RESEARCH ARTICLE

Isolation and Identification of two Cucurbitacins B and E, and Detection of Phytosterols in *Cucurbita pepo* L. var. pepo (Pumpkin) Leaves Extract

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ABSTRACT

Cucurbita pepo (pumpkin), a Cucurbitaceae membered plant, is considered one of the oldest cultivated plants; it has been cultivated about 7,000 to 5,500 BC. Traditionally, *C. pepo* is cultivated from very close to sea level in semi-dry climates, to others, which are cultivated at altitudes greater than 2,000 meters. Ethnopharmacological studies show that *C. pepo* is used in many countries for treating several diseases, e.g., as an anti-inflammatory, analgesic, urinary disorders, anti-ulcer, anti-diabetic, and anti-oxidant.

C. pepo leaves extracted with 90% methanol by maceration with continuous shaking at room temperature for three days. Thin-layer chromatography (TLC), (analytical and preparative) high-performance liquid chromatography, and liquid mass chromatography used for isolation and identification of two Cucurbitacins from *C. pepo* (pumpkin) leaves methanolic extract and detection of phytosterols. Cucurbitacins are triterpenes based structure isolated from many members of Cucurbitaceae families and other plants. Cucurbitacins exhibit polar properties, so they are isolated from plant extracts, which are extracted with methanol. Cucurbitacins exhibit anti-cancer activity, anti-atherosclerotic activity, and anti-arthritis activity, so Cucurbitacins may be an important lead molecule for future new medicinal preparation.

Keywords: *Cucurbita pepo*, Cucurbitacins, High-performance liquid chromatography, Liquid mass chromatography.

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INTRODUCTION

Cucurbitaceae, the gourd family of flowering plants, belonging to the order Cucurbitales containing 98 genera and about 975 species of food and ornamental plants. Members of this family are annual or perennial herbs native to temperate and tropical areas.

C. pepo (pumpkin), a Cucurbitaceae membered plant, is considered one of the oldest cultivated plants.[1] It has been cultivated from about 7,000 to 5,500 BC.[2] *C. pepo* cultivated from very close to sea level and in semi-dry climates, to others, which are cultivated at altitudes greater than 2,000 meters. *C. pepo* has creeping plants, which are compacted or semi-shrubby, annual, broadly ovate-cordite to triangular-cordite leaves, 20 to 30 × 20 to 35 cm, with or without white spots, often with three to five deep lobules, and with denticulate to indented-denticulate margins. The fruit is very variable in size and shape, smooth to heavily ebbed, with a rigid skin varying in color from light to deep orange.[3]

Ethnopharmacological studies show that *C. pepo* is used in many countries for treating several diseases, e.g., as an anti-inflammatory, anti-viral, analgesic, urinary disorders, anti-ulcer, anti-diabetic, and anti-oxidant.[4,5] Traditional medicine has used different parts of the plant, including the flesh of the fruits and seeds,[6] reporting that pumpkin exhibits important physiological properties, as wound healing, tumor growth inhibition, hypoglycemic effects, and immunomodulation.[7]

Pumpkin is a rich source of phytochemical compounds, like beta-sitasterol,[8] stigma sterol, charantin4, kuguacin F4, vicine, kuguacin F4, momordicaursenol, isovitexin, Cucurbitacin A,[9] Cucurbitoside F, Cucurbitoside G, Cucurbitoside H, Cucurbitoside I, Cucurbitoside J, Cucurbitoside K, Cucurbitoside L, Cucurbitoside M,[10] (+)-lariciresinol, Cucurbitoside D, Cucurbitoside C, (-)-secoisolariciresinol, isolariciresinol, lariciresinol-4′-O-β-D-glucoside 3β-hydroxycholest-7-en-24-one,[11] lariciresinol-4-O-β-D-glucoside, Cucurbitacin L 2-O-beta-D-glucopyranoide, Cucurbitacin K 2-O-beta-D-glucopyranoide Cucurbitaglycosides B,[12] Cucurbitaglycosides A, (23, 24)-dihydrocucurbitacin F, Cucurbitacin B, and Cucurbitacin E.[13]

