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Antimicrobial Potential of *Camellia sinensis* against Skin Associated Microbial Pathogens

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ABSTRACT

The present study was undertaken to assess the antimicrobial potential of leaves' extract of Green tea (*Camellia sinensis*) against skin associated microorganisms. The antimicrobial activity was studied through agar well diffusion method against Gram positive, Gram negative bacteria and fungus included *Propionibacterium acne*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans* in five different solvents viz. Methanol, Ethanol, Distilled water, Chloroform and Petroleum ether. The methanolic extract of *Camellia sinensis* showed maximum zone of inhibition against *Propionibacterium acne* (27 mm) and *S. aureus* (26 mm); ethanolic extract was most effective against *Candida albicans* (22 mm) and petroleum ether extract was most effective against *S. aureus* (20 mm). The antimicrobial analyses showed that the extracts were more effective against Gram positive bacteria than Gram negative bacteria and fungus. Comparative study of antimicrobial activity of *Camellia sinensis* extracts with antibiotics revealed that *Camellia sinensis* methanolic extract had maximum effective antimicrobial activity against all the tested microorganisms. MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) of methanolic extract of *Camellia sinensis* against the microbial agents under study ranged between 0.125 to 0.015625 mg/ml. The synergistic interaction of *Camellia sinensis* with antibiotics revealed much better results as compared to antibiotics susceptibility pattern alone. Phytochemicals analyses of *Camellia sinensis* included alkaloids, saponins, glycosides, proteins, phenols, tannins and phytosterols. The compounds identified by GC-MS analysis of methanolic extract of *Camellia sinensis* were reported to be used as water treatment, antifungals, antimicrobials and enzymatic inhibitors, improve mental alertness, antifungal, anti-HIV, anticancer, anti-inflammatory, analgesic and comonomer (e.g. for lubricating oil additives). Therefore, it may be concluded that *Camellia sinensis* leaves possess antibacterial and antifungal properties, which may be used as alternate drug of choice due to its lower side-effects to human skin as compared to presently used therapeutic agents.

Keywords : Antimicrobial potential, *Camellia sinensis*, GC-MS analysis, Phytochemicals, Skin disease.

I. INTRODUCTION

Skin is the thin layer of tissue forming the natural outer covering of the body of a person or animal. It behaves as a non-homogeneous, anisotropic, non-linear visco-elastic material subjected to a prestress. The skin is a highly organized, stratified structure consisting of three main layers, called the epidermis, dermis and hypodermis [7]. Epidermis consists of keratinocytes, which change in cellular constituents

as they move peripherally. Dermis makes up the bulk of the human skin and contributes to 15-20% of the total body weight. Hypodermis is a fibro-fatty layer, which is loosely connected to the superficial dermis. Skin microflora includes various Gram-positive bacteria, Gram-negative bacteria and the Fungi, Bacilli present in dust particles [3]. Plant derived products can be exploited to cure skin ailments with sustainable, comparative and competitive advantage. These include reduced cost, less dangerous, more effective and readily available [14]. Tribal healers in most of the countries, frequently use herbal medicine to treat cut wounds, skin infection, swelling, aging, eczema and gastric ulcer [19].

Green tea (*Camellia sinensis*) is consumed as a popular beverage worldwide, particularly in Asian countries like China, Korea and Japan. Treatment of green tea polyphenols to skin has been shown to modulate the biochemical pathways involved in inflammatory responses, cell proliferation and responses of chemical tumour promoters as well as ultraviolet light-induced inflammatory markers of skin inflammation. There is also a wide range of uses for green tea in diabetes, exercise enhancement, inflammatory bowel disease and other skin disorders. Most impressive use is the well-controlled epidemiologic studies, aimed at altering the brain ageing process, which can serve as neuroprotective agents [23]. Thus, the present study was undertaken to assess antimicrobial potential of leaves' extract of Green tea (*Camellia sinensis*) against skin disease causing microbial pathogens.

