Antibacterial effects of green tea extracts on Aggregatibacter actinomycetemcomitans (*In-Vitro* study)

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ABSTRACT

Background: Green Tea is made from the leaf of the plant "*Camellia sinensis*". Green tea is reported to contain thousands of bioactive ingredients including catechins which have shown great promise for having antimicrobial effects. Periodontal diseases represent one of the most prevalent diseases around the world and the main etiologic factor behind it, is plaque accumulation, in addition certain kinds of bacteria have been detected frequently in subjects suffering from periodontitis, Several studies suggested that the outcome of periodontal treatment is better if particular pathogens including *Aggregatibacteractinomycetemcomitans* can no longer be detected after therapy.

Materials and Methods: plaque samples were collected from 20 patients suffering from chronic periodontitis with probing pocket depth of at least 6 mm, *Aggregatibacteractinomycetemcomitans* (A.A) was isolated and diagnosed according to morphological characteristics and biochemical tests. Green tea leaves were extracted by using water and alcohol. The first experiment involved testing the sensitivity of A.A to different concentrations of the extracts using agar well diffusion method,the second experiment involved determination of the minimum inhibitory concentration and then determination of the minimum bactericidal concentration of the extract against the bacteria, laboratory analysis of green tea extracts using high pressure liquid chromatography (HPLC) was performed.

Results: Both green tea extracts were effective in inhibition of *Aggregatibacteractinomycetemcomitans* using agar well diffusion method, 90% and 100% concentrations of alcoholic extract showed larger inhibition zones than chlorhexidinegluconate 0.2% with statistically significant difference, CHX showed higher inhibition zones than all aqueous extract concentrations.The MIC (minimum inhibitory concentration) of alcoholic green tea extract that inhibit *Aggregatibacteractinomycetemcomitans* growth was 60%, The MIC of aqueous green tea extract that inhibits *Aggregatibacteractinomycetemcomitans* growth was 70%.The MBC (minimum bactericidal concentration) of alcoholic green tea extract that kills*Aggregatibacteractinomycetemcomitans* was 80%, the MBC of aqueous green tea extract that kills *Aggregatibacteractinomycetemcomitans* growth was 90%.

HPLC analysis of aqueous and alcoholic green tea extracts revealed that alcoholic extract contained higher concentration of EGCG while aqueous extract had higher content of catechin and epicatechin.

Conclusion: Green tea extracts were effective against *Aggregatibacteractinomycetemcomitans*, alcoholic green tea extract showed inhibition ability more than the aqueous green tea extract and more than CHX and it showed bactericidal activity at 80%,90% and 100% concentrations.

Key words: green tea extracts, catechins, *Aggregatibacteractinomycetemcomitans*. (J Bagh Coll Dentistry 2015; 27(3):102-108).

INTRODUCTION

Green tea is one of the most popular beverages consumed worldwide, moreover, during the last two decades it has received much attention in regard to its beneficial effects on various human health problems [(1)]. Tea prepared from Camellia sinensis is of three types: non-fermented green tea that is pan fried or steamed and dried to inactivate its enzymes, fermented black tea and semi-fermented oolong tea. Green tea with active chemical ingredients possesses diverse pharmacological properties which are linked to lower incidence of some pathological conditions including oral cancer, dental caries, stroke, cardiovascular diseases and obesity [(1-3)].

The health-promoting effects of green tea are mainly attributed to its polyphenol contents commonly referred to as catechins. There are four main types of catechins: epigallocatechin-3-gallate (EGCG), epigallocatechin, epicatechin-3-gallate and epicatechin [(2)].

The polyphenol contents of green tea have been reported to inhibit varieties of pathogenic bacterial growth such as *Helicobacter pylori, methicillin-resistant staphylococcus aureus, streptococcus mutans, streptococcus sobrinus, salmonella typhi, shigella dysentery, shigellaflexneri and vibrocholera*[(2, 4,5,6,7,8)]

Periodontal disease and bacteria:

Periodontitis is a chronic slowly progressive polymicrobial infectious disease which affects the entire tooth-supporting tissues. This infection is characterized by destruction of alveolar bone, periodontal ligaments and gingival pocket formation which consequently leads to tooth loss. Periodontitis is known to be caused by subgingival plaque bacteria including *Aggrecatibacteractinomycetemcomitans, Prevotellaintermedia, Porphyromonasgingivalis, Tannerellaforsythus*and*fusobacterium*species. These bacteriaare frequently isolated from

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gingival pocket and subgingival plaques of patients with periodontitis [9].

