Detection and Quantification of Major Phytochemical Markers for Standardization of *Talinum portulacifolium*, *Gomphrena serrata*, *Alternanthera sessilis* and *Euphorbia heterophylla* by HPLC

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ABSTRACT

**Background:** High-performance Liquid Chromatography is one of the major analytical techniques used in the quality control of phytochemicals. **Objective:** This research article presents the development of HPLC method to detect and quantify the major marker components, kaempferol, and quercetin from four plant species. **Materials and Methods:** HPLC method was developed for the qualitative and quantitative analysis of plant extracts by using orthophosphoric acid and methanol (95:5) at 370 nm for kaempferol, methanol and orthophosphoric acid (60:40) at 262 nm for quercetin. **Results:** Kaempferol was detected from the hydro alcoholic extracts of *Talinum portulacifolium* leaves (RT 13.720, concentration 1.08 mg/ml) and flowers of *Gomphrena serrata* (RT 13.758, concentration 2.13 mg/ml). Kaempferol was reported for the first time from *Gomphrena serrata*. Quercetin was separated and identified from the hydro alcoholic extracts *Alternanthera sessilis* stems (RT 6.503, concentration 0.01 mg/ml). The hydroalcoholic extract of *Euphorbia heterophylla* stems (RT 6.588, concentration 0.01 mg/ml) was also evaluated for the presence of quercetin. **Conclusion:** The method developed is a very useful tool for qualifying and quantifying the plant specimens as well as their extracts.

**Key words:** Marker, Kaempferol, Quercetin, HPLC, Quality control, Plant specimens, Extracts.

**INTRODUCTION**

Phytomedicines, refer to the medicinal products from various parts of plants that can be used to promote health and treat diseases. Despite the major advances in the modern medicine, the development of new drugs from natural products is still considered important. Since 1980, the World Health Organization has been encouraging countries to identify and exploit traditional medicine and phytotherapy.¹ There have always been concerns about the inconsistent composition of herbal medicines and occasional cases of intoxication by adulterants and/or toxic components. The development of phytotherapeutic products is dependent on their standardization, to guarantee not only the authenticity of the plant extract but also to enable a dose related use. Quality control of herbal medicines aims to ensure their consistency, safety, and efficacy.² Chromatographic methods have been recommended for the quality control of phytotherapeutic products with many advantages over other methods.³⁴⁻¹ The development of fingerprinting has been a powerful tool for the quality control of herbal medicines. A chemical fingerprint is a unique pattern that indicates the presence of multiple chemical markers existing in a sample. In the process, technologies such as high-performance liquid chromatography (HPLC), high-performance capillary electrophoresis (HPCE), and liquid chromatography–mass spectrometry (LC–MS) are often used.⁵ Chemical fingerprinting by HPLC is simple, sensitive and in expensive, and has been widely used in the pharmaceutical field. A chemical marker may or may not have the therapeutic activity, but the quantity of chemical marker can be an indicator of the quality of herbal medicine, including authentication of species or substitutes, optimization of extraction and purification methodologies, structure and purity determination. All markers may contribute to the evaluation, standardization and safety assessment of herbal medicines.⁶ The genus *Talinum* comprises about 50 species.⁷⁻⁹ Leaves of the plant *Talinum portulacifolium* Forsk. Belonging to family Portulacaceae, possess anti-diabetic and aphrodisiac properties.¹⁰ The plant was used to treat arthritis, backache, and diarrhea.¹¹ It is also used as a medicine for constipation and ulcer.¹² Previous chemical studies on this species reported that leaves and stem extracts contain quercetin, tannins, phosphates, urea, and various minerals with a

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larger amount of magnesium.\textsuperscript{13} Antiasthmatic studies on hydro alcoholic and acetone extracts of the plant along with GC-MS (Gas chromatography mass spectrometry) analysis of chloroform fraction from the acetone extract of leaves revealed the presence of major phytoconstituents namely, methoxy-bis(cyclopentadiene), 5,10-dihexyl-5,10-dihydroindolo[3,2-b] indole-2,7-dicarbaldehyde, and 1,2-bis[3,4-dimethoxy benzyl]-1,2-bis (methoxy methyl) ethane.\textsuperscript{14} HPLC analysis of quercetin was carried out on leaf and stem methanolic extracts.\textsuperscript{15} Kaempferol was isolated from chloroform and methanolic extracts of leaves.\textsuperscript{16} There were no reports available on the HPLC identification and quantification of kaempferol from this plant species.