II. MATERIALS AND METHODS

Collection of plant material and solvent extraction

The leaves of the Green tea (*Camellia sinensis*) were washed thoroughly 2-3 times with running tap water and then with sterile water followed by shade-dried, powdered and used for extraction. Five gram of shade dried powder of Green tea was put in 50 ml each of chloroform, distilled water, ethanol, methanol and petroleum ether in separate conical flasks, respectively. The solutions were placed in shaker for 24hrs so as to shake them properly. All these five extracts were filtered through Whatman filter paper no. 44 and evaporated in water bath at 65º C. The extracts were dissolved in 2% DMSO to make the final concentration (1mg /ml), which kept in refrigerator till further use [6].

Microbial strains used

The total five test organisms were used in the present study, which included Gram-positive bacteria (*Staphylococcus aureus* and *Propionibacterium acne*), Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) and fungi (*Candida albicans*).

Preparation of the Microbial inoculums

The density of human test bacteria and yeast was adjusted equal to that of the 0.5 McFarland standards (1.5×10^8 CFU/ml) by adding sterile distilled water. McFarland standards were used as a reference to adjust the turbidity of microbial suspensions so that the number of microorganisms may be within a given range. For the preparation of the 0.5 McFarland standard, 1.17%w/v $BaCl_2.2H_2O$) was added to 9.95 ml of 0.18M H_2SO_4 (1.0% w/v) with constant stirring. The McFarland standard tube was tightly sealed to prevent loss by evaporation and stored for up to 6 months [2].

Antimicrobial Activity

The antimicrobial activity of five different extracts of Green tea (*Camellia sinensis*) against five human test microorganisms was evaluated by using Agar well diffusion method.

Antibiotic susceptibility pattern of Test microorganisms used

Antibiotic susceptibility testing against test strains was done according to Kirby-Bauer Disc Diffusion assay. Autoclaved media (Nutrient Agar for gram negative bacteria i.e. *E. coli* and *P. aeruginosa*, Brain Heart Infusion Agar (BHI) for *Propionibacterium*

acne, Mannitol Salt Agar medium for *S. aureus* and Malt extract Agar medium for *C. albicans*) was poured in Petri plates thereafter the antibiotic disk diffusion assay was carried out after 24 hours.100µl standardized culture was spread on these agar plates. Antibiotic Hexa discs of Hi-Media were placed on the inoculums seeded plates. After incubation for 24 hrs at 37ºC, the plates were observed. If antimicrobial activity was present on the plates; it was indicated by an inhibition zone surrounding the disc. The zone of inhibition was measured and expressed in millimeters [4].

Determination of MIC, MBC and MFC

For MIC (Minimum Inhibitory Concentration), the macro-dilution agar method, a two-fold serial dilution of the plant extract was prepared in sterile distilled water to achieve a decreasing concentration ranging from 1mg/ml to 0.03125mg/ml in different tubes. Sterile cork borer of 6.0 mm diameter was used to bore well in pre-solidified medium agar plates and 50-100µl volume of each dilution was added aseptically into the wells made in agar plates in triplicate that had human pathogenic bacteria and yeasts seeded with the standardized inoculums (1.5×10^8 CFU /ml). 50-100µl pure extract was introduced into the well used as control. All the test plates were incubated at 37ºC for bacteria and 35ºC -37ºC for yeast and were observed for the growth after 24 hrs. The lowest concentration of an extract showed a clear zone of inhibition was considered as the MIC. The MIC plates were further incubated for 24-48hrs [10]. The lowest concentration that yields no growth following this further incubation, was the MBC (Minimum Bactericidal activity). The same method was used for antifungal activity. The standardized fungal inoculum was used. The inoculated plates were incubated at 35ºC-37ºC for the Candida albicans growth. The above mentioned procedure was done for the fungal strain. The lowest concentration that yields no growth following further incubation of MIC plate was considered as the MFC (Minimum Fungicidal Concentration).

Synergistic activity of Plant extract and commercially available Antibiotic

The bacterial cultures were grown in culture broth at 37ºC. After growth, each bacterium was inoculated on the surface of MHA (Muller-Hinton Agar) plates. Subsequently, the antibiotic disk of 6 mm diameter was placed on the surface of each inoculated plate and then added 20µl of plant extract (at a concentration of 1mg/ml), to identify synergistic effect between the plant extract and antibiotic used. The plates were incubated at 37ºC for 24 hrs .The diameter of clearing zones was measured [10].