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Minerals, elemental components: Studies determine the biochemical composition of fluted pumpkin at different stages of growth. Analyses were carried out on stems, leaves, and seeds, respectively. Pumpkin is rich in zinc, phosphorus, potassium, calcium, magnesium, iron, and copper.[14] Cucurbitacins are tetracyclic terpenes with steroidal structures that are isolated from plants of the family Cucurbitaceae, such as, pumpkins, gourds, and cucumbers.[15] Most Cucurbitacins are soluble in petroleum ether, chloroform, benzene, ethyl acetate, methanol, and ethanol, but are insoluble in ether. They are only slightly soluble in water. Cucurbitacins exhibit polar properties, so they are isolated from plant extracts, which are extracted with methanol.[16] Cucurbitacins had anti-cancer activity: Cucurbitacin actions involve growth inhibition, the arrest of the cell cycle at the g2/M phase, and induction of apoptosis in cancer cells.[17] Cucurbitacin E (Figure 1) inhibited tumor angiogenesis by inhibiting JAK-STAT3 and mitogen-activated protein kinases (MAPK) signaling pathways.[18] Cucurbitacin B (Figure 2) with docetaxel may augment the chemotherapeutic effects by suppressing STAT3 in patients with laryngeal cancer.[19] Cucurbitacin B and E in the glycosidic form to exhibit an inhibitory effect on lipid oxidation products, like malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE).[20,21] Anti-arthritis activity: Cucurbitacins B, E have been reported to inhibit cyclooxygenase (COX)-2 enzymes with no effect on COX-1 enzymes.[21] The recent studies showed that both cucurbitacin B & E have anti-atherosclerotic activity.[20]

Beta-sitosterol one of the most prevalent vegetable-derived phytosterols in the diet. β-sitosterol, has successfully lowered circulating cholesterol concentrations by decreases in LDL (low-density lipoprotein)cholesterol concentrations, Induces Apoptosis in Human Prostate Cancer Cells. β-sitosterol structure in (Figure 3).

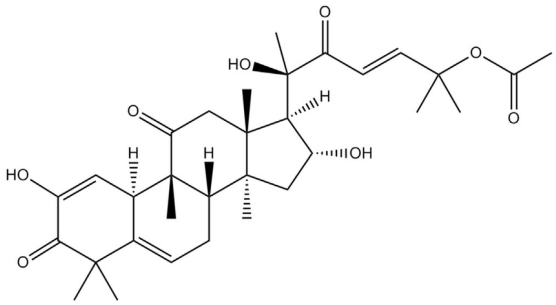


Figure 1: [(E,6R)-6-[(2S,8S,9R,10R,13R,14S,16R,17R)-2,16-dihydroxy-4,4,9,13,14-pentamethyl-3,11-dioxo-8,10,12,15,16,17-hexahydro-7H-cyclopenta[a]phenanthren-17-yl]-6-hydroxy-2-methyl-5-oxohept-3-en-2-yl] acetate; (Cucurbitacin E) structure.

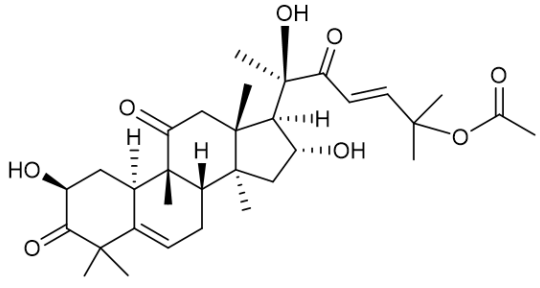


Figure 2: [(E,6R)-6-[(2S,8S,9R,10R,13R,14S,16R,17R)-2,16-dihydroxy-4,4,9,13,14-pentamethyl-3,11-dioxo-2,7,8,10,12,15,16,17-octahydro-1H-cyclopenta[a]phenanthren-17-yl]-6-hydroxy-2-methyl-5-oxohept-3-en-2-yl] acetate; (Cucurbitacin B) structure.