\textit{Gomphrena serrata} Linn, \textit{Amaranthaceae}, is a road side herb grown in temperate regions of the world. \textit{Gomphrena} species are employed in the treatment of bronchial asthma, diarrhea, and fever, and as an analgesic, tonic, or carminative.\textsuperscript{17} This species showed antimalarial and diuretic activities.\textsuperscript{17,18} Oleuropein was isolated from the chloroform extract of the whole plant.\textsuperscript{19} GC-MS analysis of chloroform fraction showed the presence of 30 bioactive compounds.\textsuperscript{20} There is little phytochemical and pharmacological screening report on this species.\textsuperscript{16,17,18,19,20,21} The phytochemical review on \textit{Gomphrena serrata} indicates that there were no reports available for the presence and detection of kaempferol from the plant species. Kaempferol was reported for the first time from the plant species.

\textit{Alternanthera sessilis} Linn. \textit{Amaranthaceae}, is used as a vegetable in Asia, traditionally used in skin diseases, to cure wounds and as an antitodal. The plant is reported for various pharmacological activities like haematonic,\textsuperscript{22} antioxidant,\textsuperscript{23} anti-inflammatory,\textsuperscript{24} hepatoprotective,\textsuperscript{25} antiulcer,\textsuperscript{26} antimicrobial,\textsuperscript{27} and wound healing.\textsuperscript{28} It was reported to contain β-carotene,\textsuperscript{29} lupeol, α and β spinasterol,\textsuperscript{30} β-sitosterol, stigmasterol,\textsuperscript{31} and campesterol.\textsuperscript{29} Antiasthmatic studies were performed on the hydro alcoholic and acetone extracts of stems along with a GC-MS analysis of chloroform fraction of acetone extract of stems, depicted following major phytoconstituents, methoxy-bis (cyclopentadiene), 5,10-dihexyl-5,10-dihydroindolo[3,2-b] indole-2,7-dicarbaldehyde and 1,2-bis[3,4-dimethoxy benzyl]-1,2-bis (methoxymethyl) ethane respectively.\textsuperscript{32} HPTLC finger printing analysis was carried out for the detection and quantification of gallic acid in methanolic extract of leaves.\textsuperscript{33} HPLC fingerprint analysis was performed for the detection and quantification of polyphenols catechin, rutin, ellagic acid and quercetin in the ethanolic extract of the whole plant.\textsuperscript{33} There were no reports available on the HPLC analysis of quercetin on stems of the plant species.

\textit{Euphorbia heterophylla} L, \textit{Euphorbiaceae}, is a branched shrub belonging to the family \textit{Euphorbiaceae}, widely distributed in the South Asian continent. The plant was reported for activities like antimicrobial and anticancer activity,\textsuperscript{34} hepatoprotective activity,\textsuperscript{35} anti-diabetic activity,\textsuperscript{36} effect of plant extract on kidney, liver and pancreatic functions was reported.\textsuperscript{37} Stigmasterol and 4-hydroxy benzoic acid were isolated from the leaf extracts showed good activity against xanthine oxidase enzymes,\textsuperscript{40} diterpenoids were isolated from roots.\textsuperscript{41} The leaves of \textit{Euphorbia heterophylla} were reported to contain quercetin.\textsuperscript{42} There were no reports of HPLC analysis on quercetin from this species.

The aim of the present study was to detect and quantify the phytochemical marker compounds kaempferol from hydro alcoholic extracts of \textit{Talinum portulacifolium} leaves and \textit{Gomphrena serrata} flowers followed by quercetin from the hydro alcoholic extracts of and \textit{Alternanthera sessilis} stems and \textit{Euphorbia heterophylla} stems by HPLC analysis respectively. The literature review presents no reports available on the HPLC determination of major photo chemicals kaempferol and quercetin present in the plant extracts, hence an effort was made to explore them for standardization of medicinal herbal extracts.