Phytochemical analysis of most potent plant extract

Freshly prepared extracts were subjected to standard phytochemicals analysis to find the presence of the phytoconstituents including phenols, flavonoids, alkaloids, glycosides, tannins, saponins, carbohydrates, phytosterols, proteins and steroids by using Mayer's test, Molisch's test, .Modified Borntrager's Test**,** Foam Test, Salkowski's Test, Ferric chloride test, Lead acetate test, Ninhydrin test and copper acetate test [10].

Purification and partial characterization of plant extract

It was done by GC-MS (Gas Chromatography-Mass Spectroscopy). The plant extract was analyzed with the help of GC-MS analyzer (GC Clarius 500 Perkin Elmer).

- On Elite-1 column the data was generated. The carrier gas helium (99.99%) was used at flow rate of 1ml per min in split mode (10: 1). Methanolic sample (2 µl) was injected to column at 250ºC injector temperature. Temperature of oven starts at 110ºC and hold for 2 min and then it was raised at rate of 10ºC per min to 200ºC without holding.
- Holding was allowed for 9 min at 280ºC at program rate of 5ºC per min. Temperature of ion source was maintained at 200ºC. The

injector temperature was set at 250ºC and detector temperature was set at 280ºC.

- The mass spectrum of compounds present in samples was obtained by electron ionization at 70 eV and detector operates in scan mode from 45 to 450 Da atomic mass units. A 0.5 seconds of scan interval and fragments from 45 to 450 Da was maintained.
- Total running time was 36 minutes [12]. The electron gun of mass detector liberated electrons having energy of about 70eV.The column employed there for the separation of components was Elite 1(100% dimethyl poly siloxane).
- The identity of the components in the extracts was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library and with published literatures. NIST08 LIB9, WILEY8 LIB10 library sources were also used for matching the identified components from the plant material [22].

III. RESULTS AND DISCUSSION

The present study revealed the scientific validation of Green tea (*Camellia sinensis*) as an antibacterial and antifungal agent. The five solvents viz. Ethanol, methanol, chloroform, distilled water and petroleum ether were used for the extraction from Green tea (*Camellia sinensis*) and used for antimicrobial activity against tested microbial strains. The methanolic extract of *Camellia sine*nsis showed maximum zone of inhibition against *Propionibacterium acne* (27 mm) and *S. aureus* (26 mm), ethanolic extract was most effective against *Candida albicans* (22 mm) and petroleum ether extract was most effective against *S. aureus (*20 mm). The antimicrobial analysis showed that the extracts were more effective against Gram positive bacteria than Gram negative bacteria and Fungus. The methanolic extract was the most promising plant having maximum effective antimicrobial activity against all tested microorganisms as shown in Table-1 and Fig.1. For the antibiotic susceptibility pattern, Gentamicin was the most effective for Gram positive test bacteria, whereas Amikacin was the most effective antibiotic for Gram negative test bacteria and Clotrimazole for test fungal strain (table 2). MIC and MBC of methanolic extract of *Camellia sinensis* against the microbial strains was ranged between 0.125 to 0.015625 mg/ml as shown in Table-3 and Fig.2. Synergistic effect of extracts of *Camellia sinensis* with antibiotics against tested microorganisms showed that the zone of inhibition was found to be greater as compared to zone of inhibition of different antibiotics used alone. As shown in Table-4, for Gram positive bacteria *S. aureus*, the methanolic extract of *Camellia sinensis* with Gentamicin showed the synergism of zone of inhibition of 22 mm. For *P. acne*, the synergism between *Camellia sinensis* with Gentamicin was observed with zone of inhibition of 30 mm. In case of Gram negative bacteria E. coli, the methanolic extract of *Camellia sinensis* with Amikacin showed the synergism of zone of inhibition of 30 mm. For *P. aeruginosa*, the synergism between *Camellia sinensis* with Amikacin was observed with zone of inhibition of 32 mm. In case of *C. albicans*, there was no synergism effect observed for Camellia sinensis to the Clotrimazole as shown in Table-4. Phytochemicals analysis of methanolic extract of *Camellia sinensis* included alkaliods, tannins, saponins, glycosides, proteins, phenols and phytosterols, respectively as shown in Fig.-3. The compounds identified by GC-MS analysis of methanolic extract of *Camellia sinensis* is used for water treatment, antifungals, antimicrobials and enzymatic inhibitors, improve mental alertness, antifungal, anti-HIV, anticancer, anti-inflammatory, analgesic and comonomer (e.g. for lubricating oil additives) (Table-5 and Fig-4).

