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Green Tea Extract-Propitious Approach to Combat Urinary Tract Pathogens

S. Valli[1]* and M. Divya[2]
[1]Assistant Professor, PG and Research Department of Microbiology, Mohamed Sathak College of Arts and Science, Chennai, Tamil Nadu, India.
[2] Research Scholar PG & Research Department of Microbiology, Mohamed Sathak College of Arts and Science, Chennai, Tamil Nadu, India.

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***Corresponding Author Email:** valli.ranjani@gmail.com

Abstract
Green tea is one of the most widely consumed beverages in the world and is currently perceived as a healthy drink now gaining worldwide popularity as a drink that is important in preventive medicine. It is derived from non-fermented leaves of the *Camellia sinensis* plant. Hence in the present work the antibacterial activity of green tea extract to combat urinary tract pathogens was assessed. Samples of urine were collected from hospital and processed for the isolation of pathogenic bacteria. Urine samples were processed and based on their morphological and biochemical characteristics the isolates were identified The effect of green tea extract was investigated against urinary tract pathogens. Ethanolic extracts of green tea were prepared by soxhlet extraction method. The antibacterial activity of ethanolic extracts were evaluated against pathogens by disc diffusion method. The maximum zone of inhibition was observed in *Proteus mirabilis* showing 39mm at 1mg/ml concentration followed by minimum zone of inhibition was observed in *Escherichia coli* (17mm) at the concentration of 1mg/ml. The phytochemicals occurring in the various solvent extracts of *Camellia sinensis* leaves were analysed quantitatively by phytochemical screening. The major phytochemicals found were saponins, flavanoids, alkaloids, proteins and steroids. GC-MS analysis was carried out on the ethanolic extract of *Camellia sinensis* and 10 different compounds were identified. The chromatogram revealed 15 peaks in the retention time range 10 to 30 minutes. The largest peak at 27.551 min with 7.54% area was identified as Tetracosane followed by Heptocosane. with 4.76% area at 26.099 minute. Screening of *Camellia sinensis* by GC-MS confirmed the plant to be a potential source for bioactive substances that supports several pharmaceutical uses and therapeutic value.

Keywords
UTI pathogens, Green tea, phytochemicals GC MS.

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INTRODUCTION
Urinary tract infections (UTIs) are most common type of infection worldwide found in any organ system, and the most common type of nosocomial infection [1].

Escherichia coli is the infective agent for 80–90% of all UTIs. Non-pathogenic strains of *E.coli* are an

important part of the normal flora in the human intestinal tract. The strains of *E.coli* that infect the urinary tract are categorized as uro pathogenic. *E.coli* (UPEC)[2] The UPEC are able to produce special surface proteins (adhesins) that allow them to attach to and invade the epithelial cells that line the urinary bladder.[3]Some strains of UPEC may then travel up the ureters to the kidneys and cause even more severe infections (complicated UTIs), which can lead to renal damage and possibly renal failure [4] The next three pathogens of importance were *Enterococcus faecalis, Klebsiella pneumoniae and Proteus mirabilis* which varied in prevalence slightly from category to category.

The antimicrobial agents that have traditionally been used to treat UTIs (β-lactams, fluoroquinolones, trimethoprim–sulfamethoxazole, nitrofurantoin, etc.) are becoming less effective in recent years, the number of antimicrobial resistant strains of *E. coli* isolated from UTIs has been increasing, including resistance to antimicrobial agents normally used to treat UTIs [5]. These isolates are also showing resistance to drug combinations such as amoxicillin/ clavulanic acid, piperacillin/ tazobactam, and trimethoprim/ sulfamethoxazole [6].

Green tea is one of the most widely consumed beverages in the world and is currently perceived as a healthy drink. It is derived from non-fermented leaves of the *Camellia sinensis* plant. Green tea contains a large amount of catechins (30 to 42% dry weight), a group of very active flavonoids The catechins, which are antioxidants, have been attributed beneficial health properties such as protection against CVD and certain types of cancer. The catechins epicatechin, epicatechin-3-gallate, epigallocatechin and epigallocatechin-3-gallate (EGCG) are the major components of green tea leaves. EGCG is the most abundant catechin and has received the most attention ... Studies using green tea have shown it to have potential benefits, most notably in cardiovascular disease, cancer, diabetes, obesity, oral health, bone health, and cognitive function [7]

