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# Antimicrobial effects of green tea extracts on Porphyromonas Gingivalis (in vitro study)

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***Abstract :***
***Background:*** *Periodontal diseases represent one of the most prevelant 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 Porphyromonas gingivalis can no longer be detected after therapy. Green Tea is reported to contain thousands of bioactive ingredients including catechins which have shown great promise for having antimicrobial effects.There are four main catechins in green tea: epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG). This study investigates the inhibitory activity of green tea extracts on Porphyromonas gingivalis .*
***Materials and Methods:*** *plaque samples were collected from patients suffering from chronic periodontitis with probing pocket depth of at least 6mm, Porphyromonas gingivalis was isolated and diagnosed according to morphological characteristic and biochemical test. Green tea leaves were extracted using water and alcohol.*
*Sensitivity of Porphyromonas gingivalis to different concentrations of the extracts was tested using agar well diffusion method, determination of the minimum inhibitory concentration and minimum bactericidal concentrations against Porphyromonas gingivalis by serial microdilution in broth media. And analysis of the extracts was performed using high performance liquid chromaticity (HPLC) technique.*
***Results:*** *Both green tea extracts were effective in inhibition of Porphyromonas gingivalis growth on agar plates but the alcoholic extract showed larger inhibition zones and contained higher percentage of epigallocatechin-3-gallate (EGCG) and it showed non-significant difference compared to chlorhexidine.,90%cconcentration of both extracts were able to demonstrate bactericidal activity against Porphyromonas gingivalis.*
***Conclusions :*** *alcoholic green tea extract was able to inhibit and kill Porphyromonas gingivalis and had higher concentration of EGCG than the aqueous extract, further studies should focus on the use of alcoholic green tea extract in treatment of periodontal disease.*
***Keywords:*** *periodontal disease, Porphyromonas gingivalis ,green tea extracts, catechins, EGCG.*

## I. Introduction

Green tea is one of the most popular beverages consumed worldwide, during the last two decades it has received much attention in regard to its beneficial effects on various human health problems [1].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 [1,2,3].

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 .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, shigella flexneri and vibrocholera [3,4,5,6,7,8].

Periodontitis is a chronic slowly progressive polymicrobial infectious disease which affects the entire tooth-supporting tissues. It is known to be caused by subgingival plaque bacteria including Aggrecatibacter actinomycetemcomitans, Prevotella intermedia, Porphyromonas gingivalis, Tannerella forsythus and fusobacterium species. These bacteria are frequently isolated from gingival pocket and subgingival plaques of patients with periodontitis[9].

Porphyromonas gingivalis which is a member of the highly investigated black pigmented bacteroids, it comprises high proportion of the subgingival microbiota in periodontal pockets [10,11].

Several studies suggested that the outcome of periodontal treatment is better if particular pathogens Porphyromonas gingivalis can no longer be detected after therapy [12,13,14,15,16].

Effective prevention of periodontal disease could be achieved by proper and regular tooth brushing, flossing and rinsing with mouthwashes containing antibacterial agents such as chlorhexidine, sodium hypochlorite, cetylpyridinium chloride and amine fluoride. Moreover, chlorhexidine gluconate was shown to have undesirable side effects such as staining, burning sensation and promoting calculus formation [17]. Considering the potential disadvantages and side effects of these chemical agents, there is a need for more agents with marked antibacterial activity and less toxicity to be used as mouthwashes and irrigating agents. During the last decade much attention has been given to the antimicrobial activities of medicinal plants and their extracts to be consumed as useful alternatives to synthetic chemical agents [18,19,20,21].

## II. materials and method

This study involved two experiments in vitro, the first experiment concerning the effects of aqueous and alcoholic green tea extracts on the sensitivity of Porphyromonas gingivalis; while the second experiment involved determination of minimum inhibitory concentration and minimum bactericidal concentration of green tea extracts against Porphyromonas gingivalis .The study also involved laboratory analysis of aqueous and alcoholic green tea extracts using high performance liquid chromaticity (HPLC) technique.

### II.1 Human sampling:

Plaque samples were collected from 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, the plaque samples were obtained from the deepest part of the periodontal pocket using a sterilized curette.The collected plaque was put on a swab that was 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 for 72 hours within a period of less than 30 minutes from taking the sample from the patient.

The plaque samples were collected from patients attending the clinic at the department of Periodontics in the teaching hospital of Dentistry College / Baghdad University, the patients were informed about the study and patients' consents and approvals were obtained prior to collecting the samples.

