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RESEARCH ARTICLE

QUANTITATIVE ANALYSIS OF ALLANTOIN IN LEAVES, STEM AND ROOTS OF *Pisonia grandis* R.Br. BY RP-HPLC

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ABSTRACT

Allantoin Allantoin is 2,5-dioxo-4-imidazolidinyl-urea that occurs naturally in plants and animals. A high-performance liquid chromatography (HPLC) method for the quantitative analysis of allantoin has been developed. Separation of allantoin from crude extract of leaves, stem and roots of *Pisonia grandis* was achieved using C_{18} column as a stationary phase and acetonitrile: phosphate buffer solution as mobile phase at a flow rate of 1 ml/min. Photo diode array detector (PDA) response was maximum at 200 nm. The calibration curve was linear in the range of 0.25 to 1.25 mg/ml. Limit of detection and limit of quantification values of allantoin were 0.435 and 1.65 mg respectively. Quantitation of allantoin in the plant extracts could be easily achieved by the proposed method with satisfactory results.

INTRODUCTION

Allantoin is 2,5-dioxo-4-imidazolidinyl-urea and is known as a keratolytic molecule that removes warts, corns and horny layer (hard layer) of the skin. It is a white, odourless, crystalline powder considered to be non-toxic, non-irritating and non-allergenic (www.balmtech.com). The U.S. Food and Drug Administration (FDA) confirmed that allantoin is a safe and effective skin protectant in the recommended dosage range of 0.5 to 2.0% (Federal Register). There are hundreds of patents published on the pharmacological activity of allantoin. Allantoin is reported to be a free radical scavenger (Guskov *et al*., 2002) as well as a wound healer (Ranson 1984., Araújo *et al*., 2010). It reduces plasma glucose in streptozotocin-induced diabetic rats (Shan Niu *et al*., 2010). Anti-inflammatory formulations (Kokai Tokkyo Koho, 1984), antimicrobial dressings (Ying Ko Sai, 1983), medicines that are used for treating gastroduodenal ulcer and chronic gastritis (Dobrescu Dumitru, 1998) and ointments for treating plaque and psoriasis (Pinheiro, 1997), contain allantoin as one of the foremost ingredients. Allantoin is a common constituent of plants. The presence of allantoin has been reported in numerous plants. Notable sources are *Symphytum officinale* (Comfrey) (Mathon *et al*., 2013), Oryza sativa L (Rice) (Wang *et al*., 2012), *Medicago sativa* (Alfalfa) (Aranjuelo *et al*., 2013), *Glycine max* (Soybean) (John Imsande, 1986) and *Coffee* (Andre *et al*., 2011). Recently allantoin has been isolated from the leaves of the plant *Pisonia grandis* (Shubashini *et al*., 2011) (Patent Pending: 3606/CHE/2010) in our laboratory and hence valid quantification is required to standardize the allantoin as a bio marker compound of *Pisonia grandis*. In this paper an RP- HPLC –PDA method of quantitation of allantoin is presented.

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MATERIALS AND METHODS

Solvents and Chemicals

HPLC grade solvents and chemicals were used. Acetonitrile, potassium dihydrogen phosphate and orthophosporic acid were purchased from Merck. Standard allantoin was purchased from Fluka.

Plant Material

Fully matured parts of *Pisonia grandis* were collected from the local areas of Coimbatore, TamilNadu, India. The parts were cleaned and shade dried. Shade dried parts were cut into small species and then used for the study.

Preparation of Extracts

De-waxed parts of *Pisonia grandis* were sequentially extracted with ethanol and water at reflux temperature for 6 hours. The extracts were concentrated under vacuum and the concentrated extracts were designated as PGSE (stem ethanol extract), PGSW (stem aqueous extract), PGRE (root ethanol extract), PGRW (root aqueous extract), PGLE (leaf ethanol extract) and PGLW (leaf aqueous extract).

Chromatographic condition

HPLC analysis was performed using Shimadzu HPLC instrument with two pumps (A and B - LC-6AD) with PDA detector (Photo Diode Array - SPD-M20A). Data collection, integration and development of analytical method was performed on class VP software. Complete separation was achieved on a reverse-phase C18 column (ODS, 250X4 60mm 5µ). A binary gradient programme was used. Acetonitrile: phosphate buffer in a ratio of 20:80 at pH = 3.5 was used as mobile phase. Flow rate was maintained as 1 ml/min and the detection was performed at 200 nm in PDA.