In case of *E. coli*, maximum zone of inhibition (17mm) was recorded by the methanolic extracts of *Camellia sinensis*. Our result substantiate the findings,

where it was demonstrated that antimicrobial activity of *Camellia sinensis* in one out of three extracts against E. coli with zone of inhibition of 13mm by taking 15 g of powered plant sample, extracted with 100 ml of methanol with concentration of 30 µl [11]. In the present study, 5g sample in 50 ml of methanol with concentration of 20 µl was taken and even then obtained better results. The methanolic and ethanolic extracts of plants parts were better because both ethanol and methanol are organic solvents and dissolve more organic compounds resulting in the liberation of greater amounts of antimicrobial constituents [5]. During the present study, the results of screening revealed that the most of the plant extracts were active against Gram positive bacteria (*S. aureus* and *P. acne*) than Gram negative bacteria (*E. coli* and *P. aeuginosa*). The reason for different sensitivity between Gram positive and Gram negative bacteria could be ascribed to the morphological differences between these microorganisms i.e. an outer polysaccharide membrane carry the structural lipopolysaccharide components, which makes cell wall impermeable to lipophilic solutes, the Gram positive are more susceptible having only an outer peptidoglycan layer which is not effective permeability barrier [21]. In case of antibiotic susceptibility pattern, our results were in agreement with the various findings including susceptibility test of ciprofloxacin and ampicillin against *S. aureus* [9]; the susceptibility test of amikacin against *E. coli* [15]; the susceptibility test of amikacin against *P. aeruginosa* [16] and the susceptibility test of clotrimazole against *C. albicans* [20].The MIC value of methanolic extract of *Camellia sinensis* ranged between 0.015 mg/ml to 0.050 mg/ml against *S. aureus* and *P. aeruginosa* [17]. The results varied because of the different solvent extraction method as they used 2.5 g of commercially available tea leaves were brewed and extracted in 100 ml of distilled water whereas in the present study, 5 g sample in 50 ml of methanol was taken. The synergistic effect of *Camellia sinensis* with the gentamicin against *S. aureus* may be varied because of the volume of 75µl of green tea extract was combined with 25µl of gentamicin [13] rather than 20 µl of green tea with 10 µg of gentamicin in the present study. In the study of synergism between *Camellia sinensis* with amikacin against E. coli revealed that the extract has antagonistic effect of amikacin by using concentration of amikacin of 10 mg [18] rather than 0.03 mg as used in the present study. The results substantiate in the antagonistic effect due to competing tea components with antimicrobial agent in binding the membrane of microorganism. The study on presence of phytochemicals in *Camellia sinensis* indicated the presence of alkaloids, flavonoids, carbohydrates, glycosides, proteins, saponins, tannins, terpenoids and anthraquinones in different solvent extracts [8]. Phytochemicals other than alkaloids, glycosides, proteins, saponins and tannins could not match with the present study perhaps due to the different solvent extraction and the phytochemical analysis method followed. The study on GC-MS analysis of the methanolic extract of *Camellia sinensis* resulted into the presence of caffeine, linoleic acid, oleic acid, palmitic acid [1], whereas in the present study only one common bioactive compound (caffeine) could be obtained along with eight other different bioactive compounds. This difference may be due to the solvent selection & extraction methodology and GC-MS analysis methodology.

