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Enrichment of Flavonoids from the Methanolic Extract of *Boerhaavia Diffusa* Roots by Partitioning Technique

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Abstract

Flavonoids(Rotenoids) present in Boerhaavia diffusa is known for its different biological activities such as antioxidant, anti-diabetic, anti-stress, anticonvulsant, antibacterial, hypoglycemic, anti-nociceptive, hepato-protective, anti-proliferative and anti-estrogenic, anti-inflammatory. The following work constitutes the enrichment of iso-flavonoids present in the methanolic extract of Boerhaavia diffusa by partition technique using various solvents such as acetone, water, Butanol, ethyl acetate, hexane, chloroform and methanol. Enrichment was determined by Thin Layer Chromatography and HPLC and the presence of flavonoids was confirmed with Shinoda test, lead acetate test and sodium hydroxide test.

Keywords: *Boerhaavia diffusa*, Flavonoids, HPLC, Thin Layer Chromatography, Shinoda test.

Introduction

It is been recorded in history that Medicinal herbs have been used as form of therapy for the relief of pain. The exploration of the chemical constituents from plants, pharmacological and phytochemical screening would provide the basis for developing the new lead molecules in strategic favor of natural product drug discovery. The aim and subject of many researchers is the discovery and development of isolating a new efficient, active and less toxic molecule for systemic activities. The biologically active agents from natural sources have always been of great interest to working on various diseases[1].

Tribal communities are using their traditional knowledge system to cure different diseases. They use plant as a source of drug through trial and error method and the process is experienced over hundreds of years, which says that the medicinal plants have been in the focus as lifesaving drugs right from the beginning of the human civilization. The medicinal plants have been the object of research in both systematic and advanced areas of plant sciences[2].

The traditional knowledge of these herbal recipes is popular among the indigenous and local communities. Even today the Tribal communities are solely dependent on plants for their medication; hence they are using them against different. They have preserved the wealth of traditional knowledge as a part of their belief and customs. They are practicing these methods generation after generation successfully[3].

Apart from medicinal uses phytochemical components which are environment friendly, economical and effectively shows anti corrosive properties[4,5] and also phyto-compounds are used as biofuels[6,7].

Boerhaavia diffusa L. (Nyctaginaceae), commonly known as 'Punarnava' in the Indian system of medicine, is a perennial creeping herb found throughout the waste land of India[8]. The *Boerhaavia* sp. has ancient medicinal use in different societies from the times of the B.C. The herbal medicine has evolved and changed through the years. A number of plant products have been identified through phyto-chemistry and the extract of their different plant parts are useful in various diseases without side effects[9].

Many Rotenoids (class of Flavanoids) have been isolated from the roots of the *Boerhaavia diffusa*[10-16]. Plant also includes a series Pharmacological Potential of *Boerhaavia diffusa* boeravinones viz., boeravinone A, boeravinone B, boeravinone C, boeravinone D, boeravinone E and boeravinone F. Punarnavoside, a phenolic glycoside, is reportedly present in roots[17,18]. C-methyl flavone also has been isolated from *Boerhaavia diffusa* roots[19].

Phytochemicals such as poly-phenols comprised principally of the flavonoids, which is chemically characterized by two benzene rings joined by a linear carbon chain. Various observational studies indicate that regular consumption of foods containing flavonoids may reduce the risk several chronic conditions, including neurodegenerative diseases, atherosclerosis, and certain forms of cancer. These results have generated considerable interest in flavonoids as it is associated with specific health outcomes[20].

The interest of the present work is to enrich the flavonoids in methanolic extract in the roots of *Boerhaavia diffusa* and determine its presence by chemical tests such as Shinoda test, lead acetate test and sodium hydroxide test. The enriched portions can be used for further studies such as the isolation of chemical entities containing flavonoids in the plant *Boerhaavia diffusa* and studies relating to various biological activity of the plant.

Material and Methods

Plant material and its extract: The plant *Boerhaavia diffusa* was collected in the month of December, 2012 from Bangalore. The shade dried plant material was grounded to coarse powder. The powdered roots of *Boerhaavia diffusa* (500 g) was refluxed with Methanol 3 times in ratio 1:6, 1:5, 1:4 respectively for 2 hours each followed by filtration using a muslin cloth and the extracted powder was discarded. The methanolic extract so obtained was further processed. The excess solvent in the extract was removed by distillation and the concentrated using rotary evaporator with controlled temperature of 50˚C. The extract was scrapped from the Buchi flask and was completely dried in a vacuum tray drier. (Extractive value 24.8g / yield = 4.96%).

