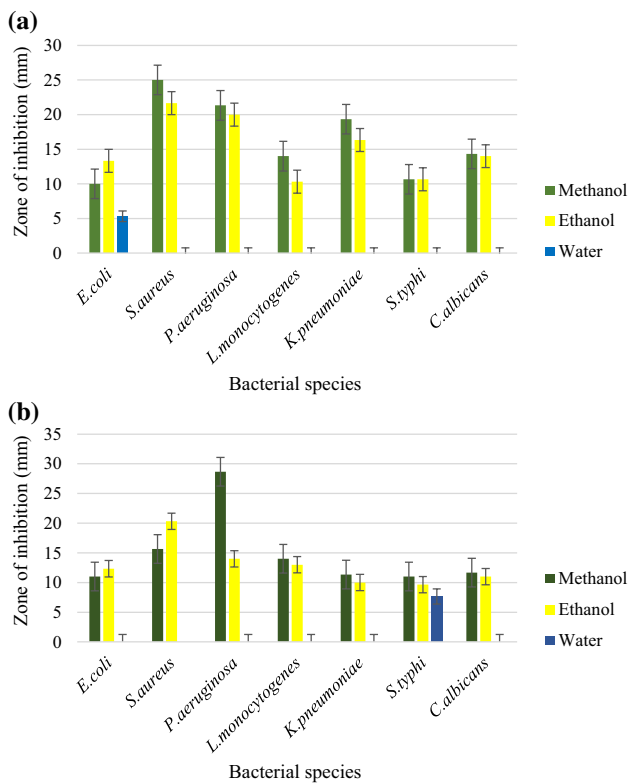
## Time-killing assay/Survival of microbial species

Qualitative analysis of bacteria survival/time-killing assay, at different time intervals, was demonstrated in Table 4. The methanolic extract of *C. sinensis* showed more efficiency against *P. aeruginosa*, *E. coli*, *C. albicans* followed by *K. pneumoniae*, *S. aureus*, *S. typhi* and *L. monocytogenes* (Table 4).

## Discussion

There are many factors that may influence the antibacterial activity of green tea leaves such as the origin of tea plants, breeding, extract preparation, and nature of tea clones and seedlings (Grange and Davey 1990) and geographic differences (Boyanova et al. 2005).

The phytochemical screening and quantitative estimation of the percentage crude yields of chemical constituents of the plants showed that the leaves and stems were rich in alkaloids, flavonoids, tannins, and saponins. They



**Fig. 2** Antimicrobial activity by disc-diffusion method: **a** *C. sinensis*; **b** *C. assamica*

**Table 4** Survival of bacteria/time-killing assay of *C. sinensis* (methanol)

<i>C. sinensis</i>				
Bacterial species	Log CFU/mL			
	0 h	2 h	4 h	6 h
<i>S. aureus</i>	4.892	4.785	4.643	4.556
<i>L. monocytogenes</i>	5.903	5.715	5.610	5.579
<i>E. coli</i>	4.806	4.380	4.041	3.477
<i>P. aeruginosa</i>	3.903	3.301	3	NG
<i>S. typhi</i>	5.037	4.880	4.845	4.770
<i>K. pneumoniae</i>	5.650	4.707	4.579	4.544
<i>C. albicans</i>	4.716	4.602	4.342	4.311

Data is the mean of triplicate

NG No growth

were known to show medicinal activity as well as exhibiting physiological activity (Edeoga et al. 2005). Therefore, both the *Camellia* species depicts the presence of phytochemical that might be responsible for medicinal and therapeutic activity (Tables 2, 3).