MATERIALS AND METHOD

Collection of Plant Materials

The pumpkin (*C. pepo*) plant is cultivated widely in Iraq. *C. pepo* is cultivated in Al-Anbakia farms near Dyala city, northeast of Baghdad, as Al-Anbakia has very good agricultural farms due to good soil, with respect to the temperature and annual course of temperature, rainfall, day length, sun's characteristics, and altitude. *C. pepo* leaves had been collected before the flower blooming; the green leaves had dried in shadow, away from light, and at room temperature. The plant was authenticated by Assistant Professor Dr. Khansaa R. Al-Joboury, Iraqi Natural History Museum Herbarium.

Extraction

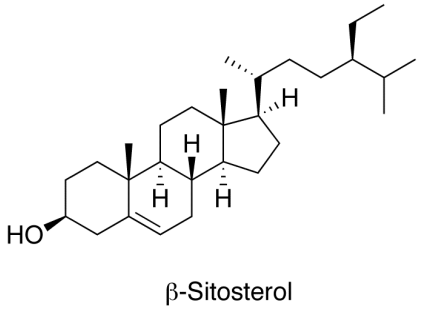
One-kilogram of pulverized plant leaves, extracted with 4.5 liters of 90% methanol by maceration, with continuous shaking, at room temperature. After 3 days, the extract was filtered off. The filtrate evaporated to dryness under vacuum, using a rotary evaporator. A dark greenish residue was obtained; the residue suspended in 500 mL deionized water and partitioned successively with hexane, chloroform, ethyl acetate, and n-butanol, respectively, three times of 500 mL for each one. The organic layer of the fractions was collected. The first three fractions (except n-butanol fraction) were dried over anhydrous sodium sulfate, filtered, and all fractions were evaporated to dryness.

Phytochemical Investigation of Triterpenes (Liebermann-Burchard's Test)

5 mL acetic anhydride and 5 mL of concentrated sulphuric acid were added carefully to 50 mL of absolute ethanol while cooling in ice. The reagent must be freshly prepared. Then, sprayed developed plate by Liebermann-Burchard's test, and warmed at 100ºC for 5 to 10 minutes; a brownish-black color indicates the presence of triterpenes, sterols.[20]

TLC Examination of Hexane Fraction

The TLC has been used to reach the best mobile phase to separate



β-Sitosterol

Figure 3: (3S,8S,9S,10R,13R,14S,17R)-17-[(2R,5R)-5-ethyl-6-methylheptan-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a] phenanthren-3-ol. (Beta-sitasterol) structure.

hexane fraction constituents; hexane:ethyl acetate (13:4) was found to be the best mobile phase.

Preparative High-Performance Liquid Chromatography (Preparative HPLC)

Preparative HPLC was carried out for isolation of present peaks of hexane fraction. Preparative HPLC conditions: HPLC system from Knauer, Germany. System components: binary high-pressure gradient pump, diode array detector, sample loop (200 µL), and injector and fraction collector. The separation was achieved on C18 (250 × 10) 5 µm particle size from Water Corporation, USA. Separation parameters: gradient of mobile phase A (0.05% trifluoroacetic acid in HPLC water) and mobile phase B (acetonitrile). Monitoring on 220 nm. Fraction collector parameters: peak recognition level 10 mAU (milli absorbance unit), slope 0.2 Au/min, fraction size: 5 mL.

Identification by LC/Mass/Mass (Liquid/mass/mass chromatography)

Sub fraction H5, H6, H7, H8, H9, and H10 subjected to LC mass at the following conditions:

Analytical LC/ mass/ mass (LC-MS) performed using a Sciex API 3200, column Zorbax-Agilent C18 150 × 4.5 mm ESI positive scan (+1); mass range 50 to 1,000 g/mol, 5 µm particles size from water corporation; mobile phase used gradient water and acetonitrile, where mobile phase A: water + 0.2% formic acid and 0.02% ammonia; mobile phase B: acetonitrile + 0.2% formic acid and 0.02% ammonia; at a flow rate of 0.7 mL/min.

