

Original Article

## Synergistic antibacterial activity of herbal extracts with antibiotics on bacteria responsible for periodontitis

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### Abstract

**Introduction:** Development of bacterial resistance and antimicrobial side-effect has shifted the focus of research toward Ethnopharmacology. A biologically active compound derived from the plants may increase the effectiveness of antibiotic when used in combination. The present study aims to determine the synergistic antibacterial effect of ethanolic extracts of *Punica granatum* (pericarp), *Commiphora molmol*, *Azadirachta indica* (bark) in combination with amoxicillin, metronidazole, tetracycline, and azithromycin on periodontopathic bacteria: *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans*.

**Methodology:** Periodontopathic bacterial strains were isolated from the plaque sample that was collected from periodontitis patients and grown under favorable conditions. Susceptibility of bacteria to the antibiotics and extracts was determined by disc diffusion method by measuring the diameter of the inhibition zones. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plant extracts were evaluated against each bacterium. Synergistic effect of plant extract in combination with antibiotics was tested against each bacterium by measuring the diameter of zone of inhibition (ZOI).

**Results:** Findings revealed that all plant extracts exhibited an inhibitory effects on the proliferation and growth of periodontopathic bacteria. The maximum antibacterial effect was exhibited by *C. molmol* on *P. gingivalis* (ZOI =  $20 \pm 0.55$  mm, MIC =  $0.53 \pm 0.24$  mg/mL and MBC =  $5.21 \pm 1.81$  mg/mL) ( $p < 0.05$ ), meanwhile, no antibacterial activity was exhibited by *P. granatum* on *T. forsythia*. Synergistic antibacterial effect was recorded when plant extracts were used in combination with antibiotics. The best synergism was exhibited by *P. granatum* with amoxicillin against *A. actinomycetemcomitans* ( $24 \pm 1.00$  mm) ( $p < 0.05$ ).

**Conclusions:** The synergistic test showed significant antibacterial activity when plant extracts were combined with antibiotics against all the experimented bacteria.

**Key words:** antibacterial activity; antibiotic; periodontopathic bacteria; plant extract; synergism.

*J Infect Dev Ctries* 2021; 15(11):1685-1693. doi:10.3855/jidc.14904

(Received 12 February 2021 – Accepted 23 May 2021)

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### Introduction

Oral health is an integral part of systemic health and may be considered as a foremost health issue that affects humans [1]. Among dental diseases, periodontal disease is an advanced lesion in the supporting periodontal tissue characterized by loss of surrounding alveolar bone, which is considered as one of the main causes of tooth loss in developing and underdeveloped countries [2,3]. Furthermore, studies from the recent past suggest a correlation between periodontitis and systemic diseases [4]. Literature from the past has confirmed that microbial plaque biofilm and their active

by-products are the primary cause of periodontal disease [5,6]. Out of all bacterial complexes present in biofilm, red complex pathogens are most commonly associated with periodontal disease. Increased levels of *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* have been reported in generalized periodontitis stage III or IV [7]. Whereas, *Aggregatibacter actinomycetemcomitans* is commonly associated with molar incisor periodontitis stage IV [8].

Periodontal therapy focuses on the removal and control of bacterial plaque by oral hygiene procedures and mechanical root debridement. The rationale for this

approach is to create “biologically acceptable” root surface by removing pathogenic bacteria and their by-products [9]. Mechanical debridement is the backbone for prevention and treatment of periodontal disease, but use of systemic or local antimicrobial agents can act as an adjuvant for maintaining long-term results. In clinical practice amoxicillin, metronidazole, tetracycline, and azithromycin are the most frequently used adjunctive therapy for the treatment of periodontitis cases [10-12]. Antimicrobial drugs play a crucial role during the management of infectious diseases. Nowadays, exponentially rising multidrug-resistant (MDR) bacteria are the predominant cause of treatment failure and increased percentage of mortality [13]. So, it becomes very crucial to develop antibacterial agents that not only inhibit the mechanisms of drug resistance but also improve the treatment outcome.

