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ORIGINAL ARTICLE

High-Performance Thin-Layer Chromatography Estimation of Boeravinone-B in *Boerhavia diffusa* L. and its Polyherbal Dosage Form (Capsule)

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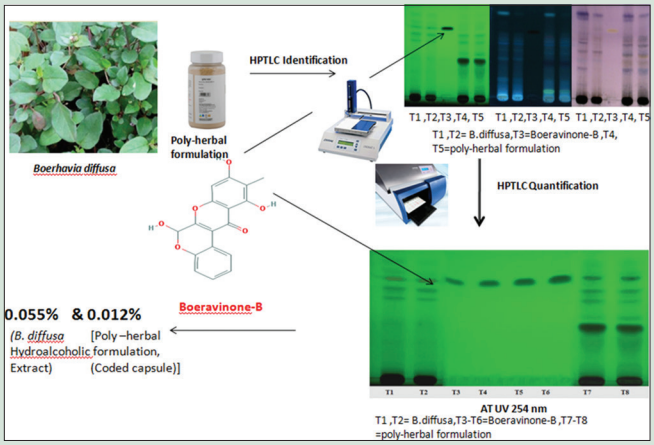
ABSTRACT

Background: Qualitative and quantitative marker estimation is the quality basis of Ayurvedic pharmaceuticals. *Boerhavia diffusa* is a widely used herb in the Indian system of medicine. **Objective:** Estimation of boeravinone-B in hydroalcoholic extract of raw drug (ingredient) as well as its poly-herbal formulation to authenticate ingredients ratio in finished product by high-performance thin layer chromatography (HPTLC) method. **Methods:** Based on planar chromatographic method (HPTLC) with optimized mobile phase toluene: ethyl acetate: formic acid: methanol (5:3:1:1 v/v). Further densitometry and marker quantification of developed plate were carried out at ultraviolet 254 nm. **Results:** Presented in the form of comparative characteristic HPTLC fingerprints and densitograms with well-resolved prominent bands for boeravinone-B at retardation factor (R_f) – 0.87 in both the samples. The linear regression by calibration plots revealed good linear relationship with 0.99953 with standard deviation 0.74% with respect to the area in the concentration range of 200–1000 ng/spot. Statistical analysis proves that the developed quantification method is reproducible and selective. Findings showed the presence of 0.055% and 0.012% w/w boeravinone-B in hydroalcoholic extract of *B. diffusa* and its polyherbal formulation, respectively, at R_f 0.87 under λ 254 nm. **Conclusion:** The study established fast, simple, precise, and cost-effective methods for qualitative and quantitative studies of Ayurvedic raw drugs, and polyherbal formulations consist of *B. diffusa*. It estimated boeravinone-B in raw drug (ingredient) as well as in its polyherbal formulation. The study qualitatively and quantitatively authenticated the presence and ratio of *B. diffusa* in polyherbal dosage form (capsule). Moreover, it suggested that the plant contains rich amount of boeravinone-B; this may be directed in selection of genuine plant species in formulation development as well as in standardization.

Key words: Boeravinone-B, *Boerhavia diffusa*, densitometry, high-performance thin-layer chromatography, polyherbal formulation

SUMMARY

- The study established fast, precise, and cost-effective HPTLC methods for qualitative and quantitative analysis of *B. diffusa* as a raw drug and its poly-herbal formulations. Where boeravinone-B was estimated to be 0.055% and 0.012% w/w in hydroalcoholic extracts of *B. diffusa* and its polyherbal formulation, respectively, at R_f 0.87 under λ 254 nm.



Abbreviations Used: TLC: Thin-layer chromatography; HPTLC: High-performance thin-layer chromatography; VSR: Vanillin sulfuric acid reagent; R_f: Retardation factor; λ_{max}: Wavelength at which absorbance is highest; SD: Standard deviation.

