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Research Article

Synergistic Antimicrobial Potential of *Pistacia atlantica* Gum Essential Oil and *Camellia sinensis* Methanolic Extract Against *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa, a versatile pathogen causing a wide range of infections, faces escalating resistance to conventional antibiotics, necessitating exploration of alternative substances. Plant extracts and essential oils, known for potent antibacterial compounds, emerge as promising candidates to combat *P. aeruginosa* effectively. Aiming to investigate the additional antibacterial effect of *Camellia sinensis* and *Pistacia atlantica* gum, this study was conducted to inhibit the growth of *P. aeruginosa*. Essential oil from *P. atlantica* gum was extracted using a Clevenger apparatus, and *C. sinensis* (green tea) extract was obtained through maceration. Gas chromatography/mass spectrometry (GC-MS) and spectrophotometry identified compounds in the essential oil and extract. Antibacterial properties were assessed using the microdilution technique, and synergistic effects of combined substances were evaluated through the modified checkerboard method, utilizing the Fractional inhibitory concentration (FIC) index. *P. aeruginosa* (ATCC27853) was the target bacteria. Alpha-pinene and polyphenols were identified as predominant compounds in *P. atlantica* gum essential oil and *C. sinensis* extract, respectively. Both compounds exhibited comparable Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) values, at 625 and 1250 µg/ml, respectively. The FIC index calculation for the combination of essential oil and extract demonstrated enhanced antibacterial activity against *P. aeruginosa*. Results affirm the inhibitory and bactericidal properties of *P. atlantica* gum essential oil and *C. sinensis* extract against *P. aeruginosa*. Concurrent use exhibited synergistic antibacterial effects. This study suggests that these natural compounds, especially when combined, could serve as potent agents in the battle against *P. aeruginosa* infections, offering a potential avenue for the development of alternative antimicrobial strategies.

1. Introduction

1.1. P. aeruginosa Antibiotic resistance

The extensive utilization of antibiotics has given rise to an escalation in microbial resistance, posing a significant challenge in the

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realm of healthcare (Gasper et al., 2013). This predicament is particularly pronounced in opportunistic bacteria like *P. aeruginosa*, intensifying concerns about effective treatment (Hirsch et al., 2010). *P. aeruginosa*, with its inherent resistance and ability to develop resistance during treatment, has emerged as a formidable threat to individuals grappling with certain diseases, immunodeficiencies, and enduring complications necessitating prolonged hospitalization (Gasper et al., 2013; Hirsch et al., 2010). Combatting infections caused by this bacterium is intricate due to its broad resistance spectrum and the capacity to acquire resistance throughout treatment (Zavascki et al., 2010; Bouki et al., 2013). Several factors contribute to the antibiotic resistance of *P. aeruginosa*, including the bacterial membrane's low permeability, the presence of purines, the existence of efflux pumps, mutations in antibiotic target sites, and a high genetic capacity for expressing various resistance mechanisms (Zavascki et al., 2010; Van et al., 2013).

1.2. Herbal medicine

In the pursuit of strategies to address infections caused by resistant bacteria, exploring the antimicrobial properties of plant extracts has garnered attention, especially in the context of Multi-Drug-Resistant (MDR) bacteria (Chand, 2013). Essential oils derived from plants, containing volatile and aromatic compounds, serve a protective role in plants against infections and exhibit potent antimicrobial properties (Hammer et al., 1999). *Pistacia atlantica*, commonly known as wild pistachio or Baneh, belongs to the Anacardiaceae family and is indigenous to Iran (Padulosi et al.,1998). The gum extracted from *P. atlantica* is a traditional medicinal resource known for its efficacy in alleviating various ailments such as abdominal pain, stomach ache, indigestion, asthma, eczema, throat infections, kidney stones, and exhibiting anti-diarrheal, astringent, anti-fever, antibacterial, and antiviral properties (Tohidi et al., 2011). *C. sinensis* (green tea) is sourced from the leaves of the *C. Sinensis* plant and is renowned for its rich content of antioxidants, and anti-cancer and anti-inflammatory compounds, including polyphenols and caffeine (Shoa Hassani et al., 2008). Numerous studies have explored the impact of different plant extracts, including *C. sinensis*, on *P. aeruginosa* (Rao et al.,2010; Silva et al., 2010). While the antibacterial effects of *P. atlantica* gum essential oil and *C. sinensis* methanolic extract have been individually investigated against various bacteria, there is a dearth of research examining the combined antibacterial effects of these two compounds. This study seeks to fill this gap by exploring the potential synergistic antibacterial efficacy of the combination of *P. atlantica* gum essential oil and *C. sinensis* methanolic extract against *P. aeruginosa*.

