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Original Article

Total Phenolic Content, Antioxidant Activity, and Cytotoxicity of Different Extractions of *Solenanthus circinnatus* Ledeb.

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ABSTRACT

Azar-Choob, scientifically known as *Solenanthus circinnatus* Ledeb., belongs to the Boraginaceae family. Traditionally, its root has been used topically for pain relief and to reduce inflammation. The present study aimed to evaluate the antioxidant and cytotoxic effects of various extracts obtained from the roots of *Solenanthus circinnatus* Ledeb. Following the collection and drying of the plant roots, phytochemical screening was conducted. The dried roots were sequentially extracted using a Soxhlet apparatus with a series of solvents with increasing polarity: n-hexane, dichloromethane, chloroform, ethyl acetate, ethanol, and methanol. The total phenolic content (TPC) of each extract was quantified using the Folin-Ciocalteu assay. Subsequently, the DPPH radical scavenging assay was performed. Finally, the cytotoxic effects of all extracts were assessed on MCF-7, MDA-MB-231, HT-29, A549, and NIH-3T3 fibroblasts via the MTT assay. The highest extraction yield was obtained with the ethanolic extract (25.2 ± 0.85 %). After phytochemical screening, the presence of pyrrolizidine alkaloids, tannins, steroids, and carbohydrates was also confirmed in the plant root. The ethyl acetate extract not only contained the highest TPC but also demonstrated the most potent antioxidant activity. Regarding cytotoxicity, most extracts had no significant effect on cell viability. However, dichloromethane and chloroform extracts at 125 µg/mL significantly reduced the viability of the MDA-MB-231 breast cancer cell line. This study showed that the ethyl acetate extract of *Solenanthus circinnatus* Ledeb. may be considered a potentially safe and effective natural antioxidant source for further applications in nutraceutical and pharmaceutical industries.

Keywords: Azar-Choob, *Solenanthus circinnatus* Ledeb., antioxidant capacity, Cytotoxic effects

INTRODUCTION

Nowadays, medicinal plants have gained increasing popularity for treating various diseases due to their favorable safety profile and fewer side effects when used appropriately [1, 2]. The World Health Organization (WHO) reports that approximately 80% of people in developing countries rely on traditional herbal medicine. Consequently, the WHO recognizes medicinal plants as a vital source for the development of novel medicines [3]. Plants can produce a large number of organic compounds using simple and inorganic precursors, including primary metabolites for plant growth, secondary metabolites that are lineage-specific, such as phenolics, which help plants interact with their environments, and finally hormones, which regulate vital processes [4]. Overall, herbs containing these natural compounds prevent and treat numerous diseases through mechanisms such as anti-inflammatory, antibacterial, antioxidant, and anticancer activities [2]. Oxidative stress contributes to the pathogenesis of numerous diseases [5]. Medicinal plants are a rich source of antioxidative compounds that can combat oxidative damage [1, 6]. Global interest in natural antioxidants has spurred extensive research into the antioxidative properties of various medicinal plants. Notable examples include *Camellia sinensis* (source of white, green, and black tea), *Teucrium polium* L., *Dracocephalum moldavica* L., *Urtica dioica* L., *Momordica charantia* L., and *Rheum species*, all of which have been identified as rich sources of natural antioxidants [7, 8].

Solenanthus circinnatus Ledeb. (S. circinnatus), commonly known as Azar-Choob, belongs to the Boraginaceae family, which comprises over 131 genera and 2500 species. S. circinnatus is a perennial plant with bluish-purple petals. This species is native to central Asia, including Iran [9, 10]. Traditionally, *S. circinnatus* has been used topically to alleviate pain and inflammation in conditions such as muscle cramps and bone fractures. The plant's root is powdered and applied topically, either alone or in combination with other ingredients such as licorice and eggs, to the affected site [11]. The roots of various genera within the Boraginaceae family, particularly those closely related to the *Solenanthus* genus, contain medicinally active compounds such as phenolic acids, saponins, phytosterols, alkaloids, naphthoquinones, and allantoins [11]. A study on 17 species of the Boraginaceae family identified allantoin, p-hydroxybenzoic acid, rutin, hydrocaffeic acid, rosmarinic acid, and chlorogenic acid in the aerial parts extract. In contrast, the root extract contained allantoin, hydrocaffeic acid, rosmarinic acid, and shikonin [11, 12]. In

