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Antibacterial Activities of Phytosterol Extracted from Kernels and Fresh Green Hulls of *Pistacia vera* L., Damghan Varieties

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Abstract

Background: Phytosterols (PSs) are plant-derived steroids known for their health-promoting effects, including anti-obesity, hypoglycemic, antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory properties. Previous studies have demonstrated that ethanolic and hydroalcoholic extracts from various parts of *Pistacia vera* L. (e.g., leaves and fruit) possess antioxidant properties. The consumption of nuts, including pistachios, appears to contribute to improved intestinal microbial composition. Furthermore, evaluations of key sterols such as campesterol, β-sitosterol, stigmasterol, and epicoprostanol against a range of bacteria have shown notable antibacterial properties.

Objectives: This is the first study to report on the antibacterial activities of PSs extracted from the kernel and pistachio green hull (PGH) of three Damghan pistachio cultivars.

Methods: The Soxhlet method was employed to extract total PSs from the kernel and PGH of the Abbasali, Akbari, and Khanjari cultivars of Damghan pistachios, sourced from Damghan Gardens. The antibacterial activities of the extracted PSs were tested against *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) bacteria in duplicate. Statistical analysis of the antioxidant activity results was conducted using SPSS software (version 27).

Results: The extracted PSs showed no antibacterial activity at concentrations ranging from 1.6 to 10,000 mg/mL.

Conclusions: In contrast to previous reports, no antibacterial effect was observed for the extracted total PSs. Further investigation is recommended to explore the reasons behind this discrepancy.

Keywords: Phytosterol, *Pistacia vera* L., Antibacterial, Damghan, Iran

1. Background

Plant sterols and stanol esters, collectively known as phytosterols (PSs), are bioactive compounds with a structural similarity to cholesterol found in plant cell membranes (1). Phytosterols are naturally present in fruits, fresh vegetables, nuts, seeds, and plant oils such as canola, corn, flaxseed, olive, and sesame oil. There are no reports of adverse side effects associated with PSs, and they are generally considered safe (2). A wide range of pharmacological and biological properties has been attributed to these plant sterols, including a reduced risk of cardiovascular diseases due to their LDL cholesterol-lowering effects, as well as anti-obesity, anti-inflammatory, hypoglycemic, and immunomodulatory activities. Due to their cholesterol- and LDL-lowering benefits, plant sterols have been approved for addition to foods by organizations such as the U.S. Food and Drug Administration (FDA) (3), the European Food Safety Authority (EFSA) (4), and other official health bodies [Reviewed in (1)].

Studies on high-fat diet mice suggest that the hypolipidemic effect of PSs is linked to the regulation of cholesterol metabolism through modulation of gut microbiota (5). In vitro and in vivo studies indicate that phytosterol-enriched diets improve gastrointestinal tract function by increasing beneficial microbiota species such as *Eubacterium halii* and decreasing the abundance of *Firmicutes* bacteria (6), which are associated with enhanced calorie absorption and weight gain (7). Furthermore, PSs contribute to reduced

appetite and food intake by promoting dietary fiber fermentation and increasing the production of short-chain fatty acids such as acetate and butyrate. Additionally, they reduce the production of cholesterol metabolites (8, 9). These findings highlight the therapeutic potential of PSs in addressing obesity, metabolic disorders, and gastrointestinal issues (10).

Phytosterols also support colonic epithelial cells by providing more energy and play a preventive role against colon cancer (8, 9). They improve immune responses in infants by modulating intestinal microbiota and increasing the diversity of bacterial genera such as *Anaerostipes*, *Bacteroidetes*, *Firmicutesin*, *Staphylococcus*, and *Streptococcus* in breast milk (9). Moreover, some studies report anticancer and antimicrobial activities of PSs (1, 2).

Global research is increasingly focused on developing novel therapies to combat antimicrobial and antiviral resistance. According to various studies, β-sitosterol extracted from Parthenium *hysterophorus* (11), avocado (12), the bark of Norway spruce (*Picea abies*) (13), and *Lycoris radiata* (14), as well as campesterol and its semi-synthetic derivatives (15), and stigmasterol (16), have demonstrated antibacterial activity. Pistachio nuts, in particular, are a rich source of healthy fats, fiber, protein, vitamins, and various phytochemicals, including anthocyanins, carotenoids, flavonoids, phenolic acids, and PSs (17).

2. Objectives

The antimicrobial activity of oleoresin (18), leaf phytochemicals (19), polyphenol-rich extracts from kernels (20), and lipophilic extracts from the leaves, stems, branches, hulls, woody shells, and kernels (21) of *Pistacia vera* L. has been previously investigated. Additionally, studies have been conducted on the antimicrobial properties of hull essential oil (22) and organic nanocomposites derived from hull extract (23) of *P. vera*. The objective of this study was to assess the antibacterial activities of total PSs extracted from the kernels and pistachio green hulls (PGHs) of *P. vera* Var. Damghan.

3. Methods

3.1. Plant Material

The kernels and PGHs of cultivars, including Abbasali, Akbari, and Khanjari, were sourced from Damghan (Iran) orchards in August 2021 and naturally sun-dried under our supervision. The cultivars were identified by Dr. Atefeh Amirahmadi (Ph.D. in Plant Biosystematics, School of Biology, Damghan University), and their specimens were preserved in the herbarium of Damghan University (Amirahmadi et al. 3086 (DU 001771), Amirahmadi et al. 3094 (DU 001780), and Amirahmadi et al. 3093 (DU 001779), respectively). High analytical grade chemicals (Merck or Sigma-Aldrich) were used in this study.

