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Original article

Toxicological, chemical and antibacterial evaluation of squill vinegar, a useful product in Persian Traditional Medicine

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Abstract

Background and objectives: Squill [*Drimia maritima* (L.) Stearn] is an important medicinal plant that has been used for medicinal purposes such as cardiovascular diseases and asthma since ancient times. Bufadienolides are the main compounds of this plant and are responsible for some reported adverse effects. In order to reduce adverse effects, different methods like boiling with vinegar were applied by traditional practitioners. In the present study, the acute oral toxicity, cytotoxic effects, proscillaridin A content and antibacterial properties of methanol and vinegar extracts of squill white variety were compared for exploring the efficacy of traditional processing method. **Methods:** Different doses of extracts (1000-5000 mg/kg) were administered during oral gavage in rats to analyze the acute oral toxicity. Cytotoxicity against HT-29, Caco-2 and NIH3T3 cell lines and antibacterial activity *(Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*) were investigated using MTT assay and conventional agar dilution method, respectively. Proscillaridin A content was evaluated in the extracts (vinager and methanol) by a validated high performance liquid chromatography method. **Results:** During the *in vivo* research no death or observed effect occurred in animals that received the extracts. Our results showed that all of the extracts exhibited no cytotoxic effects in experimented cell lines (IC_{50}>1000 µg/mL). Proscillaridin A was only detected in the methanol extract and no significant antibacterial effect was detected in methanol extract. **Conclusion:** According to results of the present study, processing squill with vinegar according to traditional experiences can reduce possible the side effects of bufadienolids.

Keywords: antibacterial effect, *Drimia maritima*, proscillaridinA, squill, toxicity

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Introduction

Squill, *Drimia maritima* L. stern, is a medicinally important plant belonging to Asparagaceae family distributed in Mediterranean countries [1,2]. It also grows in some regions of Iran. Its bulb is the main part that has been used for medicinal purposes and is usually gathered in September after the appearance of flowers while bulbs may be reaching up to 30 cm in diameter. Two varieties of *D. maritima* exist with the same morphology with white or red bulbs [2,3]. Squill has a long history in traditional medicine. Egyptian, Greek and Persian traditional practitioners applied this plant for treating various diseases such as cardiac disorders, infectious diseases, respiratory ailments, epilepsy and jaundice [4]. Cardiac glycosides, the main constituents of squill, are responsible for its cardiac effects. Cardiac glycosides are divided into cardienolides and bufadienolides based on lacton ring containing 5 or 6 atoms respectively. *D. maritima* is a source of bufadienolides and structure of these isolated compounds has been elucidated in many published researches [5]. Other constituents such as sterols, flavonides and oxalic acid have been also identified in *D. maritima* [4]. In addition to cardiovascular properties, other biological effects such as antitumor, antioxidant, insecticidal activity have been reported from this plant [6-10]. Due to the widespread use of traditional medicine in Iran in recent years, preparation of herbal medicines is elevated. Squill is one of the widely used plants and has both beneficial and adverse effects. Under the guidance of traditional Persian medicine, squill has never been used in raw form for oral consumptions. Some processing methods like boiling with vinegar with or without additives, soaking with vinegar and cooking with paste have been applied for preparation of squill containing formulations [11,12]. Nowadays application of vinegar for squill processing is the only common method. Squill vinegar is widely used for preparation of syrups which are used for medicinal purposes such as in respiratory and liver disorders. Processing methods which are based on experiences of traditional clinical scientists should be completely considered. These methods may reduce the toxicity or enhance the bioactivity of the plant compounds [13]. To the best of our knowledge, there is no published data about squill vinegar so, in the present study proscillaridin A content, acute toxicity, cytotoxic effects and antibacterial properties of the crude bulb and squill vinegar were studied.

Experimental

Plant material

Drimia maritima (white variety) was collected in September 2014 from Chenarshahijan, Kazerun, Fars province, Iran at the altitude of approximately 1050m. The plant was authenticated by Dr. Gh. Amin (Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran) and a voucher specimen (No. 6622-TEH) was deposited at the Herbarium of faculty of pharmacy. Home- made red vinegar was purchased from local herbal market, Tehran, Iran.

