



# Identification and Quantification of Boeravinone-B in Whole Plant Extract of *Boerhaavia diffusa* Linn and in its Polyherbal Formulation

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## Abstract

**Objective:** To ascertain the authenticity of Boeravinone-B in *Boerhaavia diffusa* and its poly-herbal dosage form (capsule) by identification and quantification through precise and robust HPLC method. **Materials and Methods:** The RP-HPLC analysis was accomplished employing phosphate buffer and acetonitrile as mobile phase at a flow rate of 1.5 ml/min and 270 nm as detection wavelength. **Results:** The content of Boeravinone B in ingredients (hydro-alcoholic extracts of whole plant) and poly-herbal formulation of *B. diffusa* was estimated as 0.041% w/w and 0.011% w/w, respectively with good linearity  $R^2 > 0.9997$  and retention time  $24.413 \pm 0.004$  min. **Conclusion:** The amount of Boeravinone-B present in *B. diffusa* extracts of raw drug complies with that of reported in literature. Quantification of Boeravinone-B suggested an identification mark for this drug and its different market formulations and may work as quality parameter in standardization. On the basis of percentage of Boeravinone-B the content of *B. diffusa* in poly-herbal formulation validate the ratio of ingredient in final formulation. This method proved its applicability in quality assessment via quantification of this marker in raw material as well as various phyto-formulations of *B. diffusa* and widens the acceptability criteria at international level.

**Keywords:** *Boerhaavia diffusa* Formulation Analysis, *Boerhaavia diffusa* Whole Plant Extract, Boeravinone-B Quantification, HPLC of *Boerhaavia diffusa*

## 1. Introduction

Due to the lack of scientific validity, the Ayurvedic and classical medicines have poor acceptability in world market. The acceptance of their formulations at global level highly needs evidence-based advancements of Ayurvedic research<sup>1</sup>. Now there is an urgent necessity to validate raw drugs as ingredients and various poly-herbal formulations through incorporation of advanced techniques. In view of this, HPLC is precise, robust

analytical technique for estimation of major bioactive phytoconstituents. *Boerhaavia diffusa* Linn. (*B. diffusa*) commonly known as 'Punarnava or Rakhtpunarnava in ancient medicine system due to its regeneration properties in rainy season<sup>2,3</sup> and classified as "rasayana" because of its therapeutic, chemical and ethanopharmacological values<sup>4</sup>. It is reported that root of *B. diffusa* contains some vital alkaloids, ratenoids, lignans which are named as punarnavine, boeravinones A-F, liriodendrons,

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respectively. Flavonoids, amino acids,  $\beta$ -sitosterols and tetracosanoic, esacosanoic, stearic and ursolic acids were also reported, which has wide applicability against chronic ailments<sup>4,5</sup>. The whole plant and its various parts were used in treatment of enlargement of spleen, cancer, jaundice, dyspepsia, inflammation, abdominal pain and also worked as an anti-stress, tonic and carminative agent<sup>6-10</sup>.

'*Sarangdhar Samhita*' has quoted the polyherbalism, which provide improved therapeutic efficacy by active than the individual plants. Multiple herbs composition in a fixed ratio gives better clinical results and reduce the toxic level<sup>11</sup>.

Phytochemical screening and isolation of marker compounds of this plant has confirmed the important bioactive chemicals (Boerhavin and boerhavic acid) including amino acids and alkaloids<sup>12-15</sup>. Boeravinones and other compounds in roots of *B. diffusa* were quantified and validated by analytical method like UPLC to enhance the quality and efficacy. This has been found selective and sensitive chromatographic method with good linearity, accuracy and precision<sup>22</sup>. Recently a rapid method was developed for quantitative estimation of Boeravinone-B in Punarnava. In such studies HPLC method was reported for determination of vitamins C and B contents in *B. diffusa* and *Commelina nudiflora* leaves<sup>18-20</sup>. The major constituents studied from the *B. diffusa* are punarnavine, boeravinone<sup>21</sup>, hypoxanthine 9-Larabinofuranoside, ursolic acid, punarnavoside, lirodendrin. The seeds have been studied as tonic and possess carminative properties<sup>22</sup>. Boerhadiffusene, boerhavisterol, diffusarotenoid and boerhavanostenyl benzoate and a known rotenoid, boeravinone A were identified from root part of *B. diffusa*<sup>26-28</sup>. Examination of the ether extracts of the roots of *B. diffusa* led to the separation of Boeravinone A, B, C, D, E, F, G and H by novel chromatographic method<sup>23-25</sup>.

