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Original Research Article (Experimental)

# Determination of cucurbitacin E in some selected herbs of ayurvedic importance through RP-HPLC

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ABSTRACT

*Background:* The consumption of the fruits of cucurbitaceae plants is widely popular among Indians due to their various nutritional and medicinal purposes. Some of these plants are well reported in Ayurveda due to their potential therapeutic importance. In particular, the plants of this family are well-characterized by the presence of its bitter principle, Cucurbitacin E which differs within the species due to its genetic variations.

*Objectives:* The objective of the study was to develop a validated RP-HPLC method for standardization in some widely consumed cucurbits with cucurbitacin E as a marker compound.

*Materials and methods:* The RP-HPLC method was developed with a reverse phase $C_{18}$ column, using acetonitrile and water (1% glacial acetic acid) as mobile phase (70:30 v/v). The flow rate and $\lambda_{max}$ were optimized at 1 mL/min and 230 nm respectively. The HPLC method was validated in terms of accuracy, specificity, sensitivity, and repeatability as per ICH guideline.

*Results:* The calibration curve was found linear in the concentration range of 1–100 μg/mL. The % RSD of precision and recovery was found to be <2%, which confirms high repeatability of the method. The results indicated that the content of cucurbitacin E was highest (0.0663% w/w) in *Cucurbita pepo* whereas *Lagenaria siceraria* contains the lowest (0.0356% w/w).

*Conclusion:* The study was able to explore the variation of cucurbitacin E content in some selected food plants of Cucurbitaceae family. The applicability of the method can be established in nutraceutical industry for the effective quality control of cucurbits for safe human consumption.



## 1. Introduction

Cucurbitaceae is a large plant family, consisting of about 125 genera and 960 species. The various parts (fruit, seeds, stems, leaves) of the plants belonging to the cucurbitaceae family are very popular for their uses in culinary purposes from the ancient time. It is also used in Ayurvedic and folk medicine for their several therapeutic values due to the presence of a large number of metabolites (both primary and secondary). The importance of cucurbitaceae species has been highly recognized for effective control of lifestyle diseases such as diabetes, obesity and related disorders [1]. The cucurbits are a good source of glucose, fructose, essential amino acids, vitamins, water-soluble polysaccharides, dietary fibers, phenolic glycosides, flavonoids, terpenoids, and minerals etc. Apart from the diverse chemical constituents, this family is very well characterized by their presence of cucurbitacin. Cucurbitacin consists of tetracyclic cucurbitane nucleus skeleton with a variety of oxygenation functionalities at different positions with diverse chemical categories. The cucurbitacins are present as non-glycosylated or glycosylated triterpenoids and divided into twelve categories, incorporating cucurbitacins A-T [2]. Various biochemical studies suggested that cucurbitacins have a potential cytotoxic property which is responsible for making it a prominent lead for anti-cancer drug development [3]. The hydrophobic property of the cucurbitacin nucleus is a major regulating factor for their cytotoxic effects and it increases linearly with their hydrophobicity. In particular, cucurbitacin E (Fig. 1) and their glycosides are the most widely distributed chemical constituents in food plants of Cucurbitaceae family. Cucurbitacin E has been reported to possess anti-inflammatory [4], anti-angiogenic, immunomodulatory, cytotoxic [5], cytostatic and hepatoprotective [6] properties in both *in vitro*

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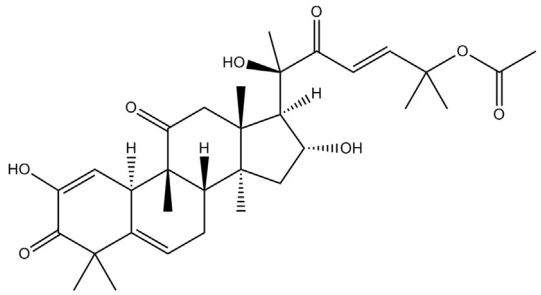


**Fig. 1.** General structure of Cucurbitacin E.

and *in vivo* model. It has been observed that the combination of cucurbitacin E with other synthetic anti-cancer drugs results in synergistic action in terms of cytotoxicity with greater efficacy in tumor growth inhibition [7]. Despite the potential therapeutic activity of Cucurbitacin E and cucurbitacin E glycoside, their chronic exposure is undesirable due to their extremely bitter and disagreeable taste as well as their toxicological effects found in experimental animals [8]. It has been presumed that back mutated fruits produce more toxicity and bitterness whereas the suppressor gene is responsible for the absence of cucurbitacins [9].

