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Antioxidant Studies and Determination of Wedelolactone in *Eclipta alba*

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Abstract: The present research reports the results of the phytochemical studies carried out to identify the diagnostic features of the *E. alba*. A high performance thin layer chromatographic method was standardized to determine the wedelolactone content in whole plant of *E. alba*. Methanolic extracts of samples from three different sources were used for analysis. The mean assay of wedelolactone was of range 0.481-0.702 mg g⁻¹ of drug powder. Radical scavenging activity of methanolic extract was evaluated by DPPH assay method, superoxide radical scavenging activity in riboflavin/light/NBT system and nitric oxide radical scavenging activity in sodium nitroprusside/Griess reagent system. The assay results indicate that the DPPH, superoxide and nitric oxide scavenging activity were intense (19.25, 39.25 and 58.26 µg mL⁻¹, respectively). The phytochemical features identified in the present study can be used as identification markers of this important analgesic agent.

Key words: *Eclipta alba*, wedelolactone, antioxidant activity, Bhrrngarajah, HPTLC

INTRODUCTION

Eclipta alba Hassk (Asteraceae) is a small genus of herbs distributed in the tropical regions of the world. It is an erect or prostrate, much branched herb with white flowers and found in almost all parts of India. It is commonly known as Trailing eclipta and Bhrrngarajah. The plant has a bitter, hot, sharp, dry taste and is used in Ayurveda (a primary health care system of India), for the treatment of vitiated conditions of Kapha and Vata. Traditionally, it is extensively used against jaundice, in treatment for night blindness, headache and diseases pertaining to hair and its growth. It is also considered as a rejuvenator (Sivarajan and Balachandran, 1994). Ethanolic extract and alkaloidal fraction of *E. alba* was found to be analgesic (Mahesh *et al.*, 2004). It has antimyotoxic, antihaemorrhagic, immunomodulatory properties and potent antihepatotoxic activity (Melo *et al.*, 1994; Jayathirtha and Mishra, 2004; Singh *et al.*, 2001; Hunda *et al.*, 1984). Aqueous extract of the plant possesses myocardial depressant and hypotensive effect unrelated to cholinergic and histaminergic effect in experimental animals (Gupta *et al.*, 1976). Alcoholic extract of the plant was found to be an antibacterial agent against *Echeria coli* and *Staphylococcus aureus* (Kurup, 1956). Important chemical constituents of the plant include wedelolactone, desmethylwedelolactone, β-amyryn, ecliptine, ecliptal, α-terthienylmethanol, stigmasterol, 2-formyl-α-terthenyl and luteolin-7-0-glucoside (Govindachari *et al.*, 1956; Krishnaswamy and Prasanna, 1970; Krishnaswamy *et al.*, 1966; Rajpal, 2002).

Pharmacological screening of various extracts of *E. alba* revealed that wedelolactone is immunomodulatory, antimyotoxic, antiphlogistic, antihaemorrhagic and antihepatotoxic (Melo *et al.*, 1994; Wong *et al.*, 1988; Jayathirtha and Mishra, 2004). It also suppresses LPS-induced caspase-11 expression by directly inhibiting the IKK complex (Kabori *et al.*, 2004).

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The present study, we describe an analytical methodology for the HPTLC analysis (fingerprint and densitometry) of the wedelolactone in the *E. alba*. HPLTC fingerprint analysis may be a powerful tool for the quality control of raw plant material. The methanolic extract of *E. alba* was also evaluated for its free radical scavenging property using different *in vitro* models.

MATERIALS AND METHODS

Plant Material

The whole plant materials of *E. alba* were collected from National Gene Bank (Anoli, Kerala, India), Herb garden (Arya Vaidya Sala, Kottakkal, Kerala) and from the Calicut University Campus (Calicut University, Kerala). The specimens were identified with the help of available literature and Department of Botany, CMPR, Kottakkal, India confirmed the identity. The voucher specimens of the same are preserved in the herbarium of CMPR.