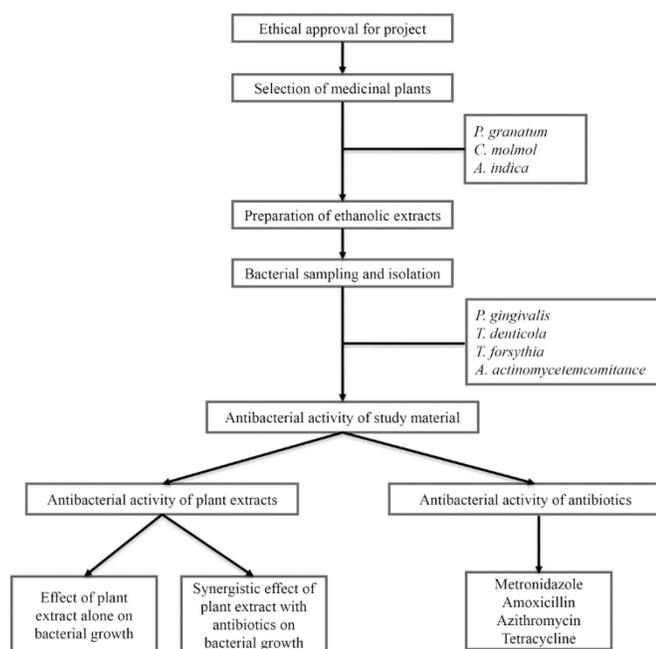
Plants and their extracts have been used for medicinal purposes since ancient era. Plants have the capacity to produce a variety of organic chemicals of high structural diversity, called secondary metabolites, such as alkaloids, terpenoids, flavonoids, and tannins, which have antimicrobial properties *in vitro* [14]. The most important advantages claimed for the use of herbal plants in various diseases are their safety, in addition to being economical, effective, and easily available [15].

Herbal extracts of *Punica granatum* (pomegranate), *Commiphora molmol* (myrrh) and *Azadirachta indica* (neem) have been shown to have good local

antibacterial and anti-inflammatory activity with no side effects [16,17]. Pomegranate is an extensively studied therapeutic plant that belongs to the family Punicaceae [18]. Results from *in vivo* and *in vitro* studies support antibacterial activity against oral and periodontal pathogens [19,20]. Myrrh is the dried gum-resin obtained from the bark of trees belonging to family Bruseraceae which is commonly found in the countries of the Arabian Peninsula. It is widely used as an antibacterial and anti-inflammatory agent in the field of Ethnopharmacology [19]. In the field of dentistry, it is commonly recognized as a remedy for spongy gums and aphthous stomatitis [21]. Neem is a fast-growing wonder plant that is most commonly used as herbal medicine. The tree belongs to the mahogany family Meliaceae species *A. indica*, and it is native to the Indian subcontinent. All the parts of the tree (leaf, bark, flower, seed, and fruit) have been documented to exhibit anti-inflammatory and antibacterial activity [22,23].

The present study aimed to evaluate the synergistic antibacterial effect of *P. granatum*, *C. molmol* and *A. indica* plant extract in combination with commercially available antibiotics amoxicillin, metronidazole, tetracycline, and azithromycin on periodontopathic bacteria such as *P. gingivalis*, *T. denticola*, *T. forsythia*, and *A. actinomycetemcomitans*. The current research was aimed at improving the antimicrobial properties of the plant extract with a view to discover new antimicrobial drugs effective against pathogenic organisms related to periodontal disease.

**Figure 1.** Flow chart of study design.



**Methodology**

*Study design and protocol*

The study employed was an *in vitro* experimental design. Ethical clearance to conduct the study was obtained from the Ethical Review Committee of the Institution (SRC/ETH/2016). Study protocols were explained to the patients and informed consent was obtained before collection of the plaque sample. Raw plant product (dried peel of *P. granatum*, gum resin of *C. molmol*, the bark of *A. indica*) was obtained from the country yard and local market in the city. Specimens were identified by a botanist and a pharmacognosist for their authenticity. Different antibiotics [metronidazole, amoxicillin, azithromycin, and tetracycline; (Sigma-Aldrich, Taufkirchen, Germany)] were used in combination with the different plant extracts, to evaluate the existence of a synergistic effect. The complete study protocol is presented in flow chart (Figure 1).