Although a large number of gourd family plants are grown and consumed, six species namely *Lagenaria siceraria, Benincasa hispida, Momordica charantia, Coccinia grandis, Cucurbita pepo,* and *Luffa acutangula* have potential nutraceutical benefits. The therapeutic benefits of these plants are also well documented in Ayurveda. *Lagenaria siceraia* (Bottle gourd) is known as Tumbini or Alabu in Ayurveda which is indicated in *Jwara* (fever), *Kasa* (cough), Svasa (respiratory distress), Visa Roga (poisoning), Sopha (inflammation/swelling), Vrana (Ulcers) *Sula* (colic pain) [10]. It is also reported as a diuretic, cardioprotective, antihyperlipidemic, antihyperglycemic, and antioxidant. The major bioactive constituents in the fruit consist of cucurbitacin B, D, E, phenolic compounds viz. phenolic glycosides, phenolic acid, flavonoids, flavon-C-glycoside such as isovitexin, isoorientin, saponarin sterols like fucosterol, campesterol etc [11,12]. In Ayurveda *B. hispida* (Wax gourd) is known as kusmanda, indicated in *Mutraghata* (Urethritis), *Prameha* (*Diabetes mellitus*), *Ashmari* (kidney stone), *Manasa Vikara* (psychological problems) [13]. It possesses several pharmacological properties including antioxidant, ACE inhibitory, anti-ulcer, anti-inflammatory, anti-obesity, anti-diarrheal activity. The presence of a large number of chemical constituents have been reported in this plant viz. lupeol, sitosterol, pentacyclic triterpenes, cucurbitacin B, E, triterpenoid (isomultiflorenol), trigonelline, β-sitosterol, alkaloids such as 5-methylcytosine, triterpenoids such as cucurbitacin

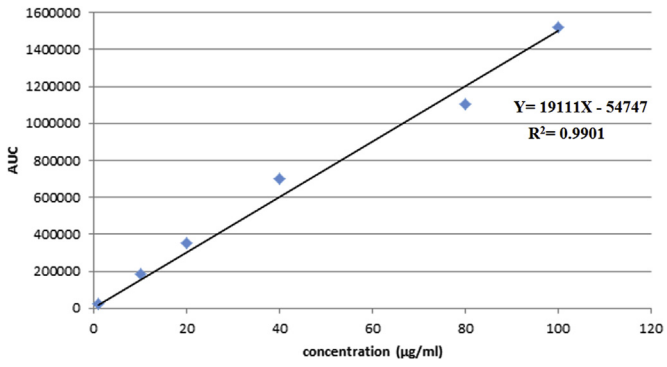


**Fig. 2.** Calibration curve of Cucurbitacin E.

**Table 1**
Cucurbitacin E content in cucurbits by RP-HPLC.

| Plant name | Voucher specimen no. | Common name | Cucurbitacin E content (%w/w) |
|---|---|---|---|
| *Lagenaria siceraria* | SNPS-1462/2016 | Bottle gourd | 0.0356 |
| *Benincasa hispida* | SNPS-1463/2016 | Wax gourd | 0.0446 |
| *Momordica charantia* | SNPS-1464/2016 | Bitter gourd | 0.0523 |
| *Coccinia grandis* | SNPS-1465/2016 | Ivy gourd | 0.0511 |
| *Cucurbita pepo* | SNPS-1466/2016 | Pumpkin | 0.0663 |
| *Luffa acutangula* | SNPS-1467/2016 | Ridge gourd | 0.0556 |

