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Antibacterial effect of green tea and pomegranate peel extracts on Streptococcus mutans of orthodontic treated patients

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ABSTRACT
The antibacterial effect of green tea and pomegranate peel extracts on Streptococcus mutans of orthodontically treated patients and its adherence to the tooth surface were investigated. Streptococcus mutans was isolated from saliva and plaque samples collected from orthodontic treated patients before the start of treatment and 3 months after the treatment. Gamma irradiated (5, and 10 kGy) and un-irradiated extracts of green tea leaves and pomegranate peels were prepared using polar (water, and 70% ethanol) and non-polar (hexane) solvents. The extraction yields, total phenol contents (TPC), and antibacterial activity of all the extracts were investigated. The irradiated (10 kGy) ethanol extracts of both pomegranate peels and green tea contained the maximum amount of TPC and showed superior antibacterial activity (maximum inhibition zone 28, and 35mm) and lowest minimum inhibitory concentration (25.0, and 12.50 mg/ml) against S. mutans, respectively when compared with the other extracts. The effect of various combinations of the irradiated (10 kGy) ethanol extracts of pomegranate peels and green tea on the adherence of S. mutans from orthodontic patients was finally evaluated.

1. Introduction
Dental caries is the most prevalent disease of mankind among all ages. The normal oral flora comprises a diverse group of microorganisms including bacteria, fungi, protozoa and even possibly viruses. The occurrence of disease results from the disturbance of the equilibrium of this complex ecosystem (Aravindraj, Preethi, & Sivapathasundharam, 2017). Streptococcus mutans is the chief oral pathogen in the etiology of dental caries. It causes demineralization of inorganic tooth structure by metabolizing sucrose to lactic acid. It can also colonize tooth surfaces and initiate plaque formation through their ability to synthesize and bind extracellular polyasaccharides (glucan) using the enzyme glucosyltransferase (Bai et al., 2016; Lalwani et al., 2014).

Orthodontic treatment may induce oral ecologic changes, leading to an increase of S. mutans in saliva. It has been found that orthodontic treatment using fixed appliance results in enamel demineralization. This demineralization of the tooth surfaces results in increase of carious lesions (Ren, Jongsma, Mei, van der Mei, & Busscher, 2014; Salehi & Sh, 2006).

S. mutans and the other microorganisms involved in the pathogenesis of dental caries have developed tolerance and resistance to many antimicrobial and antibiotic agents routinely used in the clinical practice (Krzyściak, Jurczak, Kościelniak, Bystrowska, & Skalniak, 2014).

Attention was driven to the use of natural plant extracts for their health care needs as they are effective, nontoxic, and economical and usually have no side effects. Green tea and pomegranate are among these plants (Bai et al., 2016).

Green tea (Camellia sinensis) is one of the most popular beverages; green tea contains several polyphenolic compounds, including flavins and polyphenols. Catechins are the most frequent and abundant polyphenolic compounds. The major green tea catechins include epigallocatechin-3-gallate (EGCG) and epicatechin –3-gallate (ECG), which constitute about 50 to 65% of total catechins and have a broad spectrum of antimicrobial activity against both of gram-positive and gram-negative bacteria (Chang, Huang, Lin, & Brown, 2019).

Pomegranate (Punica granatum) is the predominant member of the Punicaceae family and one of the oldest fruits known in ancient Egypt. Pomegranate is a potent antioxidant with anti-carcinogenic and anti-inflammatory properties (Lalwani et al., 2014).

Gamma irradiation is considered as an effective method of food processing to reduce microbial load and to extend the shelf life of the product without any detrimental effect on food quality. Recent studies have investigated the effect of gamma irradiation on the extraction of phenolic compounds of different types of plants using different doses of radiation (Fanaro, Hassimoto, Bastos, & Villavicencio, 2015; Khattak & Simpson, 2008; Mali, Khedkar, & Lele, 2011). Throughout
the past years, many studies were conducted on the use of natural plant extracts as an alternative method to control dental caries. However, different plants, methods, and concentrations were used and the results were controversial (Banavar Ravi, Nirupad, Chippagiri, & Pandurangappa, 2017; Chen & Wang, 2010; Cruz Martinez, Diaz Gomez, & Oh, 2017). According to our knowledge, few studies investigated the antibacterial effect of Punica granatum against oral bacteria. Moreover, none of the studies has recorded the antibacterial effect of irradiated green tea and pomegranate extracts mixture on Streptococcus mutans and/or adherence to the tooth surface of orthodontic patients. The aim of this study was to evaluate the effect of gamma irradiation on the extraction of phenolic compounds of green tea and pomegranate peels. Furthermore, to investigate the antibacterial effect of green tea and pomegranate peels (irradiated and un-irradiated) extracts on Streptococcus mutans of orthodontically treated patients and the activity of their different combinations on the adherence of S. mutans to the tooth surface.

2. Materials and methods
A total of 20 patients of age range 15–25 years, indicated for orthodontic treatment were enrolled in this study.

Inclusion criteria: included patients with permanent dentition, no clinical and/or radiographic manifestations of periodontal disease and no history of any smoking, no history of any systemic illness and no antibiotic administration at least 3 months prior to treatment.

Exclusion criteria: included smoking, pregnancy, poor general health, history of periodontal treatment, antibiotic administration.