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INTRODUCTION

Boeravinones are active marker class which is embedded in *Boerhavia diffusa* L., a medicinal plant species; chemically, it comes under rotenoids category out of various secondary metabolites, namely glycosides, flavonoid, isoflavonoids, steroids, alkaloids, phenolic, and lignan glycosides.[1] Rotenoids are potent phytochemical which includes boeravinone-B as an important member of this group. Many of them reported by the name from boeravinones-A to boeravinones-H, which have been isolated from roots and other parts of this plant.[2-7] In this sequence of investigations, nine rotenoid derivatives,[3-11] including boeravinones I-10 and J-11, were isolated from its root extract.[7] Besides this, boeravinones AI, BI, C2, D, E, and F were also previously investigated in this plant. Boeravinone has wide medicinal applications including stimulation of growth and differentiation of hematopoietic precursor cells from various lineages by binding with granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha receptors.[8] It has a high anti-inflammatory property to control autoimmune diseases, rheumatoid arthritis, osteoarthritis, and acute myoskeletal disorders.[9] Boeravinone-B is a novel dual inhibitor of bacterial Nor-A efflux pump and human P-gp

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hence used in P-gp inhibition and immunomodulation[10] and reported to work as inhibitors of breast cancer resistance protein.[11] Moreover, it significantly decreases oxidative stress, increases cell viability, and protected *Saccharomyces cerevisiae* cells from aging. It also showed noncytotoxic effects.

B. diffusa L. (*Nyctaginaceae*) is abundantly creeping weed found all over India and commonly used by name of "*Punarnava* or *Raktapunarnava*" in the traditional system of medicine.[12] It is an Ayurvedic *rasayana* herb that widely used as folk medicine and reported to be effective in controlling diabetes and many other infections such as urinary tract infection, genital tract infection, respiratory tract infection, hepatitis, inflammations, skin problems, and asthma.[13,14] Pharmacologically, it has been tested positively for various therapeutic actions such as diuretic,[15] hepatoprotective, anti-inflammatory, antifibrinolytic, anticancer, antibacterial, antifungal, adaptogenic, antiamoebic, lipotropic antistress, and anticonvulsant.[16-20] These therapeutic functions are actually regulated by several bioactive compounds embedded in seeds, leaves, stems, and root's part of *B. diffusa*. Likewise, phytoactive constituents reported in its whole plant extract were observed in the treatment of many severe ailments.[21]

Out of active markers of *B. diffusa*, boeravinones were analyzed for the development, quality control, and high efficacy of Ayurvedic and polyherbal preparations. In this regard, a rapid quantitative method was reported for the estimation of boeravinone-B in *B. diffusa*.[22-24] Under such investigations, ether and methanol extracts of root part of this plant led to the isolation of many important rotenoids followed by structure elucidation on the spectral basis which were coded as boeravinone-A, B, C, D, E, F, G, and H to evaluate their effect on intestinal motility *in vitro* and was observed that three of markers, i.e., boeravinone G, boeravinone–E, and compound 5 exhibited good spasmolytic activity.[25-28] Thin-layer chromatography (TLC) was utilized to identify analogs rotenoids, boeravinone-A, and -B in ether extract of root isolated by column chromatography, and further, their structure was elucidated on the basis of nuclear magnetic resonance evaluation.[29] Beside other analytical studies, identification of boeravinone-B in methanolic extract of its root was performed through validated primary chromatographic methods, i.e., TLC and advanced high-pressure liquid chromatography under optimized chromatographic conditions.[30] Moreover, the sophisticated form of TLC method known by high-performance thin-layer chromatography (HPTLC) technique was employed for qualitative and quantitative analysis of such active compounds in *B. diffusa*[31] to authenticate that the samples of this plant reported to contain not <0.005% boeravinone-B.[32]