B, sterols, glycosides [14]. *M. charantia* (Bitter gourd) is known as karabellak in Ayurveda indicated in *Kasa* (cough), *Svasa* (Asthma), Jvara (fever), *Raktavikara* (blood disorder), *Kamala* (jaundice), *Krmiroga* (helminthiasis), *Kustha* (skin disorder) [15] (Anonymous 1999). It consists of a wide variety of chemical constituents including triterpene (cucurbitane type), protein (Polypeptide P), steroid (diosgenin), alkaloid (vicine), inorganic and phenolic acids, phenolic glycosides, flavonoids etc. [16]. In particular, *M. charantia* extract possesses potential hypocholesterolemic, antidiabetic, antiobesity, antimicrobial, lipid-lowering properties [1,17]. Another food plant, *Coccinia indica* (Ivy gourd) is also known as Bimbi in Ayurveda, indicated in *Kasa* (cough), *Svasa* (Asthma), *Jwara* (fever), *Raktavikara* (blood disorder), *Daha* (burning sensation) [18]. *C. grandis* is used in folklore medicine as antibacterial, hepatoprotective, hypoglycemic, hypolipidemic, antioxidant properties. The fruits of this plant contain Cucurbitacin B, E, taraxerone, taraxerol, β-carotene, carotenoids, β-sitosterol, Stigma-7-en-3-one etc. as active constituents [19]. *C. pepo* is also mentioned as a variety of Kushmandu in Ayurveda and widely used in the treatment of mental disorder, epilepsy, urinary disorders, diabetes etc. [20]. It contains a large number of chemical constituents including cucurbitacin B, cucurbitacin E, dihydrocucurbitacin, acylated phenolic glycosides (cucurbitosides), spinasterol, β-sitosterol, palmitic, palmitoleic, stearic, oleic, linoleic acids etc. [21]. In Ayurveda, *Luffa acutangula* is known as Kosataki, indicated in *Kustha* (skin disorder), *Pandu* (jaundice), *Pliharoga* (Splenic disease), *Sopha* (inflammation) [22]. It has also been reported to possess several pharmacological properties like diuretic, hepatoprotective, antidiabetic etc. The fruits of *L.acutangula* contain cucurbitacin B, E as bitter principles. The plant contains a significant amount of polyphenols (mostly phenolic acids *viz.* gallic acid, p-coumaric acid, ferulic acid, protocatechuic acid, and its glycosides, flavonoids (catechin, quercetin) [23,24].

With this background, the present study was aimed to develop a validated RP-HPLC method for standardization of the selected fruits of cucurbitaceae family by using cucurbitacin E as a marker compound. The validation of RP-HPLC method was further carried out based on the ICH guidelines. This validated method can be applied for quantitative estimation of cucurbitacin E in the cucurbitaceae food plants and their related preparations.

**Table 2**
Accuracy study.

| Excess CuE added (ng) | Expected CuE in extract (ng) | Average CuE found (ng) | Average Recovery (%) | RSD (%) |
|---|---|---|---|---|
| 0 | 66.3 | 63.21 | 95.35 | 1.25 |
| 10 | 77.3 | 74.20 | 95.99 | 0.98 |
| 40 | 107.3 | 103.8 | 96.82 | 1.41 |
| 80 | 147.3 | 143.2 | 97.23 | 1.05 |

**Table 3**
Intra-day and inter-day precision study.

| Intra-day (n = 6) | | | | Inter-day (n = 6) | | | |
|---|---|---|---|---|---|---|---|
| RT (min) | | Response (AU) | | RT (min) | | Response (AU) | |
| Mean | % RSD | Mean | % RSD | Mean | % RSD | Mean | % RSD |
| 4.70 | 0.87 | 4,753,208 | 1.20 | 4.68 | 1.50 | 4,593,228 | 1.28 |
| 4.65 | 1.47 | 7,612,069 | 1.30 | 4.55 | 1.17 | 7,292,664 | 1.81 |
| 4.69 | 1.46 | 16,198,361 | 1.25 | 4.70 | 1.10 | 18,105,372 | 1.50 |

