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Research Article

ISOLATION, PURIFICATION AND CHARACTERIZATION OF BOERAVINONE B FROM *BOERHAAVIA DIFFUSA* LINN.

Praveen Kumar Posa Krishnamoorthy *, Sivanandham Muthukumaran, Akila Maheswaran, Prabhu Sukumaran
Department of Biotechnology, Sri Venkateswara College of Engineering, Pennalur, Sriperumbudur, Tamilnadu, India
*Corresponding Author Email: praveenpk@svce.ac.in

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ABSTRACT

Boerhavia diffusa is a medicinal plant used in treatment of autoimmune diseases, cancers and inflammation. In the present study, methanolic extraction of roots of *Boerhaavia diffusa* was carried out and presence of Boeravinone B was identified from Thin Layer Chromatography and reverse phase HPLC. The methanolic root extract was run in TLC containing the mobile phase as toluene: ethyl acetate: methanol in the ratio (7:1:2). Retention factor for Boeravinone B in *B.diffusa* extract was found to be 0.461. RP-HPLC was carried out from the mobile phase consisting of acetonitrile and water at the ratio of 50:50 at 35 °C with a flow rate of 1.0 mL/min in C18 reverse phase column. Retention time of Boeravinone B was found to be 13.8 min. Linearity range of the concentration of extracted Boeravinone B was 5 – 120 µg/ml and the correlation coefficient (R^2) value was found to be 0.997±0.003. Recovery was found to be 90.6% - 92.8%. The limit of detection (LOD) of Boeravinone B was found to be 2 µg/ml and limit of quantitation (LOQ) was found to be 5 µg/ml. Presence of functional groups such as phenyl groups at 1450.47 cm^{-1}, 1591.27 cm^{-1}, C=C (Carbon skeleton) at 1618.28 cm^{-1} and Isoflavonoid structure at 1649.14 cm^{-1} of absorption spectra of isolated Boeravinone B were characterized by FTIR spectroscopy through recording absorption spectra. Thus, a rapid, economical and viable method was developed for isolation, purification and characterization of Boeravinone B.

Keywords: Reverse Phase, High Performance Liquid Chromatography, Boeravinone B, Fourier Transform Infra-Red.

INTRODUCTION

India, being one of the world's 12 mega biodiversity countries, enjoys export of herbal raw material worth of U.S $100-114 million per year approximately[1]. *Boerhaavia diffusa* (Nyctaginaceae family) is an herbaceous perennial medicinal plant, native of India and Brazil[2]. This plant is commonly known as red hogweed, tar vine, red spiderling, wineflower. Roots and leaves of this plant are used by many tribes in India for their anti-diabetic[3], analgesic[4], diuretic[5], immunomodulatory[6,7,8], anti-oxidant[9], hepatoprotective[10] and anti-lymphoproliferative[11] properties. The root of *B.diffusa* contains alkaloids (punarnavine), rotenoids (boeravinones A-O), aminoacids, flavonoids, esacosanoic, stearic, β-sitosterols, tetracosanoic and ursolic acids[9,12]. In earlier study, phytochemical characterization of aqueous and alcoholic extracts of *Boerhaavia diffusa* roots are carried out and identified presence of compounds like flavonoids, saponins, proteins, carbohydrates, phenols, alkaloids, glycosides and isoflavonoids[13].

Many Boeravinones (Rotenoids) have been isolated from the roots of *B.diffusa*. Pharmacological potential of *B.diffusa* were widely studied in boeravinones. Boeravinone E binds the GM-CSF and TNF-α receptors stimulates the growth and differentiation of hematopoietic precursor cells from various lineages, including dendritic cells, granulocytes, macrophages, eosinophils and erythrocytes[4].

Autoimmune disorders are emerging non-communicable diseases and there are more than 80 different types of autoimmune disorders affecting approximately 100 million people worldwide[15]. Boeravinone B (6a, 12a – Dehydro- 6,9,11 – trihydroxy-10- methylrotenone), a bioactive marker compound from *Boerhaavia diffusa* has therapeutic effect for treating autoimmune diseases like rheumatoid arthritis, osteoarthritis, acute myoskeletal disorders, spondylosis, tendonitis, atherosclerosis, systemic lupus erythematosus and psoriasis in mammals[16]. Boeravinone B is present in higher plasma level in a lipid based formulation of rotenoid-rich fraction of phosphatidyl choline as compared with rotenoid rich fraction demonstrated an increased anti-inflammatory potential[17].

In the present study, a simple process was developed and is particularly useful for quick isolation and purification of the Boeravinone B compound after extraction and characterization from the *B.diffusa* plant roots.