Boeravinone-B is the basis for the quality analysis of *B. diffusa* (aqueous extract of fruit) and other plant-derived drugs from the herb.[33] It is difficult to identify a particular component in a polyherbal formulation containing more than two herbs or a mixture of different herbal extracts. The polyherbal formulation under study contains *B. diffusa* together with other three plant ingredients. This study was performed for the purpose of qualitative and quantitative analysis of boeravinone-B by the application of HPTLC fingerprinting in hydroalcoholic extract of *B. diffusa* (whole plant), and its processed polyherbal-coded formulation encapsulated various powder herbs such as *B. diffusa, Emblica officinalis, Tinospora cordifolia, Withania somnifera, Glycyrrhiza glabra, Bacopa monnieri,* and *Centella asiatica* in a specific combination. The aim of present study is to detect the ratio of active marker, i.e., boeravinone-B in hydroalcoholic extract of *B. diffusa* as well as its finished polyherbal formulation by comparative HPTLC profiling which is not reported so far for such formulation. This may ascertain authenticity of formulation and its efficacy. Moreover, this work may be utilized in the standardization of raw drug and various important pharmaceutical formulations composed of *B. diffusa* for curing several chronic diseases on the basis of marker evaluation and may be useful for selection of guanine raw drugs in formulation development.

MATERIALS AND METHODS

Collection of plant material

The whole plant of *B. diffusa* used for the investigation was authenticated by voucher specimen number LIH No. 6562 and was collected from an authentic supplier, i.e., M/s LailaImpex (chemiloids), 40-15-14, Brindavan Colony, Labbipet, Vijayawada 520010.

Chemicals and reagents

Boeravinone-B of purity 97.3% was procured from Natural Remedies Pvt. Ltd., Bangalore. Chemicals and solvents were of analytical grade, and double-distilled water was used in all experiments. Aluminum TLC plate (E-Merck), precoated with silica gel 60 F_{254} of 0.2 mm thickness was used as stationary phase.

Instrumentation

CAMAG HPTLC system (Muttenz, Switzerland) equipped with a semiautomatic sample applicator (spray-on technique) Linomat IV, twin trough development chamber, lighting system CAMAG TLC visualizer *Reprostar*3 integrated into winCATS software version 1.4.2 and Hamilton (Reno, Nevada, USA) Syringe (100 µl).

Sample extraction

The dry raw material was grounded into coarse powder and then extracted with 60 L of 40% ethanol (i.e., 24 l of ethanol and 36 L of water) by refluxing for 2 h at 80°C. After cooling, the hydroalcoholic extract was filtered and the residual matter extracted thrice using 45 L of 40% ethanol. The filtrate so obtained from all the four extractions is concentrated under vacuum at 70°C–80°C for 3–4 h. Finally, this extract was vacuum dried at 70°C–80°C for 14–16 h. The dried extract was milled, sieved, and packed in polythene bags for further use. The herb extract ratio was found to be 12:1, and the yield was 8% on dried basis.

Sample preparation

Plant extract

About 5 g of plant extract was taken and crushed in mortar pastel. From that, accurately weighed 650 mg powder transferred to 25 mL standard flask, i.e., 52 mg of powdered drug was taken in 2 ml methanol. Volume is made up to the mark with methanol, sonicated for 10 min. It was filtered with 0.22 µ filter to obtain sample stock solution.

Coded formulation

20 capsules (an Ayurvedic formulation composed of *Boerhavia diffusa, E. officinalis, T. cordifolia, W. somnifera, G. glabra, B. monnieri,* and *C. asiatica* in a specific ratio) were taken, opened, and crushed in mortar pastel. From that, accurately weighed 650 mg powder transferred to 10 ml standard flask, i.e., 65 mg of formulation was taken in 1 ml of methanol. Volume is made up to the mark with methanol, sonicated for 10 min. It was filtered with 0.22 µ filter to obtain sample stock solution.

Preparation of standard stock solution

10 mg boeravinone-B as reference standard was dissolved in alcohol and make up to 10 ml in a standard flask. This prepared 1 mg/ml standard solution for the marker estimation.

High-performance thin-layer chromatography analysis

Optimization of mobile phases

A mobile phase was developed on hit and trial basis with different combinations of mobile phases in fix ratios for the present study. The best separation was obtained in the mobile phase of toluene: ethyl acetate: formic acid: methanol (5:3:1:1, v/v/v/v).

