ABSTRACT

Objective: In the present paper, an accurate High Pressure Liquid Chromatography (HPLC) method was developed and validated to determine quantitatively wedelolactone (WL) and demethylwedelolactone (DWL) in herbal extract of Eclipta alba (L.) Hassk grown in the Northeast of Brazil. Consequently, an extraction technique was developed using different parts of the plant and the technique was optimized to assess the efficacy of extraction using different solvents.

Methods: This study presents the High Pressure Liquid Chromatography (HPLC) analysis of the WL and DWL contents in various parts of the plant using different solvents and extraction methods.

Results: Quantitative HPLC analyses of the methanolic extract obtained via Soxhlet extraction showed that the highest WL concentration was found in the E. alba leaves (1.152 % w/w of dry leaf) and the highest concentrations of DWL were found in the stems (0.395 % w/w of dry stem). Quantitative HPLC analyses also showed a higher concentration of WL (0.271 % w/w of dry whole plant) and DWL (0.184 % w/w of dry whole plant) using water: ethanol extraction, compared with the Soxhlet extraction in methanol, concentration of WL (0.233 % w/w of dry whole plant) and DWL (0.159 % w/w of dry whole plant).

Conclusion: HPLC analyses revealed the presence of the two coumestans in all the extracts analysed. Moreover, when analyzing the methanolic E. alba extract, the highest concentrations of WL and DWL are present in the leaves and in the stems respectively.

Keywords: Eclipta alba, Wedelolactone, Demethylwedelolactone, HPLC.

INTRODUCTION

Eclipta alba L. (syn. Eclipta erecta L. and Eclipta prostata L.) belongs to the Asteraceae family. This small annual herb with white flowers spontaneously occurs in wet locations in Brazil [1]. Regionally, it is known as “agrião do brejo”, “erva de botão”, “lanceta” and “zangara”; the local residents usually use it to treat liver, spleen and gallbladder disorders [2]. This species is one of the main plants used in the ayurvedic system of medicine and in Indian cuisine [3]. E. alba leaf powder presents diuretic, hypotensive, hypcholesterolemic and anti-inflammatory activities while significantly improving memory retrieval [4]. The main class of bioactive compounds identified in E. alba includes coumestans; these phytoestrogens are phenolic substances with structures similar to naturally occurring human steroid hormones [5]. This plant also contains other constituents, such as polyphenols, polyacetylenes, thiophenes derivatives, steroids and terpenoids [6]. Coumestans are found in various plants, and this class is present in several naturally occurring products with diverse biological activities, including antibacterial, antifungal, and myotoxic activities [7]. Wedelolactone (WL) and demethylwedelolactone (DWL) (Figure 1) are the coumestans present in E. alba; they display hepatoprotective action, enabling the regeneration of liver cells, antibacterial and antifungal activities against multi-drug resistant microorganisms [8] and topoisomerase II inhibition [9].

Natural and synthetic WL inhibit the effects of Bothrops jararacussu and Crotalus durissus terrificus [10] and Bothrops jararaca and B. jararacussu venoms, respectively, revealing a potential therapeutic application [11]. Coumestans represent an important class of natural oxygenated aromatic products, including WL and its demethylated form (DWL). Both compounds are responsible for the main medicinal effects of E. alba and are used to treat hepatitis and cirrhosis [6]. The whole plant, leaves, roots and standardized extract are used for this purpose. A literature survey reveals that various chromatographic methods are available for quantitatively estimating WL and DWL [6, 12, 13] in extracts and tinctures [6, 7, 10, 14]. However, few methods for preparing standardized extracts and tinctures are available for this plant.

In the present paper, an accurate High Pressure Liquid Chromatography (HPLC) method for quantitatively determining WL and DWL in an herbal extract of E. alba grown in the Northeast of Brazil was developed and validated. Consequently, an extraction technique was developed using different parts of the plant; this technique was optimized to assess the efficacy of extraction when using different solvents.

The aim of this study was to identify the occurrence of coumestans (wedelolactone (WL) and demethylwedelolactone (DWL)) in Eclipta alba (L.) Hassk grown in the Northeast of Brazil. A precise and accurate method for the simultaneous determination of coumestans in different parts of the plant has not been reported yet. An attempt, therefore, has been made in the present study to develop a new method for simultaneous estimation of coumestans in an herbal extract using different solvents. This method could be used in the routine estimation of coumestans in the dosage forms.