MATERIALS AND METHODS

Extraction and characterization of *Boerhaavia diffusa* roots

The roots of *Boerhaavia diffusa* shown in Figure 1 was collected from Irula Tribal Women's Welfare Society, Chengalpet and was identified, authenticated from Madras Christian College, Chennai. 20 grams of powdered roots was kept in chamber with 200 ml of solvents in a round bottom flask and refluxed for about 6 hours at 65 °C using soxhlet apparatus. The solvents used were methanol, ethanol and water for extraction. The excess solvent in the extract was removed by keeping it in hot air oven with controlled temperature of 50 °C. The extract was completely dried in a vacuum tray drier and dried extract samples were kept in an airtight container at 4 °C. Confirmation

of Isoflavonoids like Boeravinones are detected as yellow or red color through treatment of *B.diffusa* extract with sodium hydroxide solution[13]. Adding to the above solution turns colourless indicates the presence of isoflavonoids.

TLC analysis of Boeravinone B

Thin layer chromatography was used to identify the presence of Boeravinone B shown in Figure 2 from the other compounds present in the extract using silica gel loaded TLC plates. The coplin jar was equilibrated with mobile phase of Toluene: Ethyl Acetate: Methanol in the ratio (7:1:2) and left undisturbed for 30 minutes. 20 µl of methanolic extract of *B.diffusa* and the standard Boeravinone B (95% HPLC pure) compound purchased from Natural Remedy, Bengaluru at 1 mg/ml concentration was loaded onto the TLC plate. The TLC plate was dried in a hot air oven for 10 minutes after 15 minutes of run time. The plate was then sprayed with 1% HI in dilute H_2SO_4 followed by drying to be visualized.

RP-HPLC analysis of Boeravinone B

TLC separated Boeravinone B compound was quantified using HPLC (Younglin Acme 9000, Korea) on reverse phase C18 column using mobile phase of HPLC grade Acetonitrile (50 %) and High purity water (50 %) from Milli-Q water purification system in an isocratic elution mode with the flow rate of 1 ml/min[18]. Samples for HPLC analysis was filtered through a 0.45µm membrane filter purchased from Pal Life sciences. HPLC analysis was performed with the plant extract compared with Boeravinone B (95% HPLC pure) compound, after filtering through 0.45 µm membrane filter. The HPLC chromatogram peaks were monitored at 270 nm using UV-visible detector through Autochro-3000 software and the retention time was obtained. The limit of detection (LOD), limit of quantitation (LOQ) and Recovery% values were estimated for Boeravinone B.

To determine the linearity of Boeravinone B, a stock solution of Boeravinone B (1000 µg/ml) was prepared in mobile phase and the linear response was observed over a range of 1-100 µg/ml by RP-HPLC and the calibration curve was plotted. Method precision of experiment was performed by preparing the standard solution of Boeravinone B (50 µg/ml) from the stock solution of Boeravinone B (1000 µg/ml) for three times without changing the parameters of the proposed method. The results were reported in terms of percent relative standard deviation. The intra-day precision of the proposed method was determined and analyzed at three different concentrations (10 µg/ml, 50 µg/ml, 100 µg/ml) on 3 times. The accuracy of the proposed method was determined by calculating the recovery of Boeravinone B by the standard addition method. Known amounts of standard solutions was added at 10%, 50% and 100% w/w level to pre analyzed sample solutions of Boeravinone B.

FTIR analysis of Boeravinone B

FTIR absorption spectrum of purified Boeravinone B compound obtained from TLC followed by HPLC separated fraction of methanolic extract of *B.diffusa* roots was carried out[17]. The above absorption spectra was compared with Boeravinone B standard through FTIR (Shimadzu IR Tracer-100, Japan) utility.

RESULTS AND DISCUSSION

TLC Method development

TLC separation method was developed with mobile phase of Toluene: Ethyl Acetate: Methanol in the ratio of 4:4:2. Similar studies were performed in separation of Boeravinone B[19] with mobile phase of Chloroform: Ethyl Acetate: Methanol in the same ratio of 4:4:2. TLC were performed to identify the presence of Boeravinone B from the other compounds by soxhlet extraction with three different solvent extracts of *Boerhaavia diffusa* roots. Only methanolic extract of *B.diffusa* showed distinct clear layer separation in silica gel plate, as similar studies performed[20]. So methanol extract was chosen for further purification studies. A clear layer was separated and distinguished for both the *B.diffusa* extract and the Boeravinone B standard shown in Figure 3. The retention factor was calculated to be 0.461 for both the sample and standard.