Glycosides, flavonoids, tannins and saponins in the aqueous extract of leaves of *B. diffusa* exert hypoglycemic effect, were identified and assayed by means of standard TLC and HPTLC methods<sup>28</sup>. The same method was also used in isolation of Boeravinone-D, G and H. Likewise qualitative and quantitative analysis has also been reported through sophisticated and advance chromatographic technique<sup>29-30</sup>. UPLC was used for

estimation of eight Boeravinones in methanolic extract of *B. diffusa* roots using PDA detector<sup>33</sup>.

The raw drug in market cannot be authenticated on the basis of their morphological or histological characteristics and poor quality drugs are less effective or it may worsen the clinical efficacy. So TLC method was also reported for adulteration analysis of *B. diffusa* with *Trientema portulacastrum* Linn. in different Ayurvedic formulations, so it employed as an effective tool for quality control of market drugs<sup>31,32</sup>. Similarly HPLC, TLC, AAS and HNMR were used in fractions and extract of *T. portulacastrum* and *B. diffusa* to determine important bioactive compounds<sup>34</sup> and it has been found chromatographic methods are simple, rapid and inexpensive in identification and comparison of Boeravinone<sup>38</sup>. Estimation of Boeravinone B and Eupalitin-3-O- $\beta$ -Dgalactopyranosid from whole plant of *B. diffusa* and single formulation (marketed Punarnava capsule) was carried out by HPLC method in concentration range 10-120  $\mu\text{g mL}^{-1}$  and 5-60  $\mu\text{g mL}^{-1}$  respectively<sup>35</sup>. In sequence of modern analytical methods various pharmacologically bioactive chemical compounds i.e.,  $\beta$ -sitosterol, Boeravinone-B and Eupalitin-3-o- $\beta$ -d-galactopyranoside were isolated from methanolic extract of *B. diffusa* Linn., and from single drug formulations have also been reported.

Therefore after the literature review it may be concluded that *B. diffusa* has useful pharmacological activities to cure many ailments through its principal bio active chemical constituents like wise Boeravinone-B also has potent medicinal value. It also suggests that Ayurvedic formulations, ingredients and drugs of different pharmaceuticals may vary in quality, composition and properties due to lack of scientific practices and evidences. To produce effective clinical results there is need of uniform, rapid and precise analytical methods to establish the quality standards for safety and efficacy against drugs of poor quality and adulterants. Literature survey also revealed that such research work has not been published. Hence in this study an optimized RP-HPLC method tried for determination of Boeravinone-B qualitatively and quantitatively in hydro-alcoholic extract of *B. diffusa* whole plant and its processed poly-herbal formulation. It may be useful to set a quality parameter for the standardization of raw drug and various important pharmaceutical formulations of

*B. diffusa*. The recommended method may be useful in cases of *B. diffusa* that are frequently adulterated with other species which are morphologically and chemically indistinguishable.

## 2. Materials and Methods

### 2.1 Materials

#### 2.1.1 Plant Materials (extract)

The plant material in the form of powdered hydro-alcoholic extract of *B. diffusa* (whole plant) was procured from an authentic supplier i.e., M/s Laila Impex (chemiloids), 40-15-14, Brindavan Colony, Labbipet Vijayawada 520010 authenticated vide voucher specimen LIH No. 6562.