another study on the aerial parts of *S. circinnatus*, compounds such as coumaric acid, eugenol, and salicylic acid were identified [13]. Another study describes the analgesic activity of ethanolic and other extracts of *S. circinnatus* root. Ethanol extract and other extracts (petroleum ether, chloroform, ethyl acetate, and n-butanol) of *S. circinnatus* root mostly produced good analgesic and anti-inflammatory effects in rat models of pain using the formalin test and tail-flick test [11]. Given the traditional use of *S. circinnatus* root and its wide distribution in Iran, this study aimed to investigate the active constituents, antioxidant activity, and cytotoxicity of extracts prepared with solvents of varying polarity.

MATERIALS AND METHODS

Collection and Sequential Extraction of *S. circinnatus*

The ethics committee of Kerman University of Medical Sciences approved the current study's protocol (Ethical code: IR.KMU.REC.1403.285). The roots of *S. circinnatus* were collected from Shiraz, Iran, and the specimen was identified in the Herbal and Traditional Medicines Research Center (HTMRC), Kerman University of Medical Sciences, Kerman, Iran (Voucher Number: KF2220). The dried roots were ground using an electric mill. After that, the dried roots were sequentially extracted via the Soxhlet extraction method [14]. In brief, the sample was first extracted with hexane, and then the remaining residue was extracted with dichloromethane. Afterward, the remaining material was extracted with chloroform. This process was repeated with ethyl acetate, ethanol, and methanol, respectively. After concentrating in a rotary evaporator (40 ºC), the extractions were dried and kept at -20 °C for the next steps. Then, the extraction yield percentage was evaluated using the following formula: (g of extract/g of dry herb) × 100.

Phytochemical Analysis of *S. circinnatus*

The presence of secondary metabolites in *S. circinnatus,* such as alkaloids, pyrrolizidine alkaloids, tannins, flavonoids, saponins, anthraquinones, steroids, and carbohydrates, was evaluated by suitable related methods [15-17].

Folin-Ciocalteu (F-C) Assay

To determine the total phenolic content (TPC) of each extract, the F-C assay was performed. Briefly, the extract (100 µL) or standard solution of gallic acid (100 µL) was mixed with F-C reagent (500 µL; 1:10 v/v). Then, sodium carbonate solution (400 µL; 7.5% w/v) was added, and after a brief shake (1 min), the mixture was incubated in the dark at 25 ± 2 °C for 30 min. Then, the absorbance of the mixture was measured at 765 nm using a multimode reader (BioTek® Synergy HTX, USA) against blanks. The TPC of the extracts was reported as milligrams (mg) of gallic acid per gram (g) of dried extract, using a regression equation derived from a standard calibration curve of gallic acid (6.25-100 µg/mL) [18]. The data for this method were measured using the multimode reader (BioTek® Synergy HTX, USA).

α,α-diphenyl-β-picrylhydrazyl Assay (DPPH assay)

For evaluation of the free radical scavenging activity of *S. circinnatus* root extracts by the DPPH assay, briefly, 50 µL of different concentrations of *S. circinnatus* extracts or green tea methanolic extract (GTME) (as a positive control) or extract solvents (as a blank) were added to 150 µL of DPPH solution. After 30 min of incubation of the mixture in the dark at 25 ± 2 °C, absorbance was measured at 517 nm for the samples and the blank using a multimode reader (BioTek® Synergy HTX, USA). The antioxidant activity of the extracts was expressed as inhibition percentages [19].

MTT Assay

Cell lines were from the Iranian Biological Resources Center (Tehran, Iran) and the Pasteur Institute (Tehran, Iran). Cell culture materials were from Invitrogen (Carlsbad, CA, USA). High glucose DMEM medium that was supplemented with FBS (10%) and penicillin/streptomycin 1% was used for culturing of cell lines. To investigate cytotoxicity, 1×10^4 cells were seeded in 96-well culture plates and exposed to different concentrations (0-125 µg /mL) of different extractions of *S. circinnatus* for 24 hours. Finally, after 3 hours of incubation with MTT, dimethyl sulfoxide (DMSO) was added, and the absorbance was measured at 570 nm [20].

Statistical Analysis

All data are presented as mean ± standard deviation (SD), and normality was assessed using the Shapiro-Wilk test. Statistical significance for multiple comparisons was determined using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. A *p-value* of less than 0.05 was considered statistically significant.