Steroids were found in all the plants. Furthermore, some of these investigated plants contained steroidal compounds. It should be noted that steroidal compounds are of much

importance and interest in pharmacy due to their relationship with compounds as sex hormones (Okwu 1999). These phytochemicals also called secondary metabolites that are present in plant extracts. They showed potentials antibacterial activity that can be exploited as the alternatives of antibiotics in the treatment of bacterial infections (Vaghasiya et al. 2011). Antimicrobial and antioxidant potential of some medicinal plants has great attention in both food and pharmaceutical industries because it contains natural compounds that replace the synthetic antioxidant and antimicrobial substances (Deba et al. 2008). The high number of polyphenols present in plants has a great significance as a natural destroyer against pathogenic microbes (Baravalia et al. 2009). A large number of flavonoids are capable of showing antioxidant potential which is effective against broad range of microbes (Kasolo et al. 2010). It has been reported that the flavonoids possess anticarcinogenic, anti-mutagenic, anti-inflammatory, and antioxidant potential effects (Haus-teen 2005; Li-weber 2009). It helps to reduce high blood pressure as well (Ayinde et al. 2007). Tea plants could be useful to reduce disease severity.

Alkaloids are the naturally occurring nitrogen compound, commonly found to have potent antimicrobial activity because of their capability to disturb the functioning of the DNA of microorganisms (Kasolo et al. 2010). It has been reported that the phytochemicals present in plant-based food also help to improve blood pressure, glucose level, cholesterol rate etc. (Broadhurst et al. 2000; Kelble 2006). Cardiac glycoside has been pharmacologically active. It might be used to treat heart failure and give strength to the heart muscles (Sospeter et al. 2013). The role of tannins helps to prevent the degenerative diseases and exhibit antimicrobial potential against pathogenic microorganisms (Ngoci et al. 2011). Many researches reported that catechins are present in *Camellia* species are also responsible to damage bacterial membrane (Cho et al. 2008). It has been reported that extracts of tea leaves contain polyphenolic compounds with activity against a broad spectrum of microbes. Studies conducted over the last 20 years have depicted that the green tea polyphenolic catechins, including (–)-epigallocatechingallate (EGCg) and (–)-epicatechingallate (ECg), can impede the growth of wide range of Gram-positive, Gram-negative bacteria and fungal species with significant potency (Inamdar et al. 2014).

Survival of bacteria/time-killing assay outcomes showed that it depends upon the concentration of extract and time-dependent. The time kill assay show the bactericidal activity and the duration of a bacteriostatic effect of a fixed concentration of the antimicrobial agent, thereby contributes a clear analysis of the relationship between the mortality microbial population and the antimicrobial agent concentration (Igbiosa and Idemudia 2016). At a fixed dilution ( $10^{-3}$ ) methanol extract of *C. sinensis* kills *P. aeruginosa* at ( $50 \mu\text{g}$ /

mL) concentration. The survival of bacteria assay could be useful to evaluate the particular dose and time interval to inhibit the pathogen. The compound Epigallocatechin gallate in tea leaves has also been delineated to interact with the outer membrane of bacteria and may prevent the adhesion to epithelial cells (HEp-2), in mammals and probably without amendment in epithelial cells (Sharma et al. 2012). Another mechanism is *C. sinensis* extract may affect the activity of dihydrofolate reductase, as well as increase the thickness of the epidermis (Chung et al. 2003).

## Conclusion

It has been well known the rate of resistance of pathogens towards the antibiotics increasing day-by-day. Therefore, the extracts of medicinal plants and their compounds creates a new interest as an antimicrobial agent. The compounds present in the tea extracts might be pharmacologically acceptable because the natural compounds that are extracted from tea do not show any side effects on human health. Screening of phytochemical compounds in tea extract that have potent antimicrobial and antioxidant properties. The background of the manuscript indicate that *C. sinensis* and *C. assamica* phyto-complex extracts have an efficacious antimicrobial potential, as evidenced by the inhibitory effect on bacterial growth of different human pathogens.

The antimicrobial properties of *C. sinensis* and *C. assamica* extracts are of great interest in the light of the ongoing threat of microbial strains developing resistance to conventional antibiotics. These outcomes are also interesting in the perspective of biologically active molecules present in *Camellia* species from agro-industrial by-products.

## Compliance with ethical standards

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

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