Analytical High performance liquid chromatography (HPLC) for further identification

HPLC (LC-2010A HT) system from Shimadzu Japan system, UV-vis detector. The separation was achieved on ODS C18 (250 × 4.6) 5 µm particle size. Separation parameters: gradient of mobile phase A (0.01% K_2HPO_4 in HPLC grade water) and mobile phase B (acetonitrile), flow rate 1.2 mL/min, temperature 35ºC; monitoring on 220 nm.

RESULTS AND DISCUSSION

Fractionation of Extracts

For full phytochemical profile screening for a given plant, fractionation of the crude extract had been recommended so that the main class of the plant constituents will be isolated from each other according to the differences in the polarity and solubility before chromatographic analysis is performed since crude extract contains diverse classes of chemical constituents with various polarities.[20] Fractionation of crude extract is done with a series of solvents of increasing polarities (hexane, chloroform, ethyl acetate, and n-butanol).

Preliminary phytochemical examination of leaves extracts fractions revealed the presence of triterpenes in hexane fraction that is characterized by coloring with brownish-black color.

TLC Examination of Hexane Fraction

The TLC analysis revealed the presence of phytosterol and terpenes. The chromatogram is shown in Figure 6.

Isolation of Hexane Fraction Components by Preparative HPLC

Isolation of 11 sub-fractions resulted from hexane fraction chromatographed by preparative HPLC. The chromatogram is shown in Figure 7.

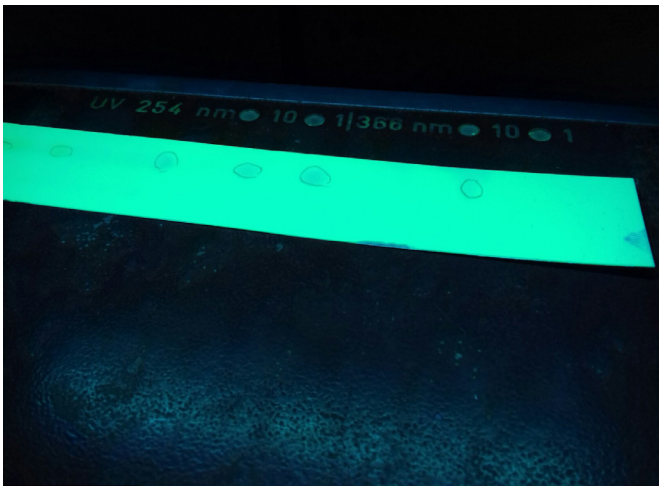


Figure 4: TLC plate of hexane fraction; mobile phase hexane:ethyl acetate (13:4); detection under UV light 254 nm

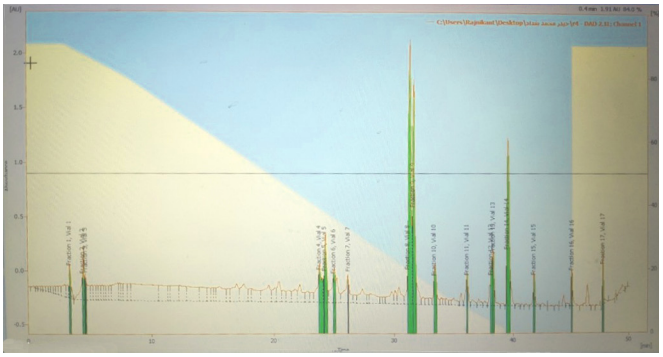


Figure 5: HPLC chromatogram of hexane fraction; mobile phase water:acetonitrile (gradient); detector: diode array detector