2.1. Sample collection
(I) Prior to orthodontic treatment.
Approximately 1 ml of unstimulated whole saliva samples was collected. All saliva samples were obtained in the morning after an overnight fast. Plaque samples were collected from the buccal and labial aspects of the anterior teeth and the four first molars to determine oral carriage of S. mutans of these patients and were recorded as P1.

(II) After placement of the orthodontic appliance.
Three months after placement of orthodontic appliance (0.22 MBT pre-adjusted Gemini stainless steel, M Unitek, CA, USA) 1 ml of unstimulated whole saliva samples and plaque samples were recollected from the same site and were recorded as P2.

Saliva and plaque samples were vortexed for two and one minutes, respectively, for dispersion, and were diluted (10⁻¹–10⁻⁵) in Brain Heart Infusion (BHI, Difco, USA) broth.

2.2. Isolation and cultivation
100 μL of each sample broth culture was spread on the surface of Mitis Salivarius Agar (MS Agar, micromaster, India) selective media plates and incubated anaerobically for 24 h at 37°C.

2.3. Identification of bacterial isolates
Purified isolates from selective media (MS agar) plates characterized by colonial shape and form, Gram staining, microscopic examination, and catalase test then confirmed using commercial kits (Remel RapID STR system, USA) and achieved using ERIC (easy-to-use, reliable electronic code compendium) system electronic program. From the bacterial culture, of the highest bioscore and typical biofrequency and implicit probability level Streptococcus mutans in ERIC system results, (100 μl) was transferred to agar plate and spread uniformly, incubated 37°C for 24h. One colony from the purified culture strain was sub-cultured overnight at 37°C in Mueller-Hilton Agar (MHA, Difco, USA) slants and stored for further studies.

2.4. Fresh bacterial culture
For preparing fresh bacterial culture, each 3 ml of Brain Heart Infusion broth (BHI, Difco, Detroit, USA) medium was inoculated with one colony of Streptococcus mutans and incubated overnight at 37°C and 5% CO₂ upon gentle shaking (Pires et al., 2018).

2.5. Microbiological procedures
2.5.1. Preparation of plant materials powder
Commercial samples of green tea bags and pomegranate fruits were purchased from local markets in the region of Nasr City, Cairo, Egypt. Pomegranate peels were washed thoroughly with distilled water and air-dried under ambient conditions, ground separately into fine powder to pass 100 mm sieve using a mixer grinder, and maintained at −20°C in vacuum-sealed packages.

2.5.2. Plant samples preparation
For each extracting solvent (distilled water, ethanol, and hexane) six samples (each 100 g) from each plant material, were divided into two replicates, the three samples of each replicate were subjected to different doses (0, 5, and 10 kGy) of gamma radiation.
2.5.3. **Gamma irradiation**

In tightly capped containers (each 100 g) the samples of green tea leaves and pomegranate peels were subjected to different doses (0, 5, and 10 kGy) of gamma radiation using the ‘Indian Gamma Chamber 4000 A’ with a 60Co source at room temperature. Irradiation was performed in the National Center for Radiation Research and Technology (NCRRT) Nasr City, Cairo, Egypt, at a dose rate of 2.492 kGy/h.

2.6. **Preparation of extracts**

2.6.1. **Cold extraction method (Maceration)**

The powder (20 g) of irradiated and un-irradiated green tea leaves and pomegranate peels were extracted separately with 200 ml of both the polar (distilled water and 70% ethanol) and nonpolar (70% hexane) solvents by maceration of the plant material with the solvents for 72 hours at 37°C with gentle shaking at 120 rpm and filtered through double layers of muslin, centrifuged at 6000 rpm for 10 min and finally filtered again through Whatman filter paper no.1 to attain a clear filtrate. The extracts were collected in Petri dishes, concentrated under low pressure, and heated at 40°C till evaporation. The obtained powder-like substance was then collected, weighed, and stored at 5°C (Borekar et al., 2018).

2.6.2. **Hot extraction method (Decoction)**

Exactly 20 g of green tea or pomegranate peels powder was mixed with 200 ml of sterile distilled water and boiled at 70–80°C for half an hour using magnetic stirrer. The mixtures were filtered by muslin cloths. The filtrates were centrifuged for 10 min at 6000 rpm. The extracts were collected in Petri dishes, concentrated under low pressure, and heated at 40°C till evaporation. The dishes content cooled, scratched, and the obtained powder-like substance was collected and stored at 5°C (Borekar et al., 2018).

2.6.3. **Yields of the extracts**

The yields of the extracts were based on dry weight and reported in percent yield. The yield percentages were calculated using the following formula:

\[
\text{Extract yield } \% = \frac{\text{weight of extracted plants residues}}{\text{weight of powder plants samples}} \times 100
\]

2.6.4. **Preparation of different concentrations from pomegranate peels and green tea powder extracts**

Different samples concentrations from pomegranate peels and green tea powder extracts were prepared by dissolving equivalent amounts of it (yielded from the 100 mg dried extract) with DEMSO 0.5% and were filter sterilized through a Millipore filter (0.45 mm) (Kuete et al., 2009).