*Plants extract preparation*

The dried plants of *P. granatum*, *C. molmol* and *A. indica* were purchased from Abha city of Asir region of Saudi Arabia. The plants were coarsely powdered with the help of Grinder for 10 seconds. The powdered material (50 g) was packed in muslin cloth and subjected to Soxhlet extractor with absolute ethanol for continuous hot extraction for 72 hours. Thereafter, ethanolic extract of plants was filtered by muslin cloth followed by whatman-1 filter paper and the filtrate was vaporized under the reduced pressure and temperature by rotary evaporator (Buchi Rotavapor R-200). The dried plant extracts were further re-dissolved in ethanol at the concentration of 2 g/mL, which was used for antibacterial susceptibility assay. Stock solutions were prepared and a final working volume was achieved by diluting 2-fold dilution of the stock ranging from 2 g/mL to 50 µg/mL, which was used later for determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

*Microbiological sample collection*

Plaque samples were collected from the deepest pockets around the tooth from the patients with established active generalized periodontitis grade III and IV for bacterial strain (*P. gingivalis*, *T. denticola* and *T. forsythia*) and molar incisor periodontitis stage IV for bacterial strain (*A. actinomycetemcomitans*). Subgingival plaque was collected by inserting Gracey-curette number 5/6 (Hu-Friedy, Chicago, USA) into the periodontal pocket. As soon as curette reaches the base of the pocket subgingival sampling was performed with one single vertical stroke. The plaque sample was transferred to the paper point #40 taper 0.02 mm/mm (Co. Roeko, Germany) and immediately immersed into the anaerobic transport media [Sodium thioglycolate (Sigma-Aldrich, Taufkirchen, Germany)]. The samples were stored at -80 °C until used for experimental purpose.

*Selective Media for Bacterial Growth*

The following specific culture media were used for the growth and isolation of bacteria from the subgingival plaque sample (Table 1). The samples were incubated in an anaerobic chamber (Don Whitley

Scientific Ltd., Shipley, West Yorkshire, United Kingdom) with an environment containing 80% nitrogen, 10% hydrogen, and 10% carbon dioxide at 37 °C for 7 days.

We used selective media for the growth of the tested bacteria. After completion of the incubation, the plates were removed and observed for suspected colony characteristics such as pigmentation. Gram staining was performed using the suspected colonies. Further, identification of species was carried out for confirmation by performing a biochemical test, which included catalase, indole and nitrate reductase along with a sugar fermentation test using key sugars like glucose, sucrose, lactose, maltose, xylose, arabinose and salicin cellobiose. The indicator used for biochemical test was bromocresol purple which imparted a purple color to the solution. Purple indicates negative and yellow indicates positive for the particular sugar. A standard table was used to compare the results of biochemical tests and identification of species was carried out [24].

*Microbiological assay*

Antimicrobial susceptibility assays of the antibiotics

Antibiotics sensitivity assay was examined using disc diffusion method. Bacterial cultures were inoculated in LB broth media at 37 °C for 3 hours and turbidity was adjusted to 0.5 McFarland’s index in phosphate-buffered saline. A bacterial lawn was spread on 90 mm Mueller Hinton Agar (MHA) plates. The selected antibiotic discs (Metronidazole, amoxicillin, azithromycin and tetracycline) were put on MHA plates and the plates were anaerobically incubated for 24 hours at 37 °C. The diameter of the inhibition zone of bacterial growth around each antibiotic was measured and recorded in millimeters as described [25].

Antimicrobial susceptibility assays of the extract

Antimicrobial activity of the extract was examined using the agar well diffusion method. The bacteria culture were inoculated in LB broth media at 37 °C for 3 hours and turbidity was adjusted to 0.5 McFarland’s index in phosphate buffered saline. Wells measuring 6 mm diameter were formed in the LB agar by the cap of sterile syringe and lawn culture was formed on the agar