During the last two decades, it has been shown that *Aggrecatibacteractinomycetemcomitans* can be regarded as a major pathogen in destructive periodontal diseases [10-12], it was also found that A.A is associated with systemic diseases [13].

Porphyromonasgingivalis which is a member of the highly investigated black pigmented bacteroids, it comprises high proportion of the subgingivalmicrobiota in periodontal pockets [11,14].

Several studies suggested that the outcome of periodontal treatment is better if particular pathogens especially *Aggrecatibacteractinomycetemcomitans* and *Porphyromonasgingivalis* can no longer be detected after therapy [15-19].

However despite the fact that non-surgical mechanical periodontal treatment as well as self performed plaque control are effective in reducing the numbers of *Aggrecatibacteractinomycetemcomitans* and *Porphyromonasgingivalis* at periodontal sites, these micro-organisms re-establish themselves rapidly in most subjects [20].

In the present study wewill investigate the inhibitory activity of green tea extract on some clinically isolated periodontopathic bacteria which is *Aggrecatibacteractinomycetemcomitans*.

MATERIALS AND METHODS:

Human sampling:

Plaque samples were collected from twenty systematically healthy patients suffering from chronic periodontitis, the plaque samples were taken from periodontal pockets with probing pocket depth (PPD) of at least six mm depth,(PPD) was measured from the gingival margin to the most apical extent of the periodontal pocket, the plaque samples were obtained from the deepest part of the periodontal pocket using a sterilized curette.

The collected plaque is put on a swab that is inserted immediately into a transfer media to preserve the sample , then the sample was spread on blood agar media and incubated anaerobically using anaerobic jar and anaerobic gas bags in the incubator for 72 hours within a period of less than 30 minutes from taking the sample from the patient.

Extraction procedures to obtain green tea extracts:

1- Aqueous extract: 100 grams of dry green tea leaves were put in a glass jar then 500ml of distilled water were added afterwards the glass jar was put in water bath (50° C) for two hours then it was left over night at room temperature, the next morning filtration was done first using gauze to get rid of the large particles of green tea leaves then the resultant liquid was filtered using a sterile Whatman filter paper No1., The filtered extract was concentrated under vacuum below 40°C using a rotaevaporator for five hours [21].

2-Alcoholic extract: 100 grams of dry green tea leaves were put in a glass jar then 500ml of alcohol (96% ethanol alcohol) were added, the infusion was put in a shaker for 48 hours after that filtration was done first using gauze to get rid of the large particles of green tea leaves then the resultant liquid was filtered using a sterile Whatman filter paper No1., The filtered extract was concentrated under vacuum below 40°C using a rotaevaporator for one hour[21].

Both extracts were kept in tightly closed screw bottles and kept in the refrigerator.

Identification and Isolation of microorganisms:

Both micro-organisms were identified according to their morphological characteristic, Gram stain, biochemical tests and their antibiotic sensitivity.

Experiment no.1:

Sensitivity of A.A to different concentrations of alcoholic and aqueous green tea extracts in vitro:

The concentrations of alcoholic green tea extract used in this experiment were: (10%,20%,30%,40%,50%,60%,70%,80%,90%,100%).

The concentrations of aqueous green tea extract used in this experiment were: (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%). CHX gluconate (0.2%) was used in this experiment as a positive control D.W (distilled water) was used in this experiment as a negative control.

Agar well diffusion method was used, using a sterile loop, three colonies were picked up and spread on blood agar plate in a mattress fashion, then wells of equal size and depth will be prepared in the agar using Pasteur pipette under aseptic conditions, afterwards each well was filled with the selected agent(100 microliter) then the plates were incubated anaerobically for 48 hours. Inhibition zone represents the clear zone across the diameter of each well where no bacterial growth is present. The inhibition zones were measured in millimeters using a ruler.

Experiment no.2:

Determination of MIC (minimum inhibitory concentration) and MBC (minimum

bactericidal concentration) of alcoholic and aqueous green tea extracts against A.A:

First serial dilution method was performed in order to standardize the bacterial inoculums. Appendroff tubes were labeled and arranged in a rack, 100 µl of bacterial suspension (10^3 concentration) were added to each tube then 50ul of the tested agent were added to its designated tube. Then the tubes were incubated anaerobically for 72 hours.

After 72 hours the tubes were examined to see if there was any turbidity (turbidity indicates bacterial growth), the tubes that showed signs of turbidity were excluded while the tubes that lack turbidity were identified as the minimum inhibitory concentration.

C-Determination of MBC:

The tubes that were identified as the MIC were then subcultured in order to determine the MBC,150µl were taken from each tube using a micropipette and then spread on a blood agar plate using a sterile spreader and incubated anaerobically for 48 hours.