Selection of mobile phase & Validation of HPLC method

The TLC fraction of separated Boeravinone B was analyzed through RP-HPLC column[18]. Several mobile phase compositions were tried to validate RP-HPLC method and to estimate Boeravinone B. Optimization of RP-HPLC chromatographic conditions are shown in Table 1. A satisfactory separation, good peak symmetry, better reproducibility and repeatability of Boeravinone B were obtained with a mobile phase of Acetonitrile: Water (50:50 v/v) at a flow rate of 1.0 mL/min. Quantification was achieved with UV visible detector at 270 nm. Figure 2, Figure 3 represents the HPLC chromatogram of identification of TLC separated Boeravinone B in methanolic extract of *B.diffusa* at 100 µg/ml concentration. Figure 4 represents the HPLC chromatogram of Boeravinone B standard at same concentration. Whereas, Figure 5 represents the HPLC chromatogram of purified Boeravinone B after recovery of same concentration. Retention time of Boeravinone B was found to be at 13.8 minute for all the extract samples and standard. The present study aimed to produce an improved, rapid, validated RP-HPLC for separation of Boeravinone B. The LOD and LOQ was found to be 2 µg/ml and 5 µg/ml for Boeravinone B. The percentage recovery of Boeravinone B from methanolic extract of *B.diffusa* and from its own comparative standard was calculated to be 90-92%. In RP-HPLC method, the linearity range was in between 5-120 µg/ml for Boeravinone B with co-efficient of correlation (R^2) = 0.997±0.003. Table 2 showed summary of validation of linear regression data, Limit of Detection (LOD), Limit of Quantitation (LOQ) and %recovery parameters. Table 3 represents the intraday precision studies of RP-HPLC separation of Boeravinone B.

Table 1: Optimization of RP-HPLC method

Method parameter	Optimized value
Column	C18
Wavelength of detection	270 nm
Mobile phase	Acetonitrile: Water (50: 50 v/v)
Pump mode	Isocratic
Flow rate	1.0 mL/min
Run time	20 minutes
Volume of Injection	20 µL
Temperature	25±2 °C
Retention time	13.8 minutes

Table 2: Summary of the validation parameters

Parameters	Boeravinone B
Linearity range (µg/ml)	5-120
Correlation coefficient (R^2) ± SD	0.997±0.003
Y= mx	Y = 5555.7x
Limit of Detection, LOD (µg/ml)	2
Limit of Quantitation, LOQ (µg/ml)	5
% Recovery	90.6 - 92.8%

Table 3: Precision studies (n=3)

Amount (µg/mL)	Area [mV.s]	Height [mV]	Mean Area±SD	%RSD
10	221.87	6.56	222.29±2.14	0.96
10	224.62	6.5		
10	220.4	6.62		
50	5245.73	25.94	5245.76±5.54	0.1
50	5240.24	25.84		
50	5251.32	26.22		
100	11362.96	291.87	11333.67±77.47	0.68
100	11245.82	296.42		
100	11392.22	288.84		