#### 2.1.2 Chemicals and Reagents

Boeravinone-B of 97.3% purity was procured from Natural Remedies Pvt. Ltd., Bangalore. HPLC grade acetonitrile and water of same grade were purchased from Rankem Ltd. for the study.

#### 2.1.3 Apparatus

Chromatographic separation was performed on Agilent 1260 infinity high-performance liquid chromatography equipped with PDA detector and EZ Chrome Elite Software was employed for this study. Agilent Zorbax HC-C18 (2) (250×4.6mm, 5 $\mu$  particle size) used as stationary phase.

### 2.2 Methods

#### 2.2.1 Preparation of Plant Material Samples

##### 2.2.1.1 Preparation of Samples for HPLC Analysis

##### 2.2.1.1.1 Batch-I and Batch-II, Ingredient (*B. diffusa* extract)

Sample stock solution was prepared by taking about 250mg procured extract of batch I and II (mentioned in subheading 2.1.1) and dissolved in methanol in 10mL standard flask. Volume was made up to the mark with HPLC grade methanol, sonicated for 10 min than filtered with 0.22 $\mu$  filter to obtain its stock solutions of both batches.

##### 2.2.1.1.1 Poly-Herbal Formulation of Batch-I and Batch-II

20 capsules (formulation composed of *Boerhaavia diffusa*, *Emblica Officinalis*, *Tinospora cordifolia*, *Withania somnifera* *Glycyrrhiza glabra* *Bacopa monnieri*, *Centella asiatica*) were taken, opened and crushed in mortar pestle. From that, weighed about 150 mg powder for batch-I and 290 mg powder for batch-II transferred to 10mL volumetric flask separately. Make up the volume with methanol to prepare standard solution of both batches in standard flask, then applied to sonicated for 10 minutes.

##### 2.2.1.2 Preparation of Stock and Working Solution of Boeravinone-B for HPLC Analysis

An accurately weighed quantity of Boeravinone-B 1.9 mg was taken and dissolved in 2mL methanol in standard flask. From the above stock solution, solution of different concentrations such as, 1.02, 2.02, 2.9 and 4.01  $\mu$ g/mL of methanol were prepared.

#### 2.2.2 Experimental Section for HPLC

The optimized parameters of HPLC method for the determination of Boeravinone-B are mentioned in the Table 1.

**Table 1.** Chromatographic conditions for HPLC of Boeravinone-B.

Instrument HPLC - Agilent Technologies 1260 Infinity with PDA Detector

with EZ Chrome Elite Software

Stationary Phase	Agilent Zorbax HC-C <sub>18</sub> (2) (250×4.6mm, particle size of 5 $\mu$ )
Wavelength	270 nm
Run time	45 min
Flow Rate	1.5 mL/min
Injection Volume	20 $\mu$ L
Temperature	27 °C
Operation Mode	Gradient elution

##### 2.2.2.1 Mobile Phase

The mobile phase consisted of water/aqueous buffer and an organic modifier (miscible with water, acetonitrile was preferred for sharper peak). Based on sample solubility, stability and suitability various mobile phase compositions were tried. Mixtures of aqueous buffer

and acetonitrile by gradient programming (Table 2.) with 0.1% acid was used as optimized mobile phases to separate the samples in desired components at pH-3.00 by O-phosphoric acid was chosen with detection wavelength 270 nm.

HPLC-type of reagents was used to minimize noise level. Freshly prepared mobile phase was used in this method to avoid the risk of contamination.

#### a. Phosphate Buffer

0.140 g of anhydrous  $\text{KH}_2\text{PO}_4$  dissolved in 900 ml of HPLC grade Water & then add 0.5 ml of orthophosphoric acid and make up the volume with water to 1000 ml. Filtration of this solution was carried out by 0.45  $\mu$  membrane filter and degassed in a sonicator for 3 minutes.