After 48 hours the plates were taken out and examined to see if there was any bacterial growth, the plates that showed no growth were identified as minimum bactericidal concentration.

Experiment no.3:

HPLC determination of green tea extracts (aqueous and alcoholic):

Both extracts were analyzed by HPLC. The samples were dissolved in water and ethanol and compared with standard Figure, the analysis performed on Shimadzu (Koyota,Japan) binary system HPLC LC-10A equipped with Shimadzu LC 10A UV spectrophotometer. The active compounds of green tea extracts were separated on FLC (Fast liquid Chromatographic) column (C 18), 3um particle size (50x4.6 mm I.D) supelco CN column, mobile phase were: 0.1% acetic acid in deionized water: acetonitrile 80:20 V/V. Detection UV set at 280 nm, flow rate 1.2 ml/min.

To calculate concentration of each constituent of water and alcohol extract this formula was used:

Concentration of sample µg /ml= area of sample/ area of standard x Concentration of standard x dilution factor.

Concentration of standard=25 mg/ml.

Dilution factor= 4 times.

RESULTS

The means of inhibition zones of the different concentrations of alcoholic and aqueous extracts are presented in figure (3.16) it clearly shows that alcoholic extract showed higher inhibition zones than aqueous extract in all concentrations.

Table 1: Descriptive statistics of inhibition zone (mm.) on AA bacteria using different types and concentrations of green tea extract and +ve and –ve control and their difference

Conc.	Inhibition zone with Alcoholic extract of green tea with +ve and –ve control				Inhibition zone with Aqueous extract of green tea with +ve and –ve control				Difference (d.f.=28)	
	Mean	S.D.	Min.	Max.	Mean	S.D.	Min.	Max.	t-test	p-value
10%	8.07	0.26	8	9	6.33	1.05	5	8	6.228	0.000 (HS)
20%	8.40	0.51	8	9	7.67	0.62	7	9	3.556	0.001 (HS)
30%	10.40	1.18	8	12	10.27	0.96	9	12	0.339	0.737 (NS)
40%	13.73	0.96	12	15	12.33	1.45	10	15	3.121	0.004 (HS)
50%	15.27	0.46	15	16	13.60	1.35	12	16	4.521	0.000 (HS)
60%	15.73	0.46	15	16	14.33	1.05	13	16	4.747	0.000 (HS)
70%	18.40	0.51	18	19	15.07	1.28	14	17	9.378	0.000 (HS)
80%	19.53	0.52	19	20	16.33	1.63	13	18	7.236	0.000 (HS)
90%	20.47	0.52	20	21	17.53	0.74	16	19	12.553	0.000 (HS)
100%	20.47	0.52	20	21	17.60	0.63	17	19	13.598	0.000 (HS)
CHX	19.07	0.70	18	20	19.13	0.74	18	20	-0.252	0.803 (NS)
D.W.	0	0	0	0	0	0	0	0	-	-

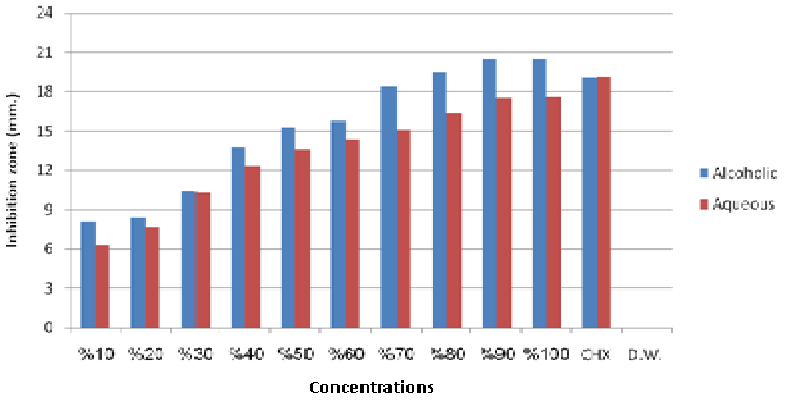

Figure 1: mean values of inhibition zones of alcoholic and aqueous extracts with +ve and –ve controls against A.A

