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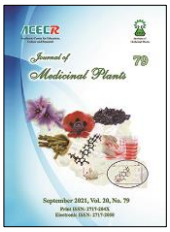

Research Article

Identification of isoflavonoids in antioxidant effective fraction of *Arum rupicola* Boiss. leaves

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ABSTRACT

Background: *Arum rupicola* Boiss. (Araceae Family) is used by the native people of southern areas of Iran as a soup called "kardeh soup". Several flavonoids and phenol compounds have been identified from *Arum* species. **Objective:** The aim of this study was to evaluate antioxidant effect and total phenol contents as well as cytotoxic activity of the leaves of *A. rupicola*. **Methods:** Antioxidant activity of total methanol extract and fractions including *n*-hexane, chloroform, ethyl acetate and water residue were evaluated using FRAP and DPPH methods. Total phenol content was measured using Folin-Ciocalteu method. Cytotoxic activity of the extract and fractions were investigated against human breast cancer MCF-7, MDA-MB-231, and T47D cell lines by MTT assay. Further phytochemical isolation was done on the water residue using column chromatography. **Results:** According to the results, water residue showed the lowest IC_{50} value (186.7 µg/ml) and the total methanol extract showed the most antioxidant power (163.62 mmol FeSO4/100 g extract) and phenol content (135 µmol Gallic acid/g extract). The hexane fraction also showed the highest cytotoxic effect against MCF-7 breast cancer cell line with IC_{50} equal to 118.9 µg/ml. Phytochemical analysis of the water residue resulted in isolation and identification of three isoflavonoids named orobol, genistein and genistein 8-*c*-glucoside. **Conclusion:** Based on the identification of isoflavonoid compounds in this plant, its ability to be used as a phytoestrogenic supplement can be considered in future studies.

Abbreviations: FRAP, Ferric Reducing Ability of Plasma; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

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1. Introduction

Arum is a genus of flowering plants belonging to family Araceae, represented by 26 species and distributed in Northern Africa, Mediterranean Region, Western Asia, and Europe [1]. Several *Arum* species are growing in Lebanon, Cyprus, Syria and Palestine and consumed in folk medicine for treatment of cancer, cough, hemorrhoids, worms, constipation and urinary tract infections. The fresh leaves of *Arum* used in cooked or roasted form after washing several times with salted water [2]. *Arum rupicola* Boiss (Syn. *Arum conophalloides* Kotschy ex Schott) is used by the native people of southern areas of Iran as a soup called "kardeh soup" [3]. Also in some areas of Turkey the aerial parts of *A. rupicola* with local name of "kahri" were used as a rice-vegetable dish [4]. Various pharmacological effects of *Arum* species have been reported such as antioxidant, and antimicrobial effects [5-7]

A variety of flavonoids including, vitexin, caffeic acid, luteolin, vicenin and 3,6,8-trimethoxy, 5,7,3',4'-tetrahydroxy flavone [8] and the alkaloid (S)-3,4,5-trihydroxy-1 H-pyrrol-2(5H)-one were isolated from *A. palaestinum* [9]. Also several flavonoids such as apigenin, luteolin, quercetin, vitexin, isoorientin, esculin, quercetin-3-O-β-glucoside, caffeic acid, and ferulic acid have been identified from *A. dioscoridis* [10, 11]. Flavonoids have antioxidant, anti-cancer, anti-allergic, anti-inflammatory and anti-viral phytochemical compounds [12]. Since several flavonoids and phenolic compounds reported in the genus *Arum*, it can be expected that the aerial parts of the plant contain interesting biological active metabolites. The aim of this study was to evaluate phytochemicals, antioxidant effect and total phenolic contents of the leaves of *Arum rupicola* collected from southern areas of Iran.

2. Materials and Methods

2.1. Chemicals

2,2-Diphenyl 1-picrylhydrazyl (DPPH; Fluka, Switzerland); butylatedhydroxylanizole (BHA), methanol, hexane, chloroform, ethyl acetate (Merck, Germany) were purchased. ^{1}H and ^{13}C-NMR spectroscopy of compounds A, B and C were performed in DMSO-d_6 on a Bruker Avance DPX 400 spectrometer (Karlsruhe, Germany) [400 MHz, tetramethylsilane (TMS) as internal standard]. Column chromatography (CC) was done by Sephadex LH-20 (lipophilic Sephadex, 25–100 μm; Sigma, Dorset, UK) columns.

