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# Extraction of Phytosterols from the Green Hull of *Pistacia vera* L. var Damghan and Optimization of Extraction Methods

**Running Title:** Phytosterols Extraction from the Green Hull of *Pistacia vera* L. Var Damghan

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## ABSTRACT

Phytosterols, as valuable compounds with known health-beneficial effects are extensively used in nutrition, pharmaceuticals, and cosmetics. Pistachio green hull (PGH) is a cost-effective agricultural waste that contains bioactive compounds. This study aimed to identify and quantify the types and levels of phytosterols in the PGH and kernel of Abbasali, Akbari, and Khanjari pistachio cultivars of Damghan (Iran) and to optimize the phytosterol extraction using the Soxhlet and Folch methods.

The phytosterol types and quantities were analyzed using gas chromatography with flame ionization detection, following the methods established by the International Olive Council. The Soxhlet and the Folch methods underwent delicate modifications, including adjustments in the concentration of potassium chloride (KCl) and the transesterification condition. A total of eleven phytosterols were identified in the oil extracted from PGHs and Kernels of the studied cultivars. β-sitosterol was the most abundant sterol in all samples. The kernel of Abbasali cultivars had the greatest phytosterol amount (1887 mg/kg), followed by Akbari (1828 mg/kg) and Khanjari (1758 mg/kg). The ratio of total phytosterol content in the kernel to that in the PGH for the studied cultivars was as follows: Abbasali: 3.3, Akbari: 2.3, and Khanjari: 2.9 .

The most effective fat removal and the maximum amount of phytosterol were observed at 25 ºC with a 1 M KCl solution using the modified Soxhlet method. The mild conditions along with the modifications to the methanol: sulfuric acid ratio and time seem to be an appropriate approach for extracting phytosterols from PGH using the Soxhlet method.

**Keywords:** Phytosterols, *Pistacia vera* L., Pistachio green hull, The Soxhlet method, The Folch method

**Statements and Declarations**

**Competing Interests and Funding:** There is no conflict of interest/financial disclosure.



## INTRODUCTION

Phytosterols or plant sterols are found in plants as crucial elements of the lipid bilayer in cell membranes and in plant tissues, especially in seeds, vegetables, and grains. These compounds exhibit structural and functional similarities to cholesterol in animals. Extensive research has demonstrated their capability to reduce serum cholesterol levels, which in turn reduces the risk of heart diseases [1]. Phytosterols are widely used in various fields, including nutrition, pharmaceuticals, and cosmetics to enrich foods and cosmetic products [2]. These valuable compounds also display other health-beneficial effects, such as anti-atherosclerotic, anti-inflammatory, anticancer, antibacterial, antioxidant, and anti-ulcerative activities comprising their merits as medically active nutraceuticals [1, 3].

Phytosterols can be extracted from vegetable oils (e.g. olive, corn, rapeseed, soya, and sunflower) or industrial by-products which are produced during the production of cocoa hulls, chocolates, and sugarcane juice), which adds to the value of the latter [2]. In the pistachio industry, both pistachio green hull (PGH) is an ample waste

[4], constituting 34-45 % of the whole fruit [5], worldwide and in Iran (>104.0 $\times 10^6$ kg). This abundant and cost-effective agricultural waste is a rich source of phenolic and bioactive compounds with high antioxidant activity and promising health advantages [4, 6-8]. Grace *et al* reported anacardic acids (31.98 g/kg), phytosterols (19.20 g/kg) and fatty acids (15.00 g/ kg) as the major components in PGH [7].

Iran is a prominent producer and exporter of *Pistacia vera* L. with an annual output of over 135,000 tons [9]. Pistachios are cultivated in the central and southern districts of Iran such as Kerman, Rafsanjan, and Damghan [10]. The most well-known commercial cultivars of Damghan pistachio include Akbari, Abbasali, Khanjari, Kallehghouchi, and Shahpasand.

Heretofore, most studies are limited to the examination of the potential for aflatoxin production in isolates of *Aspergillus flavus* from pistachio [11], the chemical composition of pistachios harvested from different regions of Iran such as East Azarbaijan province [12], and Damghan [13]. Arena et al. also compared the composition of fatty acids and phytosterols in oil extracted from pistachio seeds from countries Greece, Iran, Italy, and Turkey [14]. Although, phytosterols have been extracted from various sources such as corn, rapeseed, sunflower, grape, and wheat [15], and standards have been defined for certain vegetable oils, including pistachio kernel oil [16], limited publications are available for PGH.

The oil extraction technique determines the quantity and quality of the extracted oil and the subsequent research on plant bioactive compounds. Enhancing the yield and quality, and reducing the overall process cost, and optimizing time and heat requirements are important [17]. Various techniques for oil extraction [17, 18] and isolation and purification of sterols are used [15]. Disadvantages, such as the use of solvents and longer extraction time, have been noted for conventional methods like the Soxhlet method; however, new green extraction methods such as enzyme-assisted extraction (EAE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), pulsed electric field-assisted extraction (PEF), supercritical fluid extraction (SFE), and ultrasound-assisted extraction (UAE) are not yet commonly used on a large scale and need further optimization regarding the solubility and nature of the bioactive compounds to be extracted [18]. Conventional and classic extraction methods, such as the Soxhlet and Folch techniques, provide the foundation of widely used standard methods and patents for phytosterols isolation and identification [19-21]. In fact, the Soxhlet method has been the oldest and most referenced technique [22], while the Folch procedure using a chloroform/methanol (2:1) mixture is the most commonly used organic solvent extraction method proposed in 1959 [23]. A comparison of bioactive compounds in oils extracted from various sources by conventional and alternative green methods has been reported [24]. To the best of our knowledge, such studies are rarely available for the effects of extraction techniques -whether conventional or improved alternative methods- on the phytosterol composition and amount in *P. vera*, especially its PGH. Studies on other nuts, such as *Juglans regia* L. showed that Soxhlet extraction yielded significantly higher oil compared to cold pressing (CP) and UAE [25].

