ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

Vol 8, Issue 1, 2015

ISSN - 0974-2441

Research Article

COMPARATIVE STUDY OF ANTIBACTERIAL AND ANTI-PROLIFERATIVE POTENTIAL OF GREEN TEA FROM DIFFERENT GEOGRAPHICAL LOCATIONS IN INDIA

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Received: 29 October 2014, Revised and Accepted: 22 November 2014

ABSTRACT

Objective: The aim of this study was to investigate the *in vitro* synergistic antibacterial effect of different solvent extracts of green tea (GT) with ampicillin and interpreting the anti-proliferative activity of purified GT catechins.

Methods: The methanolic, acetone, and aqueous extracts of GT leaves consumed in Assam (AT), Himachal Pradesh (HP) and Uttarakhand regions (IP and PN) of North India were used in the study. The synergistic antibacterial properties of GT with ampicillin were determined against *Pseudomonas aeruginosa* microbial type culture collection (MTCC) 4306, *Staphylococcus aureus* MTCC 6908 and *Escherichia coli* MTCC 1698 using agar well diffusion assay. The purified catechins from different GT leaves were assessed for their anti-proliferative potential on C6 glioma cell lines using 3-(4, 5-dimethyl thiazol-2-yl)-5-diphenyltetrazolium bromide (MTT) assay.

Results: The aqueous extract of different GT alone showed no inhibition against all bacterial strains used, whereas methanolic and acetone extracts revealed significant antibacterial activity ($p<0.05$). Combination of methanolic extracts of AT with ampicillin showed highest synergistic inhibition of 39±1.414 mm and 36±0.070 mm against *S. aureus* and *P. aeruginosa* respectively with an increase of 290±0.070% and 260±0.070% inhibition with respect to their individual effect. MTT assay depicted higher growth inhibition of 95.75±0.862-89.47±0.14% on treated glial cells. However, a significant difference in the inhibitory concentration (IC_{90}) of the purified catechins from different GT leaves ($p<0.05$) was observed.

Conclusion: GT leaves have potential anti-proliferative activity and synergistic antibacterial activity. Further *in vivo* and clinical studies are required to confirm its therapeutic efficacy.

Keywords: Green tea catechins, Antibacterial, Anti-proliferative, MTT assay, Antibiotic resistance.

INTRODUCTION

Antibiotics are one of the most important weapons against microbial infections in human beings. However, in recent times, these weapons have become inadequate in the treatment. The rate at which drug resistance evolves in pathogens far outpaces the rate at which new drugs are being developed and often results in adverse effects on human health [1]. Moreover, the indiscriminate use of antibiotics has alarmingly developed resistance among micro-organisms as well as led to the re-emergence of old infectious diseases. This changing pattern of antimicrobial resistance has caused demand for exploring new and effective antimicrobial agents [2].

Plants are the natural source of valuable secondary metabolites essential for normal growth and defence against microbial infection [3]. Combinational drug therapy has emerged as a powerful strategy to extend the life of current antimicrobials [4]. It acts by minimizing the evolution of drug resistance and acting more efficiently in eradication of pathogens [5]. Green tea (GT) is an herbal beverage, which has always influenced human health from generations. It is derived from leaves of *Camellia sinensis* (L.) Kuntze, which is an angiosperm dicot plant. The benefits of GT are attributed to its huge collection of catechins, which make the major contribution toward its antimicrobial and anti- proliferative activity [6]. In several studies, the antibacterial properties of GT against *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* [7], *Micrococcus luteus*, and *Bacillus cereus* [8] have been reported.

The present investigation aims at assessing the synergistic antibacterial potentials of different solvent extract of GT leaves with commercially available antibiotics and determining the anti-proliferative effect of purified GT catechins on glial tumor cells.

METHODS

GT leaves from Assam (AT), Himachal Pradesh (HP) and Uttarakhand region (IP and PN) of North India were used in the study. The leaves were washed, dried and blended into powder and kept in sealed packets in the refrigerator at 4°C until further use.

Preparation of tea extract

A total of 30 g of dried powdered leaves were soaked in 300 ml of 70% methanol, 70% acetone and water and were allowed to macerate for consecutive 2 days. After maceration, the extracts were filtered using Whatman No.1 paper and the solvents were completely evaporated. The crude extract were weighed and were kept in sterile containers at 4°C until further use [6,9,10].