## 2. Experimental

### 2.1. Instrumentation and reagents

The RP-HPLC system (Waters, Milford, MA, USA) consisted of a 600 controller pump, a multiple-wavelength ultraviolet-visible (UV-Vis) detector equipped with an in-line degasser AF 2489 and a rheodyne 7725i injector having 20 μL loop volume. Membrane filters (0.45 μm pore size) (Millipore) were used for filtration of the mobile phase. Quantitative estimation was performed with Empower 2 software programs using the external standard calibration method. Acetonitrile (HPLC grade) and glacial acetic acid (HPLC grade) were procured from Merck (Mumbai, India). All the other solvents (AR grade) procured from Merck. Cucurbitacin E (purity ≥ 95% HPLC) was purchased from Chromadex Inc. USA. All aqueous solutions were prepared using purified water (resistivity of 18.2 MΩ cm at 25 °C) from a Mili-Q filtration system.

### 2.2. Extraction of plant material

The mature fruits of *L. siceraria, B. hispida, M. charantia, C. grandis, C. pepo*, and *L. acutangula* were collected from local market of West Bengal, India. They were authenticated and the voucher specimen of all of them has been retained in the School of Natural Product Studies, Jadavpur University, Kolkata, India vide voucher specimen numbers SNPS-1462/2016- SNPS-1467/2016 for future references. The juice was squeezed from the fruits and then filtered through Whatman no. 1 filter paper. The aqueous extract was lyophilized and stored at −20 °C for further use. The % yield of the extracts was calculated.

### 2.3. RP-HPLC conditions

The chromatographic method was developed based on the previous method with some modification [25]. The RP-HPLC method was refined by changing the mobile phase composition in a gradient manner and finally, isocratic method was optimized with the mobile phase of acetonitrile (solvent A) and water (solvent B) in the ratio of 70: 30 (v/v). The pH of the solvent B was adjusted at 3.8 by using 1% (v/v) glacial acetic acid. The mobile phase was filtered through a 0.45 μm pore size (Millipore) membrane filter followed by sonication to degas the solvent. The separation was carried out on a Waters Spherisorb 5 mm ODS2 column ($C_{18}$, 250" × 4.6", 5 μm particle size). The temperature of the column was kept at 25 °C and the injection volume was 20 μL. The total run time was set at 10 min. The flow rate was set at 1.0 mL/min and the $\lambda_{max}$ was set at 230 nm for maximum absorption of the compound. A baseline was recorded with the optimized chromatographic method for about 15 min prior to standard and sample injection. Each chromatographic analysis was followed by a blank run to wash out any carryover from the previous analysis.

### 2.4. Preparation of standard and sample solutions

A standard stock solution of Cucurbitacin E was prepared by dissolving approximately 1 mg of cucurbitacin E in 1 mL methanol. Further dilution was carried out to prepare calibration samples in the concentration range of 1–100 μg/mL. The sample solutions were prepared by taking 10 mg of extract in 1 mL methanol. The solution was filtered through 0.45 μL syringe filter prior to injection.