The primary objective of this study was to identify and quantify the types and levels of phytosterols in the PGH and kernel of Abbasali, Akbari, and Khanjari cultivars of Damghan. The second aim was to optimize the extraction method for phytosterol extraction from PGH using the Soxhlet and Folch extraction methods, which all laboratories may have the capability to carry out.

## MATERIAL AND METHODS

Damghan pistachios cultivars, including Abbasali, Akbari, and Khanjari, were sourced from Damghan orchards (August 2021) and acquired and peeled at Pistachio Processing Factory (Berom village, Damghan). The endocarps and seed coats of the Pistachios were manually removed, and the kernels and PGHs were naturally sun-dried under our supervision. Abbasali, Akbari, and Khanjari cultivars of Damghan pistachio were identified by Dr. Atefeh Amirahmadi (PhD in Plant Biosystematics, School of Biology, Damghan University), and their specimens were kept 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 in accordance with the purpose of the experiments.

### Oil Extraction from Cultivars of Damghan Pistachio's Kernel and PGH

The analysis of phytosterols types and quantities in the kernel and green hull of three pistachio cultivars was carried out in the Iranian National Standard Organization (INSO) using the methods of the International Olive Council (IOC) [20]. Briefly, kernels (10 g) and PGHs (30 g) were ground and filtered via a sieve. Subsequently, the samples' powders were mixed with 50 mL of 2 M methanolic KOH and 500 µL of 0.2% 5α-Cholestan-3β-ol as an internal standard, and the mixture was incubated for 1 h at 80 ºC. After cooling, 100 mL of distilled water ($dH_2O$) was added, and the resulting mixture was transferred to a separatory funnel. Then, 150 mL of diethyl ether was added to the separatory funnel, and the contents were vigorously shaken. The mixture was left for 8 h and then separated into two phases. The lower phase, which contained water and sample residues, was discarded, while the upper phase, which contained diethyl ether with the extracted sterols, was retained. To remove excess KOH, 800-1000 mL of deionized water was added to the organic phase to ensure complete removal. The organic phase was then separated using a separatory funnel, allowing the non-saponifiable material to remain unaltered. To confirm the removal of excess KOH, 1-2 drops of phenolphthalein were added to the outlet of the separatory funnel; a color change would indicate an alkaline environment, while no change would indicate successful removal of the excess KOH (ISO 12228-2). The ether phase was subjected to solvent evaporation using a rotary evaporator. The resulting residue consisted of a soap-free mixture containing sterols, terpenes, aliphatic alcohols, carotenes, tocopherols, and various other compounds.

### Identification of Phytosterols in Oil of Kernel and PGH Using Gas Chromatography

The analysis of phytosterols in the oil samples derived from the studied samples was conducted using a Young Lin 6000 gas chromatography (GC) with a capillary column (HP5, 30 m, 0.53 mm, and 0.2 µm) following the guidelines specified by the IOC [20]. Thin-layer chromatography plates (silica gel 60 F254) were utilized to separate the sterols and triterpene alcohols. The separated components were subsequently silylated and subjected to GC for comprehensive analysis. All chemicals and reagents used in this study were of analytical grade. The peak areas obtained from the analysis were calculated using electronic integration. The concentration of each individual sterol in milligrams (mg) per kilogram (kg) of fat was calculated using the following formula (the response factor was considered equal to 1):

$$X = \frac{A_x \times m_s \times 1000}{A_s \times m}$$

$A_x$ = area under the peak for sterol x, in computing system counts.
$A_s$ = area under the peak for the α-cholestanol, in computing system counts.
$m_s$ = mass of α-cholestanol added in mg.
m = mass of the sample used for determination, in g.

### Optimization of Phytosterol Extraction Methods

The Soxhlet [19, 21] and Folch (chloroform/methanol) [19, 23] methods underwent slight modifications to improve purity and identify the most effective approach for extracting phytosterols from Khanjari PGH for several reasons. The primary rationale is that these two techniques are extensively used to extract various compounds from plant matrices and are available in most laboratories worldwide. The selection of the Khanjari cultivar is based on several factors, including its availability as the first and most abundant cultivar in the fruit season. Additionally, it is easily distinguishable from other varieties due to its long and dagger-like shape, which gives it its name in Persian (in Persian, Khanjari means dagger-like). Lastly, PGH is an abundant and cost-effective waste compared to the valuable pistachio kernel.

### The Soxhlet Method

In this procedure, phytosterol was extracted from 30 g of Khanjari PGH powder using a soxhlet extractor and 200 mL of *n*-hexane at 69 ºC for 8 h [19, 21] with modifications. After the extraction, 70 mL of 1 M KCl solution was added to the extracted oil and stirred for 30 min at room temperature. After the separation of the resultant phases and evaporation of the solvent, the oily phase was recovered. A sulfuric acid catalyst faciliated the formation of fatty acid methyl esters by the transesterification reaction of the extracted oil with methanol. A mixture of the extracted oil with methanol/sulfuric acid in a 50:50:1 (v/v) ratio was stirred for 4 h at 500 rpm under reflux conditions. After the transesterification process, excess methanol was evaporated under vacuum conditions. A brine solution (50 mL) at 60 ºC was added to the mixture and stirred on a heater stirrer at 60 ºC for

30 min. The resulting mixture was poured into a separatory funnel, and the bottom layer was removed. The mixture was washed with 25 mL of hot $dH_2O$ and stored in a separatory funnel at room temperature for 10 h to remove the acid and salt. The esterified mixture was crystallized in a crystallizer at 4-10 °C for 10 -15 h. The residual water contained in the esterified mass promotes the crystallization of the phytosterols from the esterified mixture. After crystallization, the mixture was filtered under vacuum through a Buchner funnel, and the residue was washed with 50 mL of cooled *n*-hexane at 4 °C. The remnant was considered as total phytosterol and analyzed using GC-FID in the INSO.