Extraction

According to traditional medicine manuscripts, for preparing squill vinegar, fresh squill bulbs were cleaned thoroughly to remove any soil or debris. Bulbs were cut into small pieces, 50 g was weighted and extracted with 1000 mL of vinegar or methanol under reflux for 90 min. The suspensions were filtered and concentrated under reduced pressure. The solvent was evaporated to dryness. The dried extracts were stored at 2-8 °C with no exposure to light. They were dissolved in suitable solvents (methanol for HPLC analysis, water for acute toxicity, DMSO for antibacterial and cytotoxic assays) in order to be used in the experiments.

Proscillaridin A determination

Proscillaridin A content of squill formulation was analyzed based on our previous publication using validated high performance liquid chromatography-UV method with gradient elution (methanol: water) on a reversed phase ACE C_{18} with flow rate of 1 mL/min and UV

detection at 300 nm for 50 min[14].

Acute toxicity

The acute oral toxicity was performed as explained in OECD-423 guidelines [15]. Twenty five male Wistar rats (purchased from Pasteur institute of Iran and weighting 123±15 g) were acclimated one week in animal house prior to experiment. All animal studies were approved by the animal ethics committee of Tehran University of Medical Sciences and care was in accordance with ethics committee of Tehran University of Medical Sciences guidelines. The yield of extraction was determined as 1.6 % (w/w). The dried extracts (1 g) were dissolved in suitable volume of distilled water to administer graded amount in the range of 1000, 2500, 4000 to 5000 mg/kg body weight by oral gavage. Control animals received only distilled water. The rats were allowed free access to feed and drinking water. The clinical and behavioral signs as well as survival of animals were observed up to 72 h.

Cytotoxicity

Cell culture

Colon carcinoma (HT-29), colorectal adenocarcinoma (Caco-2) and Swiss mouse embryo fibroblast (NIH3T3) cell lines were obtained from Pasture Institute of Iran, Tehran, Iran. RPMI 1640 cell culture medium (PAA, Germany) was used to culture colon carcinoma (HT-29) and colorectal adenocarcinoma (Caco-2) cell lines. For HT-29 cells, 10% fetal bovine serum (FBS; Gibco, USA) and for Caco-2, 15% FBS was added to the medium. The Swiss mouse embryo fibroblast (NIH3T3) cell line was kept in Dulbecco's modified Eagle's medium (DMEM; PAA, Germany) supplemented with 10% FBS. 100 IU/mL Penicillinand 100 µg/mL streptomycin (Roche, Germany) were added to the media. All cell lines were incubated at 37 °C and 5% CO_2 atmosphere.

Cytotoxicity assay

The methanol or vinegar extracts were dissolved in dimethyl sulfoxide (DMSO) and further diluted with cell culture medium. Subsequently, different concentration of each extract (50, 100, 250, 500 and 1000 µg/mL) were prepared. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) assay was used to measure cytotoxic activity. Cells (1×10^4) of each cell line were seeded in 98-well plates (Nunc, Denmark) and incubated at 37 °C. The plates were allowed to proliferate and reach their exponential phase of growth. The incubation time for each cell line was assigned according to the normal growth curve of that cell line and was determined twice as long as the doubling time of each cell line [16]. Different concentrations of each extract were replaced by media after 24 h of incubation. After 72 h, 20 µL of MTT reagent (5 mg/mL) in phosphate buffered saline (PBS) was added to each well and they were incubated in 37 °C for 4 h. After evacuation of the media, the formed blue formazan crystals were dissolved in 100 µL of DMSO and were further incubated for 10 minute at 37 °C. Finally the absorbance was measured at 570 nm using a micro plate reader (Anthos, Austria). All tests were repeated three times and cytotoxicity was considered as the median growth inhibitory concentration (IC_{50}) compared to control.