Medicinal plants persist bioactive pharmacological activities which renders them as effective antioxidant, anti-infectious, and anti-cancer agents. The most essential bioactive phytoconstituents includes flavonoids, alkaloids, steroids, carotenoids, terpenoids, tannins and glycosides which serve as valuable pre-requisites for therapeutic drug development; Yadav et al., 2014). Camellia sinensis is known to possess a huge collection of phytoconstituents like catechins, alkaloids, proteins, enzymes, vitamins, carbohydrates, polyphenols, lipids, minerals. [8,9]

The analysis of volatile components is usually conducted by using gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) Gas chromatography-mass spectroscopy (GC-MS) is one of the so-called hyphenated analytical techniques. As the name implies, it is actually two techniques that are combined to form a single method of analyzing mixtures of chemicals. Gas chromatography separates the components of a mixture and mass spectroscopy characterizes each of the components individually. By combining the two techniques, an analytical chemist can both qualitatively and quantitatively evaluate a solution containing a number of chemicals. It is essential to determine the volatile as well as non-volatile components present in green tea for a better understanding of the physiological, pharmacological, and flavor-imparting properties of tea

Due to significance increase in the incidence of urinary tract infections and emergence of MDR (Multi Drug Resistant) microbes the present investigation was undertaken to study the effect of green tea extract against urinary tract pathogens and analysis of its phytoconstituents by Gas chromatography- mass spectrometry.

MATERIALS AND METHODS

Collection and Processing of Urine Samples

Clean catch and midstream urine sample was collected in a sterile wide mouthed screw capped bottle after very thorough preliminary cleaning of external genitalia with soap and water and transferred to the laboratory for processing Isolation and identification of bacterial pathogens was done by microscopy and culture methods. The urine samples were mixed thoroughly centrifuged and examined microscopically. A loopful of the well mixed urine sample was inoculated to Blood agar and MacConkey agar plates using a calibrated nichrome wire The plates were then incubated at 37ºC aerobically for 24 hrs. After incubation, the plates were observed for bacterial colonies [10].

Pure cultures of all morphologically suspected colonies were maintained in slants and stored for further characterization

PREPARATION OF EXTRACT FROM Camellia sinensis LEAF BY SOXHLET APPARATUS: [11]

Camellia sinensis tea leaves were collected from Craigmore plantations from nilgiri tea estate, Ooty. 25g of *Camellia sinensis* extract sample was weighed and extracted with 300ml of the ethanol by continuous hot percolation with the help of soxhlet

apparatus for 10hours. The extracts were filtered using Whatman filter paper, and the filtrate was concentrated under reduced pressure in vacuum at 40°C for 25 min using a rotary evaporator. and stored in the refrigerator for further use.

4.5. ANTI- BACTERIAL ACTIVITY OF Camellia sinensis LEAF EXTRACT AGAINST UTI PATHOGENS:

Muller Hinton Agar (MHA) was used as base medium for screening antibacterial activity and Muller Hinton Broth (MHB) for preparation of inoculum.

INOCULUM PREPARATION

0.85 gram of NaCl was dissolved in 100 ml of distilled water and poured onto test tubes and sterilized in an autoclave at 121ºC for 15 minutes at 15 lbs. It was allowed to cool. The culture was inoculated on each test tube mixed well and incubated at 37ºC for 1 hour. The turbidity of the cultures was adjusted to 0.5 McFarland standard ($1x10^6$ cells/ml –$5x10^6$ cells/ ml). The cultures were labeled appropriately.

Disc diffusion method [12]

The disk diffusion method was used to evaluate antimicrobial activity of green tea extract against urinary pathogens *Escherichia coli Klebsilla pneumoniae Staphylococcus aureus Pseudomonas aeroginosa* and *Proteus mirabilis* The extracts ranging in concentration 500,750 and 1000 µg/ml were loaded over sterile filter paper discs (8 mm in diameter). 10 ml of Mueller Hinton agar medium was poured into sterile Petri dishes (as a basal layer) followed with 15 ml of seeded medium previously inoculated with bacterial suspension (100 ml of medium/1 ml of 10^7 CFU) to attain 10^5 CFU/ml of medium. Sterile filter paper discs loaded with plant extract concentration of were placed on the top of Mueller-Hilton agar plates. Filter paper discs loaded with 5 µg of ampicillin was used as positive control. The plates were kept in the refrigerator at 5 °C for 2 h. to permit plant extracts diffusion then incubated at 35 °C for 24 h. The presence of inhibition zones was measured, recorded and considered as indication for antibacterial activity.