Table 2: LSD test after ANOVA test

		Alcoholic extract of green tea with +ve and –ve control		Aqueous extract of green tea with +ve and –ve control	
		Mean Difference	p-value	Mean Difference	p-value
10%	20%	-0.333	0.143 (NS)	-1.333	0.001 (HS)
	30%	-2.333	0.000 (HS)	-3.933	0.000 (HS)
	40%	-5.667	0.000 (HS)	-6.000	0.000 (HS)
	50%	-7.200	0.000 (HS)	-7.267	0.000 (HS)
	60%	-7.667	0.000 (HS)	-8.000	0.000 (HS)
	70%	-10.333	0.000 (HS)	-8.733	0.000 (HS)
	80%	-11.467	0.000 (HS)	-10.000	0.000 (HS)
	90%	-12.400	0.000 (HS)	-11.200	0.000 (HS)
	100%	-12.400	0.000 (HS)	-11.267	0.000 (HS)
	CHX	-11.000	0.000 (HS)	-12.800	0.000 (HS)
	D.W.	8.067	0.000 (HS)	6.333	0.000 (HS)
20%	30%	-2.000	0.000 (HS)	-2.600	0.000 (HS)
	40%	-5.333	0.000 (HS)	-4.667	0.000 (HS)
	50%	-6.867	0.000 (HS)	-5.933	0.000 (HS)
	60%	-7.333	0.000 (HS)	-6.667	0.000 (HS)
	70%	-10.000	0.000 (HS)	-7.400	0.000 (HS)
	80%	-11.133	0.000 (HS)	-8.667	0.000 (HS)
	90%	-12.067	0.000 (HS)	-9.867	0.000 (HS)
	100%	-12.067	0.000 (HS)	-9.933	0.000 (HS)
	CHX	-10.667	0.000 (HS)	-11.467	0.000 (HS)
	D.W.	8.400	0.000 (HS)	7.667	0.000 (HS)
30%	40%	-3.333	0.000 (HS)	-2.067	0.000 (HS)
	50%	-4.867	0.000 (HS)	-3.333	0.000 (HS)
	60%	-5.333	0.000 (HS)	-4.067	0.000 (HS)
	70%	-8.000	0.000 (HS)	-4.800	0.000 (HS)
	80%	-9.133	0.000 (HS)	-6.067	0.000 (HS)
	90%	-10.067	0.000 (HS)	-7.267	0.000 (HS)
	100%	-10.067	0.000 (HS)	-7.333	0.000 (HS)
	CHX	-8.667	0.000 (HS)	-8.867	0.000 (HS)
	D.W.	10.400	0.000 (HS)	10.267	0.000 (HS)
40%	50%	-1.533	0.000 (HS)	-1.267	0.001 (HS)
	60%	-2.000	0.000 (HS)	-2.000	0.000 (HS)
	70%	-4.667	0.000 (HS)	-2.733	0.000 (HS)
	80%	-5.800	0.000 (HS)	-4.000	0.000 (HS)
	90%	-6.733	0.000 (HS)	-5.200	0.000 (HS)
	100%	-6.733	0.000 (HS)	-5.267	0.000 (HS)
	CHX	-5.333	0.000 (HS)	-6.800	0.000 (HS)
	D.W.	13.733	0.000 (HS)	12.333	0.000 (HS)

50%	60%	-0.467	0.031 (S)	-0.733	0.062 (NS)
	70%	-3.133	0.000 (HS)	-1.467	0.000 (HS)
	80%	-4.267	0.000 (HS)	-2.733	0.000 (HS)
	90%	-5.200	0.000 (HS)	-3.933	0.000 (HS)
	100%	-5.200	0.000 (HS)	-4.000	0.000 (HS)
	CHX	-3.800	0.000 (HS)	-5.533	0.000 (HS)
	D.W.	15.267	0.000 (HS)	13.600	0.000 (HS)
60%	70%	-2.667	0.000 (HS)	-0.733	0.062 (NS)
	80%	-3.800	0.000 (HS)	-2.000	0.000 (HS)
	90%	-4.733	0.000 (HS)	-3.200	0.000 (HS)
	100%	-4.733	0.000 (HS)	-3.267	0.000 (HS)
	CHX	-3.333	0.000 (HS)	-4.800	0.000 (HS)
	D.W.	15.733	0.000 (HS)	14.333	0.000 (HS)
70%	80%	-1.133	0.000 (HS)	-1.267	0.001 (HS)
	90%	-2.067	0.000 (HS)	-2.467	0.000 (HS)
	100%	-2.067	0.000 (HS)	-2.533	0.000 (HS)
	CHX	-0.667	0.002 (HS)	-4.067	0.000 (HS)
	D.W.	18.400	0.000 (HS)	15.067	0.000 (HS)
80%	90%	-0.933	0.000 (HS)	-1.200	0.002 (HS)
	100%	-0.933	0.000 (HS)	-1.267	0.001 (HS)
	CHX	0.467	0.031 (S)	-2.800	0.000 (HS)
	D.W.	19.533	0.000 (HS)	16.333	0.000 (HS)
90%	100%	0	1 (NS)	-0.067	0.865 (NS)
	CHX	1.400	0.000 (HS)	-1.600	0.000 (HS)
	D.W.	20.467	0.000 (HS)	17.533	0.000 (HS)
100%	CHX	1.400	0.000 (HS)	-1.533	0.000 (HS)
	D.W.	20.467	0.000 (HS)	17.600	0.000 (HS)
CHX	D.W.	19.067	0.000 (HS)	19.133	0.000 (HS)