IV. CONCLUSIONS

The new, safe and more effective antibacterial and anti-fungal agents are a major challenge to the pharmaceutical industry for the cure of incurable skin diseases. To overcome the problem, scientist move forward to the uses and applications of various natural and herbal drugs. The presence of various health benefitting compounds using GC-MS and chemotherapeutic use of Green tea (*Camellia sinensis*), as revealed in the present study, indicated its antimicrobial potential and possible use in curing skin diseases.

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TABLE 1. ANTIMICROBIAL ACTIVITY OF *CAMELLIA SINENSIS* LEAVES EXTRACTS.

Microorganisms	Diameter of zone of inhibition(mm)				
	Methanolic	Ethanolic	Chloroform	Petroleum ether	Aqueous
S. aureus	26	21	11	20	NA
P. acne	27	22	9	NA	NA
E. coli	17	14	10	NA	NA
P. aeruginosa	17	16	NA	NA	NA
C. albicans	18	22	NA	12	NA

NA: No activity

TABLE 2. ANTIBIOTIC SUSCEPTIBILITY PATTERN OF TEST MICROORGANISMS.

Antibiotic- Symbol (concentration)	Zone of inhibition				
	Gram positive bacteria		Gram negative bacteria		Fungi
	P. acne	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*
Ciprofloxacin- CIP (5mcg)	32mm	19 mm	--	--	--
Gentamycin-GEN (10 mcg)	28 mm	24 mm	19 mm	20 mm	--
Vancomycin-VA (30 mcg)	26 mm	23 mm	--	--	--
Linezolid- LZ (30mcg)	11 mm	39 mm	--	--	--
Ampicillin- AMP 10 10 mcg	10 mm	8 mm	25 mm	22 mm	--
Streptomycin-S10 (10 mcg)	15 mm	18 mm	--	--	--
Amikacin- AK (30 mcg)	--	--	26 mm	30 mm	--
Tetracycline-TE (30 mcg)	--	--	21 mm	25 mm	--
Chloramphenicol-C (30 mcg)	--	--	26 mm	16 mm	--
Cotrimoxazole-CO (25 mcg)	--	--	21 mm	22 mm	--
Clotrimazole- CC (10mcg)	--	--	--	--	15 mm
Micanazole- MIC (30mcg)	--	--	--	--	No zone
Nystatin- NS (50mcg)	--	--	--	--	No zone
Ketoconazole- KT (50mcg)	--	--	--	--	No zone

TABLE 3. MINIMUM INHIBITORY CONCENTRATION (MIC), MINIMUM BACTERICIDAL CONCENTRATION (MBC) AND MINIMUM FUNGICIDAL CONCENTRATION (MFC) VALUES OF *CAMELLIA SINENSIS* METHANOLIC EXTRACT.

S.No.	Microorganisms	Plant Extract	MIC	MBC	MFC
1.	*S. aureus*	*Camellia*	0.125	0.125	-
2.	*P. acne*	*Camellia*	0.0156	0.0156	-
3.	*E. coli*	*Camellia*	0.0156	0.0156	-
4.	*P. aeuginosa*	*Camellia*	0.0156	0.0156	-
5.	*C. albicans*	*Camellia*	0.0625	-	0.125

TABLE 4. SYNERGISTIC ACTIVITY OF METHANOLIC EXTRACTS OF *CAMELLIA SINENSIS* WITH ANTIBIOTICS.

S.No.	Microorganisms	Plant extract +antibiotic	Zone of inhibition(mm)	Comparative effect on zone of inhibition
1.	*S. Aureus*	*Camellia sinensis*.+ gentamicin	22 mm	Activity decreases
2.	*P. Acne*	*Camellia sinensis* + gentamicin	30 mm	Activity increases
3.	*E. Coli*	*Camellia sinensis* + amikacin	30 mm	Activity increases
4.	*P. Aeruginosa*	*Camellia sinensis* + amikacin	32 mm	Activity increases
5.	*C. Albicans*	*Camellia sinensis* + clotrimazole	Na	Activity decreases