Antibacterial assay

Minimum inhibitory concentration (MIC) determination

The MICs of the methanol and vinegar extracts as well as vinegar were determined by conventional agar dilution method with respect to different test microorganisms including three Gram-positive (*Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633) and three Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 8739). The test microorganisms were provided from the Department of Drug and Food control, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

Two-fold dilutions of the extracts were prepared in dimethylsulfoxide (DMSO; 1 mL). Each dilute was added to molten Mueller-Hinton (MH) agar

(19 mL) at 50 °C to give the final concentrations of 1.4-46 mg/mL. The bacteria inocula were prepared by suspending colonies from Muller-Hinton (MH) agar media in 0.9% saline overnight. The inocula were adjusted photometrically at 600 nm to a cell density equivalent to approximately 0.5 McFarland standard (1.5×10^8 CFU/mL). The suspensions were then diluted in 0.9% saline to give 10^7 CFU/mL. The plates were spot-inoculated with 1 µL of each prepared bacterial suspension (10^4 CFU/spot); including a control plate containing 1 mL DMSO without any antibacterial agent to show that DMSO had not any antibacterial effect in our experiments. Ciprofloxacin was used as the positive control. The plates containing bacteria were incubated at 30-35^{o}C for 24 h. The MIC was determined as the lowest concentration of the agent that completely inhibits visible growth of the microorganisms.

Statistical analysis

Data were presented as mean±SD of three independent experiments. IC_{50} values were calculated from the dose-response curves using Sigma plot 10 software

Results and Discussion

The yield of extraction was determined as 1.6 % (w/w). The results obtained from analysis of Proscillaridin A have been showed in figure 1. In comparison with the standard sample, proscillaridin A has been detected in the methanol extract. Vinegar extract didn't have any peaks in proscillaridinA area.

No death or signs of toxicity was observed in the rats treated with different doses (mg/kg) of the extracts up to 72 h. So it was suggested that these extracts were harmless [17].

The effect of squill methanol and vinegar extracts on the proliferative response of HT-29, Caco-2 and NIH3T3 cell lines have been analyzed by treating the cells with different concentrations of the extracts. As it has been shown in table 1, no significant cytotoxic effect was observed on experimented cell lines (IC_{50}>1000 µg/mL).

The antibacterial activity of the methanol and vinegar extracts were examined on both Gram-positive (*S. aureus*, *S. epidermidis*, *B. subtilis*) and Gram-negative (*P. aeruginosa,K. pneumoniae*, *E. coli*) bacteria. The results of minimum inhibitory concentration (MIC) determination have been shown in table 1. Methanol extract didn't show significant antibacterial activity but vinegar extract and vinegar exhibited similar effects

Squill has been described for various disease such as cardiac disorders, asthma, respiratory disorders, infectious disease, jaundice and join pains by traditional practitioners in Iran. Vinegar extract of this plant is the most common product for medicinal approaches. *D. maritima* may cause poisoning symptoms similar to digitalis [18]. Lack of appetite, vomiting, diarrhea, headache and irregular heartbeat are the main adverse effects of this plant. Because of the narrow therapeutic index, side effects can occur even through therapeutic dosages [19]. Since this plant was used as a rat poison in the past, several studies have been done about its toxicity [4].There is also published report of a woman death occurred after oral consumption of two bulbs of white squill in raw form [17]. In addition, squill as a cough remedy (in combination with other components like opiate) has caused poisoning in two cases [20-21].

In the present study, toxicity chemical and antibacterial properties of methanol and vinegar extract of Iranian source of *Drimia martima* were explored. During the *in vivo* research, no death was observed in animals which consumed methanol or vinegar extracts. This finding is consistent with previous reports which revealed that only red varieties contained glycoside scilliroside which has been known as the most toxic compound [22].Although these results confirmed that integrity of squill processing method in Persian traditional medicine, patients should be informed about the main side effects before starting treatment. In addition, chronic toxicity studies should be performed for exploring the effect of squill in different organs.

All of the extracts exhibited no cytotoxic effect on normal mouse embryo fibroblast (NIH3T3)

