Original Article

Phytochemical and Biological Studies on *Muscari inconstrictum* Seeds Distributed in Iran

Abstract

Background and Purpose: *Muscari* Miller. (Asparagaceae family) contains about 50 species worldwide, which are distributed in the Central and Southeastern Europe, Southern Russia, Africa, and some area of Asia such as Iran, Iraq, Afghanistan, Anatolia, and Syria. This study was designed to assess the antioxidant and antimalarial activities of *Muscari inconstrictum* Rech. f. seeds as one of the Iranian species of *Muscari* genus. In addition, preliminary phytochemical analysis of the extracts with different polarities was performed. **Materials and Methods:** The essential oil of *M. inconstrictum* seeds was prepared using Clevenger and extracted with n-hexane, chloroform, and methanol (MeOH) by Soxhlet apparatus. Gas chromatography-mass spectrometry (GC-MS) was used for the characterization of essential oil. Total phenol and flavonoid contents were measured using Folin–Ciocalteu and aluminum chloride reagents. Free radical scavenging and antimalarial activities were investigated via 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and cell-free ß-hematin formation methods. **Results:** GC-MS analysis of the volatile oil of seeds demonstrated the presence of sesquiterpenoid, alkanes, fatty acid, and linear alcohol structures as the main constituents. Among different extracts of *M. inconstrictum* seeds, the methanolic extract showed significant antioxidant activity, which can be related to the presence of flavonoid and other phenolic structures. Furthermore, chloroform extract is introduced as the most potent antimalarial part. **Conclusion:** It seems that further studies on the *M. inconstrictum* seeds are necessary to focus on pure compounds and their biological activities.

Keywords: *Antimalarial, antioxidant, gas chromatography-mass spectrometry, Muscari*

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Introduction

Muscari Miller. (Asparagaceae family) is introduced by approximately 50 species worldwide. These ornamental and garden plants are distributed in the Central and Southeastern Europe, Southern Russia, Africa, and some areas of Asia such as Iran, Iraq, Afghanistan, Anatolia, and Syria.[1-3] These plants have pharmaceutical and economic values.[4] According to previous literature, plants of *Muscari* genus have numerous medicinal and biological activities such as antioxidant, anti-inflammatory, emetic, diuretic, hypoglycemic, and stimulant effects.[2,5,6]

A review on the chemical structures of the genus *Muscari* indicated the existence of alkaloids, flavonoids, steroids, and triterpenoids.[4] Homoisoflavonoids are an uncommon subclass of flavonoid structures with an extra carbon atom that are obtained from the plants of this genus, which are found rarely in nature. These bioactive compounds have shown a broad range of activities such as antioxidant, antiplasmodial, antimutagenic, anticlastogenic, antimicrobial, antidiabetic, anti-inflammatory, immunomodulatory, antiangiogenic, vasorelaxant, and cytotoxic.[7-10]

This study was conducted to evaluate phytoconstituents and some biological activities of *Muscari inconstrictum* Rech. f. seeds as one of the bulbous species reported from Iranian flora.[1] As per our recent studies, the chloroform and *n*-hexane extracts of the bulbs of this species showed significant antioxidant and antimalarial activities, respectively. The flavonoids and coumarins were reported as the responsible components of chloroform extract and the saponin structures were reported as the most potent antimalarial parts of *n*-hexane extract[11]

Materials and Methods

Plant material

Muscari inconstrictum Rech. f. was collected from the gardens of East Azerbaijan province of Iran (37° 53′ 51″ N, 45° 57′ 15″ E) in April 2016. It was authenticated and stored as No.8897 at

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the Herbarium of the East-Azarbaijan Agricultural and Natural Resources Research and Education Center, Tabriz, Iran.

Extraction

The seeds of *M. inconstrictum* were dried after the harvest in a shady for a week, then they were packed in paper bags and stored in a dark and dry place at the room temperature.