2. Materials and Methods

2.1. Plant Material Collection

The plant materials utilized in this study comprised *P. atlantica* gum and *C. sinensis* leaves, sourced from the Eqlid suburbs of Fars province and Ramsar farms in Iran, respectively. Authentication and confirmation of these plant compounds were conducted by a botanical expert at the Herbarium of the Plant Identification Laboratory of the Agricultural Jihad Research Center of Lorestan province in Iran.

2.2. Extraction of P. atlantica Gum Essential Oil

A precise measurement of 100 g of *P. atlantica* gum was undertaken for essential oil extraction. Distillation with water, using a Clevenger apparatus, was employed for 3 to 4 h. Subsequently, the essential oil obtained underwent extraction with sodium sulfate and was stored in a dark-colored glass container at 4°C (British Pharmacopoeia,1988).

2.3. C. sinensis Extraction

C. sinensis leaves were initially washed, shade-dried, and then powdered using an electric mill. 500 ml of 98% methanol was added to 100 g of powdered *C. sinensis* leaves, and the mixture was placed in a shaker for 72 h. After filtration and centrifugation to remove suspended particles, the clear liquid was transferred to a rotary balloon for methanol extraction under a vacuum pump. The concentrated extract was then poured onto a glass plate, air-dried, separated from the plate,

and stored in a sterile airtight container in the refrigerator (Samsam Shariat, 1992).

2.4. Analysis of P. atlantica Gum Essential Oil by to gas chromatography/mass spectrometry (GC/MS)

The essential oil was subjected to GC/MS analysis to determine the percentage and type of its constituent compounds. Parameters such as the Retention Index (RI), simultaneous injection of standard compounds, and the study of mass spectra were employed for compound identification. Comparison with standard compounds and information available in the computer library of the GC/MS device facilitated the identification process (Haghiroalsadat, 2010).

2.5. Analysis of Methanolic Extract of C. sinensis

The quantification of polyphenolic compounds in the obtained extracts was conducted through a spectrophotometric method employing the Folin-Ciocalteu reagent (Siripatrawan et al., 2010). 1 ml of the diluted extract was mixed with 5 ml of 10% Folin-Ciocalteu reagent and vortexed for 5 min. After 1h in a dark place, the developed blue color was measured at a wavelength of 765 nm using a UV-160 Shimadzu spectrophotometer. A calibration curve for gallic acid (0.005 to 0.05 mg/l) was prepared, and the results were expressed as gallic acid equivalents per g of the sample based on this curve.

R^2 = 0/996 (Y= 0.0106 + 0.041

2.6. Source of microorganisms

The bacterium utilized in this study was *Pseudomonas aeruginosa* (ATCC 27853).

2.7. Determination of Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of *C. sinensis* extract and *P. atlantica* gum essential oil was assessed through the micro-broth dilution method. Initial stocks were prepared at a concentration of 20,000 ppm by dissolving 0.2 g of extract or essential oil in DMSO 10% (100 µl of dimethyl sulfoxide and 900 µl of distilled water). Sterilization was achieved using a 0.45-micron microbe filter. In 96-well plates, 50 µl of Muller Hinton culture medium, 50 µl of extract and essential oil, and 50 µl of bacterial suspension (equivalent to 0/5 of McFarland) were added. Final concentrations for extract and essential oil were 20,000, 10,000, 5,000, 2,500, 1,250, 625, 312.5, and 156 µg/ml. The plates were then incubated at 37°C for 24 h. After incubation, 50 µl of tetrazolium chloride (TTC) salt were added to each well. The colorless salt was transformed into red triphenyl formazan by living bacteria during respiration (NCCL, 2001).

