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Isolation and quantification of bioactive compounds from *Siegesbeckia orientalis* L. using HPLC

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Abstract

A simple, precise high performance liquid chromatography method with photodiode array detection (HPLC-DAD) was developed for isolated bioactive compounds from the plant, *Siegesbeckia orientalis* L., namely Darutoside and Hythimoside-A from aerial parts exudates, collected from Talakona Forest, Chittoor District, Andhra Pradesh. Isolated methanolic extract was analyzed isocratic mode comprising of solvent A water and solvent B acetonitrile. The column used for separation was a licosphire NH_2 250×4.6mm (5µm) column. The elution was 10%A and 90%B and pH 3.2, flow rate was maintained at 1.0 ml/min. Detection was performed using a PDA detector at λ max 210 nm. The retention time for Darutoside(6.2min) and Hythimoside-A(8.2min) and the compound was resolved from other component of the extract. The amount of Darutoside and Hythimoside-A present in the aerial parts of *Siegesbeckia orientalis* L. in the month of May was 0.185% with R^2 = 0.9919 and 0.852% with R^2=0.9994, respectively. The method exhibited high accuracy, in the total run time of 12 min, it showed good separation of the peaks. We have observed that the method described was more efficient and reliable for estimation of active constituents present in this plant. At present, there is no analytical method which has been reported these compounds. We propose that the current method will be useful for identification and determination of Darutoside, Hythimoside-A content in the plants.

Keywords: *Siegesbeckia orientalis* L., method development, darutoside, hythimoside-A, HPLC

1. Introduction

As per WHO guideline (Anonymous, 2003), the plant selected for collection should be taxonomically same as recommended by the National Pharmacopoeia or other related documents. If a new plant is being selected for collection then it should be properly identified and documented.

Siegesbeckia orientalis L. (Chetty, 2007) belongs to Asteraceae family, commonly known as The Holy Herb, glutinosawall, katamapan (Anonymous, accessed on 10.12.2014) was the only species of the genus *Siegesbeckia* L. identified in India. Its various parts are used such as Juice, leaves, and whole plant. It is a small Asteraceae plant or small shrub growing in hot climate. The heads are small with an involucre of five bracts covered with very sticky glandular hairs. The active constituent present in this plant are hythimoside-A, hythimoside-B, darutoside, ent-pimarnae diterpenoid, sesquiterpenoid, diterpenoid glycosides, melomploids, *etc*. Its medicinal actions and uses for the aerial parts have been used in traditional Chinese medicine as "Xi-Xian" treats rheumatic arthritis (Anonymous, 1991), hypertension, malaria, neurasthenia and snakebite. Extracts and some chemical constituents of *Siegebeckia orientalis* L. exhibited many pharmacological actions like antiallergic, antioxidative, anti-inflammatory effects, anti-infertile effects, acute arthritis, furunculosis and impetigo. Therefore, together with the profiles on analytical techniques, there has to be proper quantification and quality control method having fingerprinting method should be developed (Rasheed *et al*., 2010; 2011; 2012 a, 2012b, 2012c).

***Siegesbeckia orientalis* L. Source:** Talakona Forest, Tirupati

Siegesbeckia orientalis L. has been traditionally taken orally as an anti-inflammation and anticancer agent (Wang *et al*., 2009), was administered to treat snakebites, cutaneous disorders, rheumatic arthritis (Qian *et al*., 2000 and Wang *et al*., 2011), allergic (Hwang

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et al., 2001), immune system (Sun and Wang, 2006), and inflammatory diseases (Wang *et al*., 2011). Wang *et al*. (2009) reported that the ethyl acetate and n-butanol extracts of *S. orientalis* markedly inhibited growth of human cervix cancer HeLa cells *in vitro*. However, to the best of our knowledge, the anticancer activity of *S. orientalis* extract on endometrial cancer has not yet been elucidated. "Orientin"(luteolin 8-c glycoside), a potentially allergic sesquiterpene lactone has been reported from this species (Hermann *et al*., 1994).

2. Materials and Methods

2.1 Standards and reagents

Standards Darutoside and Hythimoside-A isolated were used for the analysis of HPLC Purity >99% and other solvents of HPLC grade in preparation of mobile phase.

2.2 Plant materials

The aerial parts exudate of *Siegesbeckia orientalis*. L. was collected from Talakona Forest, Tirupati, Chitoor District, Andhra Pradesh, India. It was collected in the month of May 2007 and this plant was identified and confirmed by Professor Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati and further used for isolation purpose.