#### b. Acetonitrile

**Table 2.** Gradient programming

Time	A Conc. (%)	B Conc. (%)
0.01	95	0.5
18.00	55	45
25.00	20	80
28.00	20	80
35.00	55	45
41.00	95	05
45.01	Stop	

#### 2.2.2.2 Validation of the Method

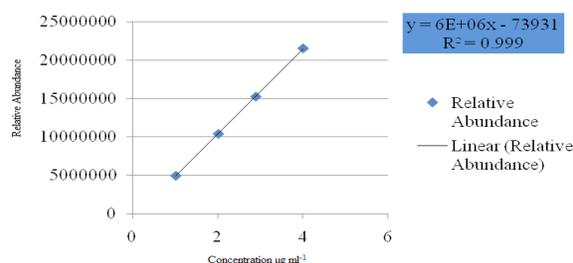
The analytical method was validated for linearity (Table 3.) and system suitability (Table 4),

Statistical calculations were carried out by Microsoft Excel 2007 of Windows software package to calculate Average, Sum, Standard Deviation (STDEV), Regression (RSQ) etc.

**Table 3.** Linearity of Boeravinone-B

For assessing the linearity, the least square regression equation and correlation of coefficient were determined.

Concentration ( $\mu\text{g/mL}$ )	Peak Area	Slope	Intercept	$R^2$
1.02	4946627	5525297.756	73931	0.999
2.02	10387124			
2.9	15211133			
4.01	21474568			



**Figure 1.** Linearity graph of Boeravinone-B for HPLC.

**Table 4.** System suitability of Boeravinone-B

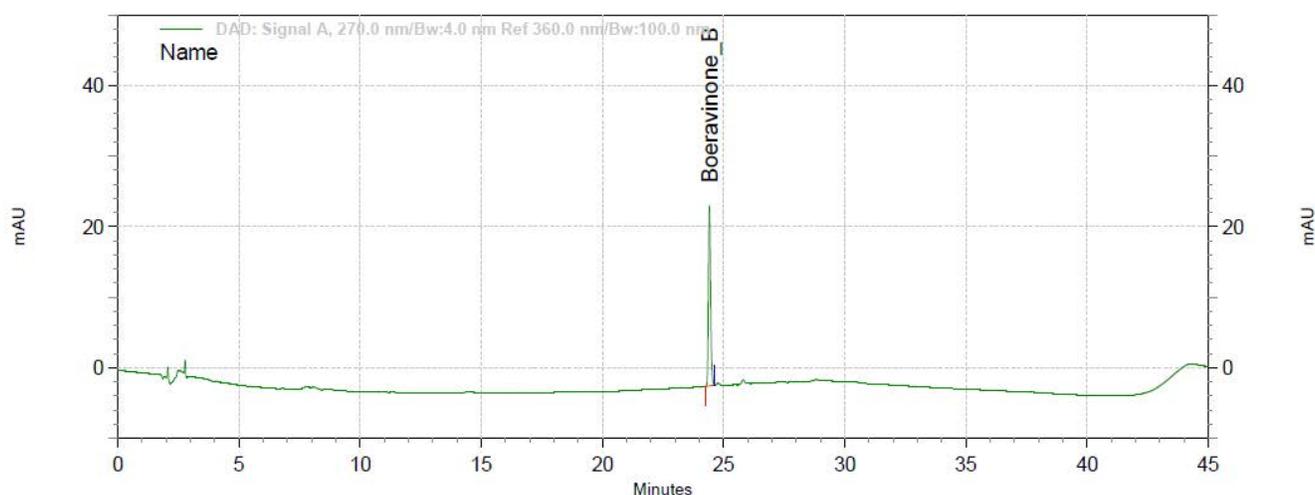
S.No	RT	Area
1	24.413	21583716
2	24.407	21604974
3	24.413	21470020
4	24.420	21482114
5	24.413	21505072
6	24.413	21474568
Average	24.413	21520077.333
STD Deviation	0.004	59161.702
%RSD	0.017	0.275

Samples were scanned in UV 200-400 nm using PDA detector was performed to reveal eventual interfering compounds and evaluating the selectivity of the method. Specificity of this method was performed by comparing the HPLC retention time and absorption spectra of target peaks from the analyzed samples with that of the standard (marker).