To enhance both the yield and purity of the extracted phytosterols, four modified methods were investigated, involving variations in the main Soxhlet method [6]. The modifications included changes in the concentration of KCl, the ratio of methanol to sulfuric acid, the transesterification time, and the mixing time with hot salt water, as outlined in Table 1.

**Table 1** Comparison of phytosterol extraction steps from 30 g of fresh PGH of Khanjari pistachios between the Soxhlet [19, 21] and its modified methods.

| | The Soxhlet method | The 1st modified method | The 2nd modified method | The 3rd modified method | The 4th modified method |
|---|---|---|---|---|---|
| KCl concentration (M) | 1 | 1 | 1 | 1.5 | 1.5 |
| Mixing time (h) | 0.5 | 2 | 2 | 2 | 2 |
| Mixing temperature with KCl (ºC) | 25 | 25 | 60 | 25 | 60 |
| Methanol to sulfuric acid ratio | 98:2 | 96:4 | 96:4 | 96:4 | 96:4 |
| Transesterification time (h) | 4 | 6 | 6 | 6 | 6 |
| Mixing time with the brine solution (h) | 0.5 | 1 | 1 | 1 | 1 |

The volume of *n*-hexane (200 mL), time (8 h) and temperature (69 ºC) of mixing with n-hexane, the volume of KCl solution (70 mL), transesterification temperature (50 ºC), the volume of brine solution (5 mL) and temperature of mixing with it (60 ºC) were the same between all examined methods. The volume of methanol and sulfuric acid mixture used was equal to that of the oil obtained from the Soxhlet extraction.

## The Folch Method

In this process, 25g of Khanjari PGH powder was stirred with a 300 mL solution of chloroform: methanol (2:1) for 2 h at room temperature. Afterward, the resulting mixture was filtered and combined with 70 mL of 1 M KCl solution, followed by an additional 30 min of stirring. Over the next 10 h, the oil and water separated, and the lower layer containing the oil was extracted using a rotary evaporator [19, 23]. Subsequently, the oil was treated with methanol: sulfuric acid solution in a 98:2 ratio and stirred for 4-6 h at 50 ºC. The transesterified mixture was then washed with the brine solution and hot $dH_2O$, and it was kept in a separatory funnel at 0-25 for 10 h. Afterward, the mixture was separated, and the oil layer was washed with *n*-hexane, the oil layer was crystallized in *n*-hexane at 4-10 ºC for 10-15 h. The obtained crystals were vacuum filtered through a Buchner funnel and washed with 50 mL of cooled *n*-hexane at 4 ºC. *n*-hexane was evaporated after about 4 h in a rotary evaporator at 69 ºC. The remnant was analyzed as a total using GC-FID in the INSO. Four modified versions of this method were used to improve the extraction process, which involved slight variations in temperature, duration, and ratios of methanol to sulfuric acid, as summarized in Table 2.

**Table 2** Comparison of phytosterol extraction steps from 30 g of fresh PGH of Khanjari pistachios between the Folch [19, 23] technique and its modified versions.

| | The Folch Method | The 1st modified method | The 2nd modified method | The 3rd modified method | The 4th modified method |
|---|---|---|---|---|---|
| KCl concentration (M) | 1 | 1 | 1 | 1.5 | 1.5 |
| Mixing time with KCl (h) | 0.5 | 2 | 2 | 2 | 2 |
| Mixing temperature with KCl (ºC) | 25 | 60 | 25 | 60 | 25 |
| Methanol to sulfuric acid ratio | 98:2 | 96:4 | 96:4 | 96:4 | 96:4 |
| Transesterification time (h) | 4 | 6 | 6 | 6 | 6 |

| | | | | | |
|---|---|---|---|---|---|
| Mixing time with the brine solution (h) | 0.5 | 1 | 1 | 1 | 1 |

The volume of methanol (100 mL) and chloroform (200 mL), the time (2 h) and temperature (25 ºC) of mixing with them, the volume of KCl solution (70 mL), transesterification temperature (50 ºC), the temperature of mixing with the brine solution (60 ºC) were the same between all examined methods. The volume of methanol and sulfuric acid mixture used was equal to that of the oil obtained from the Soxhlet extraction. The volume of the methanol and sulfuric acid mixture used was equal to that of the oil obtained from the Folch extraction.