Sample application

Application of bands of each extract was carried out (10 mm in length and 20 µl in quantity) using spray technique. Sample were applied on precoated silica gel 60 F_{254} aluminum sheets (10 cm × 10 cm) with the help of Linomat-5 applicator attached to CAMAG HPTLC system, which was programmed through WIN CATS software.[34]

Development of chromatogram

After the application of sample, the chromatogram was developed in twin trough CAMAG glass chamber 10 cm × 10 cm saturated with solvent toluene: ethyl acetate: formic acid: methanol (5:3:1:1 v/v) for 30 min and run up to 9 cm.

Detection of spots

The air-dried plates were viewed in ultraviolet radiation to midday light [Figure 1]. The chromatograms were scanned by densitometer at 420 nm after dipping in anisaldehyde sulfuric acid reagent (ASR) and heated at 105°C till the color of the spots appeared. The retardation factor (R_f) values and fingerprinting data were recorded by CAMAG WIN CATS software (Muttenz, Switzerland).

Preparation of calibration curve for high-performance thin-layer chromatography

To prepare the calibration curve for HPTLC, standard stock solution (1 mg/mL) was prepared in methanol. Standard solutions of 2 µl, 4 µl, 6 µl, and 8 µl (as standard 1, 2, 3, and 4) were each of these applied in 10 mm band length on precoated silica gel 60 F_{254} aluminum sheets (10 cm × 10 cm) with the help of linomat-5 applicator. The calibration curve was plotted depicted in Figures 2-4.

RESULTS AND DISCUSSION

Estimation of boeravinone-B in raw drug and formulated capsules was carried out using proposed HPTLC method in developed mobile phase of toluene: ethyl acetate: formic acid: methanol (5:3:1:1, v/v/v/v) in fix ratio. At optimized chromatographic conditions, ingredient (*B. diffusa*), standard, and formulation were applied and separated on TLC plate, further visualized in different illuminations by pre- and post-derivatization with 10% ASR in well-resolved distinct and prominent bands, shown in Figure 1. The plate was further scanned at wavelength 254 nm to estimate the target marker through calibration curve prepared from standards (boeravinone -B) in concentration range 200–1000 ng/spot with correlation coefficient of 0.99953, standard deviation 0.74%, observed between the concentrations of standard and the respective peak areas. Regression of standard was calculated to be Y = 5124 + 4.364 X, where "Y" is the peak area and "X" is the concentration [Figure 2]. The boeravinone-B in ingredient (hydroalcoholic extract of *B. diffusa*) and in coded formulation was calculated to be 0.055% and 0.012%, respectively, at 254 nm on the basis of peak area.

The quantitative determinations of boeravinoneB present in *B. diffusa* were reported in several literatures and official pharmacopoeias. Qualitatively, *B. diffusa* was studied by chromatographic method like TLC in selected mobile phase with better resolution.[34] This investigation proposed the HPTLC method because of its rapid, simple, cost-effective, precise features and usefulness in quality analysis of botanicals and their various pharmaceutical preparations. Here, the results presented in the form of HPTLC fingerprints [Figures 1 and 4] and densitograms [Figures 3 and 5-12] at the wavelengths 254 nm, 366 nm and under white light were well documented to comply with good manufacturing practice guideline. Preliminary confirmation of target marker in samples in correspondence of reference marker was carried