For gathering of the essential oil, approximately 100 g of powdered seeds were subjected to hydro-distillation for 4 h using a Clevenger apparatus. The obtained volatile oil was measured (V/W) and dried via anhydrous sodium sulfate and then stored in a sealed vial for further analysis.

In addition, 100 g of the seeds were powdered and were Soxhlet-extracted with approximately 1 liter *n*-hexane, chloroform, and methanol (MeOH). Subsequent extracts were evaporated at 50 ℃ using a rotary evaporator instrument.

Identification of components

Gas chromatography/mass spectrometry analysis

Gas chromatography–mass spectrometry (GC–MS) and gas chromatography with flame ionization detector (GC/FID) analysis were performed, respectively, on a Shimadzu QP5050A GC/MS system (Kyoto, Japan) and GC17A equipped with a DB-1 fused silica column (60 m × 0.25 mm i.d.; 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1.3 mL/min. The oil was diluted with a ratio of 1:10 in *n*-hexane and 1 µL of which was injected into the column. Split ratio, ionization energy, scan time, and acquisition mass range were 1:33, 70 eV, 1 s, and 30–600 amu, respectively.

The temperature program was an oven temperature of 50 ℃ rising to 260 ℃ at a rate of 3 ℃/min for a total run time of 75 min. Injector temperature was set at 220 ℃ and the detector temperature was 260 ℃.

Identification of the volatile constituents was completed based on the direct comparison of the retention times (R_t) and mass spectral data with standard alkanes (C_8–C_{20}) from Sigma Aldrich (Allentown, PA, USA), computer matching with the WILEY229, NIST21 and NIST 107 libraries, and comparing the fragmentation patterns of the mass spectra with those described in the literature[12,13].

Total phenolic content

Determination of total phenolic content of extracts was performed using modified Folin–Ciocalteu method as gallic-acid equivalents (GAE).[14] Approximately 1 mL of sample (5 mg/100 mL acetone in water 60:40) was mixed with 0.2 mL Folin–Ciocalteu and 0.5 mL Na_2CO_3 (2%) and centrifuged at 12000 rpm for 5 min. After a 30-min incubation at room temperature, the absorbance of samples was measured at 750 nm with UV/Visible Spectrophotometer (Spectronic Genesys 5 Spectrophotometer, San francisco, CA, USA). The same procedure was repeated in triplicate and average absorption was noted. Total phenolic content (TPC) was reported as GAE in mg/g of sample.

Total flavonoid content

The samples were assessed to determine their total flavonoid content (TFC) with aluminum chloride reagent and rutin as a positive control.[15] Approximately 2 mL of each sample (1 mg/1 mL methanol in water 80:20) was mixed with 1 mL reagent ($AlCl_3$ crystals and sodium acetate crystals in 100 mL of 80% of methanol in water) and 400 µL distilled water. Tubes were permitted to stay at room temperature for 30 min. Subsequently, the absorbance of samples was measured at 430 nm with UV/Visible Spectrophotometer (Spectronic Genesys Spectrophotometer). TFC was reported as rutin equivalents/g of sample.

Free radical scavenging activity

The concentrated *n*-hexane, chloroform, and methanol extracts were objected to antioxidant assay using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma, Germany) as a reagent.[16] Approximately 4 mg of DPPH was dissolved in 50 mL methanol for methanolic extract and chloroform (for *n*-hexane and chloroform extracts) to obtain the stock solution. The methanolic extract was dissolved in methanol (1 mg/mL). Next, *n*-hexane and chloroform extracts were dissolved in chloroform (1 mg/mL). Concentrations of 0.25, 0.125, 0.0625, 0.0031, and 0.0015 mg/mL were made by dilution. In the next step, 2 mL of each dilution was mixed with DPPH (2 mL). Tubes were kept at room temperature for 30 min to allow any reaction to occur. The absorbance of samples was measured using UV–Visible Spectrophotometer (Spectronic Genesys Spectrophotometer) at 517 nm. The same procedure was repeated in triplicate and the average absorbance was recorded. Reduction percentage of DPPH (R %) was calculated as below and the result was reported as sample concentration providing 50% reduction of DPPH (RC_{50}):

$$\text{Reduction percentage of DPPH (R\%)} = \left[\frac{\left(\text{Abs}_{(\text{blank})} - \text{Abs}_{(\text{sample})}\right)}{\text{Abs}_{(\text{blank})}}\right] \times 100$$

where Abs stands for absorbance of samples.