Test organisms

The microorganisms in the present study were procured from microbial type culture collection (MTCC), Institute of Microbial Technology, Chandigarh. The bacterial strains used were *P. aeruginosa* MTCC 4306, *S. aureus* MTCC 6908 and *Escherichia coli* MTCC 1698. The media used for the growth and maintenance of microorganisms were nutrient broth and nutrient agar.

Antimicrobial screening

Agar well-diffusion assay

The preliminary investigation of the antimicrobial activity of methanolic, acetone and aqueous extracts of GT leaves AT, HP, IP and PN was done against three bacterial strains using agar well diffusion assay [11]. 100 µl of 24 hrs old culture of test bacterial culture (1×10^7 cfu)was swabbed on to the surface of Muller Hinton Agar (Hi-Media). Wells (2.5 mm diameter) were prepared with the help of sterile steel cork borer. 20 µl of methanolic extract of AT (1.2 mg/ml)

and 20 µl mixture of AT and ampicillin in 1:1 ratio was added into the respective wells. 10% dimethyl sulfoxide (DMSO) was used as negative control while ampicillin solution alone was used as a positive control. Duplicate plates were used in all the experiments. The plates were kept for incubation at 37°C for 24 hrs. The antibacterial effect of acetone and aqueous extracts of AT was also determined using same method. The test was also performed for HP, IP and PN solvent extracts against all the bacterial strains.

Anti-proliferative activity of GT catechins

Purified catechins

The catechins from GT leaves AT, HP, IP and PN have been isolated by solvent partitioning method as described in our previous publication [4]. Epigallocatechin gallate (EGCG) was used as a standard to compare the anti-proliferative potential of the tested purified GT catechins of our GT leaves.

Chemicals

3-(4, 5-dimethyl thiazol-2-yl)-5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), phosphate buffered saline (PBS), Dulbecco's modified eagles medium (DMEM), trypsin, ethylenediaminetetraacetic acid (EDTA), glucose and DMSO and hydrogen peroxide were obtained from Hi-media Laboratories Ltd., Mumbai.

Cell lines and culture medium

The C6 glioma cell line was obtained from National Centre for Cell Sciences (NCCS), Pune and were regularly maintained in DMEM containing 10% FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtiter plates (Tarsons India Pvt. Ltd., Kolkata, India). Cells were seeded at a density of 1×10^5 cells/ml. The culture medium was changed twice a week.

MTT assay

The cells were seeded at density of 1.0×10^5 cells/ml in 96-well flat bottomed plates, and incubated at 37°C in a humidified incubator with 5% CO_2. Two-fold dilutions of purified GT catechins from AT, HP, IP and PN at concentration of 500 µg/ml were prepared in DMEM medium. After 24 hrs, 100 µl of different test concentrations of purified catechins ranging from 500 to 7.8 µg/ml were added on to the partial monolayer in the appropriate wells and the plate was incubated for additional 22 hrs at 37°C in 5% CO_2 atmosphere. Microscopic examination was carried out and observations were noted every 2 hrs interval. After 22 hrs, the catechin solution in the wells was discarded and 20 µl of MTT (5 mg/ml) was added to each well followed by incubation for another 4 hrs at 37°C in 5% CO_2 atmosphere. The formed formazan was solubilised by adding 100 µl of 0.04 N HCL. The plate was placed on a shaker for 20 minutes, and the absorbance was measured using a microtitre plate reader (Bio-Rad Laboratories, Lucknow) at a wavelength of 565 nm [12]. The wells containing medium only served as a blank while the wells containing untreated wells were used as control in the assay. The test was performed in duplicates.

The percentage growth inhibition was calculated using the following formula:

$$\text{Percentage inhibition } (\%) = 100 - \left\{\frac{A_t - A_b}{Ac - A_b}\right\} \times 100$$

Where, A_t=Absorbance of treated wells, A_b=Absorbance of blank, A_c=Absorbance of control wells.

The minimal dilution of the catechins that induced 90% growth inhibition of the cells were obtained as inhibitory concentration (IC_{90}) which was obtained by linear regression analysis of the dose response curve generated from the absorbance data.