2.6.5. **Total phenolic content (TPC) of the green tea leaves and pomegranate peels extracts (Quantitative methods)**

The total phenolic contents of the extracts were determined using the Folin–Ciocalteu method (Singleton & Rossi, 1965) with some modifications. Briefly, 200 µl of each extract (1 mg/ml) was mixed with 1000 µl of Folin–Ciocalteu reagent (10%) and allowed to stand for 5 min after vortexing. Then, 800 µl of aqueous sodium carbonate (Na2CO3; 75 g/l) was added and vortexed. After 2 h of incubation in the dark at room temperature, the absorbance was measured in triplicate at 760 nm against a blank (200 µl distilled deionized water, 1.0 ml Folin-Ciocalteu, and 800 µl of Na2CO3) using a UV-VIS spectrophotometer. The total phenolic content was calculated from a prepared gallic acid standard calibration curve (0–250 mg/ml). TPC content in the extracts was calculated from a prepared gallic acid standard calibration curve and expressed as gallic acid equivalent (GAE) in milligrams per gram plant extract dry weight (mg GAE/g dry weight).

2.6.6. **Determination of antibacterial activity by disc diffusion method**

Antibacterial activity of the pomegranate peels and green tea extracts were determined by the disc diffusion method (Tardugno et al., 2018). Briefly, dried and sterile filter paper discs (6 mm diameter) containing pomegranate peels and green tea extracts of known concentrations (30 µl of 3mg/ml extract) were placed on nutrient agar medium uniformly seeded with 100 µl of the *S. mutans* broth. Ampicillin discs (20 µg/disc) were used as positive controls while discs soaked in DMSO were used as negative controls. The plates were then incubated at 37°C for 24 h. The antimicrobial potency of extract samples was measured by their activity to prevent the growth of the *S. mutans* surrounding the discs which give a clear zone of inhibition. The inhibition zone diameter was measured in mm. All tests were performed in triplicate. The antibacterial activity was classified as strong (>20mm), moderate (16–19 mm) and mild (12–15mm) and less than 12 mm was taken as inactive.

2.6.7. **Determine the MIC and MBC of the ethanol extracts**

The Minimum Inhibitory Concentration (MIC) of the ethanol extracts (irradiated and un-irradiated), which exhibits a strong antibacterial activity revealed by the previous screening test (Disc Diffusion assay) were determined by Resazurin Microplate Assay (Palomino et al., 2002) with some modifications. The concentrations of the extracts were adjusted to get 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5 and 0.75 mg/ml by micro broth dilution technique and the total volume was brought up to 200 µl with Mueller-Hilton Broth (MHB). Then, 10 µl of bacterial suspensions (1-2x10^8 CFU/ml) was added. A growth positive control (containing 10 µl inoculum and 200 µl MHB) and
negative control (containing 100 μl tested extracts and 100 μl MHB) were prepared. Ampicillin and chlorhexidine 1.0% were used as antimicrobial control. The stock solutions of the antimicrobial were prepared and sterilized with a 0.45 μm Acrodisc filter. Serial two-fold dilutions ranging from 100 to 0.093 μg/ml for ampicillin and chlorhexidine were prepared in MHB. After incubation of sealed microtiter plates at 37°C for 24 h, 10μl of 0.03% resazurin solution (Sigma-Aldrich) was added per well, as an indicator of cell viability, and then incubated at 37°C for additional 5 h. After every one hour incubation time intervals plates were read for the color change from blue to pink and pink to colorless in live-bacterial containing wells. The minimal inhibitory concentration (MIC) of the extracts was defined as the lowest concentration that prevented the color change of resazurin from blue to pink. A change from blue to pink indicates a reduction of resazurin and therefore bacterial growth. All experiments were performed in triplicates.

For the Minimum Bactericidal Concentration (MBC) determination, 10 μl of broth was removed from the wells considered inhibitory and plated on MHA plates, which were incubated at 37°C for 24 h. The MBC was considered as the lowest concentration at which no growth of colonies on the surface of the culture medium was observed (Suja et al., 2017). The experiments were conducted in triplicate for each extract.

2.6.8. Determination of Fractional Inhibitory Concentration Index (FICI)

The checkerboard method was used for the determination of synergy between the combinations of the best two natural antimicrobials. The antimicrobial agents were prepared in MHB at the 2MIC, MIC, 1/2 MIC, 1/4 MIC, 1/8 MIC, 1/16 MIC, and 1/32 MIC concentrations. Different combinations of each of the different concentrations of the two extracts were assayed in 96-well, round-bottomed microtiter plates. Growth and sterility control wells (no antibiotic and no inoculum, respectively) were included in all plates. Plates were incubated at 37°C for 24 h. The effect of the individual components and each combination was determined by using the resazurin proliferation assay. The checkerboard method is often combined with the calculation of a fractional inhibitory concentration (FIC) index to test the antimicrobial potencies of drugs in medical laboratories. The fractional inhibitory concentration (FIC) was derived from the lowest concentration of green tea leaves ethanolic extract and pomegranate peels ethanolic extract combination permitting no visible growth of the test organisms on the plates (no change of resazurin blue color). The FIC value for each agent was calculated using the formula:

\[
\text{FIC (green tea leaves ethanolic extract)} = \frac{\text{MIC of green tea leaves ethanolic extract}}{\text{MIC of green tea leaves ethanolic extract}}
\]

FIC (pomegranate peels ethanolic extract) = MIC of pomegranate peels ethanolic extract in combination/ MIC pomegranate peels ethanolic extract alone.