2.2. Plant material

The leaves of *Arum rupicola* were collected from their growing habitats in Yasuj area, Iran, April 2016. The plant identified by Dr. Farideh Attar. A voucher specimen of the plant (46054 TUH) is deposited in the herbarium of the faculty of Sciences, Tehran University.

2.3. Extraction

The leaves of *A. rupicola* were air dried and grounded in the laboratory, and the obtained powder (1000 g) was extracted five times at room temperature with 80 % methanol for an interval of three days. The aqueous-methanol extract was evaporated under reduced pressure and temperature to obtain a residue of 398.63 g. The total methanol extract was suspended in water and then fractionated using hexane (21.52 g), chloroform (3.03 g), and ethyl acetate (1.67 g) according to their polarity also water residue was 160.14 g. The excessive solvents were evaporated with a rotary vacuum evaporator (60 rpm at 40 °C). All extracts were stored at 4 ºC until use.

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2.4. Total phenol contents assay

Total Phenol was determined colorimetrically using Folin-Ciocalteu reagent as described by Velioglu et al. 1998 and according to previous study [13]. The experiments were repeated three times. The phenol content calibration curves were plotted by measuring the absorption of certain concentraitions (25-150 mg/L) of gallic acid as a standard and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry matter (total extracts and fractions) as means ± SEM [13].

2.5. Antioxidant determination of DPPH assay

The total methanol extract was evaluated for its free radical scavenging activities using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method according to Brand Williams et al. (1995) [14]. Different concentrations (25, 50, 100 µg/ml) of sample solutions (1 ml) in methanol were added to DPPH methanol solution (2 ml, 40 µg/ml). Butylated hydroxyanisole (BHA) (100 µg/ml) was used as positive control. After 30 min, the absorbance was measured at 517 nm. All tests were carried out in triple replicate. Percentage of radical scavenging activity of sample was calculated according to the following equation: Inhibition % = [(A0–As)/A0] × 100 that A0 is the absorbance of the control and as is the absorbance of the sample. Half maximal inhibitory concentration (IC_{50}) value (indicate the concentration of the sample (mg/ml), required to scavenge 50 % of DPPH) was calculated from the plotted graph of scavenging activity versus the concentration of extract, using linear regression analysis.

2.6. FRAP assay

Ferric reducing antioxidant power assay is based on reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants and was performed according to previous studies [15].

2.7. Cell culture

Human breast cancer cell lines including MCF-7 (human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors), T47D (breast ductal carcinoma), and MDA-MB-231 (human breast adenocarcinoma), were supplied from the National Cell Bank of Iran Pasteur Institute, Tehran, Iran. Cancer cell lines were developed in RPMI-1640 medium accompanied with 10 % heat-inactivated fetal calf serum, 100 µg/ml streptomycin and 100 U/ml penicillin at 37 °C in a humidified atmosphere with 5 % CO_2.

2.8. Determination of cell viability by MTT assay

The in-vitro cytotoxic activity of all samples were assessed against three human breast cancer cell lines including MCF-7, T47D, and MDA-MB- 231 cell lines using MTT colorimetric assay according to previous work [16].

Concisely, the concentration of 50, 100, 200, 400 and 500 µg/ml from all samples were prepared for each cell line. At first, samples were dissolved in DMSO (Dimethyl Sulfoxide) and further diluted with cell culture medium. The final DMSO concentration used was 0.1 % of total volume of the medium in all treatments, including the control group. In each plate, there were three control wells (cells without test extracts) and three blank wells (the medium with 0.1 % DMSO) for cell viability. Etoposide was used as a positive control for cytotoxicity. The plates were incubated for 72 h. At the end of the incubation period, the medium was removed and 200 µl phenol red free medium containing MTT (1 mg/ml), was added to wells, and followed by 4 h incubation. After incubation, the culture medium was then exchanged with 100 µl of

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DMSO and the absorbance of each well was measured by using a micro plate reader at 492 nm. For each sample, the concentration causing 50 % cell growth inhibition (IC_{50}) compared with the control was calculated from concentration response curves by regression analysis [16].