RESULTS

The antibacterial potentials of GT extracts alone and in combination with antibiotic were assessed against three bacterial strains; *E. coli, S. aureus* and *P. aeruginosa*. When methanolic, acetone and aqueous extracts of different GT leaves were assessed individually by agar well diffusion assay, significant difference in the inhibitory potential was observed ($p<0.05$). The methanolic extracts were

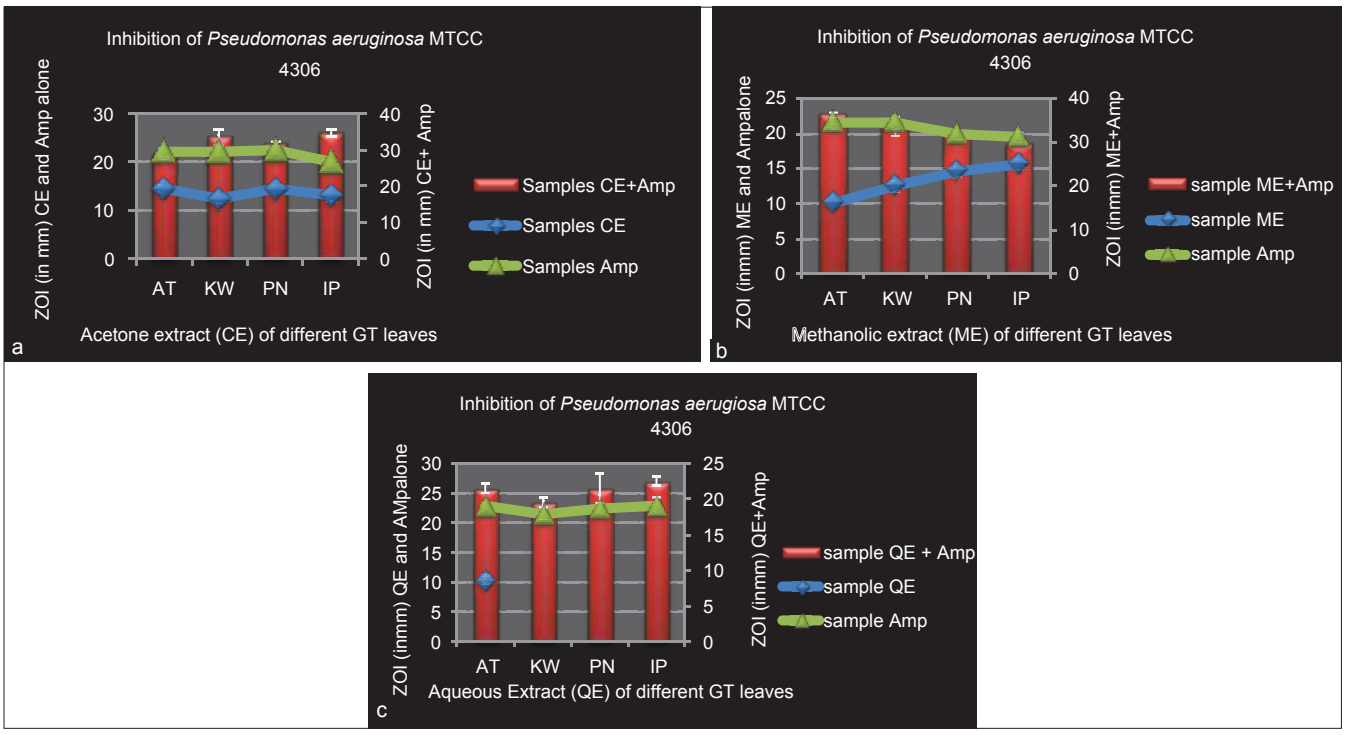

Fig. 1: (a-c) Comparative analysis of inhibitory potential of green tea solvent extracts of AT, HP, IP and PN against *Pseudomonas aeruginosa* by Agar well diffusion assay, where, CE, ME and QE are the acetone, methanolic and aqueous extracts of different GT, Amp is ampicillin

