

Determination of Juglone (5-hydroxy 1,4-naphthoquinone) in *Pterocarya fraxinifolia* by RP-HPLC

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ABSTRACT: A method for determination of juglone in leaves and hulls of *Pterocarya fraxinifolia* was developed, based on RP-HPLC. This compound was extracted from leaves and hulls by chloroform refluxing about 2 hours. The chloroform was evaporated under low pressure at 50 °C to produce a dry residue. The remained materials were dissolved in acetonitrile to be used for determination of this compound. Appropriate conditions for RP-HPLC were determined using standard of juglone. These conditions include, acetonitrile percentage in mobile phase, pH of mobile phase (phosphate buffer) and temperature of column, which were 50%, 4 and 30°C, respectively. Juglone concentration was determined using standard addition method. The concentrations of juglone were 2.15, 2.74, 1.77, 1.12 and 0.34 g in 100 g of dry leaves in May, June, July, August and September 2001, respectively. The content of juglone in hulls was 0.44 g in 100 g of dry hulls in May.

KEY WORD: *Pterocarya fraxinifolia*, Juglone, Naphthoquinone, RP-HPLC method.

INTRODUCTION

Pterocarya fraxinifolia is an indigenous plant found in north of Iran [1]. Native people use the leaves of this tree as an anesthetic agent for catching fish [2], dyeing and as antifungal agents [3]. Juglone, a naphthoquinone compound, is present in the leaves and hulls of *Pterocarya fraxinifolia* [4, 5].

It is well documented that many 1, 4- naphthoquinone derivatives show antimicrobial activity [6], specially if a hydroxyl group is present at the C-5 position [7]. Little work has been done for determination of juglone content in plants. Methods such as spectrophotometry [8],

combined spectrophotometry and preparative layer chromatography [9] and fluorescence [10] have been used for determination of juglone in plants. The main disadvantage of these methods is lack of specificity, as other constituents of the plant material can react with the reagents employed. Stensen *et al.* demonstrated the separation and determination of fifteen naphthoquinone derivatives with RP-HPLC [11]. The advantage of HPLC method is principally its greater selectivity as compared with those methods mentioned previously.

As far as we know no work has been done on

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4/\$/2.40

Table 1: Effect of pH on chromatographic efficiency, N, and asymmetry factor, B/A.

pH	N	B / A
3.5	4814	1.63
4.0	7564	1.22
4.5	3922	1.73
5.0	2744	1.78
5.5	985	2.44
6.0	82	9.00

Conditions: Mobile phase: 50% acetonitrile-water, $T = 30\text{ }^{\circ}\text{C}$; Flow rate: 1.5 ml/min;

Column: 250×4.6 mm, μ Bondapak C-18, 10 μm .

determination of juglone content in *Pterocarya fraxinifolia*, therefore in this project an RP-HPLC method was used to determine the juglone content in *Pterocarya fraxinifolia* leaves and hulls.

EXPERIMENTAL

Chemicals

Acetonitrile (HPLC grade) and Sodium dihydrogen phosphate were obtained from Fluka (Buchs, Switzerland). Water was doubly distilled, deionized and filtered through a 0.22 μm Millipore filter. Standard juglone was obtained from Aldrich (Riedstr, Germany).

Instrumentation and chromatographic conditions

The HPLC system comprised of a series 10 liquid chromatograph pump module, a model LC-95 UV detector (Perkin elmer, Norwalk, CT, USA), a STG software for data processing (Teifgostar Industry, Iran), a AR-55 linear recorder (Pye Unicam, Holland) and column C18, 250 × 4.6 mm, 10 μm (Waters Assoc.milford, MA, USA). A Jenway pH meter 3030 (Jenway Ltd., UK) was used for determination of pH.

Appropriate conditions for determination of this compound, obtained using its standard, include acetonitrile percentage in mobile phase, pH of mobile phase (using phosphate buffer) and temperature of column, which were 50%, 4 and 30 $^{\circ}\text{C}$, respectively. At these conditions juglone peak had no interferences with other compounds extracted from the leaves and hulls. At least 25-min elution time was necessary to elute non-polar compounds present in the chloroform extract prior to the next injection. Flow rate and the injected volume were 1.5 ml/min and 10 μl respectively.

Extraction of juglone from leaves and hulls

Leaves and hulls of *Pterocarya fraxinifolia* were collected from Qaemshahr, Iran in May, June, July, August and September 2001 and treated as the following. Leaves and hulls were cut into small pieces. A 0.05-0.2 g of leaves and 0.2-0.5 g of hulls were extracted separately by refluxing in 50 ml of chloroform for 2 hours which was sufficient to quantitatively extract juglone from them. The chloroform solution was filtered and evaporated under reduced pressure at 50 $^{\circ}\text{C}$ to give a dry residue. This residue was dissolved in acetonitrile and used for analysis by HPLC system.