Positive control: quercetin (0.001–0.01 mg/mL) was used as the same procedure.

Cell-free beta hematin formation assay

The antimalarial activities of three extracts (*n*-hexane, chloroform, and methanol extracts) were assessed by the heme *bio*-crystallization method.[17] Different concentrations of extracts (0.4–2 mg/mL in DMSO) were mixed with hematin (100 µL, dissolved in 0.1 M NaOH), oleic acid (10 mM), and HCl (10 µM). The reaction volume was adjusted to 1000 µL of sodium acetate buffer with a pH of 5. The samples were incubated at 37 ℃ for 24 h with consistent shaking. Then, the samples were centrifuged for 10 min in 12,000 rpm and the hemozoin sediments were continually washed by 2.5% (w/v) sodium dodecyl sulfate in phosphate-buffered saline. In the next step, they were washed in sodium bicarbonate (0.1 M and pH 9.0) until the supernatant was clear (after 4–6 washes). Afterward, the supernatant was removed and the sediments were re-suspended using 1 mL of NaOH (0.1 M). The absorbance of the samples was measured at 400 nm

with UV/Visible Spectrophotometer (Spectronic Genesys Spectrophotometer). The results were reported as inhibition percentage (I %) of heme crystallization calculated as below:

$$\text{Inhibition percentage (I \%)} = \left[\frac{\left(\text{Abs}_{(\text{blank})} - \text{Abs}_{(\text{sample})}\right)}{\text{Abs}_{(\text{blank})}} \right] \times 100$$

where Abs stands for absorbance of samples.

Positive control: chloroquine (0.01–0.5 mg/mL) was used as the same procedure.

Results

The GC–MS analysis results of the volatile oil of *M. inconstrictum* seeds (the yield of oil: 0.2%v/w) are shown in Table 1.

In addition, total phenol and flavonoid contents of three main extracts were assessed by two colorimetric methods correspondingly. Table 2 presents the TPC and TFC assay results.

In addition, antioxidant and antimalarial tests of three extracts were evaluated using DPPH and cell-free beta-hematin formation methods [Table 3].

Discussion

Based on the GC–MS analysis obtained for the essential oil of *M. inconstrictum* seeds, a total of 17 volatile constituents were recognized [Table 1]. According to the relative content of identified volatile constituents, sesquiterpenes and derivatives (49.12%) constituted the main functional groups in the seeds oil of *M. inconstrictum*. The main sesquiterpene structure of the oil is spathulenol with an abundance of 34.38%, which is categorized as a member of tricyclic sesquiterpene alcohol compounds.[18] Spathulenol could be responsible for some biological activities such as anti-inflammatory, antioxidant, antiproliferative, antimicrobial, and as a mosquito repellant agent against *Aedes aegypti* and *Anopheles stephensi* in the essential oil of various plant species.[19]

Table 1: Chemical composition of the essential oil of *Muscari inconstrictum* seeds