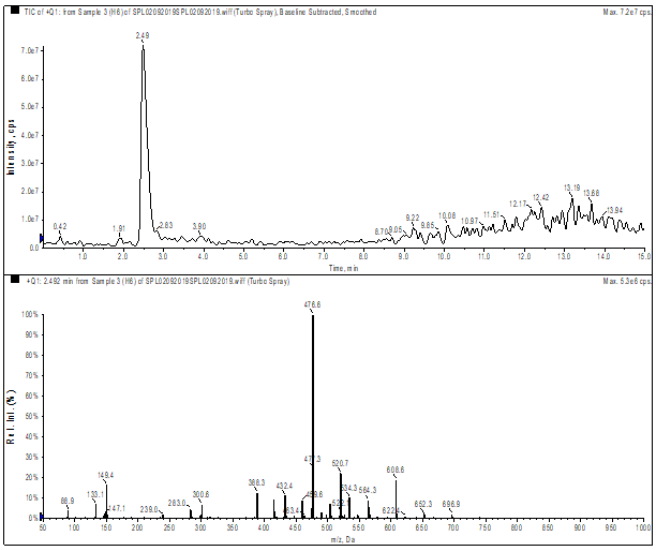


Figure 6: Liquid chromatography analysis and mass fragmentation of H6 sub-fraction by LC/mass/mass, mass fragmentation at 2.492 minutes

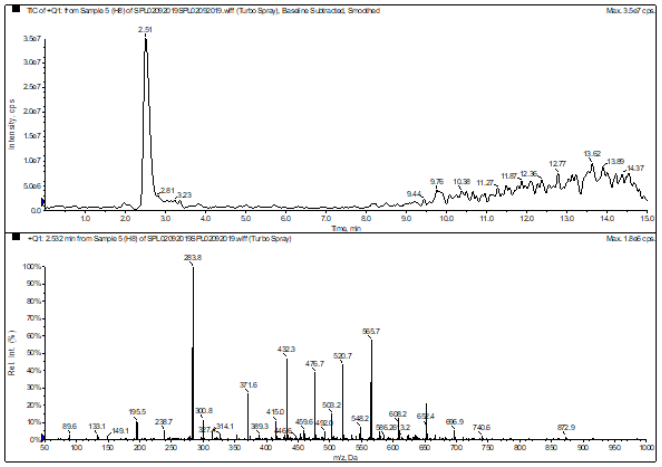


Figure 7: Liquid chromatography analysis and mass fragmentation of H8 sub-fraction by LC/mass/mass, mass fragmentation at 2.5392 minutes

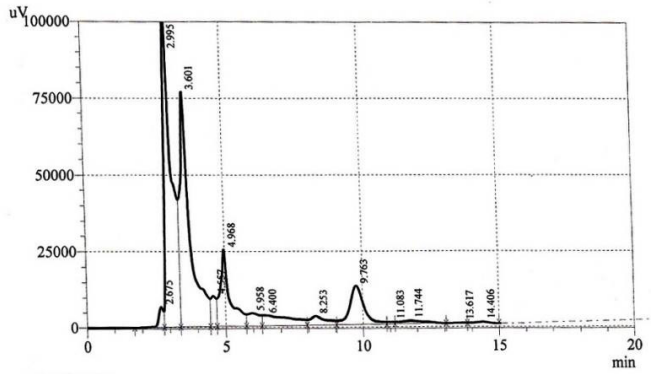


Figure 8: Analysis by HPLC of standards: Cucurbitacin B, Cucurbitacin E, beta-sitosterol, and Stigma sterol

Analysis of Sub-Fractions by Liquid/ mass/mass Chromatography (LC/MS/MS)

Analysis of the sub-fraction H6 by LC/mass/mass, mass fragmentation done at 2.492 minutes. The chromatogram is shown in Figure 3.

Analysis of sub-fraction H8 by LC/mass/mass, mass fragmentation done at 2.5392 minutes. The chromatogram is shown in Figure 4.

Result of Analytical High performance liquid chromatography (HPLC)

The four standards mentioned in Table 1 had been used for define the sub fractions H6, H8 & H9 the chromatogram of standards HPLC and their retention shown in Figure 8.

Result of H6 analytical HPLC, retention time at 3.632 minutes. The chromatogram is shown in Figure 9.

Result of H8 analytical HPLC, retention time at 3.001 minutes. The chromatogram is shown in Figure 10.