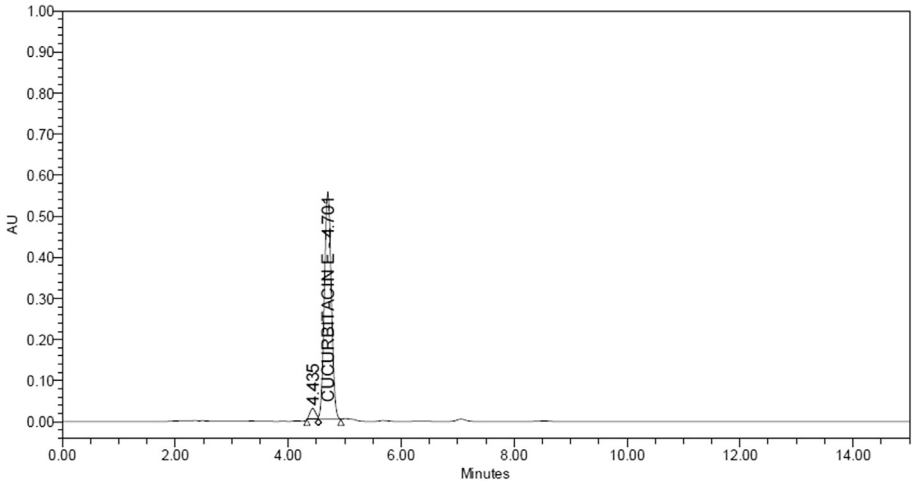


**Fig. 3.** RP-HPLC/UV chromatogram of Cucurbitacin E standard.

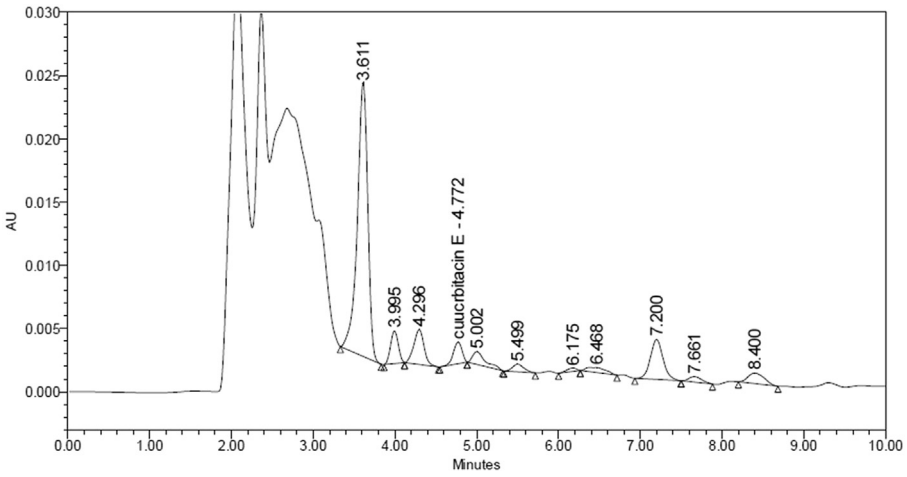


**Fig. 4.** RP-HPLC/UV chromatogram of *Lagenaria Siceraria* lyophilized extract.

### 2.5. Method validation

The RP-HPLC method validation was carried out by determining linearity, specificity, accuracy and precision, limit of quantification and limit of detection on the basis of International Conference on Harmonization guidelines [26]. Method specificity was determined by comparing the retention time of both standard and test samples. Sensitivity was evaluated by determining the Limit of Detection (LOD) and Limit of Quantification (LOQ) and calculated based on the equation: LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ is the standard deviation and S is the slope of the calibration curve. The standard deviation (σ) was calculated by measuring the deviations of the background response of an appropriate number of blank samples (n = 6). The accuracy of the method was determined by the standard addition technique and expressed in terms of % RSD for the mean recovery of the theoretical concentration. The samples were spiked with three different amounts of standard compounds in triplicate. For estimation of spike recovery, *C. pepo* extract was considered as it contains highest amount of cucurbitacin E. The precision of the method was assessed by injecting six replicates at three different concentrations, LQC (low-quality control), MQC (medium quality control) and HQC (high-quality control) for both standard and extract solutions to determine the repeatability of the method. The intra-day precision of the assay was determined by analyzing three concentrations in a day whereas the inter-day precision was carried over three successive days by analyzing the same concentrations. The robustness of the proposed method was carried out by varying different experimental conditions *viz.* flow rate, mobile phase composition, detection wavelength, column temperature and columns of the same configuration to check their influences on the retention time. Values were represented as % RSD in both cases. System suitability test was performed by using six replicates of test concentrations. A variation in the number of theoretical plates, capacity factor, and tailing factor was also calculated. Statistical analysis was performed using the Graph Pad Prism Version 5.0. The data has been represented as the mean ± % RSD.

## 3. Results

### 3.1. Extraction yield

The extracts were weighed and the percentage yields were calculated. The percentage yield (%) the aqueous extracts were found to be 5.21, 4.08, 7.25, 5.88, 3.83, 4.2% (w/w) for *L. siceraria, B. hispida, M. charantia, C. grandis, C. pepo* and *L. acutangula* respectively. The % yield was found the maximum for *M. charantia* whereas *C. pepo* was found to be lowest.