RESULTS

Evaluation of extraction yield percentage and TPC of the Different *S. circinnatus* Extracts

In this study, the *S. circinnatus* roots were extracted with different solvents such as hexane, dichloromethane, chloroform, ethyl acetate, ethanol and methanol by Soxhlet method, respectively. The yields of crude extracts obtained with each solvent are shown in Table 1. Also, chloroform yielded the lowest percentage, and ethanol yielded the highest. As shown in Table 1, ethyl acetate extract exhibited the highest TPC (254.73 ± 3.98 gallic acid (mg)/ dried extract (g), followed by ethanol, methanol, dichloromethane, chloroform, and hexane.

Table 1 Extraction yield percentage and total phenolic contents of *S. circinnatus* extracts

		Extraction yield percentage (g of extract/g of dry herb×100)	Total phenolic contents, Gallic Acid (mg)/ dried extract (g)
Extract Type	Hexane	0.41 ± 0.03	7.93 ± 0.41
	Dichloromethane	0.25 ± 0.01	21.59 ± 0.83
	Chloroform	0.13 ± 0.02	18.63 ± 0.58
	Ethyl acetate	0.28 ± 0.03	254.73 ± 3.98
	Ethanol	25.2 ± 0.85	45.65 ± 1.24
	Methanol	2.6 ± 0.02	41.62 ± 0.97

Phytochemical Analysis

According to the results of phytochemical analysis, the root of *S. circinnatus* contains pyrrolizidine alkaloids, tannins, steroids, and carbohydrate compounds (Table 2).

Table 2 Phytochemical analysis of *S. circinnatus root*

Phytochemical Constituents	Test Applied	Result
Alkaloid	Iodine, Dragendroff's, and Mayer reagent	-
Pyrrolizidine alkaloids	Ehrlich's reagent	+
Tannin	Lead acetate test, dilute ammonia test, Ferric chloride test solution	+
Flavonoid	Lead acetate test, dilute ammonia test, reduction test, Shinoda test, Wilson Tabuk, and PEW test	-
Saponin	Foam test	-
Anthraquinone	Borntragers, Sennoside, and Aloin assay	-
Steroid	Liebermann-Burchard reaction and Salkowski test	+
Carbohydrate	Molisch's, Fehling's, and Benedict's tests	+

+: Presence, -: Absence

Antioxidant Activity of Different Extracts of *S. circinnatus*

As shown in Fig. 1, the ethyl acetate extract showed the highest antioxidant activity and the hexane extract demonstrated the lowest antioxidant activity. The ethyl acetate extract displayed an antioxidant effect comparable to that of GTME at concentrations of 125, 250, 500, and 1000 µg/mL. Furthermore, all hexane extract concentrations exhibited significantly lower antioxidant activity than the corresponding GTME concentrations.

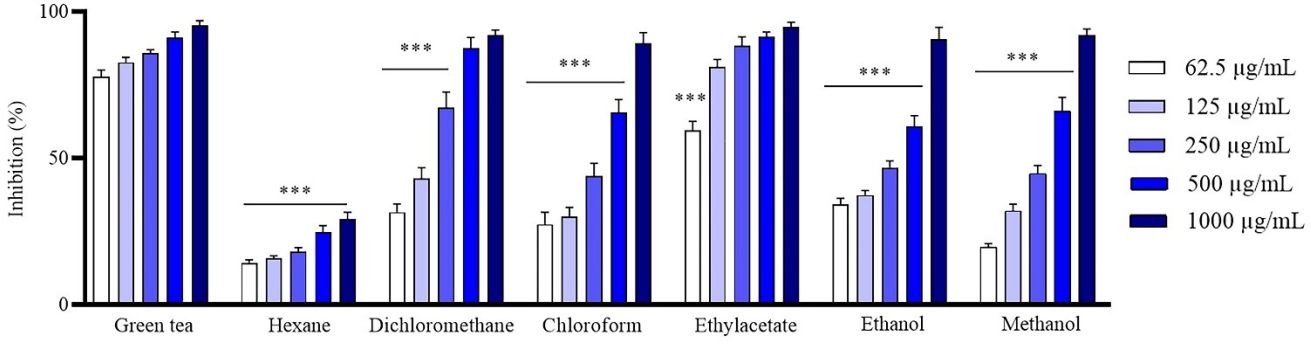

Fig. 1 DPPH of different extracts of *S. circinnatus* and green tea methanolic extracts (GTME). Results are represented as means ± SD of three independent experiments. ***$P < 0.001$ in comparison with the same concentration of GTME as standard.