### Materials and Methods

#### Collection of plant material

The plant materials were collected from local grounds of Prasadamadu, and Enikepadu coordinates 16°32′45″N 80°34′12″E of Vijayawada rural region, Krishna District, Andhra Pradesh, India. The plant specimens were identified and authenticated by Dr. P. Satya Narayana Raju, plant taxonomist, Department of Botany and Microbiology, Acharya Nagarjuna University (ANU), Guntur (District), Andhra Pradesh, India. Voucher specimens 001/VPW- \textit{Gomphrena serrata}, 002/VPW- \textit{Alternanthera sessilis}, 003/VPW- \textit{Euphorbia heterophylla}, 004/VPW- \textit{Talinum portulacifolium} were deposited in the department of Pharmacognosy, Vijaya Institute of Pharmaceutical Sciences for Women, Enikepadu, Vijayawada for future reference. The plant pictures were depicted [Figure 1, Figure 2, Figure 3, Figure 4] respectively.

#### Preparation of the extract

The plant parts were shade dried, powdered coarsely and extracted using methanol and water (50:50) by Soxhlet apparatus. The extracts were dried using vacuum evaporator. They were preserved in refrigerator till use.

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### Figures

**Figure 1:** \textit{Talinum portulacifolium} plant and leaves.

**Figure 2:** \textit{Gomphrena serrata} plant and flower.

**Figure 3:** \textit{Alternanthera sessilis} plant and twig.
**Chemicals and Reagents**
All the chemicals and reagents used were of analytical grade, purchased from Sigma-Aldrich Chemical Co.

**Thin layer chromatography**
Qualitative analysis of kaempferol and quercetin for the plant extracts was carried out by TLC (Thin layer chromatography) on silica gel using acetic acid, methanol, and water in the ratio of 5:25:75. The spots observed were studied under UV and Rₔ values were calculated. ⁴³

**Instrument and chromatographic conditions**
HPLC analysis was performed on the Shimadzu LC-20 system (Shimadzu, Kyoto, Japan) equipped with a pump (LC-20AD), auto sampler (SIL-20A), and column oven and diode array detector (SPD-M20A). The output signal of the detector was recorded using LC Solution software. The separation was executed on a YMC-Pack ODS-A C18 (250 mm×4.6 mm, 5 μm).

**Quantification of kaempferol**
Kaempferol was identified and quantified from leaf hydro alcoholic extracts of *Talinum portulacifolium* coded as TPLHAE and the flower hydro alcoholic extracts of *Gomphrena serrata*, coded as GSFHAЕ respectively. The standard sample kaempferol was coded as STDKE. The result was given [Table 2]. HPLC chromatograms for STDKF, TPLHAE, and GSFAE were produced [Figure 5, Figure 6 and Figure 7] respectively.

**Preparation of mobile phase**
The mobile phase (A) was composed of 0.136 g of anhydrous potassium dihydrogen orthophosphate was dissolved in 900 ml of HPLC grade water and 0.5ml of orthophosphoric acid was added. The volume was made up to 1000 ml. The mobile phase (B) was composed of acetonitrile. The mobile phase (A) and (B) were run with gradient elution system at 0.01-18.0 min, 95% (A), 5.0% (B); 18.0-25.0 min, 55.0% (A), 45.0% (B); 25.0-28.0 min, 20.0% (A), 80.0% (B); 28.0-35.0 min, 20% (A), 80% (B); 35.0-40.0 min, 55.0% (A), 45% (B); 40.0-45.0 min, 95.0% (A), 5% (B) and 45 min 95% (A), 5% (B) respectively [Table 1]. Both the mobile phases were run at a flow rate of 1.5 ml/min. The injection volume was 20 μl. The detection UV length was set at 370 nm. The column temperature was maintained at 35°C.

**Preparation of standard solution**
The standard stock solution was prepared by dissolving 5 mg of quercetin in 1 ml mobile phase. The standard working solution was prepared by taking 0.2 ml from stock solution and was made up to 1 ml by mobile phase to get 10 μg/ml.

**Preparation of sample solution**
1000 mg of extract was dissolved in 10 ml methanol using a standard volumetric flask, sonicated for 10 min and filtered before injection. ⁴⁴, ⁴⁵

**Quantification of quercetin**
Quercetin was identified and quantified from the stem hydro alcoholic extracts of *Euphorbia heterophylla* coded as EHSHAЕ and *Alternanthera sessilis* coded as ASSHAЕ. The standard sample quercetin was coded as STDQU. The result was produced [Table 4]. HPLC chromatograms were given [Figure 8, Figure 9, Figure 10] respectively.