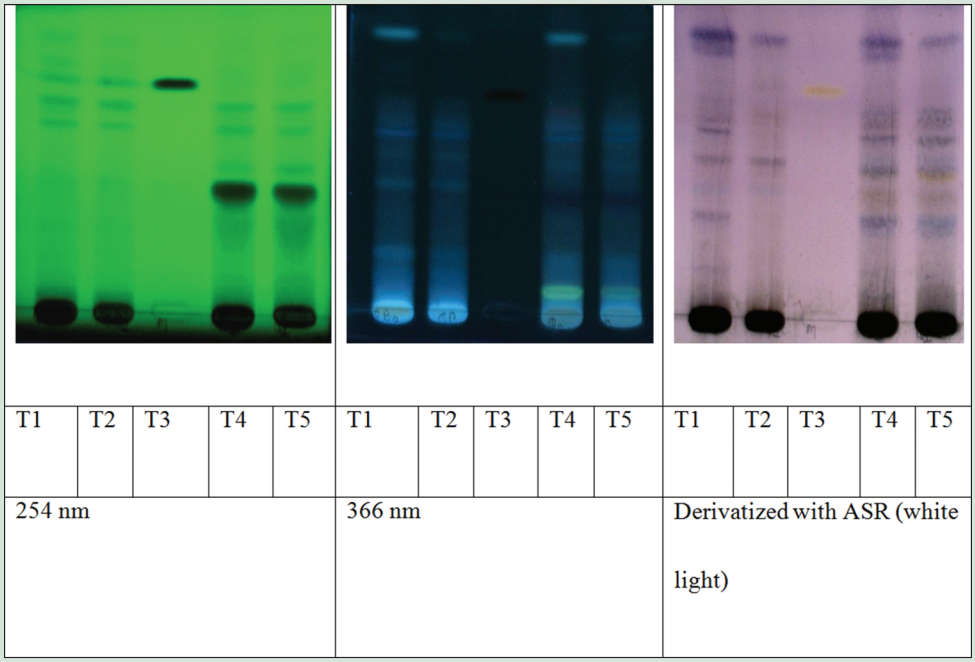

Figure 1: Comparative high-performance thin-layer chromatography profile of hydroalcohol extracts of *Borehavia diffusa* with formulation and standard under different illuminations: T1: track-1 and T-2: track-2, ingredient (*Boerhavia diffusa*); T3: track-3, standard (boeravinone-B); T4: track-4 and T-5: track-5, polyherbal formulation

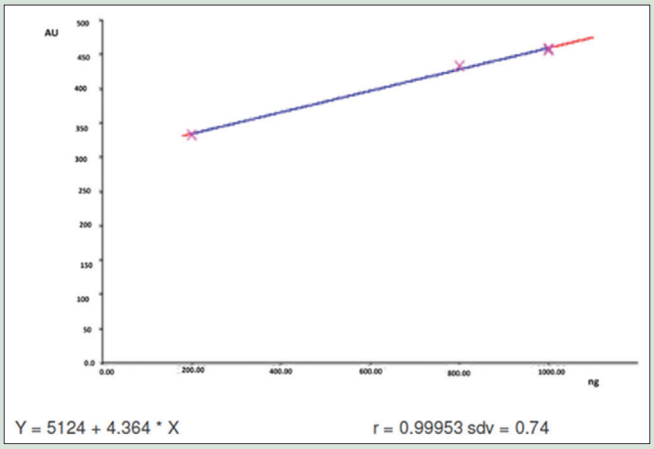

Figure 2: High-performance thin-layer chromatography calibration plot of boeravinone-B

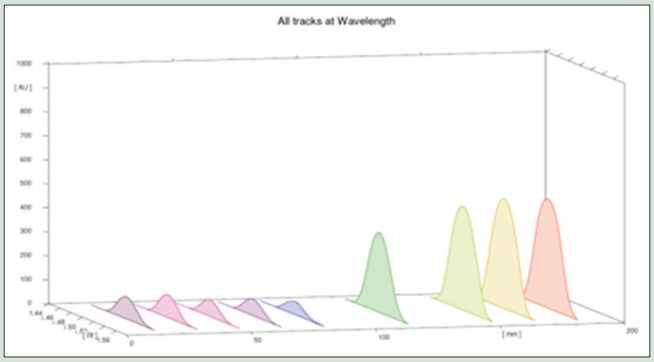

Figure 3: Three-dimensional overlay densitograms of boeravinone-B at concentrations of 2 µl, 4 µl, 6 µl, and 8 µl along ingredients and formulation with peak area and retardation factor value demonstrating system suitability of the method