Extraction

For Preliminary Phytochemical Studies

All the three samples were pooled together and 100 g of the pooled sample was extracted in methanol for 8 h and filtered and the solvent was evaporated under reduced pressure. The residue obtained was dissolved in 25 mL methanol. The resulting solution was subjected to preliminary phytochemical tests for the detection of major chemical groups.

For Estimation of Wedelolactone and Screening of Antioxidant Property

Fresh material was extracted with methanol (3×100 mL) using a reflux condenser for 8-10 h. The methanol extract was completely evaporated at 40°C under vacuum using a rotary evaporator. The residue was dissolved in 25 methanol.

Standard Solution

Stock solution of wedelolactone (Sigma-Aldrich, Germany) was prepared in methanol (analytical grade; Merck; Germany) at 500 µg mL⁻¹.

HPTLC Analysis

Chromatographic analyses of the extracts were performed on silica gel 60 F₂₅₄ HPTLC plates (10×10 cm; Merck, Darmstadt, Germany). Aliquots (3 µL) of extracts were applied on the plates as bands using a CAMAG Linomat V sample applicator. Each 10 mm-wide band was separated from its neighboring bands by a distance of 10 mm. The step volume was 5 µL, applied at a rate of 10 s µL⁻¹, with 10 sec intervals between applications. Plates were developed in a TLC chamber previously saturated (1 h) using Toluene: Acetone: Formic acid (11:6:1v/v) as the mobile phase. The development length was 80 mm (development time approximately 30 min). After development, plates were dried and derivatisation was carried out by immersion of the plates in anisaldehyde sulphuric acid reagent.

Densitometric Evaluation of HPTLC Plates

Detection and quantification was performed with a CAMAG TLC Scanner 3 at 365 nm under the following conditions: Scanning mode, re-emission-fluorescence (mercury lamp); measurement wavelength, 300 nm (emission cut-off filter 550 nm); positive signal; slit width, 0.04 mm; slit height, 6.0 mm; band optimization mode; resolution, 0.025 mm; number of measurements per position, 32; signal factor, 15. Peak area measurement was utilized.

Method Validation

In order to check the repeatability of the method, samples in triplicate were extracted and analyzed by maintaining the HPTLC conditions mentioned above. 100 $\mu\text{g mL}^{-1}$ of standard solution was applied five times on TLC plates and were analyzed to evaluate the reproducibility of the proposed method. Calibration graphs were recorded with sample amount ranging from 1-20 μg ($R = 0.991$).

Free Radical Scavenging Activity

Assay for Antiradical Activity

Antiradical activity was measured by a decrease in absorbance at 516 nm of methanolic solution of colored DPPH (Vani *et al.*, 1997). A stock solution of DPPH (1.3 mg mL^{-1} methanol) was prepared such that 75 μL of it in 3 mL methanol gave an initial absorbance of 0.9. This stock solution was used to measure the antiradical activity. Decrease in absorbance in the presence of test solution of different concentrations was noted after 15 min. EC_{50} was calculated from % inhibition. BHA and wedelolactone was used as positive control.

Super Oxide Radical Scavenging Activity

Superoxide radical generated from the photo reduction of riboflavin was detected by NBT reduction method (Beauchamp and Fridovich, 1971). The reaction mixture contained EDTA (6 mM) containing 3 μg NaCN; riboflavin (2 μM); NBT (50 μM); KH_2PO_4 - NaHPO_4 buffer (67 mM, pH 7.8) and various concentrations of the extract in a final volume of 3 mL. The tubes were illuminated under incandescent lamp for 15 min. The optical density at 530 nm was measured before and after illumination. The inhibition of superoxide radical generation was determined by comparing the absorbance values of the control with that of the treatments. Quercetin and wedelolactone were used as positive control.

Assay of Nitric Oxide Radical Scavenging Activity

Nitric oxide generated from sodium nitroprusside was measured by the Griess reagent by the method of Marcocci *et al.* (1994). Various concentrations of the extract and sodium nitroprusside (5 mM) in PBS in a final volume of 3 mL were incubated at 25°C for 150 min. After incubation, samples (0.5 mL) were removed and diluted with 0.5 mL of Griess reagent (1% sulphanamide, 2% o-phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride). The absorbance of the chromophore formed was read at 546 nm. The inhibition of nitric oxide generation was estimated by comparing the absorbance values of control with that of treatments. Quercetin and wedelolactone were used as positive control.