The following formulas were used to calculate the FIC index:

\[
\text{FICI} = \text{FIC (green tea leaves ethanolic extract)} + \text{FIC (pomegranate peels ethanolic extract)}
\]

Combinations were classified as synergistic, if the FIC indices were ≤0.5, additive if the FIC indices were >0.5 and ≤1.0, indifferent if FIC>1.0 and≤2.0, and antagonistic if FIC >2.0 (Yadav, Park, Chae, Song, & Kim, 2013). All assays were carried out in triplicate.

2.6.9. Adherence

To study the effect of green tea and pomegranate peels 10kGy irradiated ethanolic extracts on Streptococcus mutans adherence to the tooth surface, seven concentrations were used: the minimum inhibition concentration (MIC), the minimum bactericidal concentration (MBC) of each tested extract alone, and the synergistic combinations concentration, and each tested extract alone in concentration as in the synergistic combinations. These tested concentrations were compared with 1.0% chlorhexidine gluconate, control positive (broth and bacteria without the extract), and control negative (broth and the agent without bacteria). A stainless steel wire was threaded from one end in the root of a previously cleaned, polished and sterilized first molar. After the immersion for 2 minutes in 10 ml of the tested extract or chlorhexidine gluconate (except the control positive) the teeth were washed with sterilized deionized water and dried, then immersed in BHI broth (10 ml containing 5% sucrose with pH7). Each tube (treated and control tubes) inoculated with 2% S. mutans broth and incubated at 37°C for seven days. Non-effective treatment (growth of bacteria on teeth, wire, and bottle) was took a positive score and vice versa (Aldhaher, Mahmood, Taha, & Shaker, 2015).

2.6.10. Statistical analysis

All experiments were carried out in triplicates and the results were expressed as values with standard deviation (±SD). The statistical analysis ANOVA and Tukey tests were performed using in both cases the value of p < 0.05. One-way analysis of variance (ANOVA) was carried out using Minitab Statistical Software package (Minitab, version 18.0).

3. Results and discussion

3.1. Isolation, identification, and selection of the bacterial isolates

From the forty unstimulated whole saliva and plaque samples collected from twenty participants before (P1 = 20 samples) and after (P2 = 20 samples)
orthodontic treatment ten streptococcal isolates were obtained from the selective media MS agar, which promotes the growth of streptococci and suppresses other bacterial species. Single colonies from the surface of MS-agar which considered to be positive were selected for further purification by sub-culturing on the surfaces of MS-agar media. Isolates were first identified depending on their gram-staining (gram-positive), microscopic examination (cocci arranged in chains) and catalase test (catalase-negative) (Al-Mudallal et al., 2008). *S. mutans* isolates enzymatic spectra were tested by the Remel RapID STR system. From the identification results, the highest bio-score and typical bio-frequency and implicit probability level (99.9%) *Streptococcus mutans* isolate in the ERIC system result was selected for further studies (Photo 1).

### 3.2. Extraction yields

The extraction yields of un-irradiated (control samples) and gamma irradiated (5 and 10kGy) pomegranate peels and green tea in water, hexane, and ethanol were determined (Table 1). All the used solvents do not have the same extraction capacity, the control sample dry weight yields vary according to the solvents (Table 1). The ethanolic extracts showed the highest extraction yield for control samples. The extraction yields for all the test solvents from gamma irradiated samples increased in comparison with the un-irradiated samples (p < 0.05). The pomegranate peels water extract irradiated samples at 5kGy dry weight yields were found to be nearly the same as that of the control (p > 0.05). The dry weight yields of green tea leaves water extracts irradiated samples at 5kGy and 10 kGy was found to be insignificantly different (p > 0.05). There was a linear increase in the dry weight yields with the increase of gamma radiation dose from 5 to 10 kGy for all the test solvents (Table 1). The yield of the extracts with different solvents is in the order: 70% ethanol > water > 70% hexane (Table 1).

The highest yields recovery was obtained 70% ethanol (10 kGy) irradiated pomegranate peels and green tea leaves (14.445 ± 0.511 and 11.503 ± 0.197g/100gDW) and the lowest yields recovery was obtained from 70% hexane un-irradiated pomegranate peels and green tea leaves extracts (1.112 ± 0.509 and 0.491 ± 0.007 g/100gDW), respectively. ANOVA revealed that there was a significant difference between extracts yield (p < 0.05) (Table 1).

Solvent selection is an important step for obtaining extracts with high yields. From the obtained results, it was noted that the yields of extracts from different solvents were variables. The variability of yields could be explained...
Table 1. Effect of gamma irradiation and solvents on the extraction yield.

| Plants            | Radiation dose (kGy) | Water maceration | 70% Ethanol | 70% Hexane | Water decoction |
|-------------------|----------------------|------------------|-------------|------------|----------------|
| pomegranate peels | Control              | 6.454 ± 0.255<sup>j</sup> | 10.896 ± 0.509<sup>j</sup> | 1.112 ± 0.509<sup>j</sup> | 6.565 ± 0.140<sup>j</sup> |
|                   | 5 kGy                | 6.568 ± 0.123<sup>j</sup> | 12.342 ± 0.139<sup>j</sup> | 1.869 ± 0.072<sup>f</sup> | 6.779 ± 0.632<sup>j</sup> |
|                   | 10 kGy               | 7.328 ± 0.254<sup>d</sup> | 14.445 ± 0.511<sup>j</sup> | 2.625 ± 0.420<sup>g</sup> | 8.232 ± 0.255<sup>j</sup> |
| green tea leaves  | Control              | 3.866 ± 0.127<sup>i</sup> | 9.2100 ± 0.065<sup>i</sup> | 0.491 ± 0.007<sup>j</sup> | 5.008 ± 0.127<sup>g</sup> |
|                   | 5 kGy                | 4.898 ± 0.255<sup>g</sup> | 9.815 ± 0.109<sup>i</sup> | 0.972 ± 0.136<sup>j</sup> | 6.568 ± 0.382<sup>j</sup> |
|                   | 10 kGy               | 4.707 ± 0.140<sup>d</sup> | 11.503 ± 0.197<sup>j</sup> | 1.2850 ± 0.050<sup>j</sup> | 6.564 ± 0.280<sup>j</sup> |

Extraction yield (% w/w). Each value is expressed as Mean ± standard deviation (n = 3).