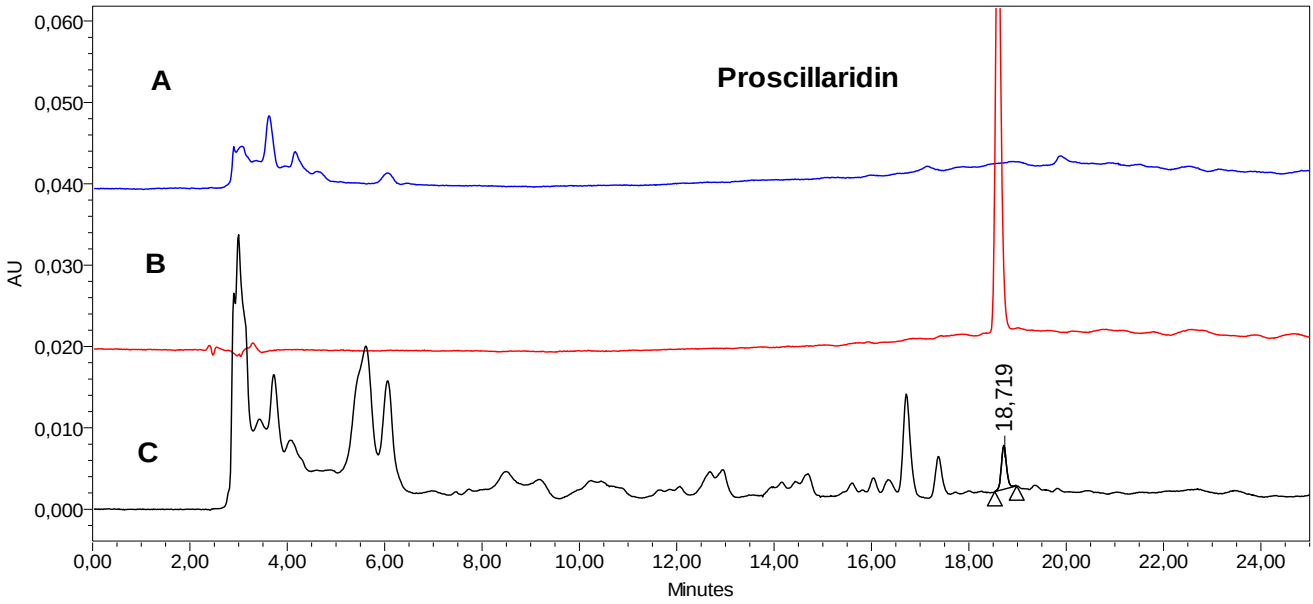

Figure 1. HPLC chromatogram of A: Squill vinegar extract, B: Proscillaridin A standard solution and C: Squill methanol extract, with chromatographic UV spectra at 200-400 nm

Table 1. Antibacterial activity (minimum inhibitory concentrations) of squill methanol extract, vinegar extracts, vinegar (mg/mL) and ciprofloxacin (µg/mL).

	MIC (mg/mL)					
Samples	[a]***S. aureus***	[b]***S. epidermidis***	[c]***E. coli***	[d]***K. pneumoniae***	[e]***B. subtilis***	[f]***P. aeruginosa***
Methanol extract	>23	>23	>23	>23	>23	>23
Vinegar extract	11.5	11.5	11.5	11.5	11.5	5.8
Vinegar	11.5	11.5	11.5	11.5	11.5	5.8
Ciprofloxacin (µg/mL)	0.19	0.39	0.01	0.003	0.19	0.39

[a] ATCC 6538,[b] ATCC 12228,[c] ATCC 8739,[d] ATCC 10031,[e]ATCC 6633 and [f] ATCC 9027

which confirmed the results of *in vivo* experiments. No activity against HT-29 and Caco-2 which are colon carcinoma and colorectal adenocarcinoma cell lines showed that these extracts are not effective against these types of cancers. Although methanol and vinegar extracts didn't show toxicity on cancer cells in this study, other cell lines may be affected by these extracts.

Proscillaridin A is one of the main cardiac glycosides which possess toxic effects via Na+/K+- ATP ase pump inhibition. Oral LD_{50} of this compound is 56.2 and 76.5 mg/kg for male and female adult rats, respectively [23]. The vinegar extract did not contain proscillaridin A. It is may be related to the hydrolysis of the sugar chain in acidic pH of vinegar. So vinegar usage in squill product may result in decreasing the adverse effects. More phytochemical analysis can be helpful to clarify the effect of vinegar in elimination of squill constituents or production of new compounds. Antibacterial activity of vinegar extract was similar to vinegar and higher than the methanol extract. Plant processing with vinegar has been common in traditional medicine of different nations and was subjected for some studies. In some vinegar containing formulations

the biological effects has improved due to processing [24,25]. Investigation of other *in vivo* and *in vitro* biological effects may lead to more elucidation of the role of vinegar in squill products.

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Declaration of interest
The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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