2.9. Purification and isolation of flavonoid compounds

In order to identify the main compounds in the plant, water residue was selected for purification due to proper weight and antioxidant effects. Fifteen grams of the water residue were loaded on a Sephadex LH-20 (25 × 5 cm) column and eluted with aqueous methanol (80 %). Fractions with a volume of 30 ml were collected from this column. The obtained fractions were compared using TLC method and the same fractions were combined. To gain more weight for each fraction, this step was performed several times, each time with about 5 g of water residue, and the same fractions were combined to afford 4 fractions. Compound **B** was isolated from the second column and for the final purification, Sephadex LH-20 column (80 × 1 cm) was used. The weight of compound **B** was about 20 mg. The R_f of compound **B** with the solvent system of formic acid/ acetic acid/water/ethyl acetate (11/11/26/100) on TLC sillicagel 60 F_{254} was about 0.7.

To separate compound **A**, the fractions No. 24-27 of the first column, the fractions 20-23 of the second column and the fractions 31-34 of the third column were combined, and first was loaded on the Sephadex LH-20 (70 × 2 cm) column and washed with aqueous methanol (80 %). Then, the fraction No. 17-24 of this column was dried and for final purification, it was loaded on a Sephadex LH-20 (80 × 1 cm) column eluted with methanol (100 %) to afford compound **A** (30 mg). The Rf of compound **A** with the solvent system of formic acid/ acetic acid/water/ethyl acetate (11/11/26/100) on TLC sillicagel 60 F_{254} was about 0.8. To separate compound **C**, the fractions No. 35-37 of the first column, the fractions 28-33 of the second column and the fractions 37-39 of the third column were merged, and then was loaded twice on a Sephadex LH-20 (80×1 cm) column eluted with methanol (100 %) to afford compound **C** (3 mg). The R_f of compound **C** with the solvent system of formic acid/ acetic acid/water/ethyl acetate (11/11/26/100) on TLC sillicagel 60 F_{254} was about 0.6.

2.10. Chemical analysis

^{1}H and ^{13}C-NMR spectroscopy of compounds **A, B** and **C** were recorded on a Brucker Avance 500 DRX (500 MHz) spectrometer. Chemical shifts are given in (ppm) DMSO using TMS as internal standard. Column chromatography (CC) was performed using Sephadex LH-20 (lipophilic Sephadex, 25–100 μm; Sigma, Dorset, UK) columns.

Compound A (Orobol): $C_{15}H_{10}O_6$. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 8.47 (1H, *s*, H-2), 7.34 (2H, *m*, H-5',6'), 6.83 (1H, *d*, *J* = 7.6 Hz, H-2'), 6.54 (1H, *s*, H-8), 6.37 (1H, *s*, H-6)

Compound B (Genistein): $C_{15}H_{10}O_5$. ^{1}H-NMR (400 MHz, DMSO-d_6): δ =8.07 (1H, *s*, H-2), 7.77 (2H, *d*, *J* = 7.2 Hz, H-2', 6'), 6.8 (2H, *d*, *J* = 7.2 Hz, H-3', 5'), 6.57 (1H, *s*, H-8), 6.46 (1H, *s*, H-6)

Compound C (Genistein 8-*C*-glucoside): $C_{21}H_{21}O_{10}$. UV (MeOH, λ_{max}, nm):265. ^{1}H-NMR (400 MHz, DMSO-d_6): δ =8.00 (1H, *s*, H-2), 7.93 (2H, *d*, *J*= 7.2 Hz, H-2',6'), 6.88 (2H, *d*, *J*= 7.2 Hz, H-3',5'), 6.59 (1H, *s*, H-6), 4.18 (1H, *d*, *J*=10 Hz, H-1")

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2.11. Statistical analyses

The values were reported as mean ± SD by SPSS and Excel 2010.

3. Results

3.1. DPPH radical scavenging activity

Free radical scavenging effects of total extract and different fractions from *Arum rupicola* leaves were assessed with 2, 2-diphenyl-1-picryl-hydrazyl (DPPH). IC_{50} values were displayed in Table 1.