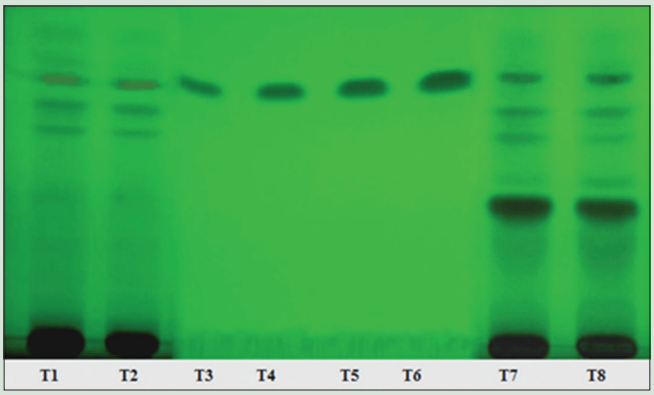

Figure 4: Boeravinone-B estimation by high-performance thin-layer chromatography fingerprint of hydroalcohol extracts at ultraviolet 254 nm: Track T1 and T2 of ingredient (*Boerhavia diffusa*), track T3–T6 of standard (2 µl, 4 µl, 6 µl, and 8 µl), and track T7-T8 of polyherbal formulation

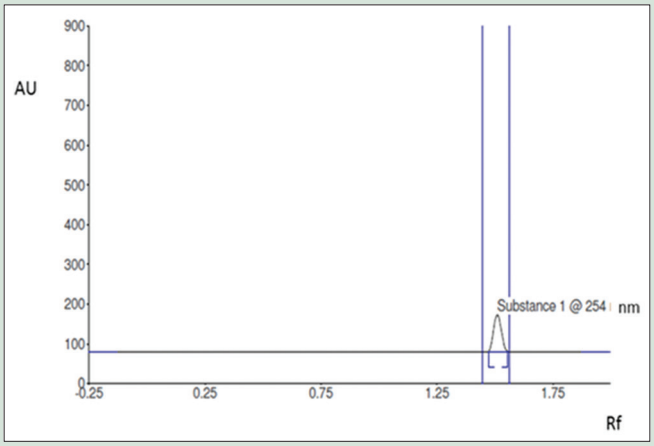

Figure 5: High-performance thin-layer chromatography chromatogram of ingredient (*Boerhavia diffusa*) 1, ID: I-1

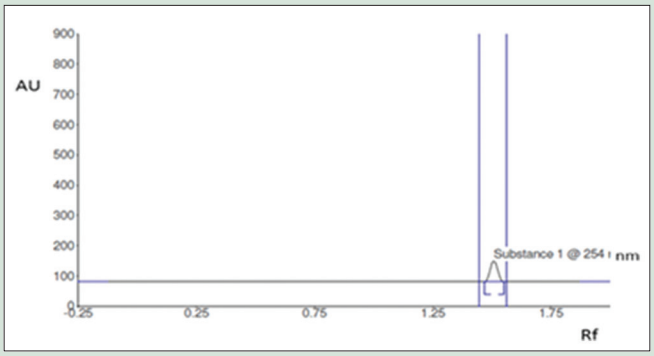

Figure 6: High-performance thin-layer chromatography chromatogram of ingredient (*Boerhavia diffusa*) 2, ID: I-2

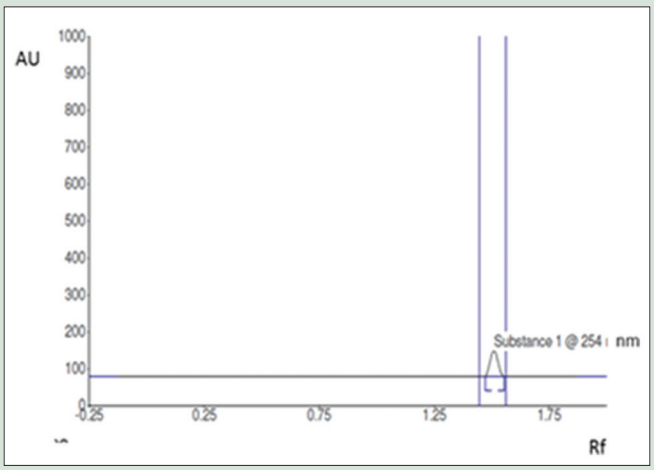

Figure 7: High-performance thin-layer chromatography chromatogram of polyherbal formulation 1, ID: F-1