### II.2 Extraction procedures to obtain green tea extracts:

**1- Aqueous extract:** 100 grams of dry green tea leaves were diffused in 500ml of distilled water then put in water bath ($50^oC$) for two hours and 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 $40^oC$ using a rotaevaporator for five hours.

**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 $40^oC$ using a rotaevaporator for one hour .

### II.3 Identification and Isolation of the micro-organism:

The micro-organism (Porphyromonas gingivalis) was identified according to the morphological characteristic, Gram stain, biochemical tests and antibiotic sensitivity.

### II.4 Sensitivity of Porphyromonas gingivalis to different concentrations of alcoholic and aqueous green tea extracts in vitro:

The concentrations of green tea extracts 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, afterwards each well was filled with the selected agent(100 microliter) then the plates were incubated anaerobically for 48 hours. The inhibition zones were measured in millimeters using a ruler.

**II.5 Determination of MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) of alcoholic and aqueous green tea extracts against Porphyromonas gingivalis:**

First serial dilution method was performed in order to standardize the bacterial inoculums

B- Determination of MIC:

Appendroff tubes were labeled and arranged in a rack, 100 µl of bacterial suspension ($10^3$ concentration) were added to each tube then 50 µl 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, 150 µl 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.

**II.6 HPLC analysis of green tea extracts (aqueous and alcoholic):**

The analysis performed on Shimadzu 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.

## III. Calculation:

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.**

**II.7 Statistical analysis:**

Data processing and analysis were carried out by using SPSS program, which provide the following:-

**1**-Calculation and presentation of statistical parameters: mean and SD of the variables in the study.

**2**- Student t-test and analysis of variance (ANOVA) for testing the significant differences among means of different groups.

**3**- For all the above mentioned tests, the analysis was accepted at $p<0.05$, as the limit of significance, when $p<0.001$ were regarded as highly significance.

## IV. Results

Aqueous and alcoholic green tea extracts showed increase in the diameter of their inhibition zones as their concentrations were increased. 90% and 100% concentrations of alcoholic extract showed the same results mean of inhibition zone for both concentrations was 19.40mm, 90% and 100% of aqueous extract also showed the same results (mean of inhibition zone for 90% and 100% concentrations was 16.73mm). Alcoholic extract showed larger inhibition zones than aqueous extract fig.1. Comparing the inhibition zones between alcoholic and aqueous extract for each concentration starting from 10% to 100% there was highly significant difference. Least significant difference (LSD) test is illustrated in table (1) which demonstrates the main finding of this study which was that 90% and 100% concentration of the alcoholic extract showed non-significant difference when compared to CHX which suggests similar antibacterial activity against Porphyromonas gingivalis .

The MIC of the alcoholic extract against Porphyromonas gingivalis was 60% while for the aqueous extract was 80%. MBC of the alcoholic extract against Porphyromonas gingivalis was 90%, MBC of the aqueous extract was also 90%.

HPLC analysis of both extract revealed that the alcoholic extract contained higher concentration of epigallocatechingallate (EGCG) which is the main active polyphenol in green tea against a wide range of micro-organisms.

## V. Discussion

The search for alternative antibacterial compounds has been a major concern in recent years because some of the drugs used have adverse effects . It was shown that herbs exhibit biochemical and pharmacological activities and can be used as mouth rinses [22], resistance also develops more slowly with natural products [23].

Effective prevention of periodontal disease could be achieved by proper and regular tooth brushing, flossing and rinsing with mouthwashes containing antibacterial agents such as chlorhexidine, sodium hypochlorite, cetylpyridinium chloride and amine fluoride. Moreover, chlorhexidine gluconate was shown to have undesirable side effects such as staining, burning sensation and promoting calculus formation [17]. Considering the potential disadvantages and side effects of these chemical agents, there is a need for more agents with marked antibacterial activity and less toxicity to be used as mouthwashes and irrigating agents. During the last decade much attention has been given to the antimicrobial activities of medicinal plants and their extracts to be consumed as useful alternatives to synthetic chemical agents .

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 other studies that supported the hypothesis that increasing concentration of green tea would increase the inhibition of bacterial growth and the highest concentration created the largest zone of inhibition [24].

when both extracts used in this study were analyzed using HPLC technique, alcoholic extract contained higher concentration of epigallocatechin gallate (EGCG), recent studies revealed that EGCG exhibited strong antimicrobial abilities,stated that the direct antimicrobial effects of green tea have been attributed to EGCG and that EGCG is the most abundant catechin in green tea [25].