3.2. Phytosterol Extraction

Total phytosterol was extracted from the kernels and PGHs of the studied cultivars using a modified Soxhlet method as reported by Hashemi et al. [(24), under review].

3.3. Antibacterial Tests

The antibacterial activity of the extracted total PSs was assessed using the disk diffusion agar method (25) and the twofold dilution method (26). The Gram-negative and Gram-positive standard strains, namely *Escherichia coli* (*E. coli*; PTCC No: 1399; ATCC 25922) and *Staphylococcus aureus* (*S. aureus*; PTCC No: 1431; ATCC 25923), were obtained from the Iranian Research Organization for Science and Technology (IROST). Each organism was tested in duplicate on different days to ensure the reproducibility of the test. Ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), and penicillin (10 µg) were purchased from PadtanTeb Company (Iran) and used as positive controls.

3.3.1. Evaluation of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The broth dilution method was used to determine the minimum inhibitory concentration (MIC) of the extracted total PSs against microorganisms. A 24-hour culture of each strain was examined microscopically, and 1.5×10^8 bacteria, prepared from morphologically similar colonies cultured overnight, were considered equivalent to 0.5 McFarland. This equivalence was confirmed by measuring absorbance in the range of 0.08 to 0.13 at a wavelength of 625 nm using a spectrophotometer (26, 27).

Fifty microliters of bacterial suspension were added to 96-well microtiter culture plates, followed by 50 µL of PSs at different concentrations (1.6 - 10,000 mg/mL). The plates were incubated for 24 hours at 37°C. The phytosterol extracts were not sterilized; however, sterile

antibiotic discs with a diameter of 6 mm were used as positive controls. Sterile distilled water served as a negative control. All tests were conducted under sterile conditions within a microbial hood. The results were evaluated by observing visible growth inhibition in the microbial tubes (absence of turbidity).

The minimum bactericidal concentration (MBC) was determined by subculture. Approximately 10 µL of each MIC tube showing no visible growth was plated onto Mueller-Hinton agar and incubated at 37°C for 24 hours. Colony growth was then examined. All tests were repeated three times to ensure consistency and reliability.

3.4. Statistical Analysis

Statistical analysis of the data was performed using SPSS software (version 27), with a significance level set at ($P < 0.05$).

4. Results

The evaluation of antibacterial activity of PSs extracted from Damghan pistachio varieties, assessed using the Kirby-Bauer disc diffusion method against both Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E. coli*), revealed that neither the kernel nor the PGH PSs exhibited any antibacterial activity against the tested bacteria.

5. Discussion

P. vera is the most economically significant species among the 11 species of the Pistacia genus (17). In this study, the antibacterial activity of total PSs extracted from the kernels and PGHs of three Damghan pistachio cultivars was examined for the first time. Our findings revealed that PSs extracted from neither the kernels nor the PGHs exhibited antibacterial activity against *S. aureus* and *E. coli*.

Various health benefits, including antimicrobial and antifungal properties, have been attributed to PSs (1, 2, 13). The antibacterial activity of PSs extracted from sources other than pistachio has been reported in several studies. For instance, the efficacy of β-sitosterol isolated from the chloroform extract of *Parthenium hysterophorus* against aquatic bacterial pathogens (11), avocado using the saponification method (12), *P. abies* using Soxhlet extraction and supercritical fluid extraction (13), methanol extract and solvent fractions from *L. radiata* (14), campesterol and its semi-synthetic derivatives (15), and stigmasterol isolated from the stem bark of *Neocarya macrophylla* (16) have been documented.

Ozcelik et al. investigated the antibacterial, antiviral, and antifungal activities of 15 lipophilic extracts obtained from different parts (branch, stem, leaf, kernel, hull, seeds) of *P. vera* and demonstrated limited antibacterial activity compared to its notable antifungal and antiviral activities (21). Smeriglio et al. found that 7.11 mg/mL of hull essential oil from *P. vera* Variety Bronte exhibited bactericidal effects (22). Bakhshi et al. synthesized copper nanoparticles (CuNPs) from hull essential oil of *P. vera* and reported their antibacterial properties (23).

To the best of our knowledge, this is the first report evaluating the antibacterial activities of total PSs extracted from the kernels and PGHs of *P. vera* Var. Damghan. It is also the first study to report the absence of antibacterial activity for total PSs. The choice of solvents for extraction significantly impacts the quantification and biological activity of extracted phytochemicals, including PSs (28).

In this study, total PSs extracted from the PGHs and kernels of three pistachio cultivars were analyzed, whereas previous studies have investigated either β-sitosterol (11-14), campesterol and its semi-synthetic derivatives (15), and stigmasterol (16) obtained from plants other than *P. vera*, or lipophilic extracts from different parts of *P. vera* (21). Other studies have shown bactericidal properties for hull essential oil from *P. vera* Variety Bronte (22) and biosynthesized CuNPs from pistachio hull essential oil (23). Further research is needed to isolate and identify extracts from *P. vera* with significant antimicrobial properties and to replicate existing findings on the antibacterial activity of PSs under comparable experimental conditions.

Footnotes

Authors' Contribution: Study concept and design: M. S. and A. R.; acquisition of data: S. K. D., N. C., E. H., S. M., and A. S.; analysis and interpretation of data: M. S., A. R., S. K. D., N. C., E. H., S. M., and A. S.; drafting of the manuscript: A. R., S. K. D., N. C., E. H., S. M., and A. S.; critical revision of the manuscript for important intellectual content: M. S.; statistical analysis: M. S. and A. R.; administrative, technical, and material support: M. S. and A. R.; study supervision: M. S. and A. R.

Conflict of Interests Statement: The authors declare that they have no conflict of interest.

Data Availability: The dataset presented in the study is available on request from the corresponding author during submission or after publication.

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