Determination of humidity

Since juglone content (as usual in pharmacology) was expressed as the weight percent of juglone in dry plant materials, determination of water content was carried out in each sample of fresh leaves and hulls [12].

RESULTS AND DISCUSSION

Table 1 gives the number of theoretical plates, N, and asymmetry factor, B/A, for juglone with mobile phase of 50% acetonitrile-water (V/V) at different pH. Results indicate that pH=4 results in better chromatographic efficiency and asymmetry factor as compared to other pH.

The juglone in leaves and hulls was extracted using different solvent such as hot water, methanol, chloroform and hexane. Fig. 1 shows content of juglone, which were extracted with these solvents at 1 hour and indicated that chloroform is the best solvent.

Fig. 2 illustrates effect of reflux time on extraction of juglone by chloroform. As can be seen, there is no significant difference between results obtained after 2

Table 2: Percentage of juglone in dry leaves and hulls at different months.

Sampling month	% Juglone in leaves	% Juglone in hulls	R ²
May	2.15 ± 0.07	0.44 ± 0.01	0.994
June	2.74 ± 0.08	-	0.993
July	1.77 ± 0.12	-	0.991
August	1.12 ± 0.06	-	0.995
September	0.34 ± 0.03	-	0.998

Conditions: Mobile phase: 50% acetonitrile-water, pH = 4, T = 30 °C; Flow rate: 1.5 ml/min;

Column: 250×4.6 mm, μ Bondapak C-18, 10 μ m

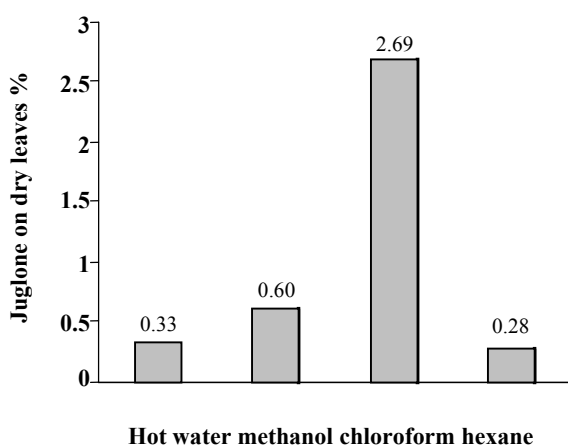


Fig. 1: Percentage of juglone extracted from leaves with different solvents for one hour extraction.

hours, therefore, 2 hours was chosen as an appropriate time for extraction.

Fig. 3 shows typical chromatograms of sample solution extracted from leaves and standard juglone. At experimental conditions used, the retention time of juglone was about 6.5 min, and its identification in the sample solution was carried out by comparison of the retention time of standard juglone. The UV spectrum of juglone that was collected from the end of HPLC column compared with standard juglone spectrum also confirmed the juglone compound at retention time of 6.5 min (Fig. 4). The λ_{\max} of standard juglone and sample collected from the end of column are close to each other. The small difference (2 nm) could be due to types of solvent used and other experimental conditions.

Determination of juglone in leaves and hulls after drying them in oven at 110 °C showed no juglone in dried materials, so the juglone content was determined in fresh leaves and hulls and used to report in percentages in dry materials.

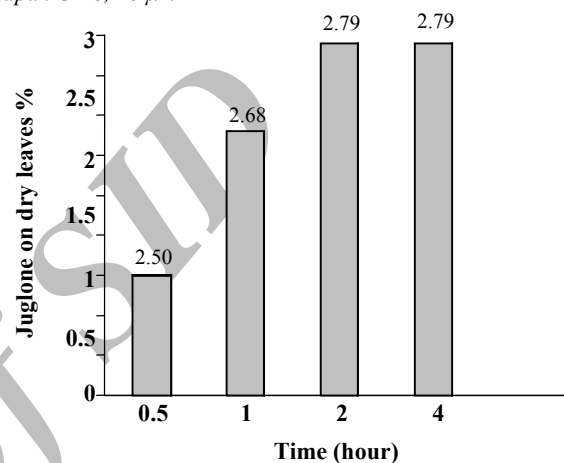


Fig. 2: Effect of reflux time on percentage of juglone extracted with chloroform in leaves.

The stability of standard juglone in acetonitrile solution was examined daily over one week period. The concentration of juglone in this solution was calculated from a standard curve derived from a freshly prepared solution. Results showed that juglone is not stable in acetonitrile solution, and therefore it is necessary to prepare the fresh standard solution just before analysis.

The correlation coefficients of acetonitrile solutions of juglone (n =5) with concentration range of 0.030-0.600 and 0.015-0.240 mg/ml were 0.992 and 0.999, respectively.