## RESULTS

### Phytosterols Identified in the Oil of Kernels and PGHs of Damghan Pistachio Cultivars

The types and amounts of phytosterols extracted from PGHs and Kernels of Damghan pistachio varieties (Akbari, Khanjari, and Abbasali) using the IOC method [20] are shown in Table 3. The GC-FID chromatograms of phytosterols extracted from the fresh hull and kernel of the studied cultivars are presented in Figure S1 (a-f). The chromatogram revealed the order of peaks corresponding to the sterol composition as cholesterol, brassicastrol, campesterol, stigmasterol, clerosterol, β-sitosterol, sitostanol, $\Delta^5$-avenasterol, $\Delta^{5,24}$-stigmastediol, $\Delta^7$-stigmastenol, $\Delta^7$-avenasterol. The sterols β-sitosterol, stigmasterol, and campesterol were the most abundant compounds extracted from Abbasali and Khanjari PGHs. However, these profiles were different from that observed in Akbari PGH which included β-sitosterol, $\Delta^7$-stigmastenol, and campesterol. The most prevalent compounds found in Akbari and Khanjari kernels were β-sitosterol, campesterol, and $\Delta^5$-avenasterol. Nevertheless, these compositions were distinct from the profile identified for the Abbasali kernel, which consisted of β-sitosterol, $\Delta^5$-avenasterol, and campesterol respectively. As results showed, β-sitosterol with a significant difference, was the most abundant sterol in all samples (GC-FID chromatograms of phytosterols extracted from 30 g of PGH of (a) Abbasali, (b) Akbari, and (c) Khanjari varieties and 10 g of pistachio kernel of (d) Abbasali, (e) Akbari, and (f) Khanjari varieties using the International Olive Council (IOC) [20] are shown in Figure S1). The types and amounts of phytosterols are presented in Table 3.

### Optimization of Phytosterol Extraction

### The Soxhlet and Its Modified Methods

The total amount of phytosterols extracted from Khanjari PGH using the Soxhlet method was 70.41 mg/kg compared to the 1st (101.21 mg/kg), 2nd (54.74 mg/kg), 3rd (20.15 mg/kg), and 4th (110.19 mg/kg) modified methods. The types and quantities of the identified phytosterols are reported in Table 4 and Figure S2 demonstrates the GC-FID chromatograms of phytosterols obtained from Khanjari PGH using the Soxhlet and its modified methods (GC-FID chromatograms of phytosterols extracted from 30 g of Khanjar PGH by the Soxhlet [19, 21] and its modified methods are shown in Figure **S1**). The types and amounts of phytosterols are presented in Table 4. For details on each method, refer to Table 1.

The main Soxhlet method which included mixing with a 1 M KCl solution for half an hour at room temperature (25 °C), using a 98:2 ratio of methanol: sulfuric acid with a reaction time of 4 h effectively eliminated lipids, but it did not yield a significant amount of sterols. The 1st modified method (mixing with a 1 M KCl solution for 2 h at 25 °C, 6-h reaction with methanol: sulfuric acid of 96:4 ratio) resulted in a small amount of fatty acids and a high yield of phytosterol. Using the 2nd modified approach (mixing with a 1 M KCl solution for 2 h at 60 °C, the 96:4 ratio of methanol: sulfuric acid with a reaction time of 6 h), neither fatty acid removal nor sterols yield was satisfactory. In the 3rd modified method (mixing with a 1.5 M KCl solution for 2 h at 25 °C, the 96:4 ratio of methanol: sulfuric acid and reaction time of 6 h), fats were successfully removed but no beneficial sterols were recovered. While the phytosterols had been effectively extracted using the 4th modified method (mixing with a 1.5 M KCl solution for 2 h at 60 °C, the 96:4 ratio of methanol: sulfuric acid and a reaction time of 6 h), but the fatty acids were not eliminated. β-sitosterol was the most abundant phytosterol extracted using all the studied soxhlet methods.

### The Folch and Its Modified Methods

The total amount of phytosterols extracted from Khanjari PGH using the Folch method was 114.01 mg/kg compared to the 1st (17.74 mg/kg), 2nd (250.07 mg/kg), 3rd (54.58 mg/kg), and 4th (9.61 mg/kg) modified methods. Table 5 provides an overview of the various types and amounts of phytosterols of each item. Figure S3 represents the GC-FID chromatograms of phytosterols extracted from Khanjari PGH using the Folch technique and its modified versions.

Based on the GC-FID analysis, in the Folch method (half an hour of mixing with a 1 M KCl solution at room temperature, 4 h reaction with methanol: sulfuric acid in a ratio of 98:2) fat removal was limited, but the amount of sterols obtained was satisfactory. The 1st modified method (mixing with a 1 M KCl solution at 60 °C, 6 h of reaction with methanol: sulfuric acid in a 96:4 ratio), effectively eliminated the fatty acids, but the amount of extracted sterols was low. The 2nd modified method (mixing with a 1 M KCl solution at 25 °C, 6 h reaction with a 96:4 ratio of methanol: sulfuric acid), slightly eliminated the fatty acids, but the extracted sterols with 250.07 mg/kg had the highest yield among the Folch and modified versions. In the 3rd and 4th modified methods (mixing with 1.5 M KCl solution at 60 and 25 °C for 2 h, respectively, and 6 h reaction with a 96:4 ratio of methanol: sulfuric acid), no acceptable sterol was obtained. In the Folch method and its modified versions, the most abundant phytosterol was β-sitosterol (Figure **S3** shows the GC-FID chromatograms of phytosterols extracted from 30 g of Khanjar PGH by the Folch [19, 23] and its modified methods). The types and amounts of phytosterols are presented in Table 5. For details on each method, refer to Table 2.