3.2. FRAP assay

Results of antioxidant effects of different fractions of the leaves extract using FRAP, are reported based on mmol Fe II/ 100 g of extract or fraction. Ferric reducing antioxidant power of the extracts calculated using the calibration curve and regression equation of ferrous sulfate (R^2 = 0.996, y = 0.0008x-0.015). According to table 1, it was presented that antioxidant property of 100 grams of hexane, chloroform, ethyl acetate, and water residue as well as total methanol extract, had antioxidant activity equivalent to 140.17 ± 4.03, 140.02 ± 4.37, 110.01 ± 3.9, 108.64 ± 4.07 and 163.62 milimol $FeSO_4$, respectively.

3.3. Total phenol content

Total phenol content (µmol of GAE/g of sample) as represented in table 2 varied from 116.55 to 135.00 µmol of GAE/g sample using the standard curve of gallic acid (R^2 = 0.9919, y = 0.0067x - 0.0194).

Table 1. DPPH radical scavenging activity (IC_{50} values)**,** total phenolic content (TPC) and antioxidant power (mmol Fe/100 g sample) of different fractions from *Arum rupicola* leaves.

Sample	IC_{50} (µg/ml)[a]	TPC (µmol Gallic acid/g sample)	Antioxidant power (mmol Fe/100 g sample)
Hexane fraction	481.5 ± 2.26[a]	120.6 ± 12.04	140.17 ± 4.03
Chloroform fraction	637.8 ± 11.3	133.2 ± 13.5	140.02 ± 4.37
Ethyl acetate fraction	441.2 ± 4.77	122.4 ± 12.38	110.01 ± 3.9
Water residue	186.7 ± 5.8	116.5 ± 9.7	108.64 ± 4.07
Total methanol extract	467.3 ± 2.19	135 ± 11.8	163.62 ± 4.42
BHA	7.9 ± 0.06	-	
Vitamin A	14.2 ± 1.23	-	

[a] IC_{50} values represent an average of three independent experiments (mean ± SD); BHA: Butylated hydroxyanisole.

3.4. In vitro cytotoxic assay

The cytotoxicity analysis of total methanolic extract, hexane, chloroform, ethyl acetate and water residue in breast cancer cell lines including MCF-7, MDA-MB, and T47D cell line was performed and the results were shown in Table 2.

Results showed that in MCF-7 cell line, hexane, chloroform fractions and total methanol extract showed cytotoxicity with IC_{50} equal to 118.9 ± 0.38, 258.5 ± 0.25, 392.7 ± 0.25 µg/ml, respectively. In this cell line, etoposide showed cytotoxicity with IC_{50} of 18.53 ± 0.24 µg/ml.

In the MDA-MB-231 cell line, hexane, chloroform fractions and total methanol extract have shown cytotoxicity with IC_{50} of 137.2 ± 0.22, 239.8 ± 0.12, 357.3 ± 0.11 µg/ml, respectively. Etoposide also showed cytotoxicity with IC_{50} of 19.9 ± 0.006 µg/ml.

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In the T47D cell line, the fractions of chloroform, hexane, and total methanol extract as well as water residue showed cytotoxicity with IC_{50} of 113.8 ± 1.25, 122.8 ± 0.2, 249.08 ± 0.14, 399.3 ± 0.6 µg/ml), respectively. In this cell line, etoposide showed cytotoxicity with IC_{50} of 23.3 ± 0.096 µg/ml.

3.5. Isolation of isoflavonoid compounds

Isolated compounds **A, B,** and **C** from the methanolic fraction of the total methanolic extract of leaves of *Arum rupicola* were identified by comparison of their NMR (^{1}H- & ^{13}C-NMR) with those reported in the literatures [17-19]. Compounds **A, B** and **C** (Fig. 1) were identified as Orobol, Genistein and Genistein 8-C-glucoside, respectively.

Table 2. In vitro cytotoxic activity (IC_{50}, µg/ml) of total extract and fractions of *Arum rupicola* leaves against cancer and normal cell lines

Sample	IC_{50} (µg/ml)[a] MCF-7	IC_{50} (µg/ml) MDA-MB-231	IC_{50} (µg/ml) T-47D
Total methanol extract	392.7 ± 0.25	357.3 ± 0.11	249.08 ± 0.14
Hexane fraction	118.9 ± 0.38	137.2 ± 0.22	122.8 ± 0.2
Chloroform fraction	258.5 ± 0.25	239.8 ± 0.12	113.8 ± 1.25
Ethyl acetate fraction	> 500	> 500	> 500
Water residue	> 500	> 500	399.3± 0.6
Etoposide	18.53 ± 0.24	19.9 ± 0.006	23.3± 0.096

[a] IC_{50} values represent an average of three independent experiments (mean ± SD).