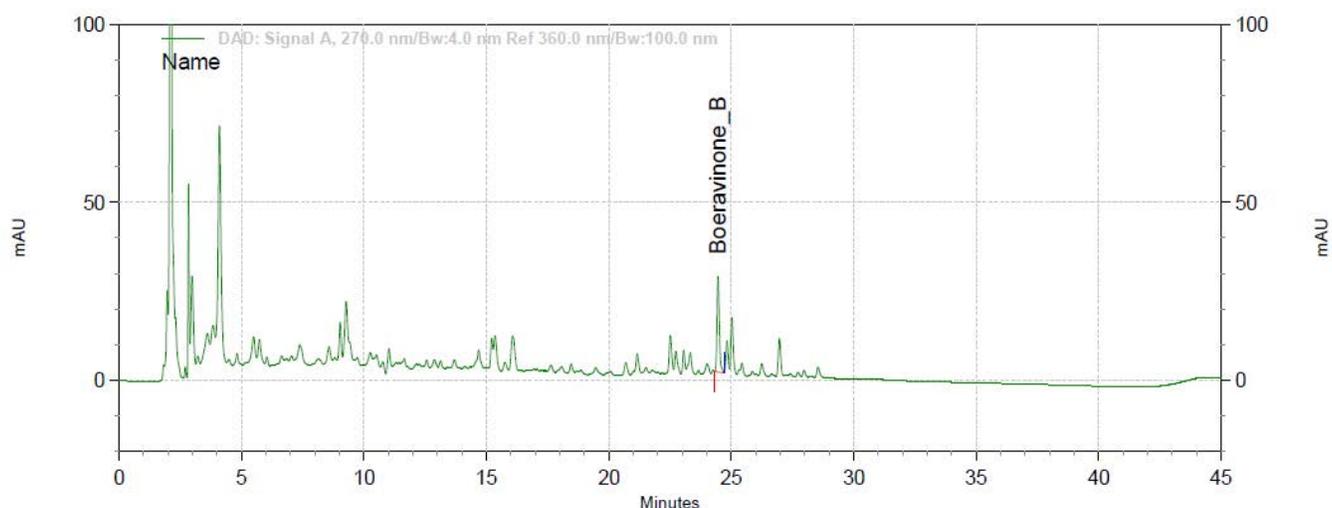
### 3. Results and Discussion

Chromatographic conditions were optimized after running different mobile phases concentrations based on sample solubility, stability & polarity with a reversed phase C18 column on hit and trial basis. A good resolution and sharp peaks of Boeravinone-B in standard, ingredient and poly-herbal formulation were obtained in gradient system in the mixture of phosphate buffer and acetonitrile. The content of Boeravinone-B in formulation and ingredient of *B. diffusa* was analyzed using optimized