Cytotoxic Effect of Different Extracts of *S. circinnatus*

The cytotoxic effects of different *S. circinnatus* extracts were evaluated in various cell lines (Fig. 2). The hexane extract was non-toxic on both cancerous and normal fibroblast cell lines. Moreover, it increased the viability of normal fibroblast cells at concentrations of 62 and 125 µg/mL (Fig. 2e). The dichloromethane extracts at the concentration of 125 µg/mL had a significant cytotoxic effect on MDA-MB-231 (Fig. 2b) and the fibroblast cell lines (Fig. 2e). Also, the chloroform extracts at the concentration of 125 µg/mL had a significant cytotoxic effect on the MDA-MB-231 cell line (Fig. 2b). Finally, the ethyl acetate, ethanol, and methanol extracts did not display a statistically significant cytotoxic effect on the viability of both cancerous and normal fibroblast cells (Fig. 2).

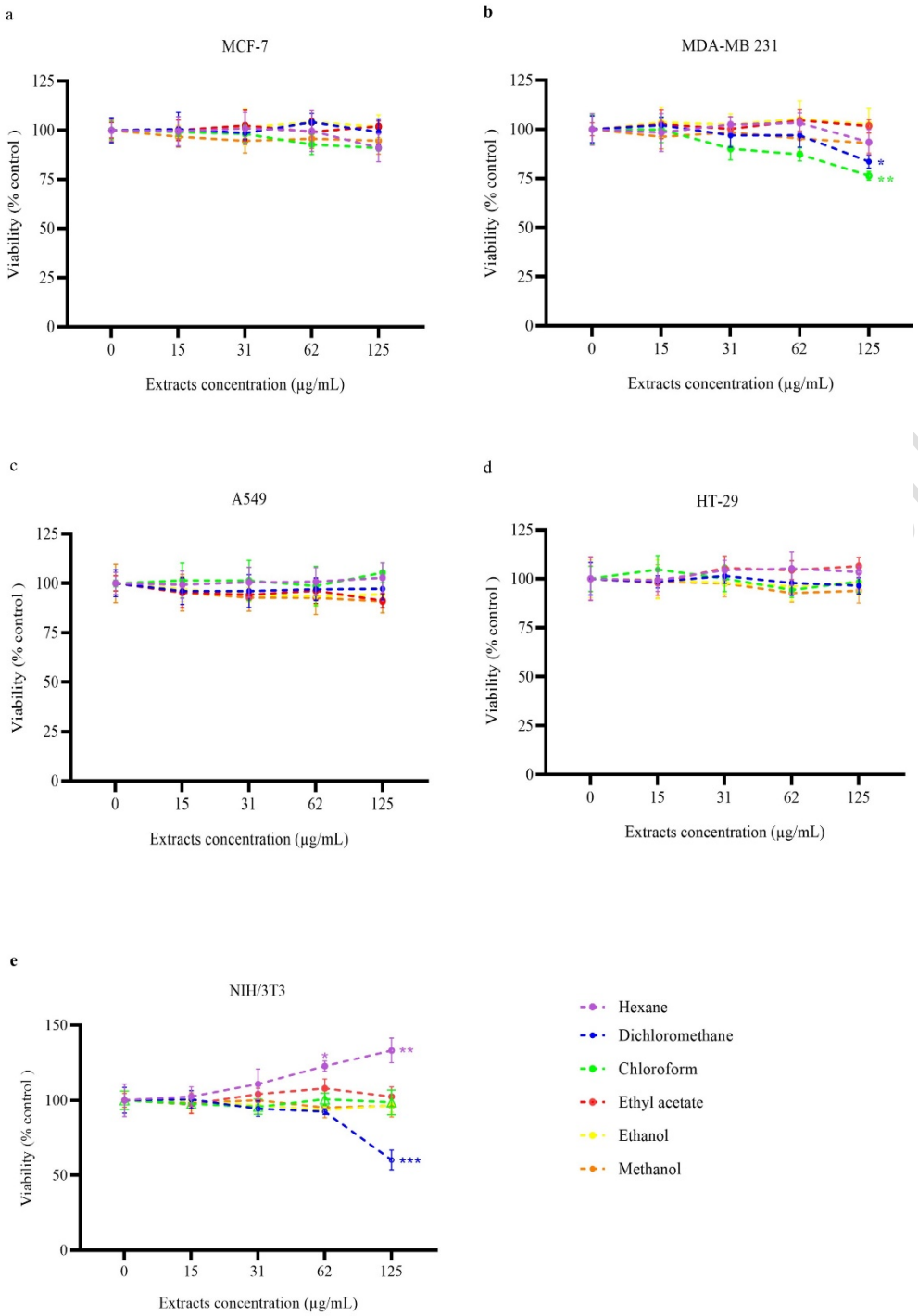

Fig. 2 Cytotoxic effect of the hexane, dichloromethane, chloroform, ethyl acetate, ethanol, and methanol extracts of *S. circinnatus* on MCF-7 (a), MDA-MB-231 (b), A549 (c), HT-29 (d), and NIH-3T3 (e) cell lines assessed by the MTT assay. Data are presented as mean ± standard deviation (SD) from three independent experiments. Statistical comparisons were made against the 0 µg/ml treated group (**P<0.05, ** P<0.01, *** P<0.001*).

DISCUSSION

Medicinal plants have been used for centuries to treat a wide range of diseases. Today, in some cases, medicinal plants have been replaced by synthetic drugs due to their fewer side effects, availability, and affordability [2]. Medicinal plants contain various bioactive chemical compounds responsible for a range of pharmacological actions, including anti-inflammatory, antibacterial, antioxidant, and cytotoxic activities [21]. According to this study's findings, both extraction yield and TPC varied significantly depending on the type of solvent. The ethyl acetate extract showed the highest antioxidant property. In the cellular assay, the extracts did not induce significant cytotoxicity in most cell lines. However, the dichloromethane and chloroform extracts at a concentration of 125 µg/mL markedly reduced cell viability of the MDA-MB-231 cell line, whereas the hexane extract produced an evident increase in cell growth in the 3T3 cell line. For the identification and isolation of phytochemicals in plant materials, optimizing the extraction process using the appropriate solvent is the primary step [22]. The particle size of