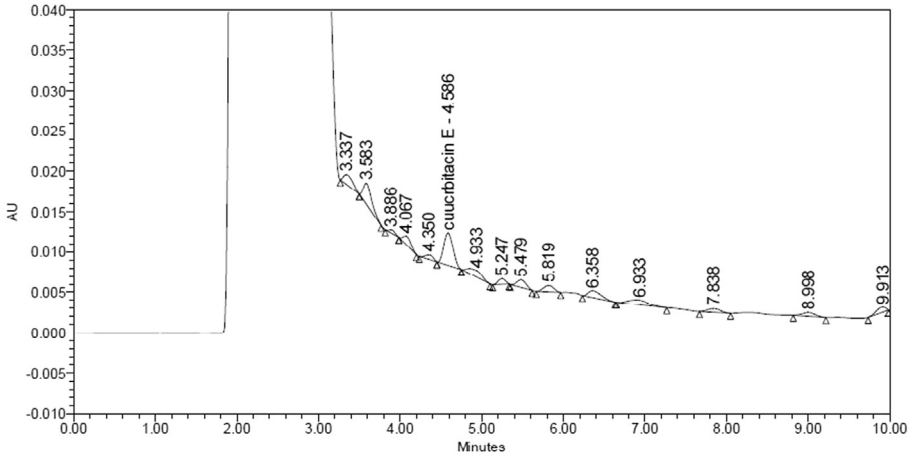


**Fig. 5.** RP-HPLC/UV chromatogram of *Benincasa hispida* lyophilized extract.

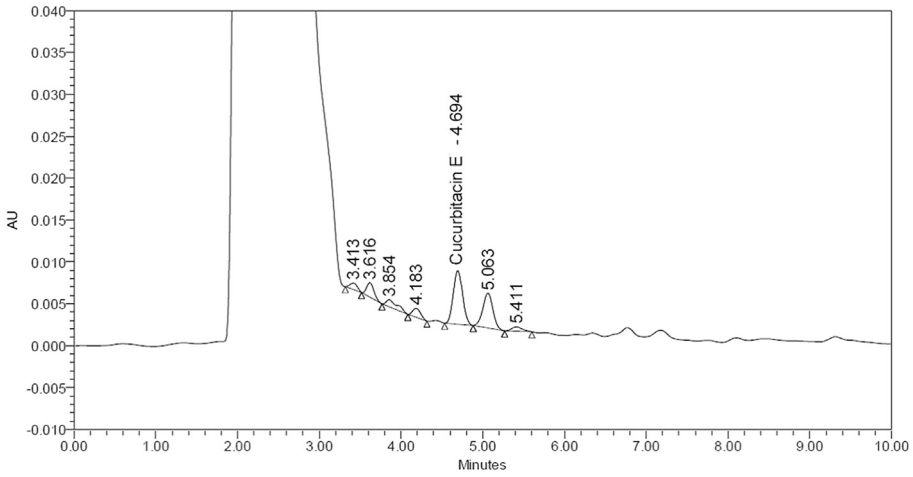


**Fig. 6.** RP-HPLC/UV chromatogram of *Momordica charantia* lyophilized extract.

### 3.2. Method validation results

In RP-HPLC, the linearity range of the response was found to be 1–100 μg/mL. The correlation coefficient was found from the calibration curve as > 0.99, which confirms that the data is closer to the line of best fit. The regression equation was found to be Y = 19111X-54747 (Fig. 2). The specificity of the proposed method confirmed no interference among the peak of standard and test samples. The limits of detection (LOD) and limit of quantification (LOQ) were estimated to be 3.45 and 8.82 μg/mL respectively, which reflect the high sensitivity of the method. The % recovery value (95.35–97.23%) indicated the good accuracy of the method (Table 2). The % RSD of intra-day and inter-day precision was reported to be <2% for in cases of both peak area (response) and retention time, which confirms high repeatability of the method (Table 3). The robustness of the experimental method was found to be in the range <2%. The number of theoretical plates, capacity factor and tailing factor were found to be 4092 (desirable > 2000), 6.72 (desirable 2–10), 1.35 (desirable < 1.5), respectively, from the mean of six determinations of test concentration.