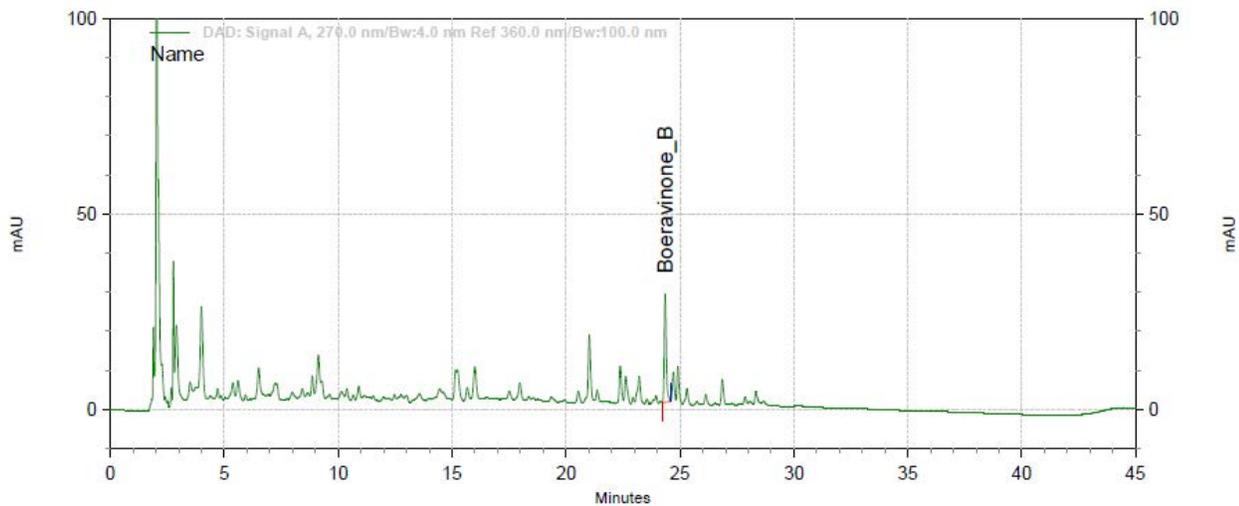
chromatographic method. Detection wavelength selected as 270 nm in an acceptable response and used for estimation of marker compound in this study. Column temperature was maintained at 27°C throughout analysis. The standards of different strengths, extracts of plant material and poly-herbal formulation were injected in the RP-HPLC column and obtained peak areas of Boeravinone-B were used for analysis of content by the regression equation. Well resolved sharp peak of Boeravinone-B obtained at  $R_t 24.413 \pm 0.004$  identified in standard (Figure 2), hydro-alcoholic extract of *B.diffusa* of Batch-I and II



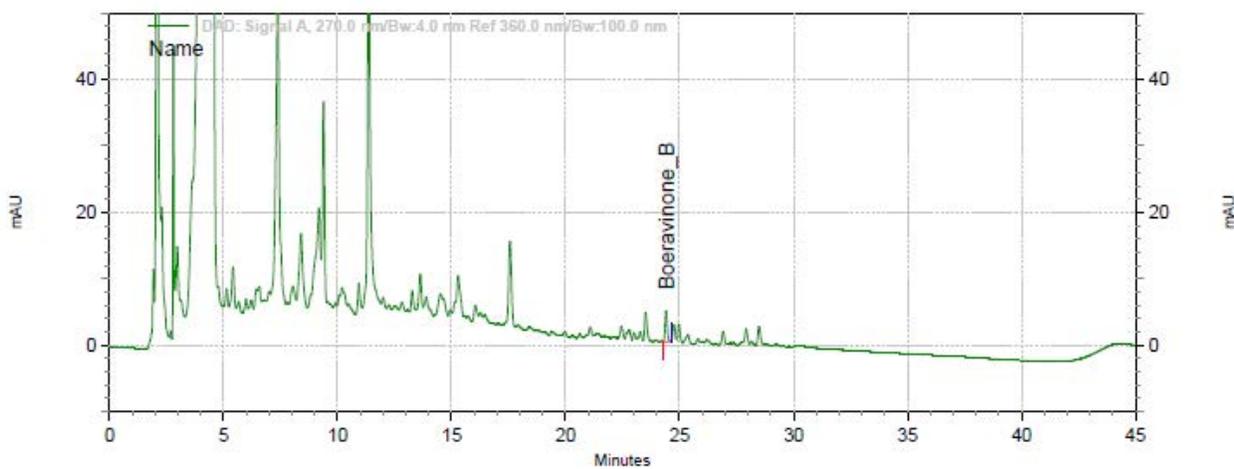
**Figure 2.** Chromatogram of Boeravinone-B standard 4.01 µg/ mL.