Sample Preparation

A known quantity of standard and each of the samples was dissolved in a known volume of mobile phase and sonicated for 2 minutes. These solutions were filtered through 0.2 µm syringe filter and the filtrates were injected for HPLC analysis.

RESULTS AND DISCUSSION

Selection of column and mobile phase determines the efficacy of separations in HPLC. Literature reports revealed the use of C18 column, phosphate buffer and UV detector for the qualitative and quantitative analysis of allantoin (Haghi *et al*., 2008, Chung Fu *et al*., 2006, Ninomiya *et al*., 2003). C18 column and acetonitrile: phosphate buffer mixture (20:80) was examined for a satisfactory separation. Linearity of the method was proved by analysing the various concentrations of the standard (Table 1). Figures 1-7 depict the HPLC profile of standard and samples obtained at 200 nm. HPLC data of the ethanolic extracts of stems (PGSE), root (PGRE) and leaves (PGLE) is presented in table 2. Table 3 represents the HPLC data of aqueous extracts of stems (PGSW), root (PGRW) and leaves (PGLW) of *Pisonia grandis.* Fig. 8 and 9 illustrate the percentage concentration of allantoin in leaves, stem and roots of ethanol and aqueous extracts of *Pisonia grandis.*

Table 1. Calibration data

Sample code	Amount per fraction (mg/ml)	R_t	Area
S1	0.25	5.45	2812253
S2	0.50	5.46	5324506
S3	0.75	5.45	8426579
S4	1.00	5.45	11249012
S5	1.25	5.45	14061265

Table 2. Retention time (R_t), area and percentage of allantoin of standard and ethanol extracts of stems, root and leaves of *Pisonia grandis* (R.Br.)

Sample code	R_t	Area	Allantoin (%)
Standard	5.451	11249012.4	98
PGSE	5.635	6382705.5	21.68
PGRE	5.629	1077847	4.16
PGLE	5.649	14053487	27.63

Table 3. Retention time (R_t), area and percentage of allantoin of standard and aqueous extracts of stems, root and leaves of *Pisonia grandis* (R.Br.)

Sample code	R_t	Area	Allantoin (%)
Standard	5.451	11249012.4	98
PGSW	5.632	1949329	7.0
PGRW	5.636	1033417	4.20
PGLW	5.615	5711930	11.23