**Preparation of mobile phase**
The mobile phase (A) was composed of 0.1% orthophosphoric acid in water. Mobile phase (B) was composed of methanol. The mobile phase (A) and (B) were run with gradient elution system at 0.01-2.5 min, 90% (A), 10% (B); 2.5-5.0 min 75% (A), 25% (B); 5.0-7.5 min 40% (A), 60% (B); 7.5-10.0 min 90% (A), 10% (B) respectively (Table 3). The mobile phase was run at a flow rate of 1.1 ml/min. The injection volume was 20 μl. The detector wave length was 262 nm. The column temperature was maintained at 35°C.

**RESULTS AND DISCUSSION**
Herbal drug standardization comprises total information on the composition of all herbal drugs including analytical operations for identification and assay of therapeutic and inactive principles. Chromatographic fingerprinting of herbal medicines is utilized for the authenticity and quality control of herbal medicines, which has become the most important tool for quality control of traditional herbal medicines (HPLC). Further, the combination of qualitative fingerprinting and quantitative analysis is a novel and rational method to address the key issues of quality control of herbal medicines. ⁴⁶ HPLC spectra showed the presence of kaempferol and quercetin in the plant extracts. The structures for kaempferol and quercetin were given [Figure 11]. ⁴⁷
In the present study, two of the phytochemicals namely kaempferol and quercetin were analyzed by TLC and HPLC methods. The Rₔ values noted for kaempferol was 0.47 and for quercetin, 0.62 after examining the yellow fluorescent spots under UV. The HPLC method was developed for the qualitative and quantitative estimation of kaempferol from hydro alcoholic extracts of *Talinum portulacifolium*, *Gomphrena serrata*, *Alternanthera sessilis* and *Euphorbia heterophylla*.

**Table 1**: Gradient conditions for mobile phase run during quantification of Kaempferol

| Time (min) | Buffer concentration (Solvent A) | Acetonitrile concentration (Solvent B) |
|------------|---------------------------------|-------------------------------------|
| 0.01       | 95.0                            | 5.0                                 |
| 18.0       | 55.0                            | 45.0                                |
| 25.0       | 20.0                            | 80.0                                |
| 28.0       | 20.0                            | 80.0                                |
| 35.0       | 55.0                            | 45.0                                |
| 40.0       | 95.0                            | 5.0                                 |
| 45.0       | 95.0                            | 5.0                                 |

**Figure 4: Euphorbia heterophylla** plant and twig
alcoholic herbal extracts of *Talinum portulacifolium*, *Gomphrena serrata* and quercetin from *Euphorbia heterophylla* and *Alternanthera sessilis* respectively. Retention time was used as a parameter for analysis of results. Many studies have suggested that flavonoid, kaempferol is well-known for its anti-inflammatory, antiallergic, antithrombotic, hepatoprotective, antispasmodic and anticancer properties. Kaempferol is a natural flavonol, a type of flavonoid, that has been isolated from tea, broccoli, Delphinium, Witch-hazel, grapefruit, cabbage, beans, endive, leek, tomato, strawberries, grapes, Brussels sprouts, apples and other plant sources. HPLC spectra of STDKF for kaempferol showed a peak at retention time of 17.276 min, TPLHAE at 13.720 min and 13.758 min was observed for GSFHAE respectively [Figure1, Figure 2 and Figure 3]. The quality control study conducted in the present investigation for *Talinum portulacifolium* leaves is well supported by the total phenolic content of plant leaf extracts expressed as equivalent to μg of gallic acid/100 gm dry weight of plant material for chloroform extract 39 μg, ethyl acetate extract 30 μg, methanolic extract 24.5 μg and aqueous extract 20 μg. The total phenolic content 9000 mg GAE/100gm (Gallic acid equivalent), total flavonol content 1840 mg GAE/100gm found in the methanolic extract of leaves whereas stem extracts showed less amount of phenolics 2880 mg GAE/100gm and flavonols 360 mg GAE/100gm respectively (Adithya 2012). The plant leaf extracts were studied for mineral content, sodium 365 mg/Kg, showed more potassium mineral content 514 mg/Kg, calcium 105 mg/Kg, phosphorous 367 mg/Kg. HPLC analysis of quercetin showed 3:30 min/sec RT with concentration 0.15mg/Kg in leaf extract and very less amount 0.08 mg/Kg in the stem extracts. Leaves possess the highest amount of phenolics, flavonoids, and flavonols. Total phenolic content of hexane extract of leaves was found to be 61 mg GAE /gm, ethanol extract 100 mg GAE /gm and aqueous extract possess more amount of phenolics 114 mg GAE /gm followed by extractive values 6.7%, 8.3% and 21%. TLC was performed using hexane and ethyl acetate (5:5) and methanol and acetonitrile (7:3), yellow spots developed.
Vani, et al.: HPLC markers for *T. portulacifolium*, *G. serrata*, *A. sessilis* and *E. heterophylla*