Result of H9 analytical HPLC, retention time at 4.952 minutes. The chromatogram is shown in Figure 11.

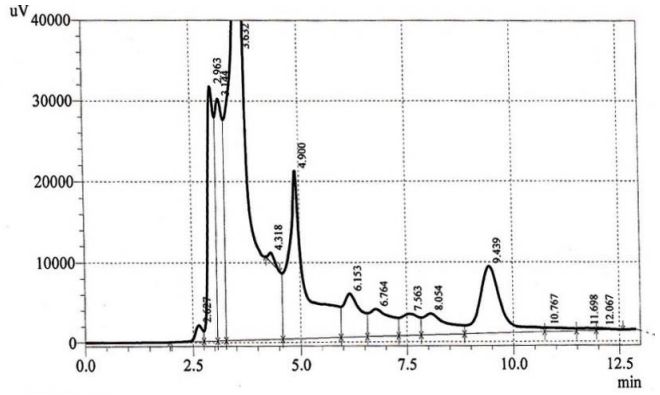


Figure 9: Analysis by HPLC of H6 sub-fraction and its retention time at 3.632 minutes

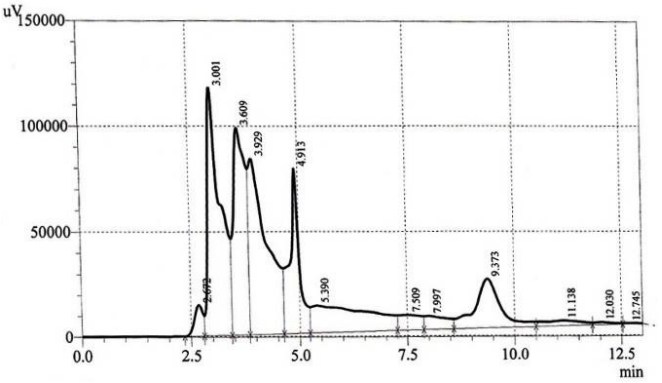


Figure 10: Analysis by HPLC of H8 sub-fraction and its retention time at 3.001 minutes

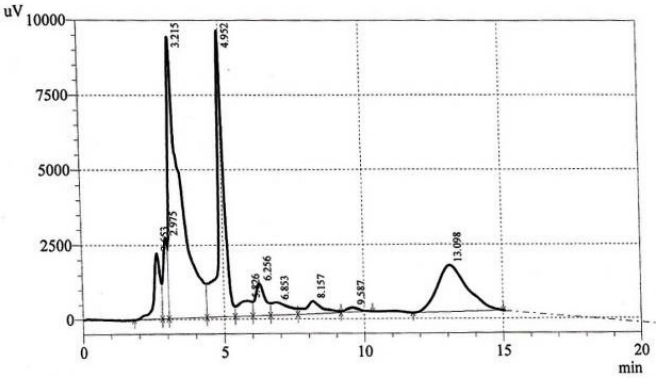


Figure 11: Analysis by HPLC of H9 sub-fraction and its retention time at 4.952 minutes

Table 1: HPLC standards analysis retention time

Peak number	*Standards name*	*Retention time (min)*
2	Cucurbitacin E	2.995
3	Cucurbitacin B	3.601
5	Beta-sitosterol	4.968
9	Stigma sterol	9.763

CONCLUSION

The results of the current study isolate and identify two cucurbitacins (cucurbitacin B and cucurbitacin E), in addition to the detection of beta-sitosterol in methanolic extract of *C. pepo* leaves, where LC mass/mass result is reasonable, and HPLC analysis showed matching between samples and standards.