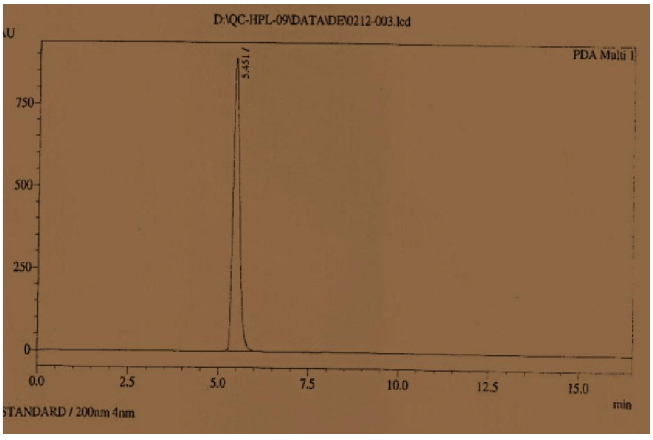

Fig. 1. HPLC Chromatogram of Standard

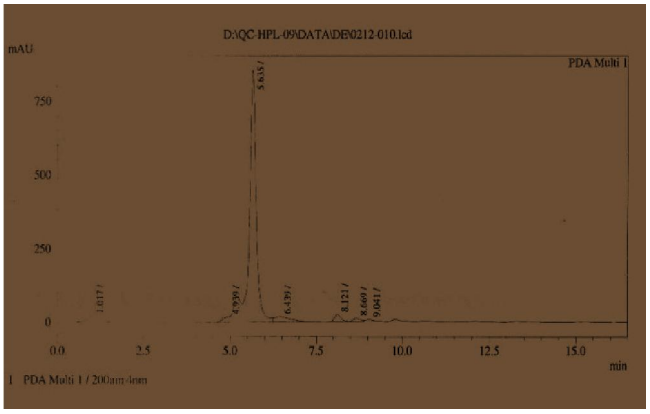

Fig. 2. HPLC Chromatogram of PGSE

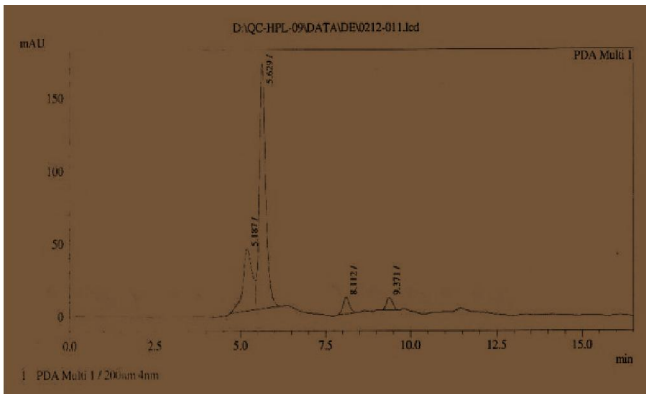

Fig. 3. HPLC Chromatogram of PGRE

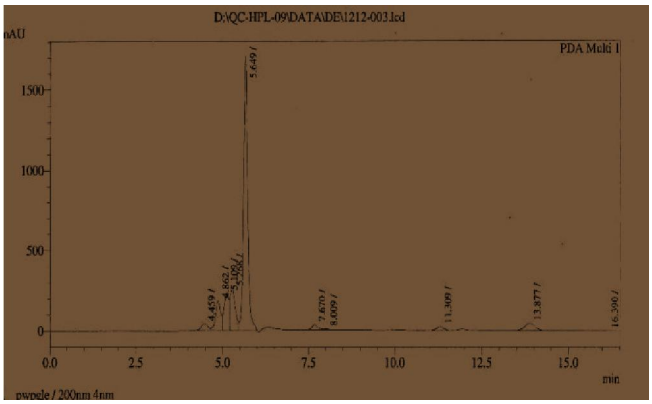

Fig. 4. HPLC Chromatogram of PGLE

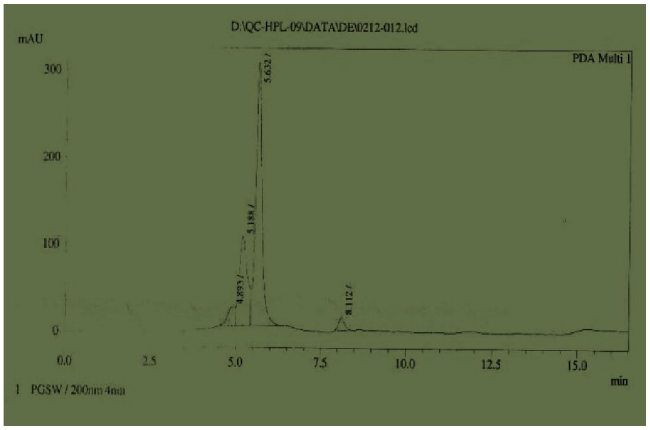

Fig. 5. HPLC Chromatogram of PGSW

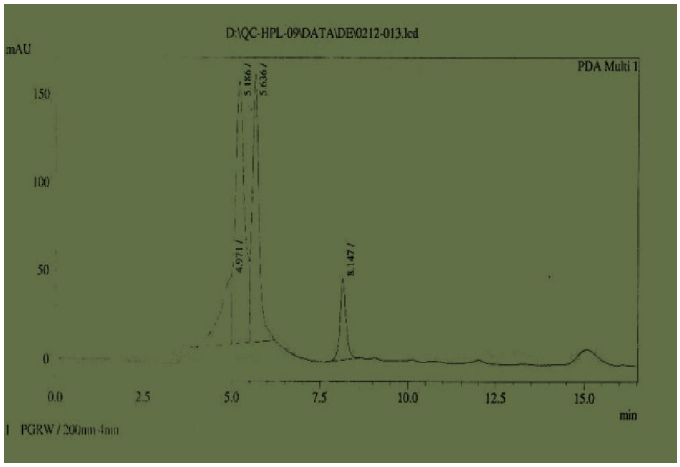

Fig. 6. HPLC Chromatogram of PGRW

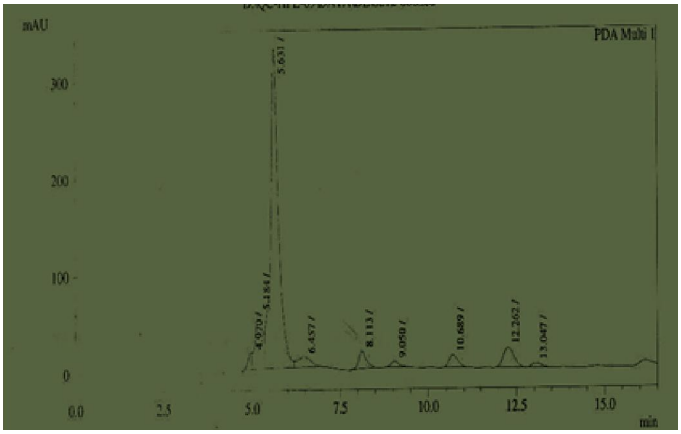

Fig. 7. HP:CChromatogram of PGLW

Quantitative analysis by HPLC revealed a greater percentage of allantoin in the leaves of *Pisonia grandis* compared to its stems and roots. The results also showed that regardless of the solvent that was used in extraction, allantoin content was found to be higher in leaf extracts PGLE and PGLW (27.63%, 11.23%). This study also reveals the efficacy of solvent in extracting allantoin from plants. Compared to water, ethanol is more efficient in extracting allantoin may be because of its poor efficacy in extracting mucilage constituents of the plant.