RESULTS AND DISCUSSION

E. alba is a widely used drug in various classical and herbal formulations. Preliminary phytochemical testing showed the presence of high amount of phenolics together with flavonoids. As wedelolactone, the bioactive marker of *E. alba*, is highly desired by the herbal drug industry for qualitative evaluation and also for making new chemical entities, a HPTLC based search has been under taken. Preliminary TLC studies revealed that the solvent system Toluene: Acetone: Formic acid (11:6:1v/v) was ideal and give a single spot with a R_f 0.39 for the marker and well resolved spots for the test samples. The spots of the chromatogram were visualized in UV-254, UV-365 and by derivatising with anisaldehyde sulphuric acid reagent. Under identical parameters, the fingerprinting patterns of the test samples were recorded. No significant variation was observed in the profiles of the three samples. The marker compound was found to be at R_f 0.39. The fingerprinting pattern of the *E. alba* collected from National Gene Bank, is given in Fig. 1. The three dimensional patterns of test

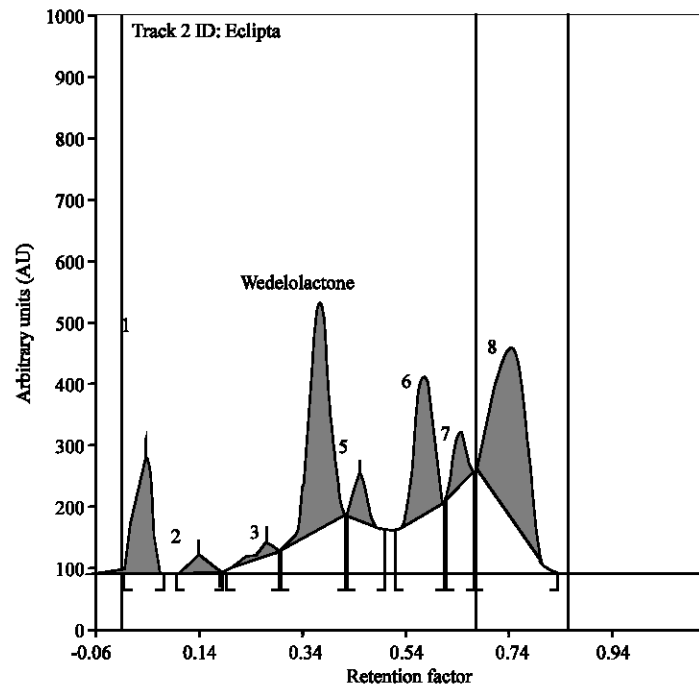


Fig. 1: TLC chromatogram of methanolic extract of whole plant of *E. alba*

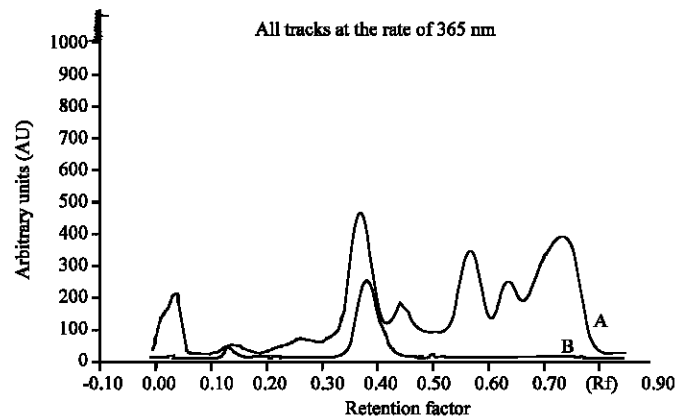


Fig. 2: TLC densitometric scan at 365 nm (A) Test solution of *E. alba* (B) Wedelolactone standard

sample revealed that the peak corresponding to Rf 0.39 is super imposable in all the samples. The spectrum characteristics corresponding to this peak were also found to be exactly matching, indicating that the compound corresponding to Rf 0.39 of the standard and test samples is identical. Linearity of the calibration curve was achieved between 1-10. The correlation coefficient for a calibration curve between 1-10 was found to be 0.991. The percentage of bioactive marker was determined with the help of calculation mode by using the peak area parameter (Fig. 2). Mean assay of wedelolactone was determined to be in the range -0.481-0.702 mg g⁻¹.