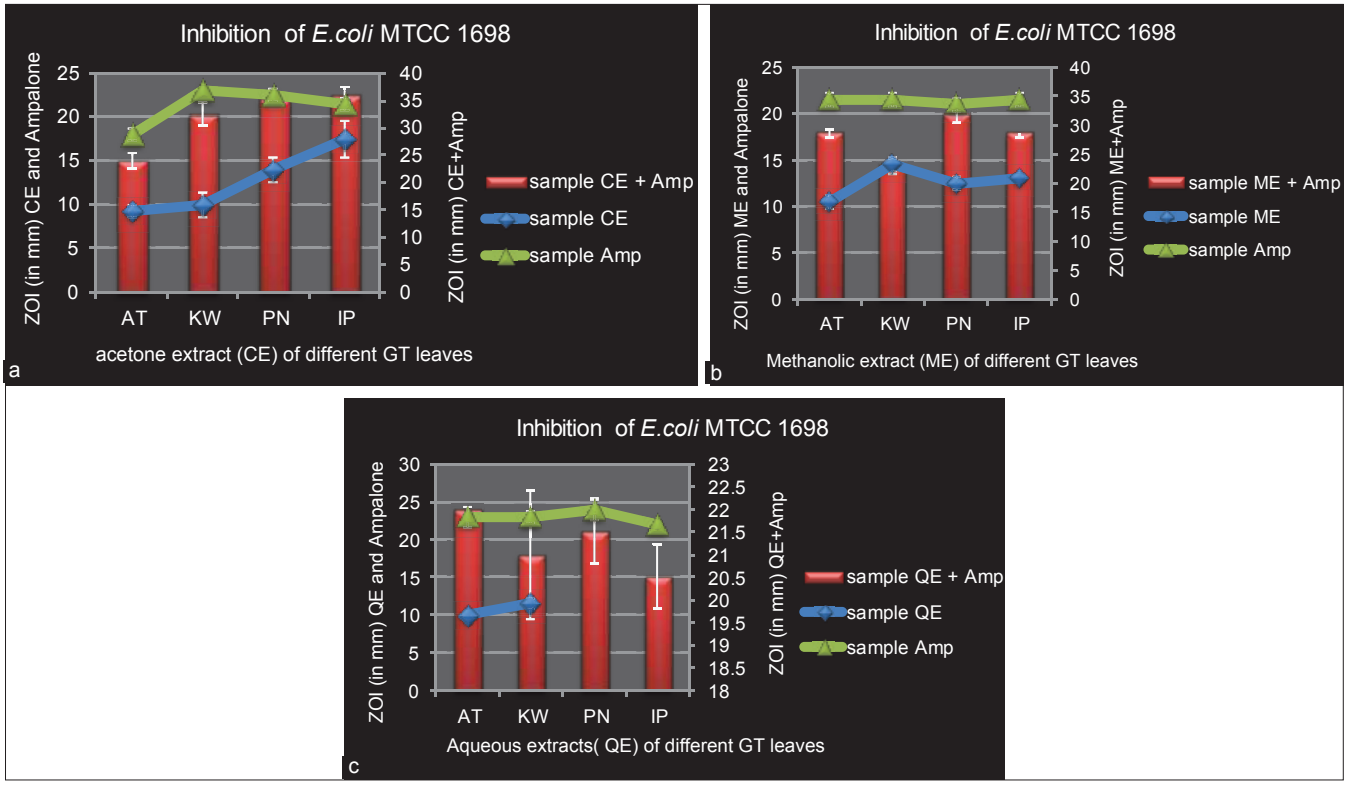

Fig. 2: (a-c) Comparative analysis of inhibitory potential of green tea (GT) solvent extracts of AT, HP, IP and PN against *Escherichia coli* by Agar well diffusion assay, where, CE, ME and QE are the acetone, methanolic and aqueous extracts of different GT, Amp is Ampicillin