**Means with different superscript letters within the same column are significantly different (p < 0.05) for (each plant extracts).

by the extraction ability of each solvent which depends firstly on the solvent affinity with the phytomolecules and on the other hand the polarity of the solvent (Dah-Nouvlessounon et al., 2015). Because of polarity differences between solvents, the solubility of the phytochemicals into the solvent is expected to be different. Ethanol and water are polar solvents, gave higher yield compared to nonpolar solvent hexane. Our findings agree in terms of solubility trend with Singh, Chidambara Murthy, and Jayaprakasha (2002) but differ in the extracted yield.

The study results show that gamma irradiation up to 10 kGy is an effective method for enhancing extract yields in pomegranate peels and green tea leaves. The increase in the dry weights of extracts following irradiation might be due to the degradation of some non-soluble components with high molecular weight to soluble ones by the test solvents. The measured effect of irradiation was stronger in the ethanolic extraction, which is able to extract both polar and semi-polar compounds. Similarly, Kim, Yook, and Byun (2000) found an increase of 5–30% in the extraction yields of Korean medicinal herbs, using various solvents, after treating with 10 kGy gamma irradiation dose. The increase in extraction yields with irradiation treatment has also been reported by Huang and Mau (2007), Khattak and Simpson (2008) study showed that at 16 kGy, increases in extraction yield were 3.7%, 4.2%, 9.0% and 5.6% for hexane, acetone, methanol, and water, respectively of gamma irradiated *Nigella sativa* seed. The difference in the extraction yields, as compared to that previously reported, maybe due to the plants' different chemical composition.

### 3.3. Estimation of Total Phenol Content (TPC)

The total phenol content (TPC) of pomegranate peels and green tea extracts were determined by the Folin Ciocalteu method and the results were expressed in mg GAE/g extract. The highest amount of TPC was found in the irradiated ethanolic extracts of both pomegranate peels and green tea followed by its aqueous extracts. On the other hand, the lowest amount of TPC was found in the hexane extract of both plants (Table 2). The irradiated (10 kGy) ethanol extracts of pomegranate peels and green tea leaves contained (247.562 ± 1.293 and 132.264 ± 0.388 mg GAE/g extract) TPC, and un-irradiated hexane extracts contained (84.954 ± 0.569 and 70.075 ± 0.679 mg GAE/g extract) TPC (Table 2). The TPC of the extracts showed the following order: 70% ethanolic extract > aqueous extracts > 70% hexane extract. For radiation-processed samples, the data (Table 2) showed significant changes in phenolic contents of ethanol extracts in the 5 kGy gamma irradiated samples, and significant (p < 0.05) increases at 10 kGy dose level as compared to control.

Three types of solvents were used in this experiment: deionized water (maceration and decoction), ethanol, and hexane. The TPC was found more in the extracts obtained from 70% ethanol and water than that of hexane. These findings demonstrate the influence of the solvents on the extractability of phenolics.

The polar nature of most of the phenolic compounds makes them widely soluble in polar solvents (as ethanol and water), so the use of nonpolar solvent hexane gave the minimum amount of phenolic compounds extracts (Wang, Pan, Ma, & Atungulu, 2011). For safety reasons, ethanol extraction of pomegranate whole fruit was preferred over methanol extraction (Rababah, Banat, Rababah, Ereifej, & Yang, 2010). Do et al. (2014) found more TPC in water and ethanol polar solvent than hexane nonpolar solvent. The international consultative group of food irradiation (ICGFI) concluded that irradiation of food at a dose level of

Table 2. Total Phenolic Compounds of pomegranate peels and green tea leaves.

| Plants            | Radiation dose (kGy) | Water maceration | 70% Ethanol | 70% Hexane | Water decoction |
|-------------------|----------------------|------------------|-------------|------------|----------------|
| pomegranate peels | Control              | 130.248 ± 0.255<sup>ad</sup> | 196.793 ± 0.842<sup>j</sup> | 84.954 ± 0.569<sup>j</sup> | 114.903 ± 1.140<sup>j</sup> |
|                   | 5 kGy                | 144.974 ± 0.847<sup>d</sup> | 224.45 ± 0.162<sup>k</sup> | 96.031 ± 0.478<sup>j</sup> | 151.779 ± 0.898<sup>j</sup> |
|                   | 10 kGy               | 161.947 ± 0.968<sup>d</sup> | 247.562 ± 1.293<sup>j</sup> | 105.38 ± 1.690<sup>j</sup> | 162.064 ± 0.445<sup>g</sup> |
| green tea leaves  | Control              | 97.276 ± 0.104<sup>j</sup> | 104.996 ± 0.929<sup>j</sup> | 70.075 ± 0.679<sup>j</sup> | 78.099 ± 0.759<sup>j</sup> |
|                   | 5 kGy                | 104.723 ± 0.781<sup>j</sup> | 116.227 ± 0.769<sup>j</sup> | 74.810 ± 0.233<sup>j</sup> | 82.911 ± 0.777<sup>j</sup> |
|                   | 10 kGy               | 116.758 ± 1.220<sup>j</sup> | 132.264 ± 0.388<sup>j</sup> | 78.980 ± 0.608<sup>j</sup> | 88.969 ± 0.303<sup>j</sup> |