Methanol extract of *E. alba* showed antiradical, superoxide radical and nitric oxide radical scavenging activities. Concentration required for 50% inhibition of DPPH radical, superoxide and nitric

oxide scavenging activities were 19.25, 39.25 and 58.26 $\mu\text{g mL}^{-1}$, respectively (Table 1-3). The Superoxide, being highly reactive oxygen species, has been implicated in pathophysiology of various clinical disorders, including ischemia, reperfusion injury, atherosclerosis, acute hypertension, haemorrhagic shock, diabetes mellitus and cancer. Also nitric oxide is implicated in inflammation, cancer and other pathological conditions (Ajith and Janardhanan, 2002). The significant *in vitro* antioxidant activity of *E. alba* was in a concentration dependent manner. Interestingly, the scavenging activities of wedelolactone was found to be less, compared to the other positive controls and the extract. The significant scavenging activity exhibited by the methanolic extract of *E. alba* is not due to wedelolactone. The activity can be attributed to the presence of other phenolic compounds and flavonoids present in the plant (Toda *et al.*, 1991; Faure and Torres, 1991). It is known that crude extracts from plants are more active pharmacologically than their isolated active principle due to the synergistic effects of various compounds present in the extracts (Hamburger and Hostettman, 1991). The significant antioxidant activity of the methanolic extract, thus suggests the therapeutic value of this endemic plant. The significant hepatoprotective effect exhibited by the plant may probably be mediated through its significant antioxidant activity (Ajith and Janardhanan, 2002).

In conclusion, from the present investigation, using *in vitro* models, methanolic extract of *E. alba* was found to scavenge superoxide and nitric oxide radicals. The preliminary chemical examination of methanol extract shows presence of phenolic constituents and flavonoids. The antioxidant activity of *E. alba* can be attributed to the presence of these compounds. The HPTLC fingerprint profile of the methanolic extract was established to characterize the extract showing antioxidant properties. The simplicity of HPTLC in the sample preparation step and the possibility of simultaneously analyzing several samples in less time have made HPTLC a better choice in routine fingerprint analyses. A sensitive HPTLC method was developed for the estimation of wedelolactone. The experimental conditions presented here should be valuable with respect to identification and quantification of wedelolactone.

Table 1: Antiradical activity of methanolic extract of *E. alba*

Sample	Conc. ($\mu\text{g mL}^{-1}$)	Inhibition (%)	EC ₅₀ ($\mu\text{g mL}^{-1}$)
Methanolic extract	12.5	32.57±0.22	19.25
	25.0	58.23±0.21	
	37.5	68.40±0.42	
	50.0	72.04±1.89	
BHA			2.9
Wedelolactone			36.5

Table 2: Superoxide anion scavenging activity of methanolic extract of *E. alba*

Sample	Conc. ($\mu\text{g mL}^{-1}$)	Inhibition (%)	EC ₅₀ ($\mu\text{g mL}^{-1}$)
Methanolic extract	12.5	22.45±1.52	39.25
	25.0	36.98±4.80	
	37.5	45.01±2.56	
	50.0	62.12±2.87	
Quercetin			13.56
Wedelolactone			106.48

Table 3: Nitric oxide scavenging activity of methanolic extract of *E. alba*

Sample	Conc. ($\mu\text{g mL}^{-1}$)	Inhibition (%)	EC ₅₀ ($\mu\text{g mL}^{-1}$)
Methanolic extract	12.5	12.35±1.56	58.26
	25.0	28.65±0.32	
	37.5	31.05±5.68	
	50.0	42.25±2.27	
	67.5	56.75±7.08	
	75.0	64.35±2.01	
Quercetin			64.00
Wedelolactone			62.48

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