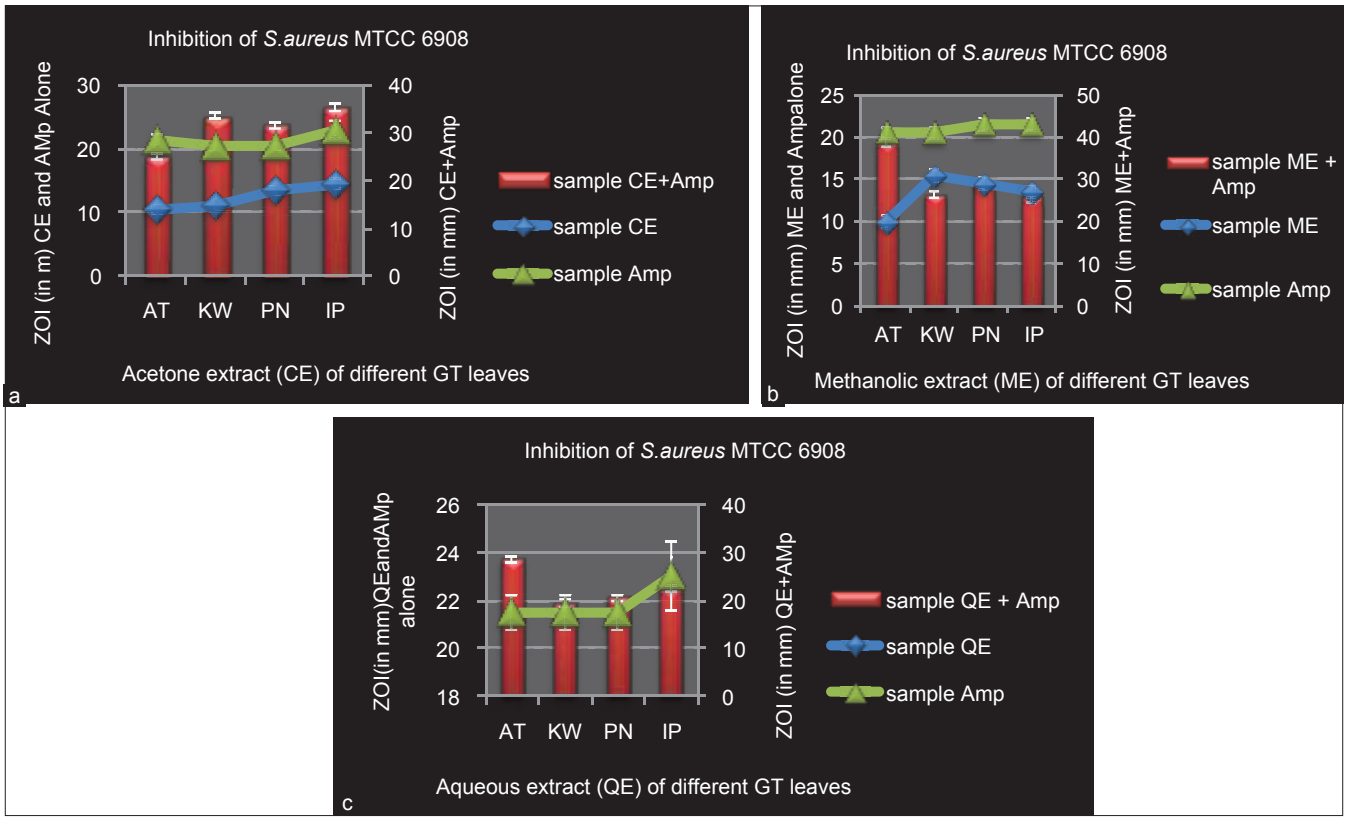

Fig. 3: (a-c) Comparative analysis of inhibitory potential of green tea (GT) solvent extracts of AT, HP, IP and PN against *Staphylococcus aureus* by Agar well diffusion assay, where, CE, ME and QE are the acetone, methanolic and aqueous extracts of different GT, Amp is ampicillin

observed as most efficient against all the tested bacterial pathogens with the zone of inhibition ranging between 15±0.707 mm and 17.5±2.12 mm. However, this zone of inhibition was less than the inhibitory zone observed as compared with the standard solution of

ampicillin (20±0.707-23±0.1.414 mm). The aqueous extracts of AT, HP, IP and PN were found to be ineffective against the tested strains.

Synergistically, high inhibitory potential was observed when the methanolic and acetone extracts of GT and ampicillin were used in combination ($p<0.05$). Remarkable increase in zone of inhibition (Figs. 1-3) was observed in the combined use of the solvent extracts and ampicillin against the tested bacterial strains. Most significant increase of 290±0.707% was observed as a result of synergistic effect of methanolic extract of AT and ampicillin against *S. aureus*. Although, the combination of aqueous extract and ampicillin showed significantly inhibition against the *S. aureus* and *Pseudomonas*, however, this significant increase in the zone of inhibition observed was lesser than the inhibitory zone observed for the standard ampicillin.