out through comparative HPTLC fingerprinting in optimized mobile phase as shown in Figure 1. Fingerprinting profile revealed prominent band of borhevion-B at R_f 0.87 matched with bands of ingredient and its polyherbal preparation; this confirms the presence of marker band in raw drug and finished product and justified use of genuine raw material in polyherbal formulation. Moreover, the similarity in bands pattern and R_f values indicated analogous chemical profile in raw drug and formulation. This may set as a characteristic parameter for tracing genuine raw drug in formulation.

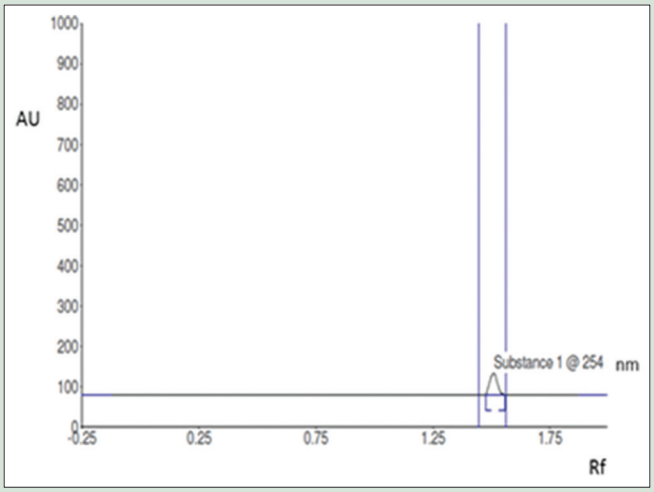

Figure 8: High-performance thin-layer chromatography chromatogram of polyherbal formulation 2, ID: F-2

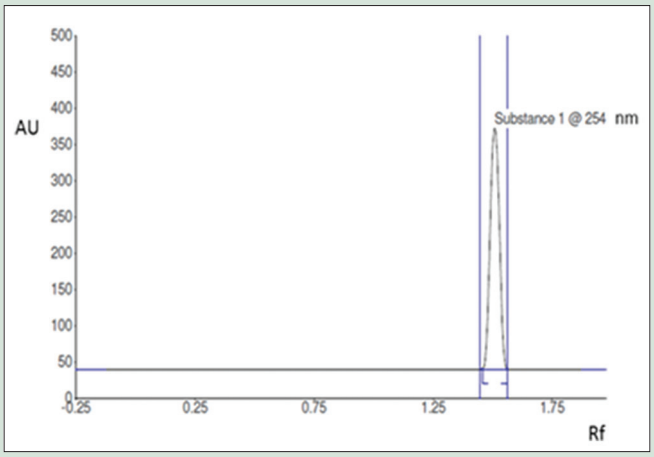

Figure 10: High-performance thin-layer chromatography chromatogram of boeravinone-B, ID: Standard 2

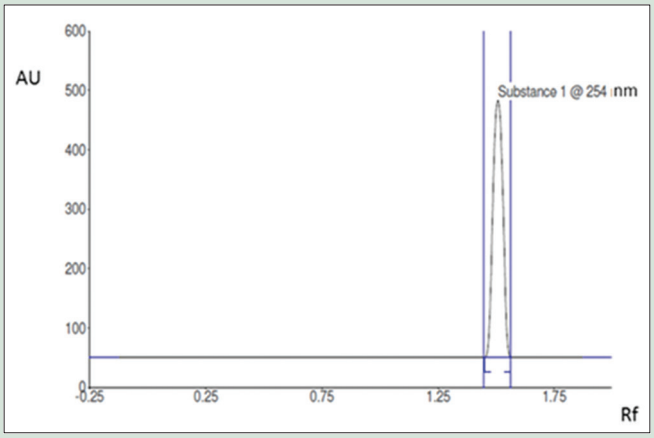

Figure 12: High-performance thin-layer chromatography chromatogram of boeravinone-B, ID: Standard 4