2.3 Isolation procedure

Procedure for isolation of the compounds from the exudates is as follows: The exudates (2 kg) was powdered and adsorbed plant material was extracted successively with pet-ether (60-80^{o}C) (3 lit), chloroform (3 lit), ethyl acetate (3 lit) and methanol (3 lit) in a soxhlet extractor. The extracts then concentrated under reduced pressure and separated. Methanolic extract on concentration yielded light yellow semi-solid (20 gm) and subjected to column chromatography (250 gm of silica gel 200 mesh) and the column was eluted successively with pet ether, followed by ethyl acetate , methanol altogether 205 fractions and (each 200 ml) were collected. The fractions were mixed based on TLC examination. The fractions 186-205 on concentration gave a white amorphous solid (0.9gm). Upon TLC examination (solvent system was ethyl acetate: methanol 8:2) showed presence of two major compounds and was, therefore, subjected to re-column chromatography over silica gel, (200 mesh), 138 fractions (each 200 ml) were collected. Fractions 19-40 on concentration white amorphous powder m.p 108-109^{0}C yield 0.0564gm. It gave pink colour to Libermann-Burchard test, also the spectral data in comparison with earlier reports in the literature, confirming it to be a terpenoid and, hence, designated as Darutoside. The fractions 70-90 on concentration white amorphous powder m. p.135-136^{0}C yield 0.396 gm. It gave pink colour to Libermann-Burchard test, also the spectral data ^{1}H,^{13}C, NMR, Mass spectroscopy in comparison with reported in the literature confirming it to be a terpenoid and, hence, designated as Hythimoside-A.

2.4 Preparation of standard solution

Accurately weighed standard-1(Darutoside) 10 mg was dissolved in methanol (10 ml) to prepare a 1 mg/ml standard solution. Other working standard solutions were prepared by dilution to obtain the concentration ranging from 1-20µg/ml.

Accurately weighed standard-2 (Hythimoside-A)10 mg was dissolved in methanol (10 ml) to prepare a 1 mg/ml standard solution. Other working standard solutions were prepared by dilution to obtain the concentration ranging from 1-20µg/mL.

Methanolic crude extract (10mg) was weighed. 10ml of methanol was added and make up the solution of 1mg/ml and then the solution was centrifuged for 20 min.The supernatant was collected in 10 ml volumetric flask and the procedure was repeated 4 times. Resultant solution was then injected1mg/ml sample.

2.5 HPLC equipment and conditions

Experiments were performed on a Agilent 1100 series HPLC equipped with a Binary pump, an auto sampler and a PDA detector, and connected to Chemistation software. The mobile phase consisted of 100 ml of water, and 900 ml acetonitrile, mix thoroughly and adjust the pH by using 0.1% trifluro acetic acid and filter through 0.45µm, using an isocratic system. The flow rate was 1ml/ min. Detection was performed using a PDA detector at λ max 210 nm. The retention time for Darutoside (6.2min) and Hythimoside-A (8.2min) structures as shown in Figure 1 and the compound was resolved from other component of the extract.

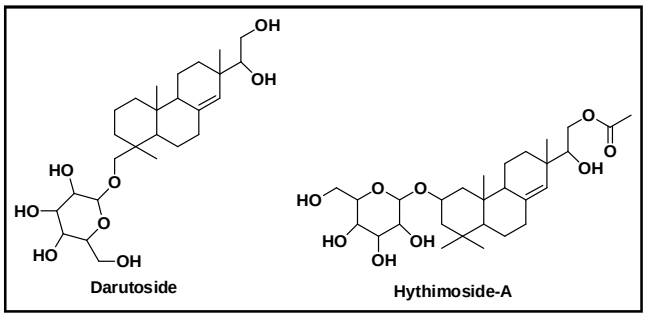

Figure 1: Structures of Darutoside and Hythimoside -A

2.6 Linearity and calibration

Calibration curve for Darutoside was drawn using different concentrations ranging from 1-20 µg/ml (Table 1) The calibration curve of the standard Darutoside was shown in Figure 2. The test sample with concentration of 1mg/ml was injected.

Calibration curve for Hythimoside-A was drawn using different concentrations ranging from 1-20 µg/ml (Table 2). The calibration curve of the standard Hythimoside-A was shown in Figure 3, the test sample with concentration of 1mg/ml was injected.