### 3.3. Estimation of cucurbitacin E by RP-HPLC

The content of cucurbitacin E in the lyophilized extract was determined using the calibration curve by plotting the mean peak area (y-axis) against the concentrations (x-axis). The study confirmed that *C. pepo* contains the highest amount of cucurbitacin E (0.0663% w/w) whereas the lowest amount of was reported in *L. siceraria* as 0.0356% (w/w). The content of cucurbitacin E in the other species varied within this range. The content of cucurbitacin E was presented in Table 1. The chromatogram of standard cucurbitacin E has been shown in Fig. 3. RP-HPLC chromatograms of the six species have been shown as *L. siceraria* (Fig. 4), *B. hispida* (Fig. 5), *M. charantia* (Fig. 6), *C. grandis* (Fig. 7), *C. pepo* (Fig. 8) and *L. acutangula* (Fig. 9).

## 4. Discussion

The aqueous extract of Cucurbitaceae fruits is widely used by practitioners of Ayurveda in India and also in other systems of Indian medicine. The juice and powder of the fruits are widely

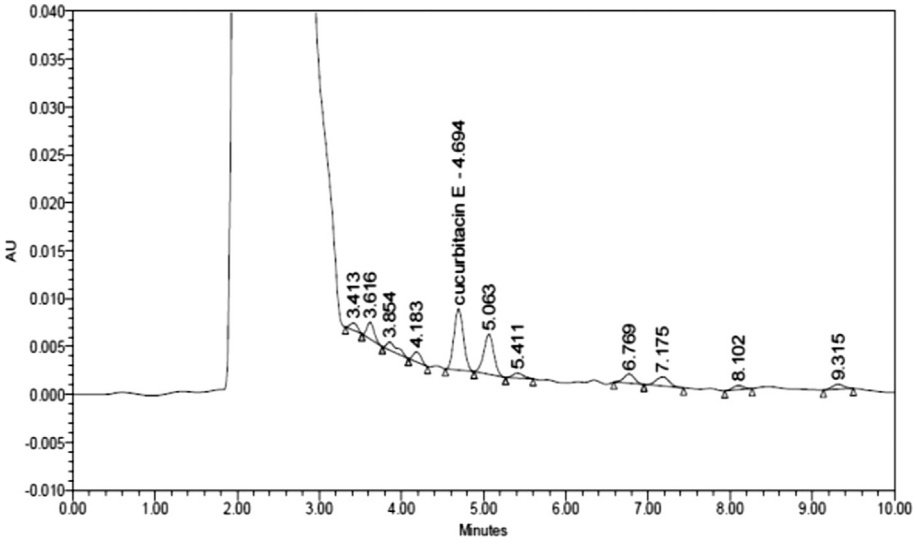


**Fig. 7.** RP-HPLC/UV chromatogram of *Coccinia grandis* lyophilized extract.

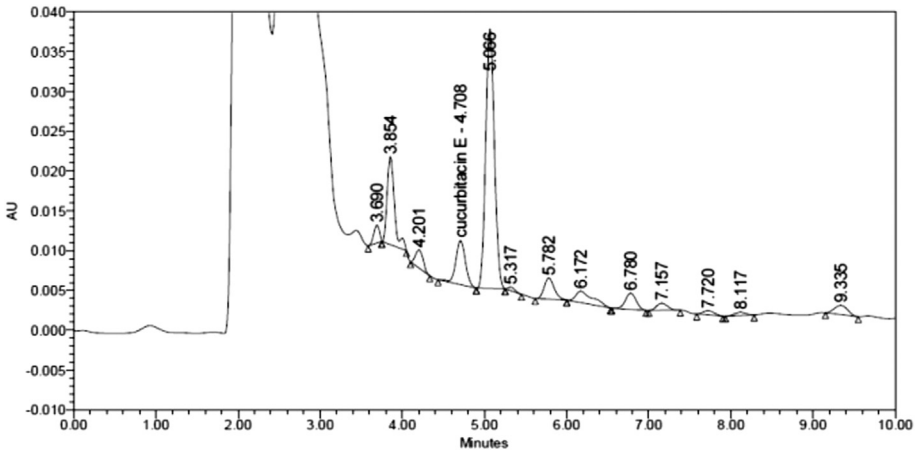


**Fig. 8.** RP-HPLC/UV chromatogram of *Cucurbita pepo* lyophilized extract.

marketed as a dietary supplement. In India, the fresh juice of *L. siceraria* and *M. charantia* are consumed for their anti-obesity and anti-diabetic properties [27,28]. Although cucurbitacin class of compounds (specifically Cucurbitacin D & E) possesses immense pharmacological potential *viz.* antitumor, hepatoprotective, anti-inflammatory etc. [29] (Miro, 2015), their unpredictable occurrence may lead to colitis with bloody diarrhea, severe abdominal cramps, vomiting, and hypotension [30]. In October 2010, Indian Council of Medical Research (ICMR), Ministry of Health & Family Welfare, Government of India conducted a pilot study on the adverse effects of *L. siceraria* after consumption of its juice. The patients were reported to have suffered from diarrhea, vomiting, elevated levels of liver enzymes and excessive ulceration in distal oesophagus [31]. There were several other cases of cucurbit toxicity which have been reported in India as well as in other countries