Quantification of target marker in *B. diffusa* and its polyherbal formulation was performed through the application of standard in

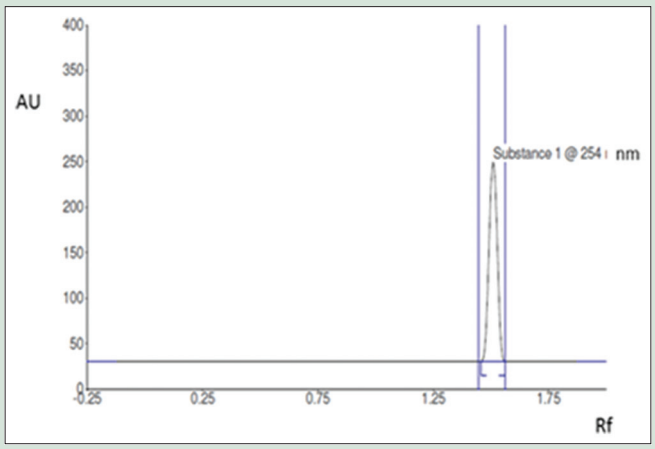

Figure 9: High-performance thin-layer chromatography chromatogram of boeravinone-B, ID: Standard 1

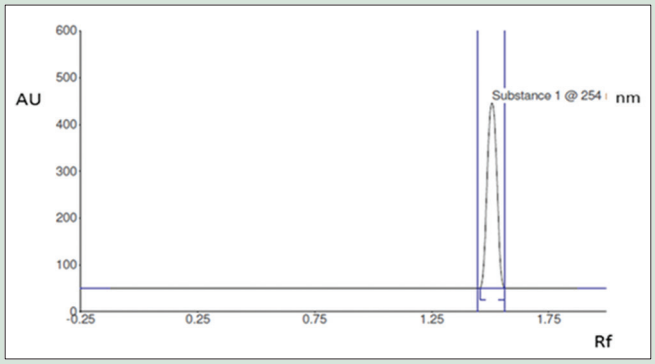

Figure 11: High-performance thin-layer chromatography chromatogram of boeravinone-B, ID: Standard 3

different concentrations which is presented as track (T3) to track (T6) in Figures 4 and 9-12 followed by HPTLC densitometry scanning at wavelength 254 nm as shown in Figures 3 and 4. The method validated on the basis of good linear relationship by calibration curve as shown in Figure 2. Three-dimensional overlay of densitograms of boeravinone-B at concentrations of 2 µl, 4 µl, 6 µl, and 8 µl along ingredients and formulation with peak area and R_f value demonstrated system suitability of method. Calculated value of 0.055% of boeravinone-B in hydroalcoholic extract of *B. diffusa* was found to be complied with reported quality standard books/official pharmacopoeias.[15,35,36] Results indicates that the ingredient of polyherbal formulation predominately contains this marker that observed as a prominent band at similar R_f of 0.87 in finally coded formulation which can surely be attributed due to *B. diffusa*.

CONCLUSION

The boeravinone-B in hydroalcoholic extract of *B. diffusa* was estimated to be 0.055% and coded formulation was 0.012% at same R_f, which reassures the fact that almost one-fourth of the formulation consists of *B. diffusa*. Study authenticated the presence of *B. diffusa* in the pharmaceutically developed polyherbal dosage form (capsule) qualitatively and quantitatively. This method may be utilized in quality control and standardization of herbal raw materials, proprietary, and official Ayurvedic formulations. The amount of boeravinone-B present in *B. diffusa* raw drug extract complies with pharmacopoeial limits.

Prominent bands of boeravinone-B in the study proved that the plant contains rich amount of target marker; this may be employed as significant tool for selection of appropriate plant species for the development of high-quality pharmaceutical formulations. Study also confirmed identity, purity, and authenticity of *B. diffusa* used in various in-house Ayurvedic preparations.

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Conflicts of interest

There are no conflicts of interest.

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