**Table 3** The types and quantity of phytosterols extracted from 30 g of PGH of Abbasali, Akbari, and Khanjari pistachio varieties of Damghan and 10 g of their kernel using the method of the International Olive Council (IOC) [20].

| Sample Identity | Abbasali – Kernel | | Abbasali - Hull | | Akbari – Kernel | | Akbari – Hull | | Khanjari – Kernel, | | Khanjari - Hull | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Repeat | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| α-Cholestan (Area) | 210.71 | 198.47 | 188.33 | 222 | 1035.69 | 216.81 | 141.04 | 143 | 190.06 | 150.97 | 484.17 | 223.83 |
| Cholesterol (%) | 0.06 | 0.04 | 0.72 | 0.58 | 0.09 | 0.08 | 0.65 | 0.55 | 0.05 | 0.05 | 0.63 | 0.71 |
| Brassicasterol (%) | 0.09 | 0.09 | 0.11 | 0.11 | 0.12 | 0.04 | 0.12 | 0.14 | 0.07 | 0.71 | 0.1 | 0.16 |
| Campesterol (%) | 6.53 | 6.92 | 6.91 | 5.49 | 6.56 | 6.28 | 5.96 | 5.88 | 6.39 | 5.94 | 6.11 | 5.45 |
| Stigmasterol (%) | 1.09 | 1.39 | 11.16 | 10.23 | 0.97 | 0.84 | 4.16 | 4.02 | 0.58 | 0.59 | 11.38 | 11.2 |
| Clerosterol (%) | 0.5 | 0.6 | 0.62 | 0.82 | 0.47 | 0.15 | 0.48 | 0.83 | 0.72 | 0.13 | 0.69 | 0.54 |
| β-Sitosterol (%) | 83.9 | 81.86 | 70.52 | 71.72 | 83.61 | 85.34 | 75.92 | 74.85 | 84.24 | 86.55 | 66.45 | 67.21 |
| Sitostanol (%) | 0.02 | ND | 0.1 | ND | 0.78 | ND | 0.44 | ND | 1.74 | ND | 0.46 | ND |
| $\Delta^5$-Avenasterol (%) | 6.62 | 6.93 | 2.06 | 3.4 | 6.35 | 6.09 | 1.49 | 2.47 | 5.59 | 4.89 | 2.46 | 3.45 |
| $\Delta^{5,24}$-Stigmastadienol (%) | 0.09 | 0.14 | 0.48 | 0.05 | 0.12 | 0.06 | 0.14 | 0.18 | 0.01 | 0.06 | 0.15 | 0.1 |
| $\Delta^7$-Stigmastenol (%) | 0.16 | 0.32 | 3.66 | 4 | 0.31 | 0.12 | 6.2 | 5.02 | 0.21 | 0.18 | 4.82 | 5.09 |
| $\Delta^7$-Avenasterol (%) | 0.19 | 0.64 | 2.66 | 3.23 | 0.28 | 0.29 | 4.32 | 5.54 | 0.19 | 0.37 | 5.75 | 5.24 |
| Others | 0.75 | 1.07 | 1 | 0.37 | 0.34 | 0.71 | 0.12 | 0.52 | 0.21 | 0.53 | 1 | 0.85 |
| Total Area | 3952.9 | 3801.5 | 2209.44 | 2754.28 | 19279.4 | 4070.5 | 3383.59 | 3310.91 | 3339.0 | 2725.31 | 5244.94 | 2377.32 |
| Total Sterols (mg/kg) * | 1876 | 1898 | 555 | 587 | 1820 | 1836 | 798 | 770 | 1734 | 1782 | 606 | 594 |

ND - non detectable, defined as ≤ 0.05 %. *The amount of each phytosterol was calculated with the formula (Total Area × IS mass (mg) ×1000) /(IS area × sample mass) [20]. For the details of the extraction method, see the Materials and Methods section 2-1 and 2-2.

**Table 4** The types and quantity of phytosterols extracted from 30 g of Khanjar PGH by the Soxhlet method [19, 21] and its modified versions.

| Method | Internal standard area | Cholesterol % | Brassicasterol % | Campesterol % | Stigmasterol % | Clerosterol % | β-Sitosterol % | $\Delta^5$-Avenasterol % | stigma estradiol % | $\Delta^7$-Stanol % | $\Delta^7$-Avenasterol % | Unknown | Recovery (%) | Total area | Total sterols (mg/kg) * |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| The main method | 541.5 | 1.8 | 2.54 | 57.6 | 2 | 1.5 | 25.83 | 3 | ND | ND | ND | 5.73 | 94.27 | 126 | 70.41 |
| The 1st modified method | 151.1 | 0.3 | 0.9 | 3.5 | 1.3 | 0.6 | 31 | 1.6 | ND | ND | ND | 60.8 | 39.2 | 460 | 101.21 |
| The 2nd modified method | 110.7 | 1.4 | 1 | 12.7 | 6.8 | 0.5 | 55 | 5 | ND | ND | ND | 17.6 | 82.4 | 180 | 54.74 |
| The 3rd modified method | 135.7 | 1.2 | 2.5 | 18 | 16 | 1.3 | 0.2 | 1.26 | ND | ND | ND | 55.94 | 44.06 | 83 | 20.15 |
| The 4th modified method | 267.4 | 1.3 | 1.5 | 29 | 6.2 | 3 | 28 | 5.17 | 0.9 | 1.4 | 0.54 | 22.99 | 77.01 | 777 | 110.19 |

ND - non-detectable, defined as ≤ 0.05 %. *The amount of each phytosterol was calculated with the formula (Total Area × IS mass (mg) ×1000) /(IS area × sample mass) [20]. For details of the extraction methods, see the Materials and Methods section 2-3-1.