like Australia, Alabama and California [32]. The probable cause of the toxicity lies in is the presence of the active principle, cucurbitacin. It was further observed that the toxicity of cucurbitacin was closely related to their chemical structure, specifically due to the presence of a double bond at C-23 and acetyl group at C-25 in their structure [33]. Reports have been found that cucurbitacin and their glycoside exerts potential cytotoxicity in several cell lines. In specific, cytotoxic behavior of cucurbitacin E was reported at lower $IC_{50}$ value, when studied in human hepatocellular carcinoma HepG2 cell line [34]. The *in-vivo* toxicity study reported the $LD_{50}$ values of cucurbitacin E at a dose of 2–12.5 mg/kg body weight in mice after oral administration of cucurbitacin derivatives [33]. The toxic effects of cucurbitacin are rendered by increasing the blood pressure and subsequently accumulates fluid in thoracic and abdominal cavities by enhancing capillary permeability in human volunteers [34]. It has been reported that maximum, tolerable limit of cucurbitacin should be restricted for human consumption, although the content of cucurbitacin may vary due to mutations, lack of irrigation and environmental factors [30]. As a large population of India consumes fruit juices of Cucurbitaceae family regularly, the standardization of these fruits with cucurbitacin E as phytomarker is very necessary. This may help in preventing toxicity associated with the Cucurbitaceae food plants at a large.

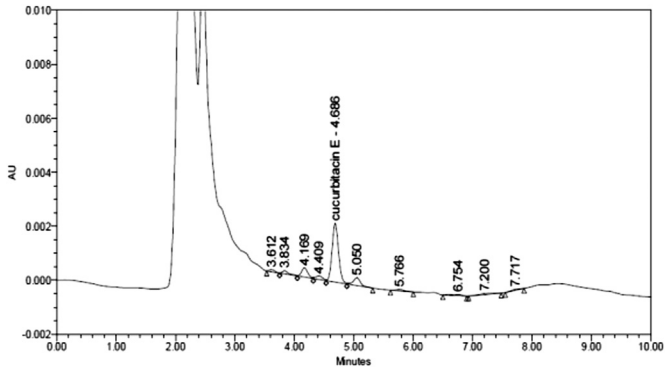


**Fig. 9.** RP-HPLC/UV chromatogram of *Luffa acutangula* lyophilized extract.

## 5. Conclusion

The RP-HPLC study confirmed the highest cucurbitacin E content in *C. pepo* whereas the lowest amount of was reported in *L. siceraria* fruit. The developed RP-HPLC method is robust, accurate, precise and reproducible for quantification of cucurbitacin E with a narrow linear range. This validated method can be beneficial for the nutraceutical industry in establishing effective quality control of these fruits for safe human consumption.

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## Conflicts of interest

None.

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