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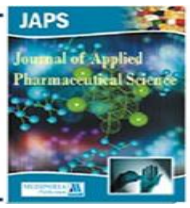

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Comparative analysis of antimicrobial activity of leaf extracts from fresh green tea, commercial green tea and black tea on pathogens

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ABSTRACT

Medicinal plants have been a major source of therapeutic agents for alleviation and cure diseases. In the present investigation comparative analysis of antimicrobial activity of green tea *Camellia sinensis* fresh leaves, commercial green tea leaves and dust tea against enteropathogens and specific fungi were carried out. The antimicrobial activity of the extracts of *Camellia sinensis* was analyzed by using well diffusion method paper disk diffusion method and minimum inhibitory concentration. Synergistic activity of green tea and commercial antibiotic chlorophenicol was analyzed. The allopathic antibacterial drugs are said to be costlier and have more side effects. Moreover multiple drug resistant strains are on the raise in this era and thus complicating treatment. On the other hand herbal preparations are comparatively cheaper and have lesser side effects. So, herbal preparations can supplement other systems of medicine for the treatment of diseases caused by bacteria and fungi.

Keywords: Antimicrobial activity, enteropathogens, synergistic activity, polyphenols.

INTRODUCTION

Green tea is non-fermented tea. The tea is an infusion of leaves that has been consumed for centuries as a beverage and is valued for its medicinal properties. The phytochemical screening of tea revealed the presence of alkaloids, saponins, tannins, catechin and polyphenols (Mbata *et al.*, 2006). Tea leaves are known for its antimicrobial activity against many microorganisms. The primary difference between green tea and black tea is in the fermentation process required to produce tea. In case of black tea the leaves and buds are fermented or oxidized after they have been dried. In green tea the leaves are steamed after they are dried. The phytochemicals present in tea leaves are highly sensitive to oxidation process. Previous works by Toda *et al.* (1989) show that moderate daily consumption of green tea killed *Staphylococcus aureus, Vibrio parahemolyticus, Clostridium perfringens, Bacillus cereus, Pleisomonas shigelloides, etc.* Green tea contains between 30 and 40 percent of water extractable polyphenols, while black tea contains between 3 and 10 percent. According to the previous studies, four polyphenol compounds, Epigallocatechin gallate (EGCG), Epicatechin gallate (ECG), Epigallocatechin (EGC) and Epicatechin (EC) are significant antioxidants constituents (Diane *et al.*, 2007). Among these EGCG is the most luxuriant component in tea extract and the most potent chemical tested for biological activity. These polyphenols may account for as much as 30% of the dry weight of fresh tea leaves. Some of the antibiotics have side effects. The synergistic antimicrobial activity of tea and antibiotics against enteropathogens are effective. The combined use of tea and antibiotics could be useful in fighting emerging drug-resistance problem especially among enteropathogens.

For example the antimicrobial effect of Chloramphenicol was 99.99% better when taken with green tea than alone. Green tea extract in combination with probiotics significantly reduced the viable count of both pathogens at 4 h and 24 h interval which had completely abolished the recovery of viable *Staphylococcus aureus* and *Streptococcus pyogenes* (Ping Su *et al.,* 2008). Kazuto Matsunaga *et al.* (2002) have reported that the tea catechin EGCG to be a potential immunomodulatory as well as antimicrobial activity. Numerous epidemiological and pharmacological studies demonstrate that green tea extract possesses strong antioxidant effects (Chang *et al.,*1999).

MATERIALS AND METHODS

Plant collection

The fresh green tea leaves used in this experiment was obtained from Ooty plantation. Commercial green tea leaves and dust tea were obtained from super market. The fresh green tea leaves were air-dried, cut into pieces and ground into powder. Commercial green tea leaves and dust tea were directly powdered.

Aqueous extraction

10g of each of the ground leaves were extracted by soaking for 2 d using 100ml of distilled water in a 250ml sterile conical flask. The extracts were filtered using Whatman filter paper No 1. The filtrates were then concentrated by using rotavapour and stored in universal bottles and refrigerated at 4°C prior to use. Aqueous extraction was carried on as preliminary study.

Methanol extraction

10g powdered samples of each were extracted with 70% methanol and 50% methanol in a successive manner to produce crude extracts containing wide range of active compounds. The extracts were prepared by maceration of the plant material with the solvents in a shaker for 2 d. The respective extracts were filtered using Whatman No.1 filter paper and dried under reduced pressure at a temperature below 45°C in rotavapour to yield a dense residue.