Both green tea extracts demonstrated bactericidal activity in high concentrations (90% and 100% concentrations), studies on the antimicrobial effects of green tea catechins on periodonto-pathogens haverevealed that the catechins showed bactericidal activity against black pigmented organism at a concentration of 1 mg/ml [26], the direct antimicrobial effects of catechins include: damage to the bacterial cell membrane ,inhibition of fatty acid synthesis and inhibition of enzyme activity [27].

## VI. Conclusion

The use of plants extracts presents a great alternative to chemical drugs. The fact that alcoholic green tea extract have shown similar antibacterial abilities to chlorhexidine against the periodontal pathogen Porphyromonas gingivalis suggests the possibility if using this extract in treating periodontitis and may provide alternative to CHX which can lead to more patient motivation and compliance since CHX had shown adverse effects such as staining and burning sensation. Further research should focus on using alcoholic green tea extract as a mouthwash or using it in local delivery systems to improve the periodontal health.

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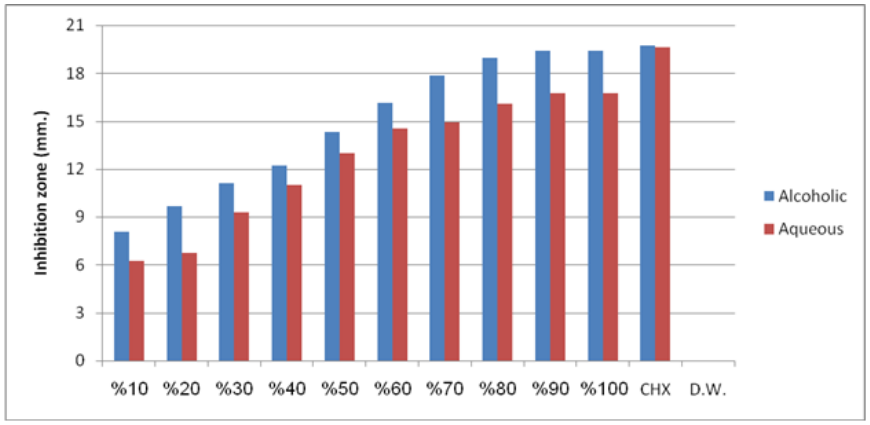


**Figure (1) mean values of inhibition zones of alcoholic and aqueous extracts with +ve and –ve controls against Porphyromonas gingivalis**