Partition using various solvents: Methods (a) 5g of methanolic extract of *Boerhaavia diffusa* was initially adsorbed with 10g of silica, the adsorbed material was washed with 50ml of acetone. Acetone soluble portion was collected separately. Acetone insoluble portion was washed with 50ml of 100% methanol; methanol soluble portion was collected separately. Methanol insoluble portion was washed with 50ml 50% methanol/water followed by 100% De-mineralized water. Methods (b) 5g of initial methanolic extract was taken in a 100ml separating funnel and 50ml of ethyl acetate was added and the contents of the separating funnel was mixed properly by shaking for around 5min and kept aside for half an hour for separation. Ethyl acetate soluble portion was collected separately. Insoluble portion was retained in the separating funnel and 50ml butanol was added, mixed and kept aside for half an hour for separation. Butanol soluble portion was collected separately and insoluble portion was dissolved in water. Methods (c) 5g of initial methanolic extract was taken in a 100ml separating funnel and 50ml of chloroform was added and the contents of the separating funnel was mixed properly by shaking for around 5min and kept aside for half an hour for separation. Chloroform soluble portion was collected separately. Insoluble portion was retained in the separating funnel and 50ml butanol was added, mixed and kept aside for half an hour for separation. Butanol soluble portion was collected separately and insoluble portion was dissolved in 50ml water. Methods (d) 5g of initial methanolic extract was taken in a 100ml separating funnel and 50ml of dichloromethane was added and the contents of the separating funnel was mixed properly by shaking for around 5min and kept aside for half an hour for separation dichloromethane soluble portion was collected separately. Insoluble portion was retained in the separating funnel and 50ml butanol was added, mixed and kept aside for half an hour for separation. Butanol soluble portion was collected separately and insoluble portion was dissolved in 50ml water. The dichloromethane soluble portion was used for further partition with 50ml of hexane followed by 50ml of methanol.

All the partitions collected were subjected to distillation and were concentrated using rotary evaporator with controlled temperature of 50˚C; the solvents were recovered.

Thin Layer Chromatography: All the partitions collected were subjected to thin layer chromatographic analysis, to find the presence of flavonoids to support the chemical test. Also an enriched portion of boeravinone was used as a standard.

The details of procedure are as following: Pre-coated silica gel on aluminum analytical TLC Plates (TLC Silica gel 60) purchased from Merck Millipore was used. Sample was prepared by dissolving 20mg of each of the partition with analytical methanol. The range of sample volume applied was controlled, spreading not more than 1 cm. Chloroform: Methanol with ratio 9:1 was used as the mobile phase. The TLC plate containing the sample spot was placed at an angle of 45˚ in the development chamber covering the bottom of the plate by the solvent up to nearly 1 mm. The ascending technique was used. The solvent front was marked and the plate was finally allowed to dry. The bands separated were detected in UV light of 254nm, 366nm and the Colorless components were detected by using visualizing agent, Anisaldehyde spray.

High Partition Liquid Chromatography: Preparation of Phosphate buffer: 136 mg of Potassium Di-hydrogen orthophosphate was dissolved in 100 ml filtered HPLC water. 500 µl of analytical grade ortho-phosphoric acid was added and volume was made up to 1000 ml using filtered HPLC water. It is sonicated for 10 min and used.

Sample preparation: 2 mg of each sample was dissolved 1.5 ml of methanol by heating and with the help of sonication. It is then filtered using HPLC filter and used.

Procedure: All the partitioned samples were analyzed by HPLC in the protocol mentioned in table 1 and the chromatogram were recorded.

Tests for flavonoids[21]: Shinoda Test: To the sample, added 5 ml of 95% ethanol and few drops of concentrated HCl. To this solution 0.5 g of magnesium turnings were added. Observance of pink coloration indicated the presence of flavonoids.

Lead Acetate Test: To the small quantity of sample, lead acetate solution was added. Formation of yellow precipitate showed the presence of flavonoid.

Sodium Hydroxide Test: On addition of an increasing amount of sodium hydroxide, the sample containing flavonoids showed yellow coloration, this decolorized after addition of acid.