The anti-proliferative effect of EGCG and purified catechins from different GT leaves at doses of 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250 and 500 µg/ml was assessed on C6 glioma cell lines by MTT assay. The microscopic examination of the treated wells observed after 2 hrs, 5 hrs and at 22 hrs revealed the remarkable decline in cell growth pattern; hence, the presence of non viable cells was found (Figs. 4.1-4.3). The percentage inhibition was evaluated and depicted the significant difference in the concentration of the purified catechins required in achieving 90% inhibition ($p<0.05$). The IC_{90} of purified GT catechins was observed between 123.84±1.16 and 396.18±2.716 µg/ml (Fig. 5). The diluted catechins were able to decline the glioma cells by 95.756±0.862-47.366±1.484% when compared with the standard EGCG which showed 98.24±0.007-68.24±0.014% inhibition. The highest dilution of HP catechins (500 µg/ml) resulted in the maximum inhibition of 95.75±0.862%, followed by the highest dilution of AT catechins with 94.73±1.315% inhibition of the glioma tumor cells (Table 1).

DISCUSSION

The evolution of drug resistance is a ubiquitous phenomenon that threatens the utility of limited availability of antimicrobial agents and has a serious impact on human health [13]. In the present study, GT leaves from different geographical locations, extracted in different solvents showed varied antibacterial activity. The antimicrobial activities of the plant extracts and their purified fractions can be attributed to their presence of secondary metabolites which includes flavanoids, steroids, alkaloids, terpenoids, saponins and glycosides [14]. GT leaves are the rich source of flavanoids which contributes to its majority of health benefits. Catechins are the flavonoids, which occupies the maximum percentage of secondary metabolites present in GT leaves. The differences in chemical composition, geographical location of leaves collection, extraction method and method used to assess the antimicrobial activity might be the reasons, which have credited to the significant differences in inhibitory potentials of the GT leaves belonging to different regions [15].

Comparing to other teas, the lesser processing of GT accounts for its high catechin concentration, which contributes in antimicrobial and anti-proliferative properties [16].

The synergistic effect of the GT and antibiotic varies depending upon the microorganism, strains and antibiotic used [17]. The known antioxidant

Table 1: Anti-proliferative effect of GT catechins and EGCG standard

Concentration (µg/ml)	Percentage inhibition of cells (%)				
	EGCG	HPp	ATp	PNp	IMp
3.9	68.24±0.18	56.29±1.41	64.95±1.82	57.65±1.68	47.36±1.47
7.8	72.68±0.93	61.4±0.438	71.46±1.56	66.37±1.13	58.75±1.70
15.6	78.43±2.22	70.7±1.725	79.82±1.612	71.92±0.83	67.54±0.96
31.25	84.18±1.02	78.76±2.45	82.4±0.80	72.8±0.28	73.12±1.30
62.5	89.08±1.01	80.7±1.73	89.4±1.80	79.82±1.78	78.87±1.13
125	93.51±0.79	85.98±1.69	92.1±1.301	82.4±0.72	81.57±0.83
250	97.36±1.47	93.85±1.209	92.8±0.52	87.7±0.29	86.84±1.82
500	98.24±0.69	95.75±0.862	94.73±1.31	89.47±0.14	90.35±0.65

Where, EGCG is the known standard. HPp, ATp, PNp and IMp are the purified catechin residues from HP, AT, PN and IM GT leaves. GT: Green tea, EGCG: Epigallocatechin gallate