Fig. 1: Chemical structures of wedelolactone (WL) and demethylwedelolactone (DWL).
MATERIALS AND METHODS

Plant material

*E. alba* plants were collected from the botanical garden and area surrounding the Federal University of Ceará – UFC, Brazil. The plant was authenticated by Herbarium Prisco Bezerra – UFC; voucher specimens are deposited under register 29456.

Extraction and isolation

The whole plant of *Eclipta alba* was air dried and ground to obtain a coarse powder. The whole plant (200 g) underwent Soxhlet extraction with MeOH for 24 h. The solvent was removed, and the residue was suspended in water (200 mL). This mixture was heated on a steam bath at 80°C for 30 min. After filtration over Celite, the aqueous phase was partitioned with EtOAc. The organic phase was dried and filtered, and the solvent was evaporated to yield a light brown powder. WL and DWL were isolated from the ethyl acetate extract of the whole plant using a silica gel column sequentially eluted with chloroform and methanol. The fraction eluted with CHCl₃:MeOH 70:30 was found to contain the coumestans WL and DWL, which were separated using preparative TLC (toluen : acetone: formic acid 11:6:1), generating 21.4 mg and 13.2 mg of WL and DWL, respectively. These compounds were identified using their melting points, UV [16], IR [6, 12] and ¹H and ¹³C NMR spectra [17], including a 2D sequence; the assignments were compared with the literature data.

Extract Preparation

Maceration followed by percolation

The whole plant of *E. alba* was air dried and ground to obtain a coarse powder. Approximately 10 g of the whole plant was extracted using 50 mL of ethanol: water (3:7) via maceration for 2 hours at temperature of 80°C. The powder was then kept for percolation until the percolate was almost colorless. The extracts were combined and evaporated to dryness using rotary evaporator at 40°C under vacuum to obtain a dark green sticky mass.

Soxhlet extraction

The coarse powder of whole plant of *E. alba* (10 g) was treated with methanol in a Soxhlet extractor for approximately 24 hours. The methanolic extract was concentrated under reduced pressure to obtain a brown sticky mass.

*E. alba* was separated in leaves, roots and stems; each part of the plant was air dried and ground to obtain a coarse powder. Each part of the plant (10 g) was Soxhlet extracted with methanol for approximately 24 hours. Each methanolic extract was concentrated under reduced pressure to obtain a brown sticky mass.

Ethyl acetate extract

Using the same procedure described above, the solvent was removed, and the residue was suspended in water (10 mL) before being heated on a steam bath at 80°C for 30 min. After filtration through Celite, the aqueous solution was partitioned with ethyl acetate. The organic phase was dried and filtered before the solvent was evaporated to yield a light brown powder.

HPLC analysis and system

To quantify the WL and DWL, the *E. alba* extracts were diluted with methanol to 10 mL, and all extracts were analyzed under the same chromatographic conditions. The solution was filtered through a 0.45 μm syringe filter before the HPLC analysis and injected in triplicate. The recovery was determined as follows: recovery (%) = \((A - B) / C \times 100\%\) where, \(A\) is the amount detected, \(B\) is the amount of sample without added standard; \(C\) is the amount of standard added. The relative standard deviations (RSD) of the recoveries were 2.4 (n= 5; mean = 98.0) for WL and 2.1 (n= 5; mean = 98.3) for DWL. HPLC was performed using a Shimadzu LC-10AD pump system equipped with a Shimadzu SPD-M10A Photodiode array detector and the detection wavelength set at 351 nm. The best separation was obtained using a reversed-phase column ([Supelco RP-C18 4.6 mm x 150 mm – particle size 5 μm) at 1.0 mL/min with an A: B solvent system ([A-acetonitrile with 1% of H₃PO₄ (solution 0.03 mol L⁻¹); B-water with 1% H₃PO₄, (solution 0.03 mol L⁻¹) with detection at 351 nm and 20 μL injections. To prepare standards solutions, accurately weigh amounts of WL (14.0 mg) and DWL (7.0 mg) isopentane and chromatographed. Each extract was dissolved in methanol (10 mL). Standard solutions were injected (2, 4, 6, 8, 10, and 20 μL, respectively) and run to generate calibration curves. Each standard was injected in triplicate, and an average of the areas was calculated for each concentration of the calibration curve (linearity range 0.14 - 1.40 μg mL⁻¹). For WL and 0.07 - 0.70 mg mL⁻¹ for DWL). The calibration graphs were plotted for a linear regression analysis of the peak area versus concentration. For WL, the regression equation of this curve and its coefficients of determination (R²) were calculated as follows: \(Y = 3E-8X - 0.003 (R² = 0.9999)\); limit of quantification 0.51 μg mL⁻¹; limit of detection 0.15 μg mL⁻¹; relative standard deviations (RSD) less than 2.0 %. For DWL, the regression equation of this curve and its coefficients of determination (R²) were as follows: \(Y = 3E-8X - 0.001 (R² = 0.9999)\); limit of quantification 0.16 μg mL⁻¹; limit of detection 0.048 μg mL⁻¹; relative standard deviations (RSD) less than 2.0 %. Regarding the extraction efficiency, repeating the work-up three times seemed sufficient because a 98.50 % extraction of WL and DWL was obtained.