**Table 5** The types and amount of phytosterols extracted from 30 g of Khanjar PGH by the Folch method [19, 23] and its modified methods.

| Method | Internal standard area | Cholesterol % | Brassicasterol % | Campesterol % | Stigmasterol % | Clerosterol % | β-Sitosterol % | $\Delta^5$-Avenasterol % | Unknown | Recovery (%) | Total area | Total sterols (mg/kg) * |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| The main method | 695.4 | 0.5 | 1.07 | 21 | 7 | 1.2 | 3.73 | 10 | 55.5 | 44.50 | 2377 | 114.01 |
| The 1st modified method | 452.2 | 0.01 | ND | 0.07 | 0.02 | ND | 0.23 | 0.05 | 79.31 | 20.69 | 220.31 | 17.74 |
| The 2nd modified method | 237.7 | 0.4 | 0.7 | 21 | 15 | 0.6 | 49 | ND | 13.3 | 86.70 | 1778 | 250.07 |
| The 3rd modified method | 282.2 | 0.5 | 1 | 9 | 6 | 1.2 | 29 | 6.8 | 46.5 | 53.50 | 429.26 | 54.58 |
| The 4th modified method | 443.1 | 0.04 | ND | 0.05 | 0.02 | ND | 0.07 | 0.04 | 85.28 | 14.72 | 140.5 | 9.61 |

ND - non-detectable, defined as ≤ 0.05 %. *The amount of each phytosterol was calculated with the formula (Total Area × IS mass (mg) ×1000) /(IS area × sample mass) [20]. For details of the extraction methods, refer to the Materials and Methods section 2-3-2.

The types and amounts of phytosterols was presented in Table 5. For details on each method refer to Table 2.

## DISCUSSION

This study is the first to identify the phytosterol composition of PGH and the kernel of three well-known pistachio cultivars, including Abbasali, Akbari, and Khanjari from Damghan, Iran. The GC-chromatogram of phytosterols extracted from three studied pistachio cultivars using the IOC method [20] this study revealed the sterol composition in the order of cholesterol, brassicasterol, campesterol, stigmasterol, clerosterol, β-sitosterol, sitostanol, $\Delta^5$-avenasterol, $\Delta^{5,24}$-stigmastadienol, $\Delta^7$-stigmastenol, $\Delta^7$-avenasterol in both PGH and kernel. According to the data, the total phytosterol content in the kernel of all cultivars (1734-1898 mg/kg) was higher than the range of 555 to 798 mg/kg found in PGHs. The PGH total phytosterol from the highest value to the lowest value, was in the following order: Akbari (784 ± 20 mg/kg), Khanjari (600 ± 8 mg/kg), and Abbasali (571 ± 23 mg/kg). Comparing the phytosterol content from different cultivars' kernels exhibited that Abbasali had the greatest amount at1887 ± 16 mg/kg, followed by Akbari at 1828 ± 11 mg/kg, while Khanjari contained the lowest level at 1758 ± 34 mg/kg. The ratio of total phytosterol content of the kernel to the content in the PGH for the studied cultivars was as follows: Abbasali: 3.3, Akbari: 2.3, and Khanjari: 2.9. The ratio of β-sitosterol content of kernel to PGH in the examined cultivars was as follows: Abbasali: 1.2, Akbari: 1.1, and Khanjari: 1.3. The percentages of cholesterol and some phytosterols, including stigmasterol, $\Delta^7$-stigmastenol, and $\Delta^7$avenasterol in PGHs were greater than those in the kernels of the studied cultivars.

The Codex Alimentarius Commission (CAC) founded by the Food and Agriculture Organisation (FAO) and the World Health Organization (WHO) to protect consumer health and promote fair practices in food trade [26] does not provide a Codex Alimentarius report on the levels of phytosterols in PGH. The presence of components such as anacardic acids (31.98 g/kg), phytosterols (19.20 g/kg), and fatty acids (15.00 g/kg) has been reported in PGH by Grace et al. [7]. Chahed et al. reported alpha-terpinolene, alpha-pinene, and monoterpene hydrocarbons in the essential oil extracted using water distillation and liquid-liquid extraction from PGH [27].

Compared to our findings of 1734-1898 mg/kg total phytosterol in the kernels of all the studied cultivars, Codex Alimentarius has reported the levels of desmethylsterols as 1840-4500 mg/kg in crude oil derived from the kernel of *P. vera* fruit [28]. The percentage of total sterols of pistachio kernel in the Codex Alimentarius is as follows: cholesterol (ND–1.0 %), brassicasterol (ND), campesterol (4.0–6.5 %), stigmasterol (0.5–7.5 %), β-sitosterol (75.0–94.0 %), $\Delta^5$-avenasterol (6.0–8.0 %), $\Delta^7$-stigmastenol (ND–0.7 %), $\Delta^7$-avenasterol (ND–0.5 %), others (ND) [16] compared to cholesterol (0.05-0.09 %), brassicasterol (0.08-0.39), campesterol (6.17-6.42 %), stigmasterol (0.5–7.5 %), β-sitosterol (82.88-85.40 %), $\Delta^5$-avenasterol (5.34-6.53 %), $\Delta^7$-stigmastenol (0.22-0.24 %), $\Delta^7$-avenasterol (0.28-0.42 %). Further, we identified sitostanol and $\Delta^{5,24}$-stigmastadienol in both PGH and kernels that were not specified in the Codex Alimentarius guidelines for *P. vera* kernels.

Ling et al. [28] and Al Juhaimi [29] reported no phytosterols in their study on the physicochemical properties, volatile compounds, and antioxidant properties of CP kernel oils from *P. vera*. According to Ling et al. the 36% loss of the oil in CP compared to the Soxhlet is an inescapable problem associated with CP [28]. The relative ratios of 0.19 free sterols and 0.03 sterol esters have been reported by Celenk VU et al. for the CP pistachio oil. The concentrations of β-sitosterol, stigmasterol, and campesterol in pistachio oil were determined as 4685.9, 663.3, and 236.8 µg/g oil, respectively [30]. In another study, the percentage of sterol constituents in freshly extracted CP pistachio oil was reported as follows: apparent β-sitosterol (94.1 ± 0.2 %), campesterol (4.2 ± 0.1 %), stigmasterol (0.6 ± 0.0 %), and $\Delta^7$-stigmastenol (0.4 ± 0.0 %) [31]. Yuenyong et al. analyzed the fatty acid profile and functional phytochemicals of fifty CP plant oils, including *P. vera*, using GC-MS and HPLC with diode-array detection (HPLC -DAD) in Thailand. They reported the total phytosterols of 1200 ± 0.76 µg/g (1063 ± 0.22 µg/g of β-sitosterol and 137 ± 0.55 µg/g of stigmasterol and campesterol) in the CP oil of *P. vera* Kernel [32].