2.8. Determination of Minimum Bactericidal Concentration (MBC)

5 µl from wells with no observed growth were transferred to Muller Hinton Agar culture medium and incubated for 24 h at 37°C. The lowest concentration without observable growth was considered the minimum bactericidal concentration (NCCL, 2001).

2.9. Checkerboard dilution test

The antibacterial effect and synergistic properties of the extract and essential oil combination were examined using the checkerboard dilution modified (FIC) method (NCCL, 2001). The FIC index was calculated for *C. sinensis* methanolic extract and *P. atlantica* gum essential oil. Values less than 0.5 indicated a synergistic effect, values between 0.5 and 4 indicated an additive effect and values greater than 4 suggested an antagonistic effect. Successive double concentrations of essential oil and extract were added to 96-well microplates, and bacterial suspension was introduced. Plates were incubated at 37°C for 24 h. After incubation, 50 µl of triphenyl tetrazolium chloride (TTC) reagent (5 mg/ml) were added to all wells, and the plates were incubated for an additional 20 min. The MIC of the bacteria was determined as the concentration higher than the last concentration that exhibited the tetrazolium color (Eliopoulos et al., 1996).

FIC_{index}=FIC of drug A + FIC of drug B

FIC of drug A=MIC of drug A in combination/MIC of drug A alone

FIC of drug B=MIC of drug B in combination/MIC of drug B alone

FIC Values:

Synergy <.0.5
Antagonism > 4
Additive or indifference 4-0.5

The mean results of triplicate experiments were statistically analyzed using SPSS software.

3. Results

3.1. GC/MS Analysis of P. atlantica Gum Essential Oil

The analysis revealed that alpha-pinene and beta-pinene are the predominant compounds in *P. atlantica* gum essential oil (Table 1).

3.2. C. sinensis Extract Composition

The examination of *C. sinensis* extract indicated that polyphenols are the most abundant compounds in *C . sinensis*.

3.3. MIC and MBC Results

Both *P. atlantica* gum essential oil and *C. sinensis* extract exhibited inhibitory effects on *P. aeruginosa* growth at a concentration of 625 µg/ml. At a concentration of 1250 µg/ml, they demonstrated bactericidal effects on *P. aeruginosa*.

3.4. Synergistic Effect of Combination

The checkerboard method was employed to investigate the combined effect of *C. sinensis* extract and *P. atlantica* gum essential oil on *P. aeruginosa*. The results indicated that the simultaneous use of these two compounds has an enhancing effect on microbial growth inhibition. The Fractional inhibitory concentration (FIC) obtained in this study was 1. According to the checkerboard rule, a value between 0.5 and 4 (0.5>1>4) suggests a simultaneous increasing effect of these two compounds on bacterial inhibition (Table 2).

In summary, the combined use of *C. sinensis* extract and *P. atlantica* gum essential oil demonstrated an additive effect, enhancing their ability to inhibit the growth of *P. aeruginosa*.

Table 1. Chemical components of *P. atlantica* gum essential oil

Peak	Compound	Area (%)	RI
1	Alpha-pinene	92.91	939
2	Camphene	0.92	953
3	Sabinene	0.49	976
4	BETA-PINENE	2.69	983
5	beta- Myrcene	0.35	991
6	DELTA-3-Carene	0.41	1011
7	PARA CYMENE	0.2	1026
8	D-Limomene	0.53	1031
9	1,8-Cineole	0.19	1033
10	ALPHA-TERPINOLENE	0.2	1088
11	alpha-Pinene oxide	0.31	1105
12	cis –Verbenol	0.24	1123
13	L-trans-Pinocarveol	0.22	1166
14	Trans-Verbenol	0.34	1145

Table 2. MIC combination and FIC *P. atlantica* gum essential oil and *C. sinensis* Extract