Apparatus

The melting points were determined using a melting point apparatus (Microquimica MQAPP-301), and the NMR spectra were recorded using a Bruker DRX 500 [500 MHz (H) and 125 MHz (¹³C)] spectrometer. The chemical shifts were recorded (ppm) relative to the residual solvent peak (2.49 and 39.5 ppm). Multiple-pulse experiments (COSY and HMBC) were performed on a Bruker Avance DRX-500 using the Bruker standard micro programs.

RESULT AND DISCUSSION

*E. alba* is reportedly the best drug for treating liver cirrhosis and infective hepatitis; wodelolactone and demethylwodelolactone are considered as the active principles responsible for the use of these drugs in liver disorders [6]. The use of various parts of the plant, methods of preparation for the extracts and solvents and analytical methods have been described in the literature for preparing extracts with a hepatoprotective effect [2]. Several reports describe methods for preparing these extracts [6, 10, 12, 16]; no studies have determined the amount of active principles responsible for the use of these extracts in liver disorders. In this study, a method to determine the amount of the active principles in *E. alba* extract phytotherapics produced in the Northeast of Brazil was developed to standardize the use of these plant extracts. The usual forms of *E. alba* are aqueous ethanolic extracts (1:1), tinctures, decoctions and the powdered plant in juice or honey [2]. To evaluate the efficiency of different extraction methods for WL and DWL from *E. alba* and to prepare a standardized extract of whole *E. alba* with a high concentration of coumestans, we compared the methanolic extracts produced using Soxhlet extraction with material produced by maceration followed by percolation using water: ethanol (7:3). WL and DWL were isolated using the procedure described by Zafar and Sagar [14]. Pure WL and DWL were used as the standards for further research work. The quantitative analysis of the WL and DWL by HPLC was performed using a procedure described by Wagner et al., [6], and a calibration curve was generated with an external standard. WL and DW were identified in the extracts by comparing the retention time and UV curve with data from the standards (Figures 2 and 3).

To optimize the different extraction conditions of WL obtained from *E. alba*, Savita and Prakashchandra [12] showed that the highest percentage of WL was found in an extract prepared through maceration in methanol with ethanol. In present work, a methanolic extract was prepared using this method with various parts of *E. alba*. The quantitative HPLC analyses of the methanolic extract obtained via Soxhlet extraction showed the presence of two coumestans (WL and DWL) in all of the analyzed extracts (table 1).
Table 1: Coumestan content in the methanol Soxhlet extracts of *Eclipta alba*

<table>
<thead>
<tr>
<th>Plant parts</th>
<th>% WL*</th>
<th>SD**</th>
<th>% DWL*</th>
<th>SD**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roots</td>
<td>0.001</td>
<td>± 0.001</td>
<td>0.003</td>
<td>± 0.001</td>
</tr>
<tr>
<td>Stems</td>
<td>0.055</td>
<td>± 0.004</td>
<td>0.395</td>
<td>± 0.176</td>
</tr>
<tr>
<td>Leaves</td>
<td>1.152</td>
<td>± 0.059</td>
<td>0.021</td>
<td>± 0.003</td>
</tr>
</tbody>
</table>

* % w/w of dry part of plant, ** SD standard deviation

The highest WL concentration was found in the *E. alba* leaves (1.152 % w/w of dry leaf) followed by the stems (0.055 % w/w of dry stem) and roots (0.001 % w/w of dry root). The highest concentrations of DWL were found in the stems (0.395 % w/w of dry stem), followed by the leaves (0.021 % w/w of dry leaf) and roots (0.003 % w/w of dry root). The percentage of WL obtained from the *E. alba* leaves are higher than the results described by Savita and Prakashchandra [12] for whole plant. The data obtained by HPLC are presented in Table 2 and Figure 4.