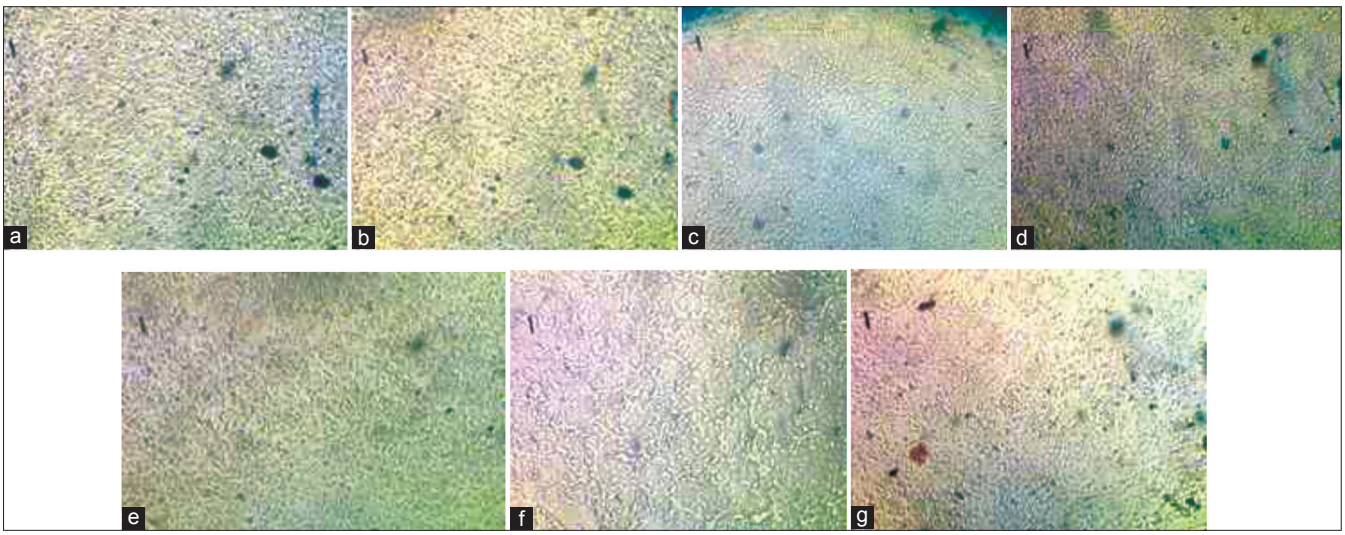

Fig. 4.1: Anti-proliferative activity of purified catechin residues of different green tea on C6 glial cell line after 2 hrs of treatment, (a) AT, (b) HP, (c) PN, (d) IP, (e) postive control, (f) blank, (g) epigallocatechin gallate standard

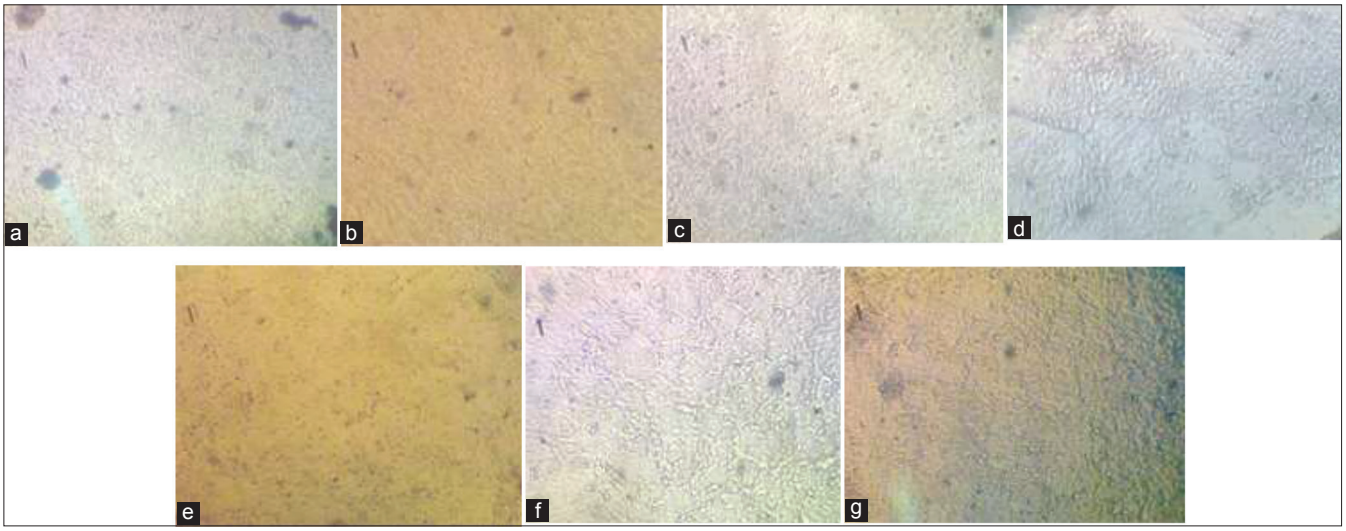

Fig. 4.2: Anti-proliferative activity of purified catechin residues of different green tea on C6 glial cell line after 5 hrs of treatment, (a) AT, (b) HP, (c) PN, (d) IP, (e) positive control, (f) blank, (g) epigallocatechin gallate standard