Each value is expressed as Mean ± standard deviation (n = 3). Total Phenols (mgGAE/g extract)

**Means with different superscript letters within the same column are significantly different (p < 0.05) for (each plant extracts).
10 kGy or below was toxicologically safe and nutritionally adequate (WHO, 1981, 1994). Irradiation exerts its effects as direct and indirect mechanisms. In the case of direct mechanism, the increase in total phenols and total flavonoids is due to the release of phenolic compounds from glycosidic components and the degradation of larger phenolic compounds into smaller ones by gamma irradiation treatment as suggested by Harrison and Were (2007). In the case of indirect mechanism, the radiolysis of water results in the production of free radicals such as hydroxyl radicals, hydroperoxide radicals, and hydrated electrons. These radicals may break the glycosidic bonds of procyanidin trimer, tetramer, and hexamer that are present in plants, leading to the formation of procyanidin monomers, which increase the total phenolic and total flavonoids content in irradiated plants (Lee et al., 2009).

The increase in total phenolic content of pomegranate peel powder could be attributed to the break of high molecular weight tannins present in the peel powder into simple phenolic compounds such as tannic acid, gallic acid, and other active ingredients (Kumari et al., 2009).

The tea catechin and polyphenols have been found to possess antibacterial effect against *H. pylori* and may have a therapeutic effect against gastric mucosal injury induced by this organism (Mabe, Yamada, Oguni, & Takahashi, 1999). The differences in effects were attributed to the different phenolic compounds present in the various plants (Naveed, Rizwan, & Sajid, 2017).

In the present study, the radiation dose of 10 kGy was chosen since the dose of radiation recommended by the FAO/WHO Codex Alimentarius Commission for use in food irradiation does not exceed 10 kGy (WHO, 1998).

The irradiated green tea and pomegranate groups resulted in inhibition of *S. mutans* adhesion to the tooth surface. Fanaro et al. (2015) recorded an increase in the phenolic content of irradiated green tea with different doses of gamma irradiation from 1 to 10 kGy. Mali et al. (2011) found a significant increase in the total phenolic content of 10.0 kGy irradiated pomegranate immediately after irradiation and 60 days of post-irradiation storage. This could be attributed to the biological effect of gamma radiation which is mainly due to hydrolysis of the water content of irradiated plants and the formation of free radicals. Formed free radicals will react with other components of plant cells and results in the accumulation of phenolic compounds thus increase its antibacterial effect.

### 3.4. Antimicrobial activity

All the pomegranate peels and green tea extracts were investigated for their antimicrobial activity against *S. mutans* gram-positive bacteria by simple agar diffusion method Table 3. It is clear that the irradiation treatment was found to be superior for improving the quantity and the antimicrobial effect of the green tea leaf and pomegranate peels extracts Table 3. Ethanol irradiated extracts of both plants, and pomegranate peels water decoction extract showed strong (the zone of inhibition >20mm) activity against *S. mutans*. Pomegranate peels water maceration and irradiated green tea leaves water decoction extracts showed moderate (the zone of inhibition between 16–19 mm) activity. Irradiated green tea leaves water maceration and pomegranate peels hexane extracts have mild activity (the zone of inhibition between 12–15mm) against the pathogenic organism, and hexane extracts were inactive (the zone of inhibition less than 12mm). All the activities were determined by measuring the zone of inhibition (in mm) compared with the standard antibiotic (ampicillin) Table 3.

The antibacterial activity of plant extracts vary according to the concentrations of the active secondary metabolites presents in the extracts, the extraction method, chemical constituents of the plant, and the used bacterial strains (Qader, Khalid, & Abdullah, 2013). Hydrophilic solvents are better for hydrophilic polyphenols recovery from plants. The highest antibacterial activity of the ethanolic extracts of pomegranate peels and green tea is because of the presence of tannin, saponin, and glycosides which are known as potential antimicrobial agents. In pomegranate fruits, tannins constituents representing 25% of phenolic content (Voravuthikunchai et al., 2005) and green tea polyphenols (30% W/DW) are the major components (An et al., 2004).