In all examined cultivars of our study, β-sitosterol was the predominant phytosterol identified in PGH and kernel when extracted using the IOC, Soxhlet, and Folch methods. Campesterol and $\Delta^5$-avenasterol were found to be the most abundant phytosterol in the studied kernels, whereas campesterol and stigmasterol were the most prevalent in PGHs. On the contrary, brassicasterol and $\Delta^{5,24}$-stigmastadienol were the least prevalent phytosterols. However, some sterols, including stigmastediol, $\Delta^7$-stigmastenol, and $\Delta^7$-avenasterol were not detected in samples extracted using the Folch and its modified methods and were only found in certain samples extracted

using the Soxhlet technique and its modified methods. These phytosterols may present in small amounts (< 0.1%) and have been lost during the longer steps of the Soxhlet and Folch extractions compared to the used IOC method. Phytosterols extracted from Khanjari PGH using both the Soxhlet and the Folch methods were rich in β-sitosterol, campesterol, and stigmasterol. β-sitosterol, along with campesterol and sitostanol, holds the utmost significance as a phytosterol utilized in the food and various other industries [2]. Except for sitostanol, which was not extracted through both the Soxhlet and the Folch methods, β-sitosterol and campesterol were successfully obtained using all extraction methods. More phytosterols were obtained using the Soxhlet method compared to the Folch method. Yahyavi et al. evaluated the chemical profile of pistachios harvested from various areas in East Azerbaijan province in Iran and found a total sterol content ranging from 1125- to 2784 mg/kg oil in the samples. The oil extracted using *n*-hexane and analyzed by GC-FID showed β-sitosterol as the predominant sterol (2419-966 mg/kg oil), followed by $\Delta^5$-avenasterol (170-72.6 mg/kg oil), campesterol (47.4-128 mg/kg oil), stigmasterol (12.3 -27.8 mg/kg oil) and cholesterol (8.3-39 mg/kg oil) [12]. These phytosterols were also identified in our studied methods. However, we also assessed the presence of brassicasterols, chlorosterol, sitostanol, $\Delta^{5,24}$-stigmastadienol, $\Delta^7$-stigmastanol, and $\Delta^7$-avenasterol using the IOC method. In addition, when compared to Yahiavi's findings, the lowest and the highest amounts of total sterols were 1887 and 1758 mg/kg respectively.

Arena et al. used petroleum ether as a solvent to extract ground pistachio oil for 6 h in a Soxhlet extractor. In their GC/MS identification of pistachio kernels phytosterols from different countries (Italy, Turkey, Greece, and Iran), nine various phytosterols, including β-sitosterol, $\Delta^5$-avenasterol, campesterol, and stigmasterol (as main phytosterols) and clerosterol, $\Delta^{5,24}$-stigmastadienol, $\Delta^7$-stigmastenol, $\Delta^7$-avenasterol, and cholesterol (as the minor sterols) were identified. According to Arena et al. study, pistachio kernels from Iran had a higher amount of phytosterols compared to those from other countries [14]. Although, they did not indicate the specific origin in Iran where the pistachios were studied, we identified two other phytosterols (brassicasterol and sitostanol) in addition to the ones they identified. The most abundant phytosterol in their report was β-sitosterol (88% in Iranian samples), similar to our findings (Kernel and PGH phytosterols: 82.88 and 71.12 in Abbasali cultivar; 84.48 and 75.39% in Akbari cultivar; 85.40 and 66.83% in Khanjari cultivar). Arena reported 5.7% of $\Delta^5$-avenasterol in Iranian samples [14]. We observed the percentage of $\Delta^5$-avenasterol in the total phytosterol of Kernel and PGH as follows: Abbasali (6.78 and 2.73%), Akbari (6.22 and 1.98%), and Khanjari (5.24 and 2.96%) cultivars. In our results, the percentage of campesterol in kernels and PGHs of Abbasali (6.73, 6.2 %), Akbari (6.42, 5.92 %), and Khanjari (6.17, 5.78 %) was greater than the 4.55% reported by Arena et al. for Iranian sample. Arena et al. reported 0.64 % of stigmasterol [14] compared to our report for kernels and PGHs, namely Abbasali (1.24 and 10.70) %), Akbari (0.91 and 4.09 %), and Khanjari (0.59 and 11.29 %). $\Delta^7$-stigmastanol was not indicated in Iranian samples in their study, while we detected 0.24 and 3.83% in Abbasali, 0.22 and 5.61% in Akbari, and 0.20 and 4.96% in Khanjari cultivars' kernels and PGHs, respectively.

In the Soxhlet modified extraction methods, the best fat removal was observed at 25 ºC with both 1 M and 1.5 M KCl concentrations (the 1st and 3rd modified methods, respectively). However, the increase in KCl concentration at room temperature decreased the obtained phytosterol from 101.21 mg/kg in the 1st modified method to 20.15 mg/kg in the 3rd modified method. Fat removal was limited at 60 ºC with both 1 M and 1.5 M KCl concentrations (the 2nd and 4th modified methods, respectively). In contrast to room temperature, the increase in KCl concentration at 60 ºC increased the obtained phytosterol from 54.74 mg/kg in the 2nd modified method to 110.19 mg/kg in the 4th modified method.