TABLE 5. GC-MS PEAK REPORT TIC OF PHYTOCOMPOUNDS PRESENT IN THE METHANOL EXTRACT OF THE *CAMELLIA SINENSIS.*

Peak	Retention	Chemical formula	Compound	Uses	Cas #
1	9.57	C7H13NO2	2Hydroxymethyl-2methylpyrrolidine1carboxaldehyde	Water treatement	NA
1	9.57	C4H8N4	4H1,2,4Triazol3amine, 4ethyl	antifungals, antimicrobials, and enzymatic inhibitors	42786061

1	9.57	C8H15NO	1Propyl-4piperidone	pyrrolidine alkaloid	23133371
2	15.54	C15H28O2	Dodecyl acrylate	feedstock for chemical syntheses	2156970
2	15.54	C20H39ClO2	3Chloropropionic acid, heptadecyl ester	Use for manufacture of intermediates for pyrroles,pyrrolines and pyrrolizidines.	NA
2	15.54	C16H30O2	2Propenoicacid, tridecyl ester	Comonomer (e.g. for lubricating oils)	3076048
3	17.43	C8H10N4O2	Caffeine	improve mental alertness.	58082
3	17.43	C8H10N4O2	1,4Dimethyl4,5,7,8tetrahydroimidazo[4,5E]1,4diazepin5,8 (6H)dione	antifungal, anti-HIV, anticancer, antiinflammatory, analgesic	130063159
3	17.43	C15H24FN	2Fluorobenzylamine, N,Ndibutyl	Synthesis of anticonvulsant.	NA

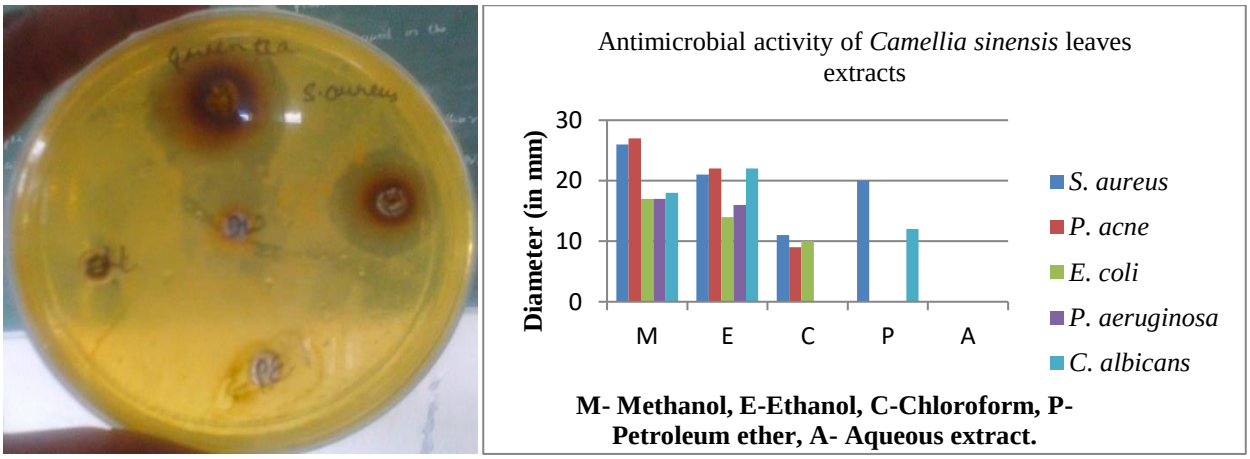
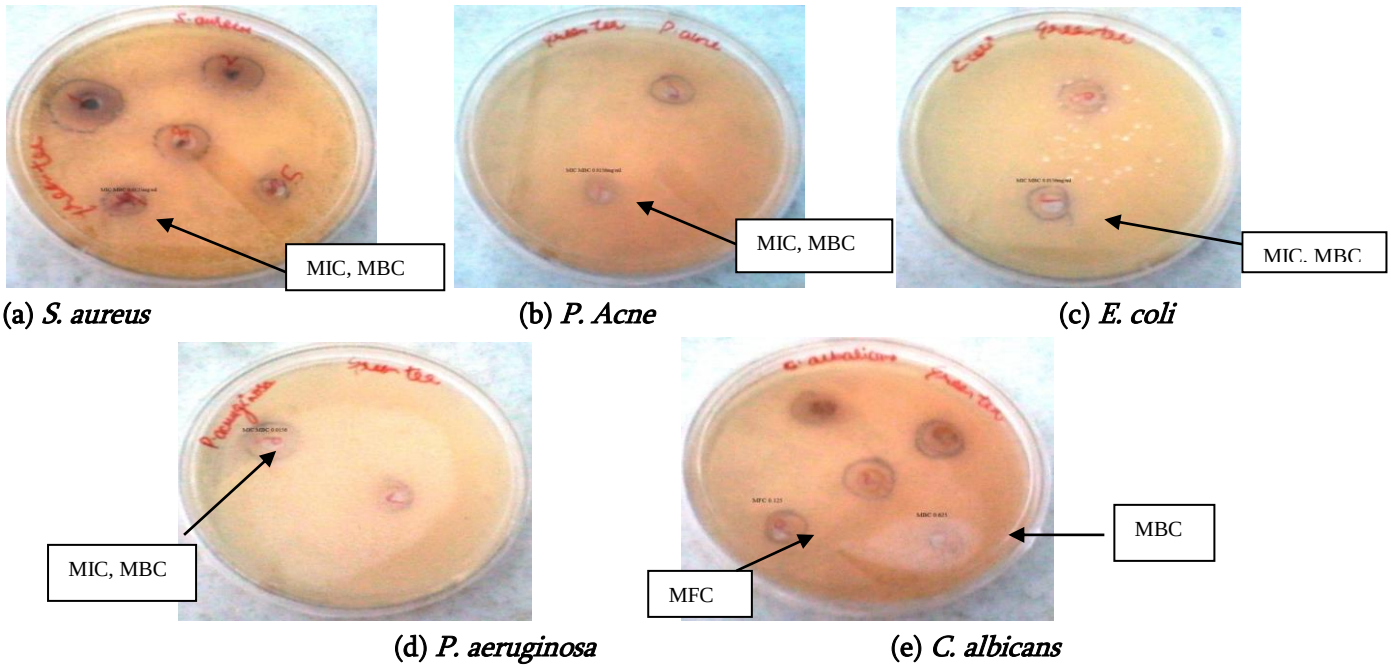