Statistical Analysis: -

All the above assays were conducted in triplicate and repeated thrice for consistency of results and statistical purpose. The mean zone of inhibition and standard deviations were calculated for all treatments.

PHYTOCHEMICAL ANALYSIS OF *Camillia sinensis* LEAF EXTRACT*:* -

Specific qualitative tests were performed for the presence and absence of phytochemicals viz, alkaloids, tannins, flavanoids, saponins, steroids etc., in tea extract to identify the constituents using standard procedures.

GAS CHROMATOGRAPHY – MASS SPECTRUM ANALYSIS (GC-MS): -

GC-MS analysis of this extract was performed using GC SHIMADZU QP2010 system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with Elite-1 fused silica capillary column (Length: 30.0 m, Diameter: 0.25 mm, Film thickness: 0.25 is Composed of 100% Dimethyl poly siloxane). An electron ionization energy system with ionization energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1.51ml/min and an injection volume of 2ìl was employed (split ratio: 20). Injector temperature 200°C; Ion-source temperature 200°C. The oven temperature was programmed from 70°C (isothermal for 2 min.), with an increase of 300°C for 10 min. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds with scan range of 40 – 1000 m/z. Total GC running time was 35 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was GC MS solution ver. 2.53.

Identification of components

Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technique (NIST08s), WILEY8 and FAME having more patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST08s, WILEY8 and FAME library. The Name, Molecular weight, Molecular formula and Structure of the component of the test material were identified

RESULTS

Samples of urine were collected as per standard procedures and processed for the isolation of pathogens. The collected urine samples were directly streaked onto the MacConkey agar, Nutrient agar and Blood agar. Based on the morphological and biochemical characteristics the isolates were identified as ***Escherichia.coli***. ***Staphylococcus aureus Pseudomonas aeruginosa Klebsiella pneumonia*** and ***Proteus mirabilis***.

EXTRACTION OF *Camellia sinensis* LEAF: -

The ethanolic extract of green tea sample were prepared, filtered and evaporated as per standard methods. Maximum percentage yield of 5% was observed in ethanolic extract of *Camellia sinensis* leaves.

EVALUATION OF ANTIBACTERIAL ACTIVITY OF *Camellia sinensis* LEAF EXTRACT: -

The ethanolic extract of the plant *Camellia sinensis* was evaluated for antibacterial activity against the urinary tract pathogens. Their antibacterial potency was assessed by the presence or absence of inhibition zones and zone diameters (mm). Maximum zone of inhibition was observed in *Proteus mirabilis* showing 21mm at 1mg/ml concentration followed by *Staphylococcus aureus* 20mm at the concentration of 1mg/ ml of the *Camellia sinensis* leaf extract and *Klebsilla pneumonia* 10mm. The diameter of inhibition zone exhibited by *Escherichia coli* was 9mm and 15mm by *Pseudomonas aeroginosa*. There was a significant increase in zone diameter with increasing concentration of the extract. Results are presented in (Table 2).

EVALUATION OF PHYTOCHEMICAL ANALYSIS OF *Camellia sinensis* LEAF EXTRACT: -

The phytochemicals occurring in the various solvent extracts of *Camellia sinensis* leaves extract ethanol were analysed quantitatively by phytochemical screening. The results revealed the presence of various secondary metabolites of therapeutical importance. The major phytochemicals found were saponins, flavanoids, alkaloids, proteins and steroids. All extracts showed the absence of tannins, phenols, anthroquinones was show in Table 2

GC-MS:

GC-MS analysis was carried out on the ethanolic extract of *Camellia sinensis* and 10 different compounds were identified. The chromatogram revealed 15 peaks in the retention time range 10 to 30 minutes. The largest peak at 27.551 min with 7.54% area was identified as Tetracosane. The second less prominent peak at 26.099 minutes with 4.76% area corresponds to the compounds Heptocosane. The third predominant peak at 29.300 minutes with 7.53% area Tetracosane &Eicosane followed by a peak at RT 19.793 minutes with 10.26% area corresponds to the compound Hexadecanic acid. The other compounds identified were presented in (Table 3)