the sample, the chemical nature of the phytochemicals, the extraction method, the nature of the solvent, and the presence of interfering substances can affect extraction efficiency. The extraction yield also depends on key parameters, such as the solvent nature and polarity, extraction time, pH, temperature, and sample composition. When the time and temperature of extraction are the same, the sample composition and the nature of the solvent are considered the most important parameters [23]. To identify appropriate extraction solvents, a wide range of solvents with varying polarities should be investigated [22].

In this work, *S. circinnatus* extracts were obtained using hexane, dichloromethane, chloroform, ethyl acetate, ethanol, and methanol, respectively. Extraction yields ranged from 0.13 ± 0.02% for chloroform to 25.2 ± 0.85% for ethanol. The ethanol extraction yield is higher than that with other solvents. In addition to increasing polarity, ethanol has a polar hydroxyl group and a non-polar alkyl group, allowing it to extract compounds of varying polarity more effectively [24]. This may explain why the ethanol yield was higher than others'. However, Chatha *et al*. reported that the highest extraction yield from rice bran was obtained with methanol compared with acetone [25]. This discrepancy may be related to plant composition and extraction methods. Furthermore, in our study, we used a sequential extraction method.

According to Table 1, the TPC of the different extracts of *S. circinnatus* root ranged from 7.93 ± 0.41 to 254.73 ± 3.98 mg GAE/g dried extract. The ethyl acetate extract exhibited the highest TPC, followed by the ethanol extract. In general, the extraction efficiency of phenolic compounds is influenced by the nature of the extraction solvent, particularly its polarity index (PI) and its capacity to dissolve phenolic compounds. The solubility of polyphenols is closely associated with the number and position of hydroxyl groups as well as the size and molecular structure of their hydrocarbon chains. Similar findings have been reported by Bui *et al*. [22] for leaves of *Avicennia officinalis* and by Li Hai-yun *et al*. [26] about *Lysimachia foenum-graecum* Hance, where ethyl acetate was identified as a suitable solvent for recovering phenolic and polyphenolic constituents. These observations indicate that, although high-polarity solvents often yield higher total phenolic content and antioxidant activity, the optimal solvent for extracting phenolic compounds can vary substantially among plant species due to differences in phytochemical composition and molecular characteristics.