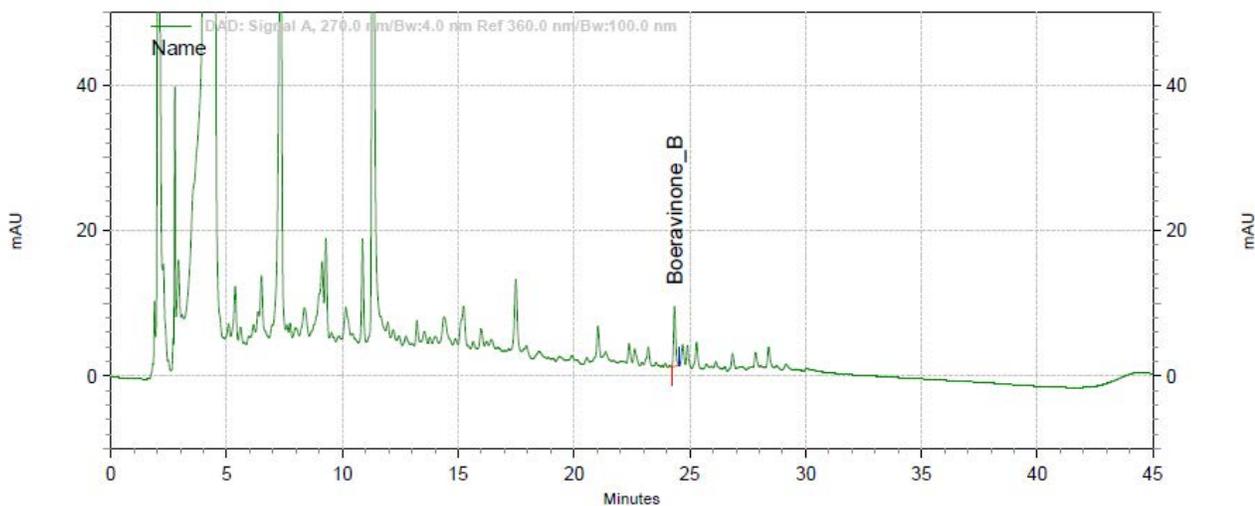
**Figure 3.** Chromatogram of hydro-alcoholic extract of *B.diffusa*: Batch-I.



**Figure 4.** Chromatogram of hydro-alcoholic extract from *B. diffusa*: Batch-II.



**Figure 5.** Chromatogram of poly-herbal formulation: Batch-I.



**Figure 6.** Chromatogram of poly-herbal formulation: Batch-II.

(Figure 3-4) and poly-herbal formulation of batch-I and II (Figure 5, 6).

The graphical study of Figure 3-6 has revealed that peak height in terms of concentration of Boeravinone-B is more in ingredients than formulations. Percentage of Boeravinone B in ingredients (hydro-alcoholic extracts) and poly-herbal formulation was calculated to be 0.041% w/w and 0.011% w/w respectively, this validates the composition ratio of ingredient in formulation i.e., less in formulation than ingredient. It complies with the API limit and reassures the fact that almost one fourth of the formulation consists of *B. diffusa*. This signifies precision of present study and suggested that ingredient of poly-herbal formulation predominately contains Boeravinone-B in a perfect ratio. Such research study may be recommended for quality control in various pharmaceuticals to make a uniform approach in clinical research. HPLC chromatograms may also help to evaluate the adulterants and identification of standard and pure raw material.

## 4. Conclusions

Results of this study have revealed that Boeravinone-B is present in ingredient (*B. diffusa* extract) and poly-herbal formulation i.e. 0.041% w/w and 0.011% w/w respectively which scientifically validate the ratio of ingredient in finished drug. It may become a versatile method for qualitative (identity) and quantitative analysis in quality control of various market formulations containing *B. diffusa* to enhance their efficacy. The results of analysis comply with several Quality standards books/official pharmacopoeias<sup>16,17,36</sup>. It presents a very good resolution with greater sensitivity and linearity associated with the coulometric detector than the other conventional methods<sup>28</sup>. This study may widen the acceptability criteria of ayurvedic drugs containing *B.diffusa* globally through estimation of marker(Boeravinone-B) in raw material, intermediate as well as various finished phyto-formulations.

## 5. Conflict of Interests

No competing financial interests exist.

## 6. Acknowledgements

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