No.		KI	Compounds
1	1.56	821	2,4-Dimethylheptane
2	1.20	1104	5-Methylundecane
3	3.38	1174	Hexylbutyrate
4	2.24	1193	Octyl acetate
5	4.59	1284	Undecane, 3,8-dimethyl
6	2.21	1329	Octane, 2,3,6,7-tetramethyl
7	5.76	1458	*n*-Dodecanol
8	5.04	1502	Dodecane, 2,6,11-trimethyl
9	3.21	1516	Delta-Cadinene
10	2.88	1546	Pentadecane
11	34.38	1567	Spathulenol
12	5.08	1574	β-Caryophyllene oxide
13	1.49	1582	Salvial-4(14)-en-1-one
14	3.36	1671	Aromadendrene oxide II
15	2.51	1720	Heptadecane
16	1.60	1830	Hexahydrofarnesyl acetone
17	7.52	1941	n-Hexadecanoic acid

Total compounds: 17
Total identified: 88.01%
Sesquiterpenes and derivatives: 49.12%
Alkanes: 19.99%
Fatty acids and derivatives: 13.14%
Linear alcohol: 5.76%

The presence of *n*-hexadecanoic acid (7.52%) as the second most abundant component of *M. inconstrictum* seeds was also prominent. In addition, *n*-hexadecanoic acid or palmitic acid is one of the common saturated fatty acids in plants.[20,21] Previous studies have reported the anti-inflammatory effect of n-hexadecanoic acid via inhibition of phospholipase A_2 and the results validated the use of the *n*-hexadecanoic acid-rich medicinal oils for management of rheumatic symptoms in traditional medicine.[21] In addition to the mentioned chief constituents, alkanes (19.99%) and linear alcohol (5.76%) were identified as the other functional groups.

Moreover, antioxidant and antimalarial activities of three extract of *M. inconstrictum* seeds were evaluated [Table 3]. The methanol extract showed the highest antioxidant activity, which was confirmed by total phenol and flavonoid contents [Table 2]. Earlier investigations on *Muscari* genus showed potent antioxidant activities of *Muscari racemosum*, which are related to the presence of homoisoflavonoid structures.[9] Furthermore, the antimalarial assay showed a moderate effect of chloroform extract of the seeds of *M. inconstrictum* in comparison with chloroquine as the positive control of the test. Researchers have shown strong antimalarial effects of purified flavonoid and the potential synergistic effects between artemisinin and flavonoid structures.[22,23] In this regard, Midiwo *et al.*[10] reported antiplasmodial activities of a homoisoflavonoid structure against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*.

However, according to previous literature and our findings, it is necessary to conduct further studies on *M. inconstrictum* pure compounds and their biological activities.

Conclusion

Overall, according to this introductory biological and phytochemical investigation, among different extracts of *M. inconstrictum* seeds, the methanolic extract showed significant antioxidant activity, which can be related to the existence of flavonoid and other phenolic structures. Moreover, the chloroform extract with the moderate antimalarial effect is introduced as the most potent antimalarial part of *M. inconstrictum* seeds.

The GC–MS analysis of the volatile oil of *M. inconstrictum* seeds showed the presence of sesquiterpenes as the main constituents.

Table 2: TPC and TFC results of *n*-hexane, chloroform, and MeOH extracts of *Muscari inconstrictum* seeds

Samples and tests	*n*-Hexane extract	Chloroform extract	Methanol extract
TPC (mg/g)*	–	5.65 ± 0.3	124.96 ± 1.21
TFC (mg/g)**	–	1.59 ± 0.04	4.99 ± 0.15

*Galic acid was used as standard
**Rutin was used as standard

Table 3: Antioxidant and antimalarial results of *n*-hexane, chloroform, and MeOH extracts of *Muscari inconstrictum* seeds

Samples and tests	*n*-Hexane extract	Chloroform extract	Methanol extract	Control positive
RC_{50} (mg/mL) (antioxidant test)*	–	–	0.53 ± 0.045	0.0028 ± 0.0004
IC_{50} (mg/mL) (antimalarial test)**	–	7.57 ± 0.036	–	0.04 ± 0.006

*Quercetin was used as control positive in DPPH assay
**Chloroquine was used as a control positive in cell-free β-hematin formation assay

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Conflicts of interest

There are no conflicts of interest.

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