**Figure 7:** HPLC chromatogram indicating detection of kaempferol in GSFHAE

**Figure 8:** HPLC chromatogram indicating detection of quercetin in STDQU

**Figure 9:** HPLC chromatogram indicating detection of quercetin in ALSHAE

**Figure 10:** HPLC chromatogram indicating detection of quercetin in EHSHAE

**Figure 11:** Structures of kaempferol and quercetin

**Table 3:** Gradient conditions for mobile phase run during quantification of quercetin

| Time (min) | Ortho phosphoric acid concentration (Solvent A) | Methanol concentration (Solvent B) |
|-----------|-----------------------------------------------|-----------------------------------|
| 0.01      | 90                                            | 10                                |
| 2.5       | 75                                            | 25                                |
| 5.0       | 40                                            | 60                                |
| 7.5       | 80                                            | 20                                |
| 10.00     | 90                                            | 10                                |

**Table 4:** Quantification of quercetin in STDQU, EHSHAE, and ALSHAE

| Sample   | Concentration injected (mg/ml) | Retention time (min) | Name of the compound | Area     | % Peak area | Concentration detected (mg/ml) |
|----------|--------------------------------|----------------------|----------------------|----------|-------------|------------------------------|
| STDQU    | 1                              | 6.527                | Quercetin            | 27251716 | 98          | 1                           |
| ASHRAE   | 0.5                            | 6.503                | Quercetin            | 142247   | 1.0         | 0.01                         |
| EHSHAE   | 0.5                            | 6.588                | Quercetin            | 240927   | 1.7         | 0.01                         |
against purple background indicating antioxidant compounds after spraying with DPPH reagent. Luteolin and kaempferol were isolated from the methanolic extract of leaves, showed the presence of luteolin and kaempferol by TLC, IR (Infrared) Mass and NMR (Nuclear magnetic resonance) spectral studies.

Partial support for the standardization studies of Gomphrena serrata can be attributed to the morphological description of various parts of the plant, often the species is confused with Gomphrena celosioides, which can be differentiated by oblanceolate, acute apex leaf being present in G. serrata while oblong obovate, obtuse apex leaf can be seen in G. celosioides.

HPLC spectra of STDQU for quercetin showed a peak at retention time of 6.527 min, and 6.503 min and spectra of EHSHEA at 6.588 min observed for ALSHAHE respectively [Figure 8, Figure 9 and Figure 10]. The mobile phase consists of methanol: 0.1% orthophosphoric acid in water (60:40 v/v). Percentage of quercetin present in the herbal extract was calculated. The quantified amount for ALSHAHE was found to be 0.01mg/ml of plant extract. The quantified amount was found to be 0.01 mg/ml for EHSHEA [Table 4].

Quercetin is present in different parts of plants not only as aglycones but also as glycosides and is known to impart profuse color to the fruits, flowers, leafy parts etc. It is reported to display anti-histamine, anti-cancer as also anti-inflammatory activities which mostly follow its antioxidant traits. The dietary sources of quercetin include citrus fruits, apples, onions, parsley, sage, tea and red wine. In fact, onion (Allium cepa L) is the richest source of quercetin (2.60 mg/gm) whereas a very good amount of this flavonol was found in hydro alcoholic extracts of ALSHAE 0.81 μg/g tannins was determined.