Figure 1. Roots of *Boerhaavia diffusa*

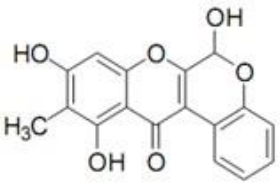

Figure 2. Boeravinone B

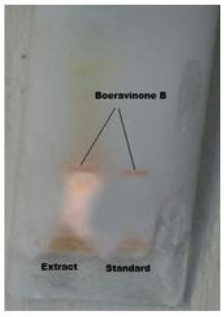

Figure 3. TLCs of methanolic extract of *B.diffusa* and Boeravinone B Standard

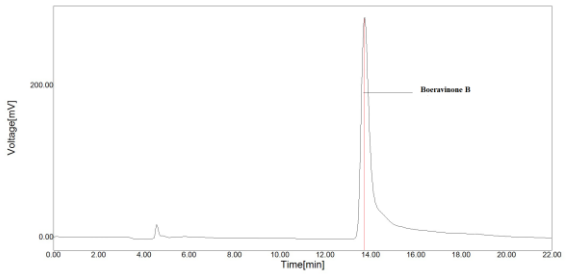

Figure 4. Chromatogram of TLC separated Boeravinone B at 100 µg/ml concentration

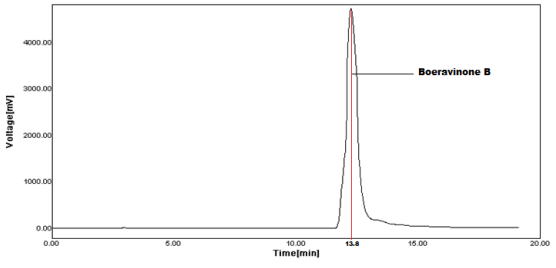

Figure 5. Chromatogram of Boeravinone B standard at 100 µg/ml concentration

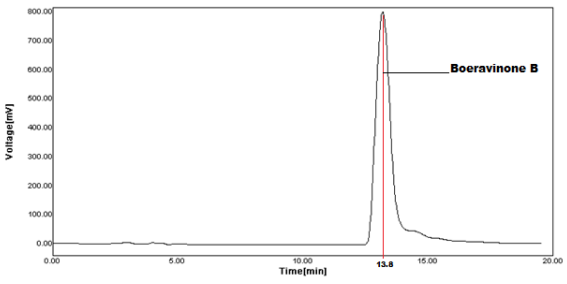

Figure 6. Chromatogram of recovered sample of Boeravinone B

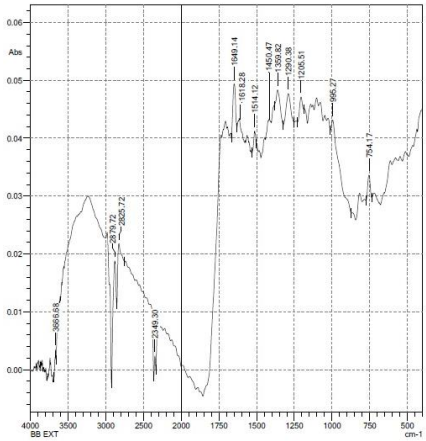

Figure 7. FTIR spectra of TLC separated and Boeravinone B standard

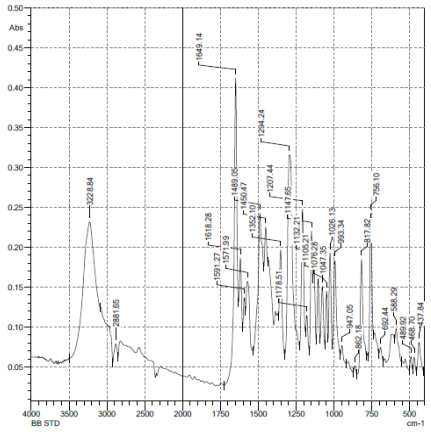

Figure 8. FTIR spectra of obtained HPLC fractionated Boeravinone B

FTIR spectral analysis

FTIR absorption spectra characterization analysis of functional groups are carried out with TLC separated fraction of Boeravinone B from methanolic extract of *B.diffusa*. The spectra obtained was further compared with Boeravinone B standard as similar type of studies were performed in structure determination of Boeravinones D, E and F[21] through FTIR. Also, structure elucidation of other boeravinones like Boeravinone K, L, M, N and O was earlier determined by IR (KBr)[17]. Figure 7 shows the FTIR spectra of Boeravinone B, obtained from TLC separation of methanolic extract of *B.diffusa* roots. Whereas, Figure 8 shows the FTIR spectra of standard Boeravinone B. Boeravinone B compound showed the presence of various functional groups such as phenyl groups at 1450.47 cm^{-1}, 1591.27 cm^{-1}, C=C (Carbon skeleton) at 1618.28 cm^{-1} and Isoflavonoid structure at 1649.14 cm^{-1} of absorption spectra. However, TLC separated fraction of Boeravinone B showed mild peaks of phenyl group at 1450.47 cm^{-1}, C=C (Carbon skeleton) at 1618.28 cm^{-1} and strong peak in Isoflavonoid structure at 1649.14 cm^{-1} of absorption spectra. FTIR results of Boeravinone B extracted and purified from *B.diffusa* showed more or less similar peaks as compared to standard Boeravinone B. FTIR absorption spectra analysis of extracted Boeravinone B from roots of *B.diffusa* compared with standard Boeravinone B showed high significant peaks in isoflavonoid structure at 1649.14 cm^{-1}.

CONCLUSION

A method established for extraction, isolation and purification of Boeravinone B from roots of *B.diffusa*. The method developed utilizes highly economical raw material derived from roots source of *B.diffusa* which is abundantly present in Tamilnadu. The developed and validated RP-HPLC method for Boeravinone B estimation was found in precision and accurate. The simplicity of the method, economical availability and low limit of detection and quantification makes the method superior to the other reported HPLC methods. FTIR results of extracted Boeravinone B showed promising peaks as compared with extracted Boeravinone B from roots of *B.diffusa*. The purified Boeravinone B compound thus obtained can be used as drug or drug adjuvant for application in manufacture of various vaccines for the treatment of autoimmune diseases like asthma, arthritis, anti-inflammatory diseases and solid tumors like breast cancer, prostate cancer, etc.

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