**Table 1.** Specific media used for bacterial growth.

Bacteria	Media
<i>P. gingivalis</i>	[Columbia agar base*, Bacitracin*, Colistin*, Nalidixic acid*]
<i>A. actinomycetemcomitans</i>	[Trypticase soy*, Bacitracin*, Vancomycin** (TSBV)]
<i>T. forsythia</i>	[Tryptic soy broth*, Yeast extract**, Vit. K*, N-Acetylmuramic acid*]
<i>T. denticola</i>	[Oral bacteria growth medium (OBGM)]

\* Sigma Aldrich Co Ltd, St. Louis, Missouri, United States; \*\* HiMedia Laboratories Pvt. Ltd, Mumbai-86, India.

using the sterile cotton swab from diluted culture. 20 µL extract from different plants (2g/mL) was transferred into each well and plates were anaerobically incubated for 24 hours at 37 °C. The diameter of the inhibition zone of bacterial growth around each well was measured and recorded in millimeters as described [25]. 20 µL of ethanol without any extracts was transferred into the well for each bacteria considered as control. The zone of inhibition produced by ethanol was subtracted by zone of inhibition produced by plant extracts.

**Determination of minimum inhibitory concentration (MIC)**

MICs of the plant extracts against bacterial strains were determined by micro-broth dilution assays using Muller Hinton broth. The concentrations of the extracts were ranged from 2 g/mL to 50 µg/mL. Consequently, 180µl culture of all bacterial strain was transferred into the wells of polystyrene sterile flat-bottom 96-well plates. 20 µl from the 2-fold dilution of the plant extract were loaded in triplicate wells for each strain. 20 µl of ethanol (5%) was loaded in triplicate wells considered as control. The starting inoculum for each strain was 1.5×10<sup>5</sup> CFU/mL. After 24 hours of anaerobic incubation at 37 °C, the lowest concentration of compounds that showed neither visible bacterial growth nor turbidity in micro-broth dilution assay was considered as MIC.

**Determination of minimum bactericidal concentration (MBC)**

To examine minimum bactericidal concentration (MBC) of the extracts, 100 µL of the culture from each well of the micro-broth assay was sub-cultured on Muller Hinton agar (MHA) plates anaerobically for 24 hours at 37 °C. The lowest concentration of extracts that showed no bacterial growth was considered as MBC. The experiments were performed in triplicate for each strain.

**Synergistic antimicrobial assays**

Different antibiotics were used alternatively in combination with plant extracts to evaluate the synergistic antimicrobial activity. The suspension of

bacterial strain was spread on MHA plates with a turbidity of 0.5 McFarland. The discs were kept under anaerobic conditions for 24 hours at 37 °C. For the evaluations of the synergistic effects, selected antibiotic discs were separately impregnated with 5 µL of different plant extracts (at the MBC value) and employed on the inoculated agar plates. The zones of inhibition produced by the plant extract in combination with standard antibiotics after overnight incubation were estimated as described by [26]: if zones of combination treatment > zone of plant extract + zone of the corresponding antibiotic, was interpreted as synergism; if zone of combination treatment = zone of plant extract + zone of correspondence antibiotic, was interpreted as additive; if zone of combination treatment < zone of plant extract + zone of the corresponding antibiotic, was interpreted as antagonism.

**Statistical Analysis**

All the experiments were performed in triplicates. For each result, data were summarized as mean ± standard deviation (SD). Statistical analysis was performed using SPSS (version 11.5, Chicago). One tail Student’s *t*-test was used to calculate the significance of the difference between the mean expression of given experimental sample and the control sample. A *p*-value of < 0.05 was considered significant.

**Results**

*Antibacterial activity of antibiotics and plant extracts*

In the present study, before evaluation of antibacterial activity of the ethanolic extracts from different plants, we first screened activity of selected antibiotics against the bacterial strains. Table 2 presented the result of the antibiotic sensitivity of experimented bacteria against the antibiotics used in the study. During the experimental period, azithromycin was the only antibiotic that reported activity against all the bacteria.