Table 2: Coumestan content of the dried whole plant using different solvents

<table>
<thead>
<tr>
<th>Extract</th>
<th>% WL*</th>
<th>SD</th>
<th>% DWL*</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>0.150</td>
<td>± 0.038</td>
<td>0.080</td>
<td>± 0.005</td>
</tr>
<tr>
<td>Methanol</td>
<td>0.233</td>
<td>± 0.023</td>
<td>0.159</td>
<td>± 0.011</td>
</tr>
<tr>
<td>Water: ethanol (7:3)</td>
<td>0.271</td>
<td>± 0.002</td>
<td>0.184</td>
<td>± 0.006</td>
</tr>
</tbody>
</table>

* % w/w of dry part of plant, ** SD standard deviation

The data obtained by HPLC are presented in Table 2 and Figure 4.

Fig. 2: HPLC chromatograms of standard wedelolactone (WL) at 351 nm and the UV spectrum of wedelolactone (WL).

Fig. 3: HPLC chromatograms of standard demethylwedelolactone (DWL) at 351 nm and UV spectrum of demethylwedelolactone (DWL).

Fig. 4: HPLC chromatogram of the methanolic extracts of *Eclipta alba* roots at 351 nm.
With the results obtained in table 2 and the isolation method used to obtain pure coumestans, the leaves were the best source of WL, while the stems were the best for DWL. The extract contains a large amount of one coumestan relative to the other, facilitating the final preparative TLC process.

A quantitative analyses by HPLC (table 2) for the water: ethanol extract obtained after a second sequence of extractions showed the highest concentration of WL (0.271 % w/w of dry whole plant) and DWL (0.184 % w/w of dry whole plant) relative to the Soxhlet extraction in methanol (WL (0.233 % w/w of dry whole plant) and DWL (0.159 % w/w of dry whole plant)). These results differed from the work of Savita and Prakashchandra [12]; they estimated the wadeolactone content to be 0.38% w/w for maceration followed by percolation with methanol, and approximately 0.49% w/w for Soxhlet extraction with methanol after extracting whole E. alba. The differences in the results could be explained by the differences in the locations where the plants were grown. In conclusion, using solvent water: ethanol (7:3) with maceration followed by percolation produced better results than the same method using methanol. The quantitative HPLC analyses of the methanolic extract of the whole E alba showed highest concentration. Therefore, partitioning with ethyl acetate led to a large loss of the coumestans.

Pure coumestans (wadeolactone and demethylwadeolactone) were isolated and characterized from the ethyl acetate-soluble fraction of Eclipta alba. The isolated WL and DWL samples were used as standards, and a quantification method using HPLC was developed and validated. The HPLC analyses revealed the presence of the two coumestans in all of the analyzed extracts; the highest concentrations of WL and DWL are present in the leaves and stems, respectively, when analyzing the methanolic E. alba extract. Similar to previous reports, the content of these metabolites appeared very low in the roots [14]. Therefore, the yield of WL and DWL for E. alba grown in Northeastern Brazil agrees with those detected in previous works using the same species grown in India [12]. The HPLC method was very effective for determining the coumestans present in various plant parts and obtaining a standardized hydroethanolic extract of the whole plant. This study should help to develop standardized extracts prepared from various plants grown in northeastern Brazil [17].

CONCLUSION

Pure coumestans (wadeolactone and demethylwadeolactone) were isolated and characterized from the ethyl acetate-soluble fraction of Eclipta alba. The isolated WL and DWL samples were used as standards, and a quantification method using HPLC was developed and validated. The HPLC analyses revealed the presence of the two coumestans in all of the analyzed extracts; the highest concentrations of WL and DWL are present in the leaves and stems, respectively, when analyzing the methanolic E. alba extract. Similar to previous reports, the content of these metabolites appeared very low in the roots [14]. Therefore, the yield of WL and DWL for E. alba grown in Northeastern Brazil agrees with those detected in previous works using the same species grown in India [12]. The HPLC method was very effective for determining the coumestans present in various plant parts and obtaining a standardized hydroethanolic extract of the whole plant. This study should help to develop standardized extracts prepared from various plants grown in northeastern Brazil [17].

ACKNOWLEDGEMENT

The authors are grateful to the following: CNPq, CAPES, FUNCAP and BNB (Brazilian agencies) for financial support and research fellowships (CNPq); CENAUREMN / UFC for providing the NMR spectra; and Herbarium Prisco Bezerra-UFC for plant authentication.

CONFLICT OF INTEREST

There are no conflicts of interest to declare

REFERENCES