Nonpolar solvents plant extracts (hexane), were inactive compared to polar solvents (ethanol and methanol) due to the presence of a very little amount of glycosides and the absence of antimicrobial tannin, and saponin (Khali, Khan, & Shabbir, 2018). Hexane did

### Table 3. Antimicrobial activity of different pomegranate peels and green tea leaves extracts using disc-diffusion method

| Plants          | Radiation dose (kGy) | Water maceration | 70% Ethanol    | 70% Hexane | Water decoction |
|-----------------|----------------------|------------------|----------------|------------|----------------|
| *S. mutans*     |                      |                  |                |            |                |
| Pomegranate     | Control              | 16.59 ± 0.350**  | 26.19 ± 0.11    | 8.54 ± 0.14  | 20.40 ± 0.430   |
|                 | 5 kGy                | 17.60 ± 0.16     | 32.48 ± 0.17    | 12.03 ± 0.94  | 26.23 ± 0.89    |
|                 | 10 kGy               | 20.29 ± 0.42     | 35.56 ± 0.12    | 13.44 ± 0.08  | 30.45 ± 0.11    |
| Green tea       | Control              | 8.43 ± 0.11h     | 20.50 ± 0.22    | Nl ± 0.00    | 13.60 ± 0.051   |
|                 | 5 kGy                | 12.13 ± 0.07g    | 25.73 ± 0.09    | 8.46 ± 0.26   | 16.42 ± 0.088   |
|                 | 10 kGy               | 14.06 ± 0.02f    | 28.76 ± 0.32    | 10.78 ± 0.19  | 18.47 ± 0.403   |

Each value is expressed as Mean ± standard deviation (n = 3). **Diameter of inhibition zone (mm).**

**Means with different superscript letters within the same column are significantly different (p < 0.05) for each plant extracts.**

Extract Conc (3mg/disc). Nl = No Inhibition ampicillin (20 μg/disc.).
not extract active principles comparing to methanol and water, and phenolics and flavonoids were only present in the water-methanol extract which increase its broad-spectrum activity against microorganisms (Al-Zoreky, 2009).

The irradiated polyphenol might not change its chemical structure but some change in activity is expected of functional groups such as – OH or – COOH, resulting in higher antimicrobial activity (Park, So, & Bahk, 2015). The previous results indicate that the polyphenols of green tea inhibit the growth of tested microorganisms at a lower concentration. Biological and antimicrobial activities of the polyphenol isolated from green tea leaves were not changed by irradiation at 40 kGy (An et al., 2004). The extract seems to be thermostable because the hot water extract (using boiling water) still retained the activity nearly the same as other extracts. This was also found by Al-Zoreky (2009).

The MIC values for test bacteria seemed to correlate with the total phenolic content found in the extracts. The total phenolic content of ethanol extracts was the highest, followed by the water and hexane extracts, respectively. Generally, irradiated ethanolic extracts had lower MIC than MIC values of un-irradiated samples, against S. mutans. The MIC values for active extracts ranged between 100 and 12.5 mg/ml for pomegranate peels and between 200 and 25 mg/ml for green tea leaves, respectively Table (4). The MIC of green tea extracts and pomegranate peels decreased by irradiation. As shown in Table 4, the MIC and MBC of 10 kGy irradiated pomegranate peel ethanolic extract were at least two-fold and four-fold less than that of 10 kGy irradiated green tea leaves ethanolic extract, respectively. Results indicate that the irradiated samples showed higher antimicrobial activity than did the un-irradiated (P < 0.05). The highest dilution that yielded no bacterial growth on solid medium was taken as MBC.

The microbial toxicity of phenolic compounds was related to reaction with sulfhydryl protein groups and the unavailability of substrates to the microorganism (Machado et al., 2002). Extracts from pomegranate were interfered with protein secretions by bacteria. Different inhibition profiles of peel extracts against microorganisms may in part due to the different extraction methods, the freshness of fruits used and variations in the season and region of growth (McCarrell et al., 2008). Bactericidal and antibacterial effect of green tea on various bacterial isolates from infected root canal was reported (Oak, El Bedoui, & Schini-Kerth, 2005).

3.5. Determination of Fractional Inhibitory Concentration (FIC) and Fractional Inhibitory Concentration Index (FICI) values

The interactive inhibition commonly measured by the checkerboard method, which was used for the determination of synergy between the antibiotics and natural antimicrobials. Each combination (from 2MIC to 1/32 MIC) of irradiated (10kGy) pomegranate peels and green tea leaves extracts was tested. The most effective combination regimens were (6.25 green tea and 1.562 peels mg/ml), which showed a strong synergistic interaction (FICI value was 0.375) against S. mutans. Not all of the regimens exhibiting a synergistic effect (Table 5).

This study was the first of its kind assessing the antimicrobial efficacy of the combinations of irradiated pomegranate peels and green tea extracts irradiated leaves extract on S. mutans oral bacteria. Hence, these results could not be compared with other studies. A lot of plant extracts have been reported to have antimicrobial effects with minimal side effects. It may be possible to maximize the antimicrobial effect of the plant extracts by using them in combination. The combinations of plant extracts may yield significant benefits owing to the synergistic action of components present in them (Shah & Chen, 2017). A combination of plant extracts has dual benefits as it may increase the therapeutic efficacy and at the same time lower the toxic effect, and lower the rate of resistance development because of their phytochemical constituents (complex mixtures) which make microbial adaptability very difficult (Shekar, Nagarajappa, Singh, Suma, & Thakur, 2014). In principle, the synergistic mechanism behind some antimicrobial combination is relatively simple through physicochemical effects: where one drug facilitates the bioavailability of other drug or synergistic mechanism by dual inhibition on the same pathway (van Geelen, Meier, Rehberg, & Kalscheuer, 2018).