All four bacterial strains were treated with different plant extracts to evaluate the antibacterial activity of the extracts. A zone size of more than 8 mm was considered significant in terms of susceptibility of the strain to the

**Table 2.** Antibiotic sensitivity tests against experimental bacteria.

Antibiotics	Zone of inhibition (mm)			
	<i>P. gingivalis</i>	<i>T. denticola</i>	<i>T. forsythia</i>	<i>A. actinomycetemcomitans</i>
Metronidazole (20 µg)	4 ± 0.2 (R)	15 ± 0.3 (S)	18 ± 0.9 (S)	10 ± 1.13 (IS)
Amoxicillin (30 µg)	12 ± 0.36 (IS)	15 ± 0.35 (S)	13 ± 0.78 (S)	6 ± 1.28 (R)
Azithromycin (10 µg)	19 ± 1.56 (S)	17 ± 1.5 (S)	13 ± 1.3 (S)	15 ± 3.6 (S)
Tetracycline (10 µg)	15 ± 0.1 (S)	12 ± 1.5 (S)	8 ± 0.26 (R)	13 ± 0.2 (S)
Significance	0.001*	0.001*	0.001*	0.003*

\* *p* < 0.05 = significant difference; S = Sensitive; R = Resistance; IS = Intermediate Sensitive.

tested plant extract (Figure 2). Table 3 presented the result of antibacterial sensitivity of experimented bacteria to different plant extracts. All the plant extracts showed a significant zone of inhibition against the experimented bacteria, except for *P. granatum* to *T. forsythia*, which showed a non-significant zone of inhibition.

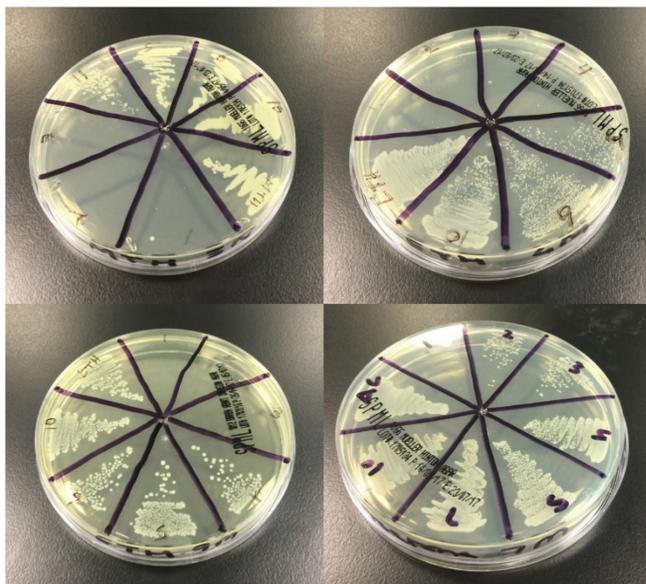
*MIC and MBC of plant extracts*

To compare the effect of different plant extracts on the growth of microorganisms tested in this study, we took MIC and MBC of all three extracts into consideration. As the absorption and diffusion of the extract-bioactive compounds that limit the effect on microbial growth in agar medium are ruled out in the liquid dilution method used for MIC and MBC determination. It is evident from Table 3 that the majority of bacterial strains were susceptible to ethanolic plant extract, except for *P. granatum* to *T. forsythia* showed no activity.

*Synergistic activity of plant extracts with antimicrobial agents*

When *P. granatum* was combined with amoxicillin, it was able to inhibit *T. forsythia* and *A. actinomycetemcomitans*, and the size of inhibition zone was suggestive of the strong synergy ( $p < 0.05$ ). Combination of *P. granatum* with metronidazole and tetracycline showed synergistic outcomes against *P. gingivalis* and *T. denticola* respectively (Table 4). Combination of *C. molmol* with tetracycline showed a synergistic effect against *P. gingivalis* and *T. denticola* ( $p < 0.05$ ). Whereas combination of *C. molmol* with metronidazole showed synergistic outcomes against *T. forsythia*, and a combination with amoxicillin showed synergy against *A. actinomycetemcomitans* (Table 5).

**Figure 2.** Determination of zone of inhibition by disc diffusion method.



*A. indica* showed dual synergism with azithromycin and tetracycline against *A. actinomycetemcomitans* ( $P < 0.05$ ). It showed synergistic activity in combination with amoxicillin, tetracycline and metronidazole against *P. gingivalis*, *T. denticola* and *T. forsythia* respectively (Table 6).