Test organism

The pathogens used in this experiment were obtained from Thanjavur Medical college, Thanjavur. Bacterial strains *Escherichia coli, Enterococcus faecalis, Salmonella typhi, Streptococcus aureus, Pseudomonas aeruginosa, Vibrio cholerae* and fungal strains *Fusarium, Aspergillus fumigatus, Aspergillus niger* and *Candida albicans* were used. The bacterial strains were maintained by transferring them weekly in nutrient broth and fungal strains in Sabouraud's dextrose broth on weekly basis.

Antibacterial and antifungal activity

The antibacterial and antifungal tests of the leaves extract were tested on the test strains using the agar-gel diffusion inhibition test and paper disk diffusion inhibition test. In the agar-gel diffusion test 0.2 ml of a 24 h broth culture of the bacterial and fungal test organism was aseptically introduced and evenly spread using sterile 'L' rod on the surface of sterile Mueller Hinton agar plates. Three wells of about 0.6 mm diameter were aseptically cut on agar-plate using a sterile cork borer allowing at least 30 mm between adjacent wells and between peripheral wells and the edge of the petri dish. Fixed volumes (0.1 ml) of the leaves extract were then introduced into the wells in the plates. A control well was made in the centre with the extracting solvent.

In the paper disk diffusion test, sterile paper discs were soaked in the leaves extract for 2 h. The test bacteria of 0.2 ml were spread on the surface of Mueller Hinton agar plates and the disk were placed at different areas on the plate. They were incubated at 37°C for 24h for the test bacteria and at room temperature for 2 to 4 d for fungal strains. The plates were duplicated in all the experiments.

Synergistic activity of tea and antibiotics

Antibiotic discs were soaked in leaves extract for few minutes. Bacterial and fungal cultures were swabbed on their respective plates Mueller Hinton agar plates and Sabouraud's Dextrose agar plates. Antibiotic disc chloramphenicol was used. The soaked disc was placed in the petriplate and incubated and synergistic activity was noted.

Minimum inhibitory concentration

The MIC of crude extracts was determined by broth dilution method. 5ml of sterile nutrient broth was taken in sterile test tubes and to this a loopful of test bacterial strains was inoculated. The leaf extract was added to each test tube in increasing concentration (20µl, 40µl, 60µl, 80µl, 100µl). The contents of the tubes are subjected to gentle shaking for proper mixing of the leaves extract. The test tubes were incubated at 37˚C for 24 h. A control tube was kept without the test organism.

Determination of phenolics in tea extract

Thin layer chromatographic method

The TLC sheet acts as a stationary phase support. The solvent used was petroleum ether: ethyl acetate in the ratio 7:3 respectively which acted as mobile phase. 2µl of each sample (fresh leaves, commercial tea leaves and dust tea extract), control (solvent) were taken and spot in TLC sheet and kept in a beaker containing mobile phase. This set up was kept aside for 10-15 min. After that the TLC sheet was taken out and the bands were visualized. According to the number of component present bands were noted (Amico *et al.,* 2008).

UV Spectrometric method

This method involves the analysis of absorbance by polyphenols in extract at wavelength of 725nm. 10µl of the test solution was mixed with 20µl of Folin-Ciocalteu reagent and 50µl of 25% sodium carbonate solution. The mixture was shaken thoroughly and the volume was made up to 1 ml. The mixture was allowed to stand for 1h in dark. Then the absorbance at 725nm was determined. These data were used to estimate the total phenolic content using the standard curve which was obtained using various concentrations of Gallic acid (Amico *et al.,* 2008, Singleton and Rossi 1965).

RESULTS AND DISSCUSSION

The results of the study showed that the leaves extract of *Camellia sinensis* indicates the presence of potent antibacterial activity, which confirms its use against infection. 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 organisms found to be sensitive to fresh green tea extracts were *E.coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Candida albicans* and *Pseudomonas aeruginosa* (Table 1).

Table 1. Antimicrobial activity of methanol extract of fresh green tea, commercial green tea leaves and dust tea by well diffusion method.

S.No	Organisms	Fresh green tea leaves Zone of inhibition(mm)	Commercial green tea Zone of inhibition(mm)	Dust tea Zone of inhibition(mm)
1.	*E.coli*	6	6	4
2.	*Enterococcus faecalis*	22	12	-
3.	*Salmonella typhi*	24	12	6
4.	*Staphyloccus aureus*	16	14	6
5.	*Pseudomonas aeruginosa*	12	18	-
6.	*Vibrio cholerae*	10	10	18
7.	*Fusarium*	6	18	4
8.	*Aspergillus fumigatus*	30	24	10
9.	*Aspergillus niger*	-	-	-
10.	*Candida albicans*	24	20	-

Table 2. Antimicrobial activity of methanol extract of commercial green tea, commercial green tea leaves and dust tea by minimum inhibitory concentration (mic- by broth dilution method).