TABLE:1 ANTIBACTERIAL ACTIVITY OF GREEN TEA EXTRACT OF *Camellia sinensis* AGAINST UTI PATHOGENS

S.NO	Organisms	Concentration(µg/ml)			Antibiotic (1mg/ml)
		1000	750	500	
1.	*Escherichia coli*	9±0.3	9±0.3	9 ±0.3	17 ±0.4
2.	*Klebsilla pneumoniae*	10±0.25	9±0.3	9 ±0.3	21 ±0.4
3.	*Staphylococcus aureus*	20±0.2	16±0.25	11 ±0.25	37 ±0.45
4.	*Pseudomonas aeroginosa*	15±0.35	14± 0.25	13± 0.25	25 ±0.4
5.	*Proteus mirabilis*	21±0.4	18±0.4	13±0.25	39±0.5

Note: Mean values of triplicates (zone of inhibition) ±S.D

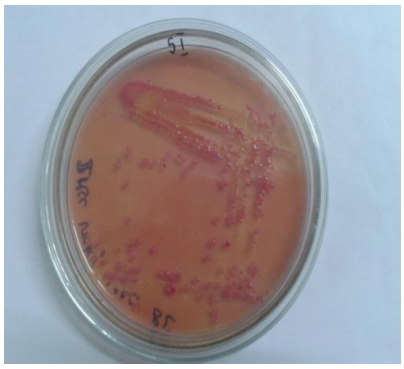
TABLE: 2 QUANTIFICATION OF PHYTOCHEMICAL ANALYSIS OF *Camellia sinensis* EXTRACT.

S.NO	TEST	ETHANOLIC EXTRACT	INFERENCE
1.	TANNINS	Absent	Absent
2.	SAPONIS	Pressence of emulsion	Strongly present
3.	FLAVONOIDS	Yellow	Strongly present
4.	ALKALOIDS	Precipitate	Strongly present
5.	PROTEINS	Blue	Strongly present
6.	STEROIDS	Brownish	Strongly present
7.	ANTHROQUINONES	Absent	Absent
8.	PHENOL	Absent	Absent

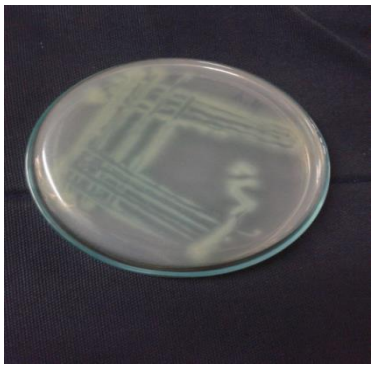
TABLE: 3 Identification of compounds present in ethanolic extract of *Camellia sinensis* by GC-MS.

S.NO	Peak No	Ret time	Area%	Name of the compound
1.	1.	14.731	2.20	Phenol,2,4-bis(1,1-dimethylethyl)
2.	2.	16.932	2.73	Cyclododecane
3.	3.	1.8.500	2.75	1,4-Eicosadiene
4.	4.	19.793	10.26	n-Hexadecanoic acid
5.	5.	21.213	3.11	Phytol
6.	6.	24.562	9.11	Octacosane
7.	7.	25.343	4.47	Heptacosane
8.	8.	26.099	4.76	Tricosane
9.	9.	26.353	5.94	Pregma-4,16-diene-3,20-dione
10.	10.	25.824	7.79	Heptacosane
11.	11.	27.552	7.54	Tetracosane
12.	12.	27.669	4.38	Hexacosane
13.	13.	28.364	7.49	Tetracosane
14.	14.	29.300	7.53	Hexacosane
15.	15.	29.667	2.87	Cholestrol
16.	16.	30.402	11.30	Nonadecane
17.	17.	31.708	5.78	Eicosane

PLATE-1: ISOLATION OF BACTERIAL PATHOGEN FROM URINE SAMPLE



Klebsiella pneumoniae

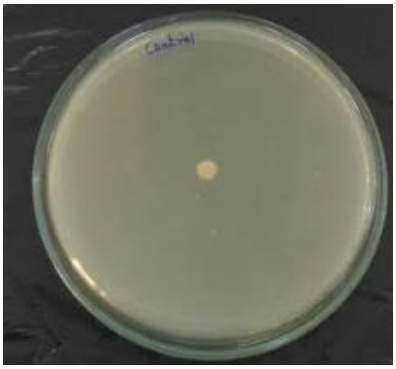


Pseudomonas aeruginosa

EMB with *E.coli*

Proteus mirabilis

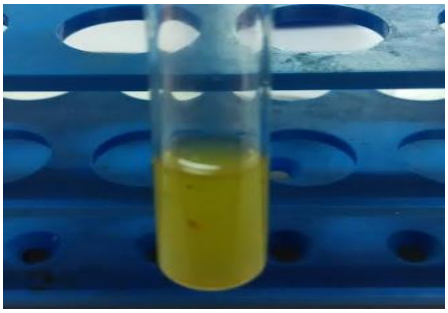
Staphylococcus aureus



CONTROL

PLATE-2:

Collected leaves of *Camellia sinensis* **Ethanolic extraction of *C.sinensis***

PHYTOCHEMICAL ANALYSIS OF *Camellia sinensis* EXTRACT

1 2 3 4 5 6 7 8

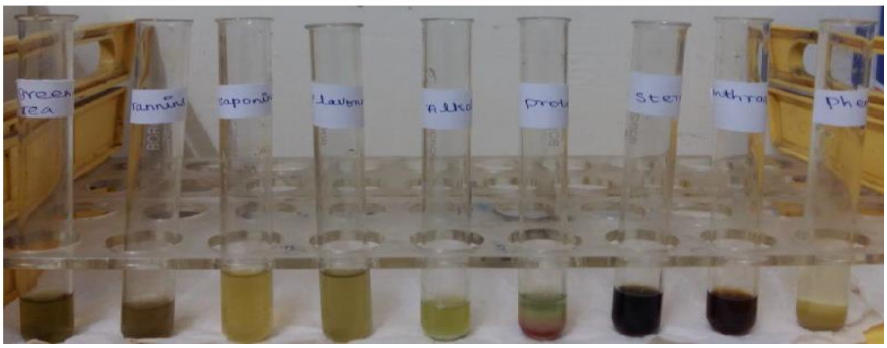


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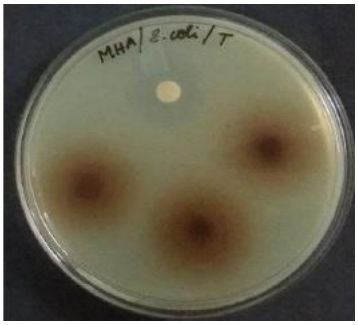
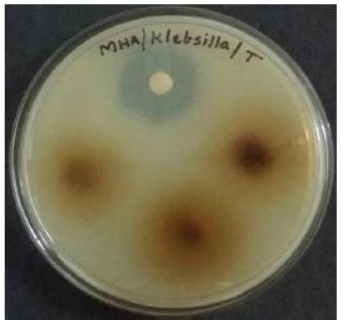
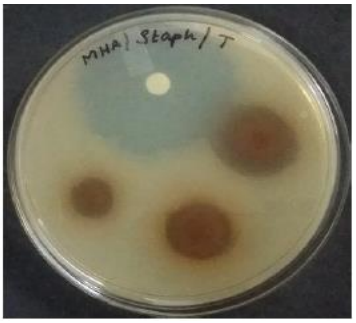
PLATE-3: ANTIBACTERIAL ACTIVITY OF *Camellia sinensis* EXTRACT AGAINST UTI PATHOGENS

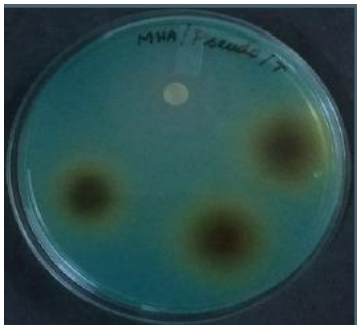
Escherichia coli ***Klebsilla pneumoniae*** ***Staphylococcus aureus***







Pseudomonas aeroginosa ***Proteus mirabilis*** **CONTROL**



DISCUSSION

Urinary tract infections (UTI) are most common form of bacterial infections, affecting people throughout their lifespan. The pathogenesis of complicated and uncomplicated UTI is complex and influenced by many host biological behavioral factors and properties of the infecting uropathogens. Despite the existence of potent antibiotics, resistant or multi resistant strains are continuously appearing, imposing the need for a permanent search and development of new drugs. For Centuries plants have been used throughout the world as drugs and remedies for various diseases. These drugs serve as prototype to develop more effective and less toxic medicines. Hence, an attempt has been made to evaluate antibacterial activity of green tea extract against urinary tract pathogens.

Samples of urine were collected from hospital and processed for the isolation of pathogenic bacteria. Urine sample were processed and based on their morphological and biochemical characteristics the isolates were identified as *E.coli*, *Proteus mirabilis, Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

A variety of enteropathogenic bacteria are known to cause UTI worldwide. *E.coli* being the predominant aetiological agent. The present study demonstrated *E. coli, Klebsiella pneumonia, Pseudomonas aeruginosa,* and *Proteus mirabilis* as the causative agent of UTI. These findings are similar to studies conducted by Nerukar *et al*., (2012). As drug resistance among bacterial pathogens is an evolving process regular surveillance and monitoring is necessary to provide treatment to UTI.