**Discussion**

Nowadays, many researchers around the world are working on plant extracts, to develop new antimicrobial agents with enhanced safety and efficiency [27-30]. The prime objective of ethnopharmacology is to identify plants for medicinal importance with minimal side effects. Additionally, active compounds from the plant extracts with antibacterial activity can be transformed into possible medication. Research to develop efficient

**Table 3.** Antimicrobial activity exhibited by ethanolic extract of *P. granatum*, *C. molmol* and *A. indica* against experimental bacteria.

Plant extract	Zone of inhibition (mm)			
	<i>P. gingivalis</i>	<i>T. denticola</i>	<i>T. forsythia</i>	<i>A. actinomycetemcomitans</i>
<i>P. granatum</i>	14 ± 0.45	19 ± 0.55	5 ± 1.15	15 ± 0.46
<i>C. molmol</i>	20 ± 0.55	16 ± 0.55	13 ± 0.26	18 ± 0.1
<i>A. indica</i>	13 ± 0.36	14 ± 0.5	12 ± 0.53	9 ± 0.45
Significance	0.013*	0.031*	0.012*	0.025*
<b>Minimum inhibitory concentration MIC (mg/mL)</b>				
<i>P. granatum</i>	5.21 ± 1.80	2.08 ± 0.72	-	8.30 ± 3.61
<i>C. molmol</i>	0.53 ± 0.24	2.56 ± 0.78	8.33 ± 3.61	2.08 ± 0.90
<i>A. indica</i>	0.64 ± 0.24	2.60 ± 0.90	5.35 ± 1.55	10.42 ± 3.61
Significance	0.019*	0.230	0.201	0.251
<b>Minimum bactericidal concentration MBC (mg/mL)</b>				
<i>P. granatum</i>	41.67 ± 14.43	21.45 ± 4.72	-	40.47 ± 14.23
<i>C. molmol</i>	5.21 ± 1.81	20.83 ± 7.22	78.33 ± 28.87	20.83 ± 7.22
<i>A. indica</i>	10.42 ± 3.61	41.67 ± 14.43	81.25 ± 38.65	95.67 ± 8.74
Significance	0.023*	0.270	0.210	0.280

\*  $p < 0.05$  = significant difference; - = No activity at the concentration of the extract used.

and accessible medication from active plant compounds in the interest of public health is the need of the present world. The present *in vitro* experimental study explored the synergistic effect of plant extracts *A. indica*, *P. granatum* and *C. molmol* in combination with commonly used antibiotics against the target periodontopathic microorganisms like *P. gingivalis*, *T. denticola*, *T. forsythia* and *A. actinomycetemcomitans*.

However, despite optimum periodontal therapy adjunctive use of systemic antibiotics are indicated in some individuals who showed continued attachment loss may be because of invasion of pathogenic bacteria in the tissue or due to poor host defense response [31]. In the present study, we had selected the group of antibiotics (metronidazole, amoxicillin, azithromycin, and tetracycline) which are commonly employed in the treatment regime of periodontal diseases [31-33].

In the present study, based on the facts from the previous studies we have selected ethanol as a solvent to extract active compounds from the plant products [34-36]. Active ingredients from the pericarp (peel) of Pomegranate are known to have antibacterial, anti-inflammatory and antifungal properties. *In-vitro* studies

from the literature revealed that cariogenic bacteria and primary colonizers of plaque are sensitive to the ethanolic extracts of *P. granatum* [37,38]. In one clinical study, the author recommended use of *P. granatum* mouthwash twice a day for fifteen days, as it showed more reduction in gingival inflammation when compared to 0.2% chlorhexidine mouthwash [39]. In another clinical study, authors found that *P. granatum* extract mouthwash has inhibitory action against *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* [40]. Limited *in vitro* studies are available in the literature evaluating the antibacterial effect of *P. granatum* on periodontopathic bacteria. The result of the present *in vitro* study demonstrated the antibacterial activity of *P. granatum* on the periodontopathic bacteria, except for *T. forsythia*. The antibacterial activity was highest against *T. denticola* (MIC = 2.08 ± 0.90 mg/mL, MBC = 20.83 ± 7.22 mg/mL) (*p* < 0.05) followed by *P. gingivalis* (MIC = 5.21 ± 1.80 mg/mL, MBC = 41.67 ± 14.43 mg/mL) and *A. actinomycetemcomitans* (MIC = 41.67 ± 14.43 mg/mL, MBC = 50 mg/mL). The results of present study are in