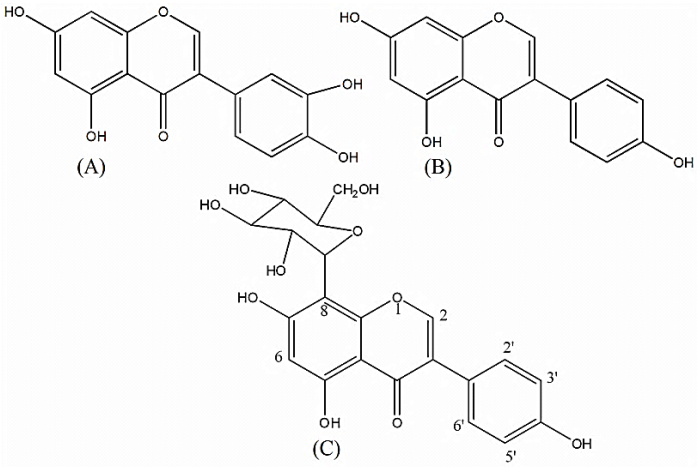

Fig. 1. Chemical structure of isoflavonos isolated from Arum rupicola leaves. A: Orobol, B: Genistein, and C: Genistein 8-C-glucoside

4. Discussion

Various flavonoids and phenolic compounds have been identified and reported in different species of *Arum* [20, 21], but no phytochemical studies have been performed on *A. rupicola*. In this study, antioxidant effects, cytotoxicity as well as purification and identification of secondary metabolites of *A. rupicola* leaves were investigated using different chromatographic and spectroscopic methods.

According to the results, methanolic fraction showed higher radical scavenging effect and the total methanol extract of the plant exhibited higher antioxidant power and total phenol content than other fractions. In a study, antioxidant and antitumor activities of the

ethanol extracts from different parts of six plants growing in Palestine, including *A. palaestinum* were evaluated and concluded that the studied plants showed different antioxidant abilities which were strongly associated with their phenolic contents [22].

The results of cytotoxicity study of plant samples on breast cancer cell lines showed that hexane fraction due to more toxicity in breast cancer cell lines could be considered for future studies. One study reported *in vitro* cytotoxic activity of different fractions and four flavonoid compounds isolated (luteolin, chrysoeriol, isoorientin, isovitexin) from the diethyl ether and ethyl acetate fraction of *Arum palaestinum* extract were investigated against four human carcinoma cell lines, epidermal carcinoma of larynx (Hep2), cervix (HeLa), liver (HepG2) and breast (MCF-7). Results showed that the fractionated extract and the isolated compounds showed significant antitumor activity against the four cell lines [1].

Phytochemical study of water residue of *A. rupicola*, using chromatographic methods, led to the isolation and identification of three isoflavonoid compounds, Orobol, Genistein and Genistein 8-C-glucoside. This is the first report on the occurrence of isoflavonoids in the genus *Arum*. Isoflavones, coumestans, stilbenes, and lignans are important subclasses of phytoestrogens. Isoflavones, which are more studied than other phytoestrogens, have estrogen-like properties in mammals and are found in large quantities in soybean and its products [23, 24]. Isoflavonoids have also been reported in several plant families in addition to the leguminous family and attracted the attention of many specialists from phytochemistry and plant physiology to medicine and nutrition [25]. Several beneficial effects of isoflavones have been reported, such as cardioprotection, osteoporosis prevention and antioxidant effects, which may be related to their phytoestrogenic effects [26]. Orobol is an isoflavone that is in small amount in soybean and is structurally similar to genistein, a largest isoflavone in soybean. Orobiol and genistein and their derivatives are known as multifunctional isoflavones with biological activities like neuroprotective, anti-obesity and anti-cancer [27, 28].

The presence of these isoflavones in *A. rupicola* can be introduced it as a good source of phytoestrogens and antioxidant.