**Table-1: 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% | -1.600 | 0.000 (HS) | -0.467 | 0.056 (NS) |
| | 30% | -3.067 | 0.000 (HS) | -3.000 | 0.000 (HS) |
| | 40% | -4.133 | 0.000 (HS) | -4.733 | 0.000 (HS) |
| | 50% | -6.267 | 0.000 (HS) | -6.733 | 0.000 (HS) |
| | 60% | -8.067 | 0.000 (HS) | -8.267 | 0.000 (HS) |
| | 70% | -9.800 | 0.000 (HS) | -8.667 | 0.000 (HS) |
| | 80% | -10.867 | 0.000 (HS) | -9.800 | 0.000 (HS) |
| | 90% | -11.333 | 0.000 (HS) | -10.467 | 0.000 (HS) |
| | 100% | -11.333 | 0.000 (HS) | -10.467 | 0.000 (HS) |
| | CHX | -11.667 | 0.000 (HS) | -13.333 | 0.000 (HS) |
| | D.W. | 8.067 | 0.000 (HS) | 6.267 | 0.000 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | (HS) |
| 20% | 30% | -1.467 | 0.000 (HS) | -2.533 | 0.000 (HS) |
| | 40% | -2.533 | 0.000 (HS) | -4.267 | 0.000 (HS) |
| | 50% | -4.667 | 0.000 (HS) | -6.267 | 0.000 (HS) |
| | 60% | -6.467 | 0.000 (HS) | -7.800 | 0.000 (HS) |
| | 70% | -8.200 | 0.000 (HS) | -8.200 | 0.000 (HS) |
| | 80% | -9.267 | 0.000 (HS) | -9.333 | 0.000 (HS) |
| | 90% | -9.733 | 0.000 (HS) | -10.000 | 0.000 (HS) |
| | 100% | -9.733 | 0.000 (HS) | -10.000 | 0.000 (HS) |
| | CHX | -10.067 | 0.000 (HS) | -12.867 | 0.000 (HS) |
| | D.W. | 9.667 | 0.000 (HS) | 6.733 | 0.000 (HS) |
| 30% | 40% | -1.067 | 0.000 (HS) | -1.733 | 0.000 (HS) |
| | 50% | -3.200 | 0.000 (HS) | -3.733 | 0.000 (HS) |
| | 60% | -5.000 | 0.000 (HS) | -5.267 | 0.000 (HS) |
| | 70% | -6.733 | 0.000 (HS) | -5.667 | 0.000 (HS) |
| | 80% | -7.800 | 0.000 (HS) | -6.800 | 0.000 (HS) |
| | 90% | -8.267 | 0.000 (HS) | -7.467 | 0.000 (HS) |
| | 100% | -8.267 | 0.000 (HS) | -7.467 | 0.000 (HS) |
| | CHX | -8.600 | 0.000 (HS) | -10.333 | 0.000 (HS) |
| | D.W. | 11.133 | 0.000 (HS) | 9.267 | 0.000 (HS) |
| 40% | 50% | -2.133 | 0.000 (HS) | -2.000 | 0.000 (HS) |
| | 60% | -3.933 | 0.000 (HS) | -3.533 | 0.000 (HS) |
| | 70% | -5.667 | 0.000 (HS) | -3.933 | 0.000 (HS) |
| | 80% | -6.733 | 0.000 (HS) | -5.067 | 0.000 (HS) |
| | 90% | -7.200 | 0.000 (HS) | -5.733 | 0.000 (HS) |
| | 100% | -7.200 | 0.000 (HS) | -5.733 | 0.000 (HS) |
| | CHX | -7.533 | 0.000 (HS) | -8.600 | 0.000 (HS) |
| | D.W. | 12.200 | 0.000 (HS) | 11.000 | 0.000 (HS) |
| 50% | 60% | -1.800 | 0.000 (HS) | -1.533 | 0.000 (HS) |
| | 70% | -3.533 | 0.000 (HS) | -1.933 | 0.000 (HS) |
| | 80% | -4.600 | 0.000 (HS) | -3.067 | 0.000 (HS) |
| | 90% | -5.067 | 0.000 (HS) | -3.733 | 0.000 (HS) |
| | 100% | -5.067 | 0.000 (HS) | -3.733 | 0.000 (HS) |
| | CHX | -5.400 | 0.000 (HS) | -6.600 | 0.000 (HS) |
| | D.W. | 14.333 | 0.000 (HS) | 13.000 | 0.000 (HS) |

| | | | | | |
|---|---|---|---|---|---|
| 60% | 70% | -1.733 | 0.000 (HS) | -0.400 | 0.1 (NS) |
| | 80% | -2.800 | 0.000 (HS) | -1.533 | 0.000 (HS) |
| | 90% | -3.267 | 0.000 (HS) | -2.200 | 0.000 (HS) |
| | 100% | -3.267 | 0.000 (HS) | -2.200 | 0.000 (HS) |
| | CHX | -3.600 | 0.000 (HS) | -5.067 | 0.000 (HS) |
| | D.W. | 16.133 | 0.000 (HS) | 14.533 | 0.000 (HS) |
| 70% | 80% | -1.067 | 0.000 (HS) | -1.133 | 0.000 (HS) |
| | 90% | -1.533 | 0.000 (HS) | -1.800 | 0.000 (HS) |
| | 100% | -1.533 | 0.000 (HS) | -1.800 | 0.000 (HS) |
| | CHX | -1.867 | 0.000 (HS) | -4.667 | 0.000 (HS) |
| | D.W. | 17.867 | 0.000 (HS) | 14.933 | 0.000 (HS) |
| 80% | 90% | -0.467 | 0.107 (NS) | -0.667 | 0.007 (HS) |
| | 100% | -0.467 | 0.107 (NS) | -0.667 | 0.007 (HS) |
| | CHX | -0.800 | 0.006 (HS) | -3.533 | 0.000 (HS) |
| | D.W. | 18.933 | 0.000 (HS) | 16.067 | 0.000 (HS) |
| 90% | 100% | 0 | 1 (NS) | 0 | 1 (NS) |
| | CHX | -0.333 | 0.248 (NS) | -2.867 | 0.000 (HS) |
| | D.W. | 19.400 | 0.000 (HS) | 16.733 | 0.000 (HS) |
| 100% | CHX | -0.333 | 0.248 (NS) | -2.867 | 0.000 (HS) |
| | D.W. | 19.400 | 0.000 (HS) | 16.733 | 0.000 (HS) |
| CHX | D.W. | 19.733 | 0.000 (HS) | 19.600 | 0.000 (HS) |