FIG.1. ANTIMICROBIAL ACTIVITY OF *CAMELLIA SINENSIS* EXTRACTS AGAINST TEST MICROORGANISMS WITH GRAPHICAL REPRESENTATION.

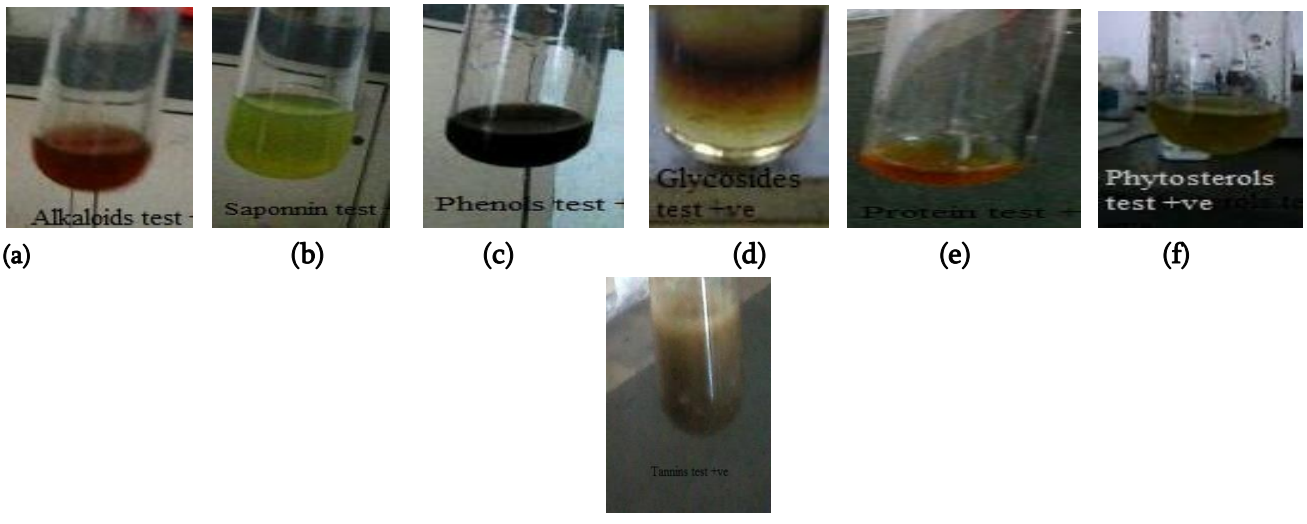


(a) *S. aureus* (b) *P. Acne* (c) *E. coli*

(d) *P. aeruginosa* (e) *C. albicans*

FIG. 2. MIC, MBC AND MFC OF METHANOLIC EXTRACT OF *CAMELLIA SINENSIS* AGAINST TEST MICROORGANISMS

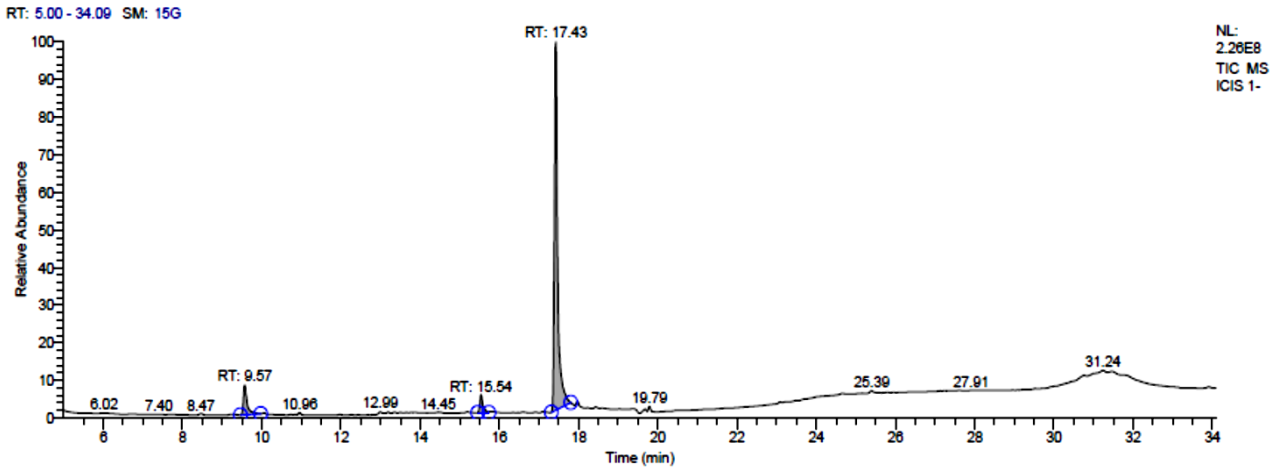
(a) *S. Aureus,* (b) *P. acne,* (c) *E. coli, (d)P. aeruginosa* and (e) *C. albicans.*



(a) (b) (c) (d) (e) (f)

(g)

FIG. 3. PHYTOCHEMICAL ANALYSIS OF METHANOLIC EXTRACTS OF *CAMELLIA SINENSIS* (a) ALKALOIDS TEST (b) SAPONIN TEST (c) PHENOL TEST (d) GLYCOSIDES TEST (e) PROTEIN TEST (f) PHYTOSTEROLS TEST (g) TANNINS TEST



Qual Peak Table

RT	Peak Area	Area %	Peak Height
9.57	120301522.28	8.10	17811458.85
15.54	58979345.32	3.97	10703312.95
17.43	1305141685.08	87.92	220902906.85

FIG 4. TOTAL ION CHROMATOGRAM (TIC) OF METHANOL EXTRACT OF *CAMELLIA SINENSIS.*