Pathogen	MIC combination (*mg*/*ml*) *P. atlantica* gum	MIC combination (*mg*/*ml*) *C. sinensis*	FIC *P. atlantica* gum	FIC *C. sinensis*	FIC Index
p. aeruginosa	3.12 ± 0.9	3.12 ± 0.9	0.5±0.04	0.5±0.04	1 ±0.8

4. Disscusion

The results of this study demonstrated that *P. atlantica* gum and *C. sinensis* extract possess antimicrobial properties due to the presence of bioactive compounds such as alpha-pinene and polyphenols (Fazly Bazzaz et al., 2016; Elahi et al., 2019). Mihalik et al. (2008) showed the inhibitory effect of green tea extract on the Quorum Sensing of *P. aeruginosa* in their study conducted in the United States. In the current study, green tea extract exhibited bacteriostatic and bactericidal properties against the mentioned bacteria (Mihalik et al., 2008). Maksum Radji et al. (2013) confirmed the antimicrobial effect of green tea extract on resistant *P. aeruginosa*, aligning with the results of the present study, indicating the antimicrobial effect of green tea extract on standard and resistant *P. aeruginosa* strains (Maksum Radji et al., 2013). Additionally, Aseel and May (2021) demonstrated the antimicrobial property of green tea on *P. aeruginosa*, consistent with the findings of the current study and previous research highlighting the presence of polyphenolic compounds in green tea with antimicrobial and anti- *P. aeruginosa* properties (Aseel and May., 2021).

Ghalem and Mohamed (2009) identified the antimicrobial effect of *P. atlantica* gum essential oil, and in the current study, *P. atlantica* gum essential oil exhibited antimicrobial effects against *P. aeruginosa*, confirming its antimicrobial properties (Ghalem and Mohamed., 2009). Salimi et al. (2011) analyzed the essential oil of *P. atlantica* gum using GC-MS and reported alpha-pinene as the main component at 81.9%. In the present study, the percentage of alpha-pinene in *P. atlantica* gum essential oil was 92.9%, aligning with the study by Elahi et al. (2019), reporting alpha-pinene content in *P. atlantica* gum as 92.5%, demonstrating the variability in the main component of essential oil and its antimicrobial properties (Salimi et al., 2011; Elahi et al., 2019). Parvin et al. (2012), Hosseini et al. (2013), Doosti (2019), and HamaAmin et al. (2022) confirmed the antimicrobial activity of *P . atlantica* gum essential oil. Among these studies, Doosti's results on the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *P. atlantica* gum on *P. aeruginosa* are consistent with the current study's findings (Parvin et al., 2012; Hosseini et al., 2013; Doosti., 2019; HamaAmin et al., 2022). The results of past research align with the present study, with alpha-pinene reported as the main component in all studies. In our research, alpha-pinene content was found to be 92.9%, the highest reported compared to previous studies. The consistency in previous and current research results confirms the presence of antimicrobial compounds such as alpha-pinene in *P. atlantica* gum and polyphenols in green tea extract. The combination of these two antimicrobial compounds, as demonstrated in the current study, can serve as an effective suppressive drug in the future against drug-resistant nosocomial infections caused by the significant bacterium *P. aeruginosa*.

Conclusion

In conclusion, the addetive effect discovered in this study by combining *P. atlantica* gum and green tea extract against *P. aeruginosa* highlights a significant advancement in the field of antimicrobial research. The observed enhancement in inhibiting and restraining the growth of *P. aeruginosa*, a crucial pathogen responsible for drug-resistant hospital-acquired infections, underscores the potential of harnessing the combined antimicrobial properties of these two plant-derived compounds.

The findings suggest that the addetive between *P. atlantica* gum and green tea extract can pave the way for the development of a potent therapeutic agent. This collaborative antimicrobial effect becomes particularly noteworthy in the context of combating drug-resistant strains of *P. aeruginosa*, addressing a critical concern in healthcare settings. The discovery opens avenues for further exploration and development of novel treatments, offering promising possibilities in the ongoing battle against nosocomial infections.

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