Green tea is derived from non-fermented leaves of the *Camellia sinensis* plant. Green tea has been a favored drink, traditionally, in Asian countries. Because of studies that have shown the potential health benefits of green tea, it is now gaining worldwide popularity as a drink that is important in preventative medicine. Studies using green tea have shown it to have potential benefits, most notably in: cardiovascular disease, cancer, diabetes, obesity, oral health, bone health, and cognitive function [13]. In addition, green tea has been shown to have antimicrobial effects

In the present study the effect of green tea extract was investigated against urinary tract infections. Ethanolic extracts of green tea were prepared by soxhlet extraction method. The antibacterial activity of ethanolic extracts were evaluated against pathogens by disc diffusion method. Maximum zone of inhibition was observed in *Proteus mirabilis* showing 18mm at 1mg/ml concentration followed by *Staphylococcus aureus* 20mm at the concentration of 1mg/ ml. Similar reports were given by [14].

Among the health benefits that have been studied using green tea are: as an antioxidant, anti-inflammatory, anticarcinogenic, in cardiovascular health, oral health, and as an antimicrobial. Green tea has been shown to have antimicrobial effects against a variety of gram positive and gram negative bacteria (*e.g., Escherichia coli, Salmonella spp., Staphylococcus aureus, Enterococcus.spp*), some fungi, and a virus.

The phytochemicals occurring in the ethanolic extracts of *Camellia sinensis* leaf were analysed quantitatively by phytochemical screening. The results revealed the presence of secondary metabolites of therapeutical importance. The major phytochemicals found were saponins, flavonoids, alkaloids, proteins and steroids.

GC-MS analysis was carried out on the ethanolic extract of *Camellia sinensis* 24 different compounds were identified. The chromatogram revealed 15 peaks in the retention time range 10 to 30 minutes. The maximum peak was obtained at 27.551 min with 7.54% area and the compound was identified as Tetracosane and the minimum peak was obtained at 29.300 minutes with 7.53% area Tetracosane and Eicosane.

GC-MS analysis was done to identify the components responsible for antibacterial activity. The compounds such as 1-Octadecene and 1-Heptadeceane possess anticancer, antioxidant and antimicrobial activity compounds such as squalene, tetracosane, ascorbic acid, and stigmasterol can be responsible for the antioxidant activities which are present in the ethanolic extract of *Camellia sinensis* leaves. Tetracosane with anticancer and antioxidant properties have been reported by [15]. Eicosane with antitumour and cytotoxic property was reported by [16] which was recovered from green tea leaves in the present study.

There are different mechanisms for antimicrobial effects of green tea such as:

- Polyphenols are anti-inflammatory agents that inhibit clinical symptoms of UTIs.
- Catechins, induce production of cytokines such as IL-12 and IL-10.7.
- Green tea polyphenols decrease tumor necrosis factor-α gene expression, which is important in pathogenesis of *E. coli* infection.
- Catechins, by blocking the connection of conjugated R plasmid in *E. coli*, have bactericidal and antitoxin effects.
- Catechin-copper (II) complexes damage the cytoplasmic membrane *of E. coli.*

- EGC can bind to the ATP site of the DNA gyrase β subunit of bacteria and inhibit the activity of the gyrase enzyme.
- The bactericidal action of catechin is due to its hydrogen peroxide generation.
- The highest antimicrobial activity of tea is due to presence of catechins and polyphenols which damage the bacterial cell membrane.
- Catechins interfere with the expression of β-lactamases in staphylococci and inhibit the extract.

CONCLUSION:

The present investigation aimed the antioxidant, the antimicrobial potential of phytoconstituents present in the ethanolic extract of *Camellia sinensis* and identification of chemical compounds by GC-MS analysis. The presence of phytoconstituents such as long-chain fatty acids, steroids, terpenoids, aliphatic, and aromatic hydrocarbons is responsible for the antimicrobial activity and antioxidant potential. Screening of *Camellia sinensis* by GC-MS confirmed the plant to be a potential source for bioactive substances that supports several pharmaceutical uses and therapeutic value. Therefore, it is essential to have *in vivo* studies on antibacterial effects of green tea and evaluated the efficacy of its catechins in the treatment of Urinary tract infections in the future.

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