Experiment no.2: Determination of minimum inhibitory and minimum bactericidal concentrations of aqueous and alcoholic green tea extracts against A.A and P.G:

1-Determination ofMinimum inhibitory concentration (MIC):

The MIC for alcoholic green tea extract that inhibit *Aggregatibacteractinomycetemcomitans* growth was 60%.The MIC for aqueous green tea extract that inhibits *Aggregatibacteractinomycetemcomitans* growth was 70%.

2-**Determination ofMinimum bactericidal concentration (MBC)**:

The MBC for alcoholic green tea extract that kills *Aggregatibacteractinomycetemcomitans* was 80%.The MBC for aqueous green tea extract that kills *Aggregatibacteractinomycetemcomitans* growth was 90%.

Experiment no.3: HPLC determination of green tea extracts (aqueous and alcoholic):

Table 3: descriptive data for concentration of each constituent of aqueous and alcoholic green tea extracts:

Subjects	Conc. of standard	Conc. of water extract/ µg	Conc. of alcoholic extract/ µg
Caffeine	25	72.19	141.63
Epicatechin	25	123.73	101.15
Epicatechingallate (ECG)	25	183.37	111.36
Epigallocatechingallate (EGCG)	25	132.13	174.96

By viewing the results of HPLC analysis of both extracts, it was revealed that alcoholic extract had higher content of epigallocatechingallate (EGCG) which is the main active polyphenol in green tea, while aqueous extract had higher content of epicatechin and epicatechingallate.

DISCUSSION

There are four main catechins found in green tea: epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG).Three of these (ECG, EGC and EGCG) have shown to have antimicrobial effects against a variety of organisms [22]. The results of studies on the antimicrobial effects of green tea have shown that the potential for preventive and therapeutic purposes is present.

The search for alternative antibacterial compounds has been a major concern in recent years because some of the drugs used haveadverse effects and high cost. It was shown that herbs exhibit biochemical and pharmacological activities and can be used as mouth rinses [23], resistance also develops more slowly with natural products [24].

Sensitivity of *Aggregatibacteractinomycetemcomitans* to different concentrations of green tea extracts (alcoholic and aqueous) in vitro (agar well diffusion):

Results showed that alcoholic and aqueous green tea extracts were able to inhibit the growth of A.A, this finding was in coincidence with other studies [25-27].

The diameter of inhibition zones were increased as the concentration of both green tea extracts increased from 10% to 90%, this was in agreement with

It was reported that that increasing concentration of green tea would increase the inhibition of bacterial growth and the highest concentration created the largest zone of inhibition [28].

Alcoholic extract 80%, 90% and 100% concentrations showed larger inhibition zones than chlorhexidine , and by using LSD test 80% conc. showed significant difference, 90% and 100% conc. showed highly significant difference which suggests that they have shown higher antimicrobial activity than chlorhexidine. This finding presents a great promise to use green tea extract as an alternative to chlorhexidine.

Meanwhile chlorhexidine showed larger inhibition zones than all aqueous extract concentrations.

Recent studies revealed that EGCG exhibited strong antimicrobial abilities, the direct antimicrobial effects of green tea have been attributed to EGCG and that EGCG is the most abundant catechin in green tea [29]. ECg and EGCg strongly inhibited the cytotoxic effects of Aggregatibacteractinomycetemcomitans–lipo-poly saccharide on each cell [27].

It was stated that initial screenings of plants for possible antimicrobial activities typically begin by using crude aqueous or alcohol extractions and can be followed by various organic extraction methods. Since nearly all of the identified components from plants active against microrganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction and this may explain the greater antimicrobial activity exhibited by the alcoholic extract compared to the aqueous extract [21].

High EGCG concentrations irreversibly damage the bacterial cytoplasmic membrane by generating hydrogen peroxide within the bilayer or by inhibiting the cytoplasmic enzymes and type II fatty acid synthesis system [30-32].

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