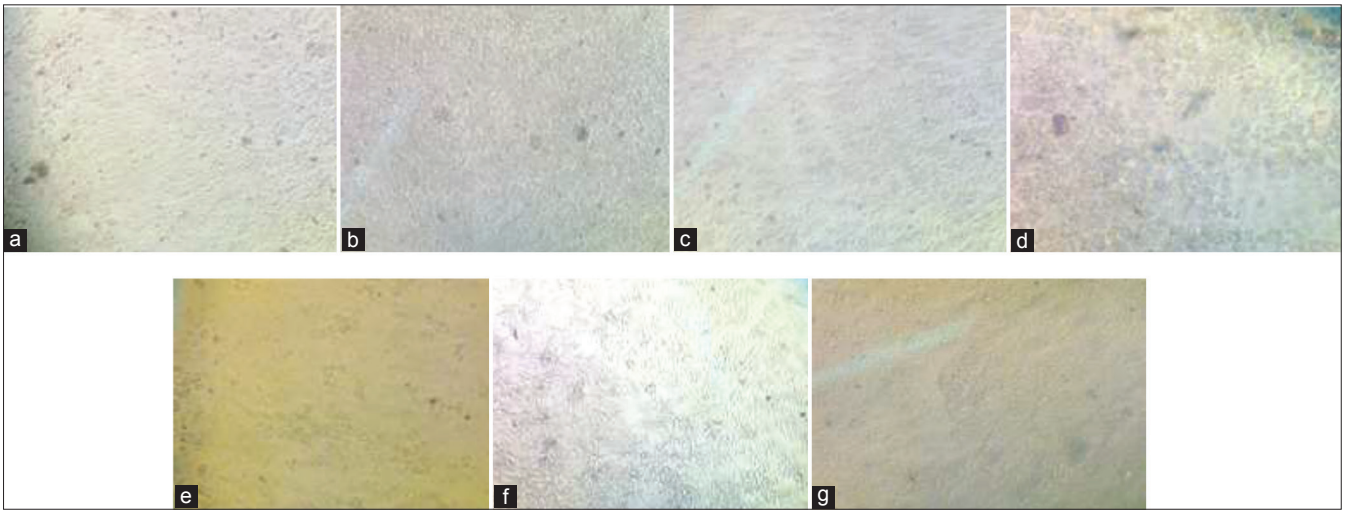

Fig. 4.3: Anti-proliferative activity of purified catechin residues of different green tea on C6 glioma cell line after 22 hrs of treatment, (a) AT, (b) HP, (c) PN, (d) IP, (e) positive control, (f) blank, (g) epigallocatechin gallate standard

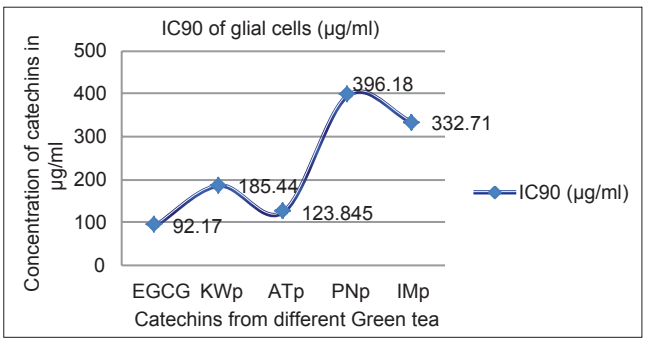

Fig. 5: Comparative analysis of inhibitory concentration (IC_{90}) dose of epigallocatechin gallate (EGCG) standard and purified green tea (GT) catechins, where, EGCG is the known standard. HPp, ATp, PNp and IMp are the purified catechin residues from HP, AT, PN and IM GT leaves

potential of GT catechins offers protection against the occurrence of cancer and several studies have explored the antioxidant effect of GT catechins on different cell lines [18]. EGCG has been explored to induce disparity between tumor cells and normal cells and have undefined phenomenon by which normal epithelial cells responds to the catechin concentration for which tumor cells undergo apoptosis [19].

CONCLUSION

Different solvent extracts of the GT leaves exhibited difference in their antibacterial and anti-proliferative activity. Methanolic extract of GT leaves (AT) exhibited the most efficient synergistic inhibitory activity with ampicillin against *S. aureus* and *P. aeruginosa.* The purified catechins from different GT depicted the significant anti-proliferative activity with growth inhibition of 95.75±0.862-89.47±0.14% on treated glioma cells. GT leaves could serve as a natural alternative with anti-proliferative activity and can synergistically overcome the antibiotic resistance in bacterial pathogens. Further *in vivo* study need to explore its anticancer potentials. Further in vivo studies as per standard guidelines are required to explore its anticancer potentials.

ACKNOWLEDGMENT

We acknowledge Department of Biotechnology, Graphic Era University, Dehradun, for providing all laboratory facilities.

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