Furthermore, the ethyl acetate extract exhibited the strongest antioxidant activity. In contrast, the hexane extract showed weak antioxidant activity, so that its radical-scavenging activity failing to reach 50% inhibition at any concentration. Chavez *et al*. described phenolic compounds as primary antioxidants and effective free radical scavengers [27]. Similarly, Gopinathan *et al.* have extracted the root of *Heliotropium indicum L*. using various solvents (hexane, chloroform, ethyl acetate, methanol, and water) via the Soxhlet method. They reported that the ethyl acetate extract showed greater DPPH radical inhibition compared with the other extracts [28]. These findings are consistent with our results, suggesting that the ethyl acetate extract of *S. circinnatus* root contains a higher concentration of active antioxidant constituents, mostly phenolic compounds, than the other extracts, thereby contributing to its superior DPPH radical scavenging activity.

The cytotoxicity assessment revealed that most tested concentrations of *S. circinnatus* extracts did not exert significant cytotoxicity on the different cell lines. Interestingly, chloroform and dichloromethane extracts at 125 µg/mL demonstrated significant cytotoxicity against MDA-MB-231 cells. To the best of our knowledge, no cytotoxic studies have been previously reported for the *Solenanthus* genus. However, several studies have reported the cytotoxic effect of different genera within this family [29-34]. These effects were generally dose-dependent and varied according to the type of extract and cell line. Overall, despite these reports, the current study showed that *S. circinnatus* extracts did not have a notable cytotoxic effect against cancerous cell lines. Furthermore, the hexane extract exhibited a stimulatory effect on fibroblast viability rate. Considering the positive effect of steroidal compounds on the growth of a fibroblast cell line in a previous study [35], the observed effect in the current study might be related to plant steroidal constituents. Although the hexane extract appeared to increase NIH-3T3 fibroblast viability at certain concentrations, this observation warrants further investigation with complementary assays (e.g., a BrdU proliferation assay or real-time cell analysis).

CONCLUSION

In this study, six solvents were sequentially used to extract active compounds from the roots of *S. circinnatus* to investigate how solvent polarity affects the TPC, antioxidant activity, and cytotoxicity of the extracts. The highest TPC and antioxidant activity were observed for the ethyl acetate extract. Thus, ethyl acetate was identified as the optimal solvent for polyphenol extraction from *S.circinnatus*. Furthermore, dichloromethane and chloroform extracts exhibited significant antiproliferative effects against the MDA-MB-231 cancer cell line at 125 µg/mL. In contrast, the hexane extract seems to promote the viability of 3T3 mouse embryonic fibroblast cells. The apparent stimulatory effect of the hexane extract on fibroblast viability should be interpreted with caution. Additional experiments are necessary to confirm this effect.

Consent for Publication

Not applicable.

Data Availability

The data that support this study will be shared upon reasonable request made to the corresponding author.

Conflict of Interests

The authors declared no conflict of interest.

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Authors' Contribution

Mitra Mehrabani and Mehrzad Mehrbani contributed to the conceptualization of the study. Data curation was performed by Mahboobeh Kavirimanesh Khanaman, Mitra Mehrabani, Mehrnaz Mehrabani, Mahboobeh Raeiszadeh, and Faegheh Farhadi. Formal analysis and software implementation were conducted by Mehrnaz Mehrabani. Funding acquisition and supervision were undertaken by Mitra Mehrabani. Investigation was carried out by Mehrnaz Mehrabani, Arian Amirkhosravi, Mahboobeh Kavirimanesh Khanaman, and Mahboobeh Raeiszadeh. The methodology was developed by Mitra Mehrabani, Mehrzad Mehrbani, Mahboobeh Raeiszadeh, and Faegheh Farhadi. Validation was performed by Mitra Mehrabani and Mehrnaz Mehrabani. The original draft of the manuscript was prepared by Mahboobeh Kavirimanesh Khanaman and Mehrnaz Mehrabani. Review and editing of the manuscript were performed by Mitra Mehrabani, Mehrnaz Mehrabani, Mahboobeh Raeiszadeh, and Mehrzad Mehrbani. All authors read and approved the final manuscript.

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