Table 4. Minimum inhibitory concentrations (MIC) and Minimum Bactericidal Concentration (MBC) of un-irradiated and irradiated pomegranate peels and green tea leaves extracts on S. mutans microbial growth.

| Plants            | Ethanol Extract | MIC (μg/ml) | MBC (μg/ml) |
|-------------------|-----------------|-------------|-------------|
|                   | Control         | 100 ± 0.00* | 200 ± 0.00  |
|                   | 5 kGy           | 50 ± 0.00   | 100 ± 0.00  |
|                   | 10 kGy          | 12.5 ± 0.00*| 12.5 ± 0.00 |
|                   | 5 kGy           | 100 ± 0.00* | 100 ± 0.00  |
|                   | 10 kGy          | 25 ± 0.00*  | 50 ± 0.00   |
|                   | Control         | 200 ± 0.00  | 200 ± 0.00  |

*Means with different superscript letters within the same column are significantly different (p < 0.05) for each plant extracts.

Table 5. Fractional inhibitory concentration (FIC) and Fractional inhibitory concentration index (FICI) values for the combination between the selected extracts against the tested strains.

| Extracts     | MIC combination | FIC | FICI | Effect     |
|--------------|-----------------|-----|------|------------|
| Pomegranate peels | 12.5            | 1.562 | 0.125 | 0.375 | Synergism |
| Green tea leaves | 25              | 6.25 | 0.250 |        |

FICI classified as follows: synergistic if FICI ≤ 0.5, additive if FICI > 0.5 and ≤ 1.0, in different if FICI > 1.0 and ≤ 2.0, and antagonistic if FICI >2.0. (Yadav et al., 2013) [21]. * (mg/ml)
3.6. Adhesion of bacteria to the cells

The effect of different concentrations of pomegranate peels and green tea leaves irradiated (10kGy) ethanolic extracts on the adherence of S. mutans to tooth surface was studied. The results (Table 6 and Photo 2) showed that the synergistic combinations concentration (SCC), and MBC of the tested extracts were prevented the colonization and adherence of S. mutans to the tooth surface as 1.0% chlorhexidine gluconate.

(Ferrazzano et al., 2017) demonstrated that the hydroalcoholic extracts of pomegranate juice and peel were able to contrast the main cariogenic bacteria involved in tooth decay. The recorded results could be due to the presence of phyto-compounds in the pomegranate peel extracts like hydrolysable tannins, polyphenolics, and flavonoids. Tannins may act on the cell wall and across the cell membrane because they can precipitate proteins. Moreover, it suppresses many enzymes such as glycosyltransferases. Similarly, polyphenols may affect the bacterial cell wall, inhibit enzymes by oxidized agents, form high molecular weight complexes with soluble proteins, increase lyses of bacteria and interfere with bacterial adherence and disturb its co-aggregation (Machado et al., 2002; Widyarman, Suhalim, Nandary, & Theodorea, 2018).

The bacterial adhesion to the cells can alter by the irreversible damage of the bacterial cytoplasmic membrane by catechins (Sharma, Gupta, Sarethy, Dang, & Gabrani, 2012). Asahi et al. (2014) suggested that the antimicrobial activity of tea catechins might be attributable to the inhibition of nucleic acid synthesis. Fikrika, Ambarsari, and Sumaryada (2016) used a molecular docking simulation of catechins on Glucosamine-6-Phosphate Synthase, and they demonstrated that glucosamine-6-phosphate synthase

Table 6. The effect of the different tested concentrations on S. mutans adhesion.

| Extracts (10kGy) | SCC | MBC | MIC | Each extract alone | Chlorhexidine 1.0% | Control positive | Control negative |
|------------------|-----|-----|-----|-------------------|-------------------|-----------------|-----------------|
| Pomegranate peels| -ve | -ve | +ve | +ve               | -ve               | +ve             | -ve             |
| Green tea leaves | -ve | -ve | +ve | +ve               | -ve               | +ve             | -ve             |

*Synergistic Combinations Concentration
**Each extract alone in concentration as in the synergistic combinations

![Photo 2](Image). The effect of the different tested concentrations on S. mutans adhesion.
inhibition by catechin suppressed the production of a bacterial cell wall and reduced the population of invading bacteria.

Multifactorial stages could be used for the explanation of green tea antibacterial effect on caries formation. It has been proved that green tea leaf catechins play an important role in the bacterial phenotype modification thus prevented S. mutans attachment to saliva-coated hydroxyapatite discs. Moreover, it may inhibit the streptococcal agent proliferation and interfere with the adhesion to tooth enamel and act as inhibitors for glucosyl transferase and salivary amylase (Joiner, Muller, Elofsson, & Arnebrant, 2004; Vukosavljevic, Custodio, Buzalaf, Hara, & Siqueira, 2014).

4. Conclusion
The results of the present investigation indicate that gamma radiation assisted solvents extraction of green tea and pomegranate peels besides improving its yield and quality. Gamma-irradiated (10kGy) ethanol extracts of green tea and pomegranate peels have a high inhibition effect on S. mutans bacteria and decrease its adherence to the tooth surface. The efficacy of irradiated (10kGy) pomegranate peels and green tea leaves extracts combinations against S. mutans was significantly higher compared to each one alone. The combinations of plant extracts offer enhanced antimicrobial efficacy due to the synergistic effects besides slowing the development of resistance.

Highlights
- Controlling S. mutans oral pathogen by green tea and pomegranate peel extracts for caries prevention.
- Green tea leaves and pomegranate peels extracts could be effective in inhibiting the growth of S. mutans.
- Gamma irradiation enhanced the antimicrobial efficacy of plants extracts.
- The combinations of plants extracts offer enhanced antimicrobial efficacy.

Disclosure statement
No potential conflict of interest was reported by the authors.

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