5. Conclusion

According to the results of this study, it can be concluded that the stronger antioxidant effect of water residue of *Arum rupicola* could be due to the presence of three isoflavonoid compounds called orobol, genistein and genistein 8-C-glucoside. Considering the biological effects of isoflavons, which are a class of phytoestrogens, it is recommended to study the effects of this plant on diseases related to estrogen deficiency and also to expand its use as a food source containing phytoestrogens.

Author contributions

Substantial contributions to design, analysis and interpretation of data: Z. T., M. V., M. E., and SN. SL; investigation: Y. S., A. T., S. T., and R. A.; drafting the article or revising: Y. S. and SN. SL.; final approval of the version to be published: Z. T. and MR. SA.

Conflict of interest

We declare that there is no conflict of interest.

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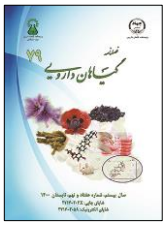

مقاله تحقیقاتی

شناسایی ایزوفلاونوییدهای فراکسیون موثر آنتی‌اکسیدانی برگ‌های گونه‌ای شیپوری (*Arum rupicola*)

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اطلاعات مقاله

گل‌واژگان:
شیپوری
آنتی‌اکسیدان
اثر سمیت سلولی
جنیستئین
ایزوفلاونویید

چکیده

مقدمه: گیاه *Arum rupicola* Boiss. (خانواده گل شیپوری) گونه‌ای شیپوری است که توسط مردم بومی مناطق جنوبی ایران در آشی به نام "آش کاره" استفاده می‌شود. تاکنون ترکیبات فنلی متعددی از گونه‌های شیپوری شناسایی شده است. **هدف:** هدف از این مطالعه ارزیابی اثر آنتی‌اکسیدانی و تعیین محتوای تام فنلی و بررسی فعالیت سمیت سلولی عصاره برگ *A. rupicola* می‌باشد. **روش بررسی:** فعالیت آنتی‌اکسیدانی عصاره تام متانولی و فراکسیون‌های هگزانی، کلروفرمی، اتیل استاتی و باقیمانده آبی به روش FRAP و DPPH ارزیابی شد. محتوای تام فنلی با استفاده از روش فولین سیوکالتئو مورد سنجش قرار گرفت. اثر سمیت سلولی عصاره بر رده‌های سلولی سرطان پستان انسان MCF-7 و MDA-MB-231 و رده سلولی T47D با روش MTT مورد بررسی قرار گرفت. جداسازی ترکیبات فیتوشیمیایی باقیمانده آبی با استفاده از کروماتوگرافی ستونی انجام شد. **نتایج:** بر اساس نتایج مشاهده شده، باقیمانده آبی کمترین مقدار IC_{50} برابر با ۱۸۶/۷ میکروگرم در میلی‌لیتر را در تست DPPH نشان داد و عصاره متانولی تام نیز بیشترین قدرت آنتی‌اکسیدانی برابر با ۱۶۳/۶۲ میلی‌مول سولفات آهن در صد گرم عصاره و محتوای تام فنلی برابر با ۱۳۵ میکرومول گالیک اسید در گرم عصاره را نشان داد. فراکسیون هگزانی نیز بیشترین اثر سیتوتوکسیک را بر رده سلولی سرطان پستان MCF-7 (IC_{50} برابر با ۱۱۸/۹ میکروگرم در میلی‌لیتر) نشان داد. بررسی فیتوشیمیایی باقیمانده آبی منجر به جداسازی و شناسایی سه ایزوفلاونوئید با نام‌های اوربول، جنیستئین و جنیستئین-۸- سی گلوکوزید شد. **نتیجه‌گیری:** بر اساس شناسایی ترکیبات ایزوفلاونوییدی در این گیاه، می‌توان قابلیت مصرف آن را به عنوان مکمل فیتواستروژنی در مطالعات آینده در نظر گرفت.

مخففها: FRAP، قدرت احیاکنندگی آهن پلاسما؛ DPPH، ۲،۲- دی فنیل -۱- پیکریل هیدرازیل؛ MTT، ۳-(۵،۴- دی متیل تیازول-۲- ایل)-۵،۲- دی فنیل تترازولیوم بروماید

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