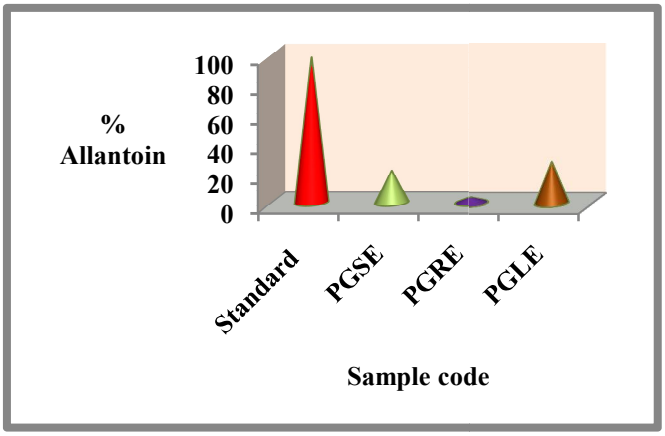

Fig. 8. Percentage concentration of allantoin in leaves, stem and roots of ethanol extracts of *Pisonia grandis* (R.Br.)

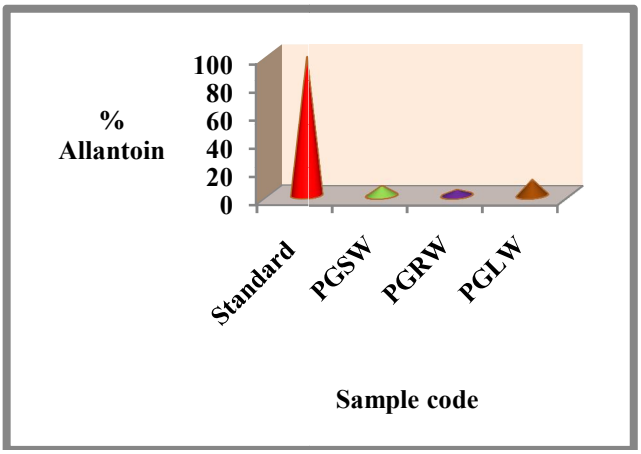

Fig. 9. Percentage concentration of allantoin in leaves, stem and roots of aqueous extracts of *Pisonia grandis* (R.Br.)

Conclusion

Allantoin is a common constituent of plants and is generally used for the storage of nitrogen in plant cells. A simple and versatile RP-HPLC –PDA method for quantification of allantoin is described. Though several methods are known for the determination of allantoin yet a small modification in the existing method with careful analysis confirmed that the method adopted here is linear, rapid and reliable. This study showed that allantoin is present in stems, roots and leaves of the plant *Pisonia grandis* but its leaves are found to a good source of allantoin. The amount of allantoin ranged from 4 % to 27%.

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