**Table 4.** Synergistic antimicrobial activity of *P. granatum* in combination with different antibiotics.

Bacteria	Antibiotics	MIZ antibiotics (mm)	MIZ <i>P. granatum</i> (mm)	MIZ antibiotics + <i>P. granatum</i> (mm)	Outcome	Significance
<i>P. gingivalis</i>	Metronidazole	4 ± 0.2	14 ± 0.45	20 ± 1.25	Synergism	* <i>p</i> < 0.05
<i>T. denticola</i>	Tetracycline	12 ± 0.35	19 ± 0.55	34 ± 4.00	Synergism	
<i>T. forsythia</i>	Amoxicillin	13 ± 0.78	5 ± 1.15	20 ± 1.73	Synergism	
<i>A. actinomycetemcomitans</i>	Amoxicillin	6 ± 1.28	15 ± 0.46	24 ± 1.00	Synergism	

\* *p* < 0.05 = significant difference.

**Table 5.** Synergistic antimicrobial activity of *C. molmol* in combination with different antibiotics.

Bacteria	Antibiotics	MIZ antibiotics (mm)	MIZ <i>P. granatum</i> (mm)	MIZ antibiotics + <i>P. granatum</i> (mm)	Outcome	Significance
<i>P. gingivalis</i>	Tetracycline	15 ± 0.36	20 ± 0.55	37 ± 1.23	Synergism	* <i>p</i> < 0.05
<i>T. denticola</i>	Tetracycline	12 ± 0.35	16 ± 0.55	30 ± 1.46	Synergism	
<i>T. forsythia</i>	Metronidazole	18 ± 0.9	13 ± 0.26	33 ± 1.38	Synergism	
<i>A. actinomycetemcomitans</i>	Amoxicillin	6 ± 1.28	18 ± 0.1	26 ± 2.05	Synergism	

\* *p* < 0.05 = significant difference.

**Table 6.** Synergistic antimicrobial activity of *A. indica* in combination with different antibiotics.

Bacteria	Antibiotics	MIZ antibiotics (mm)	MIZ <i>P. granatum</i> (mm)	MIZ antibiotics + <i>P. granatum</i> (mm)	Outcome	Significance
<i>P. gingivalis</i>	Amoxicillin	12 ± 0.36	13 ± 0.36	26 ± 0.28	Synergism	* <i>p</i> < 0.05
<i>T. denticola</i>	Tetracycline	12 ± 1.5	14 ± 0.5	28 ± 0.14	Synergism	
<i>T. forsythia</i>	Metronidazole	18 ± 0.9	12 ± 0.53	32 ± 1.48	Synergism	
<i>A. actinomycetemcomitans</i>	Azithromycin	15 ± 3.6	9 ± 0.45	26 ± 1.25	Synergism	
	Tetracycline	13 ± 0.2		25 ± 1.56	Synergism	

\* *p* < 0.05 = significant difference.

agreement with the findings from previous studies [38-40].

*C. molmol* is regularly used as a traditional medicine against a variety of diseases among the Saudi population of the Southern region [31]. Evidence from the clinical trials revealed that *C. molmol* containing mouthwash is effective in treating the gingival inflammation, and inhibition of biofilm formation [42,43]. To the best of authors' knowledge, there is no reported study which evaluates the *in vitro* effect of *C. molmol* on periodontopathic bacteria. In the present study, the extract of *C. molmol* showed antibacterial activity against all the studied periodontopathic bacteria. The highest antibacterial activity was reported against *P. gingivalis* (MIC =  $0.53 \pm 0.24$  mg/mL, MBC =  $65.21 \pm 1.81$  mg/mL) ( $p < 0.05$ ), followed by *A. actinomycetemcomitans* (MIC =  $2.08 \pm 0.90$  mg/mL, MBC =  $20.83 \pm 7.22$  mg/mL), *T. denticola* (MIC =  $2.60 \pm 0.90$  mg/mL, MBC =  $20.83 \pm 7.22$  mg/mL), and minimum against *T. forsythia* (MIC =  $8.33 \pm 3.61$  mg/mL, MBC =  $83.33 \pm 28.87$  mg/mL).