The salting-out effect of KCl may play a role in this observation. In biphasic systems, proteins and sugars preferentially partition into the aqueous layer and lipids into the organic layer. The addition of KCl in the separation step can change the distribution of lipids between the two phases such that it aids lipid exchange between the aqueous phase and the organic phase. It is supposed that the cations produced by KCl reduce the separation of lipids by a mass effect and result in the lipids transferring to the organic phase while keeping the salts in the aqueous phase [33]. It seems that the increased temperature has intensified the salting-out effect of KCl.

In the Folch modified methods, the best fat removal was obtained at 60 ºC with a 1 M KCl solution (the 1st modified method), but the phytosterol yield was low (17.74 mg/kg). In comparison, the phytosterol extracted increased to 54.58 mg/kg usin ag 1.5 M KCl solution at this temperature (the 3rd modified method), though the

fat contamination increased, too. At room temperature, a 1.5 M KCl solution yielded a lower phytosterol amount (9.61 mg/kg, the 4th modified method) compared to 250.07 mg/kg of phytosterol obtained using 1 M KCl solution (the 2nd modified method), both with high-fat contamination. In addition to the salting-out effect of KCl, the results observed in the Folch modified methods can be attributed to the polar nature of methanol/chloroform compared to non-polar *n*-hexane, which does not dissolve large fatty acids or polar compounds.

In the transesterification of sterol esters, free sterols and the methylated fatty acids are produced [34]. In the transesterification step of both the Soxhlet and the Folch modified methods, we adjusted the ratio of methanol to sulfuric acid from 98:2 in the original methods to 96:4. Additionally, the duration of transesterification was extended from 4 h to 6 h. The original Soxhlet method yielded a low quantity of phytosterols (70.41 mg/kg) with lower fatty acid content compared to its modified methods. Comparatively, the Folch method yielded 114.01 mg/kg of phytosterol, placing it second after the 2nd modified Folch method which yielded 250.07 mg/kg of phytosterol, both with substantial fat contamination. However, the 1st modified Soxhlet method and the 2nd modified Folch method, both of which used a 1 M KCl solution at 25 ºC, similar to their original methods, resulted in higher phytosterols yields compared to the original methods with values of 101.21 and 250.07 mg/kg, respectively. Therefore, it can be concluded that the mild extraction conditions we used (e.g., room temperature) with the changes in the methanol to sulfuric acid ratio and time helped produce free sterols. Although the 2nd modified Folch method has limitations in terms of fat removal, the use of one-step transesterification seems to be an appropriate approach for the phytosterol extraction from PGH utilizing the Soxhlet method.

The impact of the extraction method on the oil yield and phytosterol composition is well acknowledged. Jafarian Asl et al. compared the techniques of supercritical $CO_2$ extraction using solvent and Soxhlet to extract phytosterols and tocopherols from rapeseed oil and reported the advantages of supercritical $CO_2$ extraction technique over the Soxhlet method. The reduction of sample handling steps leads to minimum loss of analytes, resulting in shorter analysis times. Moreover, the purification steps were unnecessary because the extracts obtained from the supercritical method were shown to be free of impurities [35]. However, supercritical $CO_2$ extraction may not be available in all laboratories.

The solvent-extraction methods such as the Soxhlet and Folch methods are cost-efficient and easy to use. The Folch method is one of the oldest approaches for lipids extraction. The Soxhlet method takes longer than the Folch method in terms of time and heat energy consumption, and the researcher also deals with more laboratory equipment. However, the steps involved in transesterification remain consistent across both methods. Haitham et al. observed *n*-hexane yielded higher oil-extracting values (37.03%) from sesame seeds compared to chloroform (6.73%) and acetone (4.37%) [33]. The notable advantage of the Soxhlet method over the Folch method is the utilization of a single solvent type (*n*-hexane), which can be recycled. The Folch method requires a greater volume of solvent to treat an equal amount of sample, which further makes complicates separating and recycling the two solvents. According to our results, the Soxhlet method yields a greater variety and quantity of isolated phytosterols. The longer contact time of samples with the heated solvent may account for the increased oil obtained, thus resulting in a higher yield. Hexane and chloroform are both non-polar solvents. Chloroform is more cost-effective but encounters higher environmental hazards; its excessive use results in contamination of air and water as well as toxic effects on humans and animals. On the other hand, hexane has fewer environmental concerns compared to chloroform [36].

Nonconventional or improved techniques such as CP, UAE, EAE, and SFE are introduced as more environmentally friendly alternatives to the classic Soxhlet and Folch methods because flammable solvents with environmental and health problems are used in latter methods [17, 37]. It is a known fact that the production processes of pure enzymes as well as devices and instruments utilized in the mentioned methods, incur energy expenditure. The objective of the present study was to isolate and identify the phytosterol composition of agricultural waste (PGH) using classic techniques available in all laboratories. The isolation and purification of phytosterols from PGH and any sources using conventional or improved methods on an industrial scale requires further studies.

## CONCLUSION

The present study is one of the few and first studies on the assessment of the phytosterols in pistachio hulls. We showed that PGH, a cost-effective waste material that poses various environmental challenges, can be considered

a valuable source of phytosterol, just as the pistachio kernel. Among the various methods and techniques for phytosterol extraction, the Soxhlet and the Folch methods are the most widely accessible to all laboratories. With the changes made in the extraction steps, the 1st modified soxhlet method (extended mixing time with KCl, less methanol to sulfuric acid ratio, but longer transesterification time compared to the main method) was considered as the most efficient method among the total of 11 examined methods (five Soxhlet methods and five Folch methods) in the sense that the extracted phytosterol had less fatty acid. The Folch approach with modification improved fatty acid removal but could not provide high-quality sterol compared to the Soxhlet method.


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