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REFERENCES

1. Ratnam N, Naijibullah M, Ibrahim MD. A review on Cucurbita pepo. Int J Pharm Phytochem Res. 2017;9:1190–1194.
2. Hrabovski N, Sinadinović-Fišer S, Nikolovski B, Sovilj M, Borota O. Phytosterols in pumpkin seed oil extracted by organic solvents and supercritical CO2. Eur J Lipid Sci Technol. 2012;114(10):1204–1211.
3. Smith BD. The initial domestication of Cucurbita pepo in the Americas 10,000 years ago. Science (80-). 1997;276(5314): 932–934.
4. Wang XL, Liu J, Chen ZHB, Gao F, Liu JX, Wang XL. Preliminary study on pharmacologically effect of Curcurbita pepo cv Dayanggua. J Tradit Chin Vet Med. 2001;20:6–9.
5. Caili FU, Huan S, Quanhong LI. A review on pharmacological activities and utilization technologies of pumpkin. Plant foods Hum Nutr. 2006;61(2):70–77.
6. Chaturvedi P. Antidiabetic potentials of Momordica charantia: multiple mechanisms behind the effects. J Med Food. 2012;15(2):101–107.
7. Li W, Koike K, Tatsuzaki M, Koide A, Nikaido T. Cucurbitosides F-M, acylated phenolic glycosides from the seeds of Cucurbita pepo. J Nat Prod. 2005;
8. Kikuchi T, Ando H, Maekawa K, Arie H, Yamada T, Tanaka R. Two new ent-kaurane-type diterpene glycosides from zucchini (Cucurbita pepo L.) seeds. Fitoterapia. 2015;107:69–76.
9. Wang D-C, Pan H-Y, Deng X-M, Xiang H, Gao H-Y, Cai H, et al. Cucurbitane and hexanorcucurbitane glycosides from the fruits of Cucurbita pepo cv dayangua. J Asian Nat Prod Res. 2007;9(6):525–529.
10. Feng XS, Wang DC, Cai H, Deng XM, Liu YR. Determination of the cucurbitacins from Cucubita pepo cv dayangua by HPLC. Zhong yao cai= Zhongyaocai= J Chinese Med Mater. 2007;30(4):418–420.
11. Bardaa S, Halima N Ben, Aloui F, Mansour R Ben, Jabeur H, Bouaziz M, et al. Oil from pumpkin (Cucurbita pepo L.) seeds: evaluation of its functional properties on wound healing in rats. Lipids Health Dis. 2016;15(1):1–12.
12. Roux A, Lison D, Junot C, Heilier J-F. Applications of liquid chromatography coupled to mass spectrometry-based metabolomics in clinical chemistry and toxicology: A review. Clin Biochem. 2011;44(1):119–135.
13. Pitt JJ. Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. Clin Biochem Rev. 2009;30(1):19.
14. Kaushik U, Aeri V, Mir SR. Cucurbitacins–an insight into medicinal leads from nature. Pharmacogn Rev. 2015;9(17):12.
15. Dong Y, Lu B, Zhang X, Zhang J, Lai L, Li D, et al. Cucurbitacin E, a tetracyclic triterpenes compound from Chinese medicine, inhibits tumor angiogenesis through VEGFR2-mediated Jak2–STAT3 signaling pathway. Carcinogenesis. 2010;31(12):2097–2104.
16. Liu T, Zhang M, Zhang H, Sun C, Deng Y. Inhibitory effects of cucurbitacin B on laryngeal squamous cell carcinoma. Eur Arch Oto-rhino-laryngology. 2008;265(10):1225–1232.
17. Esterbauer H. Cytotoxicity and genotoxicity of lipid-oxidation products. Am J Clin Nutr. 1993;57(5):779S-786S.
18. Tannin-Spitz T, Bergman M, Grossman S. Cucurbitacin glucosides: Antioxidant and free-radical scavenging activities. Biochem Biophys Res Commun. 2007;364(1):181–186.
19. Jayaprakasam B, Seeram NP, Nair MG. Anticancer and antiinflammatory activities of cucurbitacins from Cucurbita andreana. Cancer Lett. 2003;189(1):11–16.
20. Wagner H, Bladt S. Plant drug analysis: a thin layer chromatography atlas. Springer Science & Business Media; 1996.
21. Gutierrez RMP. Review of Cucurbita pepo (pumpkin) its phytochemistry and pharmacology. Med Chem. 2016;6: 12–21.