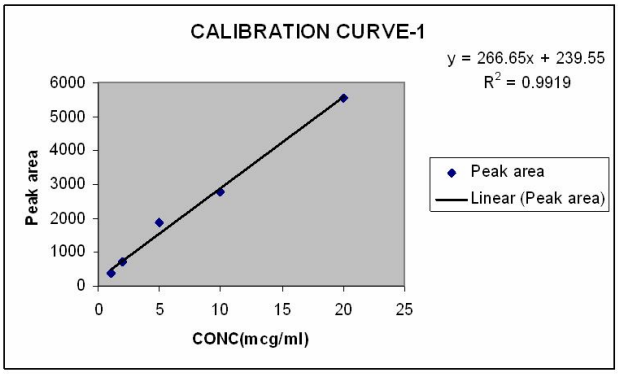

Figure 2: Calibration curve for Darutoside

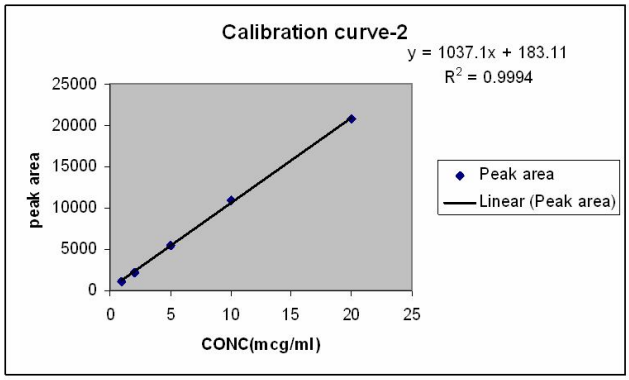

Figure 3: Calibration curve for Hythimoside-A

2.7 Accuracy

Accuracy was measured by analysis of standard solutions at different concentrations several times on the same day. The RSD was <3.1%, which is acceptable as shown in Table 1.

Table 1: Accuracy data for the HPLC method

Standard	Concentration taken (µg/ml)	Measured conc. ± S.D	% RSD
1	5	5.12 ± 0.16	3.11
	10	9.99 ± 0.23	2.32
2	5	5.02±0.4	2.00
	10	10.07±0.2	1.98

2.8 Repeatability

Different samples were chromatographed in six times by using the same equipment on different days. The RSD was < 3.11%, which is acceptable as shown in Table 2.

Table 2: Repeatability data for the HPLC method

		Intra day		Inter day	
Standard	Concentration taken (µg/ml)	Measured conc. ± S.D	% RSD	Measured conc. ± S.D	% RSD
1	5	5.07 ± 0.12	2.36	5.06 ± 0.14	2.64
	10	10.06 ± 0.2	1.98	10.05 ± 0.2	3.11
2	5	5.2 ± 0.4	2.00	5.7 ± 0.12	2.64
	10	10.07 ± 0.12	2.36	10.05 ± 0.2	3.00

2.9 System suitability parameters

System suitability tests are integral part of chromatographic method. They used to verify the reproducibility of the chromatographic system was adequate for analysis. To ascertain its effectiveness, system suitability tests were carried out by prepared standard stock solution of standard-Darutoside, standard- Hythimoside-A as shown in Table 3.

Table 3: System suitability parameters

S.No.	Parameter	Darutoside	Hythimoside-A
1	Retention time(min)	6.62	8.2
2	Theoretical plates	11750.54	9308.3
3	Capacity factor	2.47	2.37
4	Relative standard deviation	0.874	0.143

3. Results and Discussion

Suitable method was developed for the isolated compounds to get the best resolution for Darutoside (6.2min) and Hythimoside-A in aerial parts of *Siegesbeckia orientalis*. L. Experimental conditions were optimized for separation of Darutoside and Hythimoside-A from other components of the plant extract. The solvent system used was an isocratic mode comprising of solvent A water and solvent B acetonitrile. The column used for separation was a licosphire NH_2 250×4.6mm (5µm) column. The elution was 10%A and 90%B. The pH is 3.2, and flow rate was maintained at 1.0 ml/ min. Detection was performed using a PDA detector at λmax 210 nm. The retention time for Darutoside(6.2min) and Hythimoside-A (8.2min) and the compound was resolved from other components of the extract. The amount of Darutoside present in the aerial parts of *Siegesbeckia orientalis*. L in the month of May was found to be 0.185 % (using the equation Y =266.65X + 239.5). The correlation coefficient was 0.9919. The amount of Hythimoside-A in the aerial parts of *Siegesbeckia orientalis*. L in the month of May was found to be 0.852% (using the equation Y =1037.1X + 183.11) the correlation coefficient was 0.9994.

Conclusions

In this present study, an attempt was made to develop method for isolated bioactive compounds from the plant *Siegesbeckia orientalis*. L. namely Darutoside and Hythimoside-A. The aerial parts of plant material extracted successively by using organic solvents. The methanol crude extract (20 gm) subjected to column chromatography by using silica gel (200 mesh size), the column was eluted successively with pet-ether followed by ethyl acetate, ethyl acetate: methanol (9:1). The structure elucidation of the isolated compound was based on the chemical tests and spectroscopic data. The IR,MASS, NMR data indicating that compounds were characterized as Darutoside and Hythimoside-A. These two compounds have the activity of antirheumatic arthritis, anti-inflammatory activity. The current method was accurate and shorter run time 12 min, having good separation of the peaks. It is more efficient reliable method for estimation of active constituents present in the plant. There is no analytical method yet been reported for these compounds. This method will be useful for identification and determination of Darutoside and Hythimoside-A content in the plant.

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Conflict of interest

We declare that we have no conflict of interest.

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