The leaf, bark, and seed of *A. indica* are known to exhibit antibacterial and antifungal properties against a wide variety of pathogens. Findings from the previous clinical studies conclude that *A. indica* based mouth rinse and toothpaste are highly efficacious as anti-plaque and anti-gingivitis agents [44,45]. The result of the present study showed the highest antibacterial activity of *A. indica* extract against *P. gingivalis* (MIC =  $0.64 \pm 0.24$  mg/mL, MBC =  $10.42 \pm 3.61$  mg/mL) ( $p < 0.05$ ). These findings are in accordance with the results of the study conducted by Heyman *et al.*, which revealed the good antibacterial activity of ethanolic leaf extract of *A. indica* against *P. gingivalis* [46]. *A. actinomycetemcomitans* was found to be the least susceptible bacteria to the extract of *A. indica* (MIC =  $10.42 \pm 3.61$  mg/mL, MBC =  $166.67 \pm 57.74$  mg/mL). The contrasting result was reported in comparison with the previous study, which showed no effect of leaf extract of *A. indica* on the growth of *F. nucleatum* [46], whereas in the present study we found antibacterial activity against *T. forsythia* (MIC =  $5.35 \pm 1.55$  mg/mL, MBC =  $83.33 \pm 28.87$  mg/mL).

The resultant outcome of combining two drugs can be synergistic, additive or antagonistic. Various *in vitro* experiments have established the fact that a combination of plant extracts and antibiotics possess a synergistic effect, which results in a significant decrease in levels of MIC for the antibiotics [47,48]. One of the main objectives of the present study was to evaluate and establish the combined effects of antimicrobial agents with plant extracts against clinical

strains of periodontopathic bacteria. The findings from the present study may help to understand the synergistic effect of combination therapy, which in the recent future could provide a new strategy for the treatment of periodontal infections. In the present study, a combination of *P. granatum* with amoxicillin and tetracycline revealed synergistic activity with a wider zone of inhibition against periodontal pathogens, with maximum synergism reported against *A. actinomycetemcomitans* ( $24 \pm 1.00$  mm). *C. molmol* in combination with tetracycline showed the highest synergism and widest zone of inhibition against *P. gingivalis* and *T. denticola* as compared to the other experimental groups. Combination of *A. indica* with tetracycline and azithromycin revealed the highest synergism against *A. actinomycetemcomitans*  $25 \pm 1.56$ mm and  $26 \pm 1.25$ mm respectively. The above-discussed findings are in accordance with the previous study, in which the author had reported synergism between ethanol plant extract and gentamycin against *P. gingivalis*, *T. denticola* and *T. forsythia* [49]. Similar study in the recent past also concluded that plant extracts enhanced the activity of tetracycline two-to four-fold, against resistant strains of periodontal bacteria [50].

## Conclusions

The issue of bacterial resistance is emerging rapidly in the medical field. Recent ethnopharmacological studies revealed that plants are a good source of antibacterial compounds. This activity can be enhanced by the synergism between herbal extracts and known antibiotics. Findings from the current study revealed that ethanolic extracts from all three plants *A. indica*, *P. granatum*, and *C. molmol* exhibit antibacterial activity against periodontopathic bacteria, except for *P. granatum* that showed no activity against *T. forsythia*. The synergistic test results showed significant antibacterial effects when plant extracts were combined with the antibiotics. The best synergism was exhibited by *P. granatum* with amoxicillin against *A. actinomycetemcomitans*, followed by *A. indica* with tetracycline against *A. actinomycetemcomitans*. The reported synergism between herbal extract and antibiotics will help to improve the treatment strategies against periodontal infections. Further researches are needed to find out the active compound in these plant extracts. However, the mechanisms involved in these synergistic effects are still poorly understood.

## Acknowledgements

The authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through General Research Project under grant number (GRP-164/1441).

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**Conflict of interests:** No conflict of interests is declared.