The juglone content was determined on leaves and hulls using standard addition method reported in table 2. The limit of detection was 1.03×10^{-4} mg/ml using $3S_b/m$ equation.

CONCLUSIONS

A simple, rapid, specific and isocratic RP-HPLC method for determination of juglone content in leaves and hulls of *Pterocarya fraxinifolia* tree of Qaemshahr, Iran

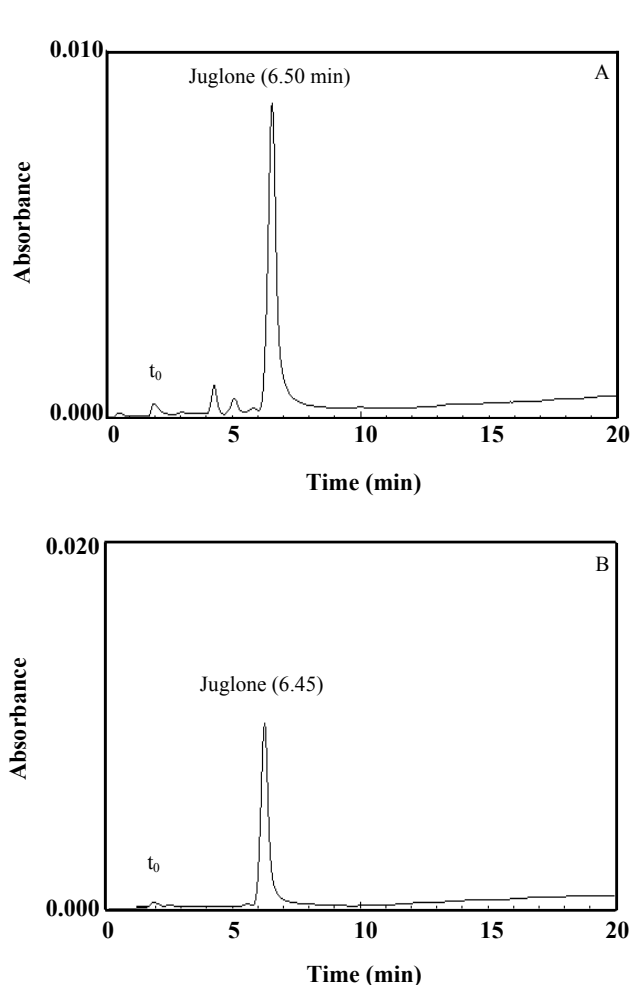


Fig. 3: Chromatogram of chloroform extract from leaves (A) and a standard solution of juglone (B).

were developed using standard addition method. The limit of detection was 1.03×10^{-4} mg / ml using UV detector.

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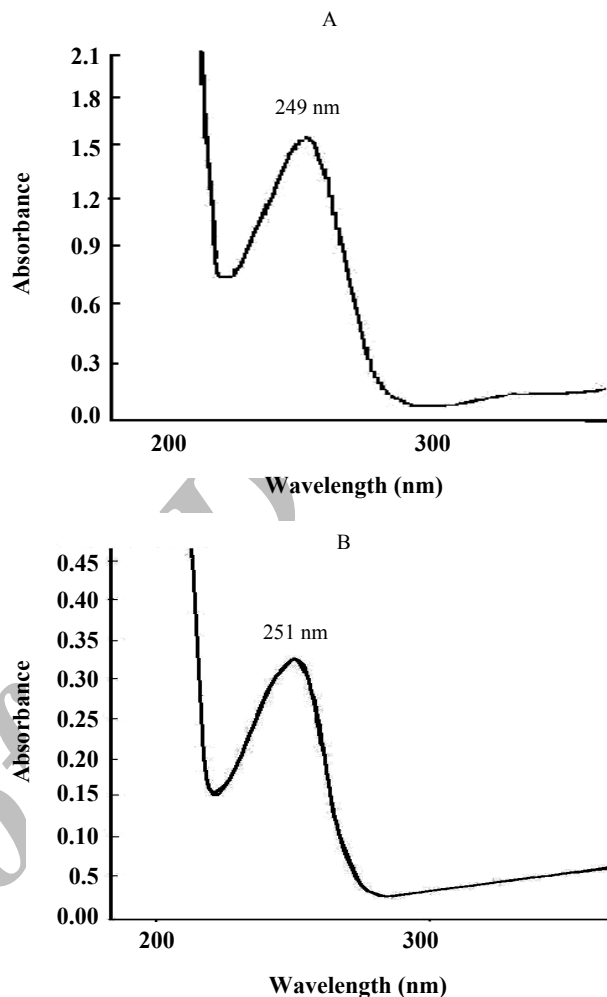


Fig. 4: UV spectrum of standard juglone (A) and juglone collected from the end of HPLC column (B).