S.No	Organisms	Fresh green tea leaves MIC (µl)	Commercial green tea MIC (µl)	Dust tea MIC (µl)
1.	*E.coli*	40	-	100
2.	*Enterococcus faecalis*	80	-	-
3.	*Salmonella typhi*	60	60	40
4.	*Staphyloccus aureus*	40	40	80
5.	*Pseudomonas aeruginosa*	-	-	-
6.	*Vibrio cholerae*	-	-	-
7.	*Fusarium*	-	-	-
8.	*Aspergillus fumigatus*	2	-	-
9.	*Aspergillus niger*	-	-	-
10.	*Candida albicans*	-	-	-

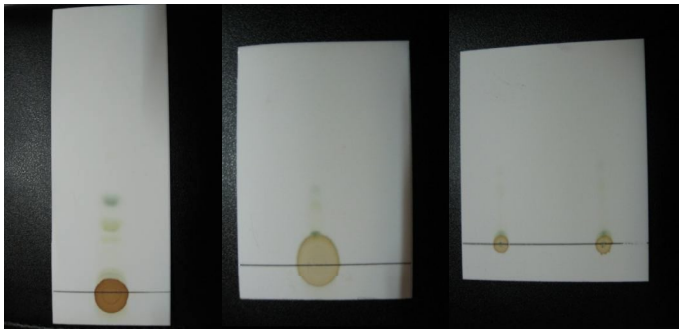
Five different concentration of MIC: 20µl, 40µl, 60µl, 80µl, 100µl)

The fresh green tea extract with methanol was found to have high antimicrobial activity followed by commercial green tea leaves and the least activity was found in dust tea.There were no significant results recorded in disk diffusion method against pathogens. In an earlier study *Candida albicans*, green tea extracts are effective only when the pH was adjusted to 6.5 (Masatomo and Kazuko 2004). The minimum inhibitory concentration of methanol extracts of fresh green tea leaves has shown good results (Table 2). Synergistic activity is effective for specific organisms like *E.coli*, *E. faecalis*, *Aspergillus fumigatus*. With dust tea *Aspergillus niger* did not show any positive results. The Minimum inhibitory concentration for dust tea is effective for *Staphylococcus aureus*, *Salmonella typhi and E.coli* which were found to be at higher concentrations when compared to fresh green tea leaves (Table 2). The combined activity of tea leaves and antibiotic chlorophenicol was found to be effective in green tea leaves followed by commercial green tea leaves and dust tea (Table 3).

Table 3. Synergistic antimicrobial activity of methanol extract of commercial green tea, commercial green tea leaves and dust tea with antibotic disc. Zone of inhibition(mm).

S.No	Organisms	Chloramphenicol Disk	Fresh green tea leaves and Chloramphenicol disk	Commercial green tea and Chloramphenicol disk	Dust tea and Chloramphenicol disk
1.	*E.coli*	16	16	15	15
2.	*Enterococcus faecalis*	24	28	26	15
3.	*Salmonella typhi*	12	16	11	10
4.	*Staphyloccus aureus*	6	12	6	6
5.	*Pseudomonas aeruginosa*	10	16	4	1
6.	*Vibrio cholerae*	-	-	3	-
7.	*Fusarium*	-	6	-	-
8.	*Aspergillus fumigatus*	14	31	25	8
9.	*Aspergillus niger*	-	-	-	-
10	*Candida albicans*	-	-	-	-

Plate 1: TLC of fresh greentea, commercial green tea and black tea extracts .



Green tea (fresh leaves) Green tea (commercial leaves) Black tea

The presence of phytochemicals in tea extracts were analyzed by thin layer chromatographic method. The solvents used were petroleum ether and ethyl acetate in the ratio 7:3 respectively which acted as mobile phase. According to the number of components (polyphenols) in tea extract, the bands were clearly visible. The results are as follows: in green tea fresh leaves extract shows prominent seven bands and three bands, green tea

commercial leaves extract shows three bands and one band in dust tea extract (Plate 1). From the results obtained it is understood that dust tea undergoes oxidation process while manufacturing and it looses the polyphenols (catechin) compounds and so only one band is seen. This can be attributed to the lesser antimicrobial activity of dust tea the concentration of these compounds is less, due to the oxidation process they have undergone.

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. The earlier works by Katsuhiro 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 presence of polyphenols was confirmed in the present study however, concentration of polyphenols did not show much variation between different green tea leaves analysed. The concentration of polyphenols in green tea fresh leaves was 105 mg/mL, 86 mg/mL for commercial green tea and 62 mg/mL for dust tea. The lowest concentration in dust tea could be attributed to the fact of oxidation during processing process.

CONCLUSION

It is hoped that this may help to avoid the side effects of antibiotics. In future, the combined use of tea and antibiotics could be also useful in fighting emerging drug-resistant problem especially among enteropathogens.

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