Additional support to the current study can be obtained from the concentration of gallic acid by HPTLC analysis for the methanolic extract of leaves of Alternanthera sessilis was found to be 8.022 μg/ml which would be useful in establishing the standardization parameters and act as a reference in detecting adulteration or substitution (Rajiv Gupta 2016). HPLC analysis of various polyphenols, catechin, rutin, ellagic acid and quercetin found at 117.72, 490.74, 3007.26 and 13.85 mg/100gm of a dry extract from ethanolic extract of the whole plant was carried out. Further, morphological and microscopic descriptions of various parts of the plant also contribute to the current experimentation.

The scientific data reported on quality control studies of Euphorbia heterophylla includes the plant species Euphorbia heterophylla is confused with Euphorbia cyathophora. They can be identified through plant characteristics. E cyathophora is distinguished by the presence of unstalked ovary where the stalked ovary is seen in E.heterophylla. The percentage of total alkaloids 2.9 μg/g, saponins 3.72 μg/g, saponins, 0.17 μg/g flavonoids, 0.81 μg/g tannins was determined. Quantification of primary metabolites were studied, where total carbohydrate was found to be 10.29mg/g, total protein 7.43 mg/g, fat 3.5 mg/g followed by total phenolic content 8.26mg/g and ascorbic acid 1.14% from the aqueous extract of leaves of the plant. The standardization parameters favoring the quality control of aqueous extract of leaves, studied were moisture content 11.79% and ash value 2.57%, moreover, the plant was supposed to possess high carbohydrate content with more number of reducing sugars and rich nutrient status.

The retention times of both kaempferol and quercetin detected in the plant extracts were comparable to standard drugs. The developed method offers rapid detection and quantification of kaempferol and quercetin in plant extracts and therefore can be used as a reference for carrying out the standardization studies.

CONCLUSION

The study confirmed the presence of biologically and pharmacologically important flavonoids as markers, kaempferol, and quercetin in a quantifiable amount in the plant extracts. The developed method has enabled rapid analysis of two flavonoids with marked retention time. The results of the study can be used for developing the quality control profile, qualitative and quantitative analysis of extracts and other plant species containing these two flavonoids.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATION USED

HPLC: High performance liquid chromatography; HPCE: High performance capillary electrophoresis; LC-MS: Liquid chromatography-mass spectrometry; GC-MS: Gas chromatography-Mass spectrometry; HPTLC: High performance thin layer chromatography; ANU: Acharya Nagarjuna University; TPLHAE: Talinum portulacifolium hydro alcoholic extract; GSFAHE: Gomphrena serrata hydro alcoholic extract; STDKF: Standard Kaempferol; EHSHEA: Euphorbia heterophylla hydro alcoholic extract; ASSHAE: Alternanthera sessilis hydro alcoholic extract; STDQU: Standard quercetin; RF: Retention factor; TLC: Thin layer Chromatography; UV: Ultra violet; GAE: Gallic Acid Equivalent; DPPH: Diphenyl picryl hydrazyl; IR: Infrared; NMR: Nuclear magnetic Resonance; RT: Retention time; Kg: Kilogram; µg: Microgram; mg: Milligram; ml: Milli litre; min: Minutes; gm: Gram; °C: Degree Celsius; nm: Nanometre; %: Percentage.

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World Health Organization has been encouraging countries to identify and exploit traditional medicine and phytotherapy. Chromatographic fingerprinting of herbal medicines is utilized for the authenticity and quality control of herbal medicines, which has become the most important tool for quality control of traditional herbal medicines (HPLC). The present study was to detect and quantify the phytochemical marker compounds kaempferol through HPLC analysis. HPLC spectra showed the presence of kaempferol from hydro alcoholic extracts of Talinum portulacifolium leaves and Gomphrena serrata flowers. Quercetin was phytochemical marker from the hydro alcoholic extracts of Alternanthera sessilis stems and Euphorbia heterophylla stems. Quercetin in the plant extracts. The developed method has enabled rapid analysis of two flavonoid markers kaempferol and quercetin with marked retention time 13.72 minutes and 6.5 minutes compared to standard drug. Therefore the developed method can be used as a reference for carrying out the standardization studies, and in detecting adulteration or substitution.

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