Results and Discussion

Extraction: The roots were extracted using solvent 100% methanol in the above mentioned method. The semi-solid extract so obtained was aromatic and brownish-black in color. The extractive value was found 24.8g and 4.96% was the yield. The extractive value was found the partitioned samples is been shown in the table 2.

Thin Layer Chromatography: The bands separated were detected in UV light of 254nm, 366nm and the Colorless

components were detected by using visualizing agent, Anisaldehyde spray. Shown in figure 1 and the sampling codes as given in table 03.

Compared to the standard enriched fraction of boeravinones (flavonoids of *B.diffusa*), sample no: 3, 6, 9, 14 and 15 showed the presence of flavonoids.

High Partition Liquid Chromatography: The chromatograms of the partitioned samples are given in the figure 02 and the sampling codes in table 3.

Compared to the HPLC chromatogram of the standard enriched fraction of boeravinones (flavonoids of *B.diffusa*), sample no: 6, 14 and 15 showed the presence of flavonoids.

Tests for Flavanoids: The results for tests for flavonoids viz., Shinoda test Lead Acetate test and Sodium hydroxide test are shown in the table 4.

Sample numbers: 4, 6, 9, 14 and 15 gave the positive results for flavonoids.

Table-1
Protocol of HPLC of All the Samples

Instrument	SHIMADZU HPLC system with LC 10A pump		
Column	KROMOSIL RP C-18 (250 X 4.6 X 7µ)		
Flow rate	1.2ml/min		
Detection wavelength	280 nm		
Mobile phase	Pump A	Phosphate buffer	
	Pump B	Acetonitrile	
Method name	Boerhaavia Diffusa – 4 method		
Gradient Time program	Time	B. conc.	A. conc.
	0.01	05	95
	18.00	30	70
	25.00	55	45
	28.00	55	45
	35.00	30	70
	45.00	05	95
	52.00	Stop	-
Injection volume	20µl		
Detector	SPD-M10Avp photodiode array detector at 280 nm.		

Table-2
Extractive Value and yield of the Partitioned Samples

S No.	Sample	Extractive value	Yield	Method No.
1	Acetone soluble	540mg	0.108%	a
2	Methanol Soluble	270mg	0.054%	
3	50% Methanol/Water Soluble	2.82g	0.564%	
4	Water Soluble	570mg	0.114%	
5	Butanol Soluble	660mg	0.132%	b
6	Ethyl Acetate Soluble	510mg	0.102%	
7	Water Soluble	2.86g	0.572%	
8	Chloroform	510mg	0.102%	c
9	Butanol Soluble	175mg	0.035%	
10	Water Soluble	3g	0.6%	
11	Butanol Soluble	510mg	0.102%	d
12	Water Soluble	2.85mg	0.57%	
13	Hexane Soluble	240mg	0.048%	
14	Methanol Soluble	140mg	0.028%	

Table-3
Sampling Codes for TLC and HPLC Graphs (figure-1 and 2)

Sample code	Sample	Method Number
STD	Standard Enriched fraction boeravinone B and E	-
1	Acetone soluble	a
2	Methanol Soluble	
3	50% Methanol/Water Soluble	
4	Water Soluble	
5	Butanol Soluble	b
6	Ethyl Acetate Soluble	
7	Water Soluble	
8	Chloroform	c
9	Butanol Soluble	
10	Water Soluble	
11	Butanol Soluble	d
12	Water Soluble	
13	Hexane Soluble	
14	Methanol Soluble	
15	Initial Methanolic Extract	-

Table-4
Results for the Chemical Tests for Flavonoids

Sl no	Sample Code	Shinoda Test	Lead Acetate Test	Sodium hydroxide test
1	STD	+	+	+
2	1	-	-	-
3	2	-	-	-
4	3	-	-	-
5	4	+	+	+
6	5	-	-	-
7	6	+	+	+
8	7	-	-	-
9	8	-	-	-
10	9	+	+	+
11	10	-	-	-
12	11	-	-	-
13	12	-	-	-
14	13	-	-	-
15	14	+	+	+
16	15	+	+	+

Conclusion

With the compiled results including TLC, HPLC and the chemical tests for flavonoids, Ethyl Acetate partition of the methanolic extract of B.diffusa in method-B showed the best enrichment of the flavonoids. The yield of ethyl acetate partition was found to be 0.102%. The partitioned sample can be further used for determination of various biological activities as well as isolation of different flavonoids present in B.diffusa.

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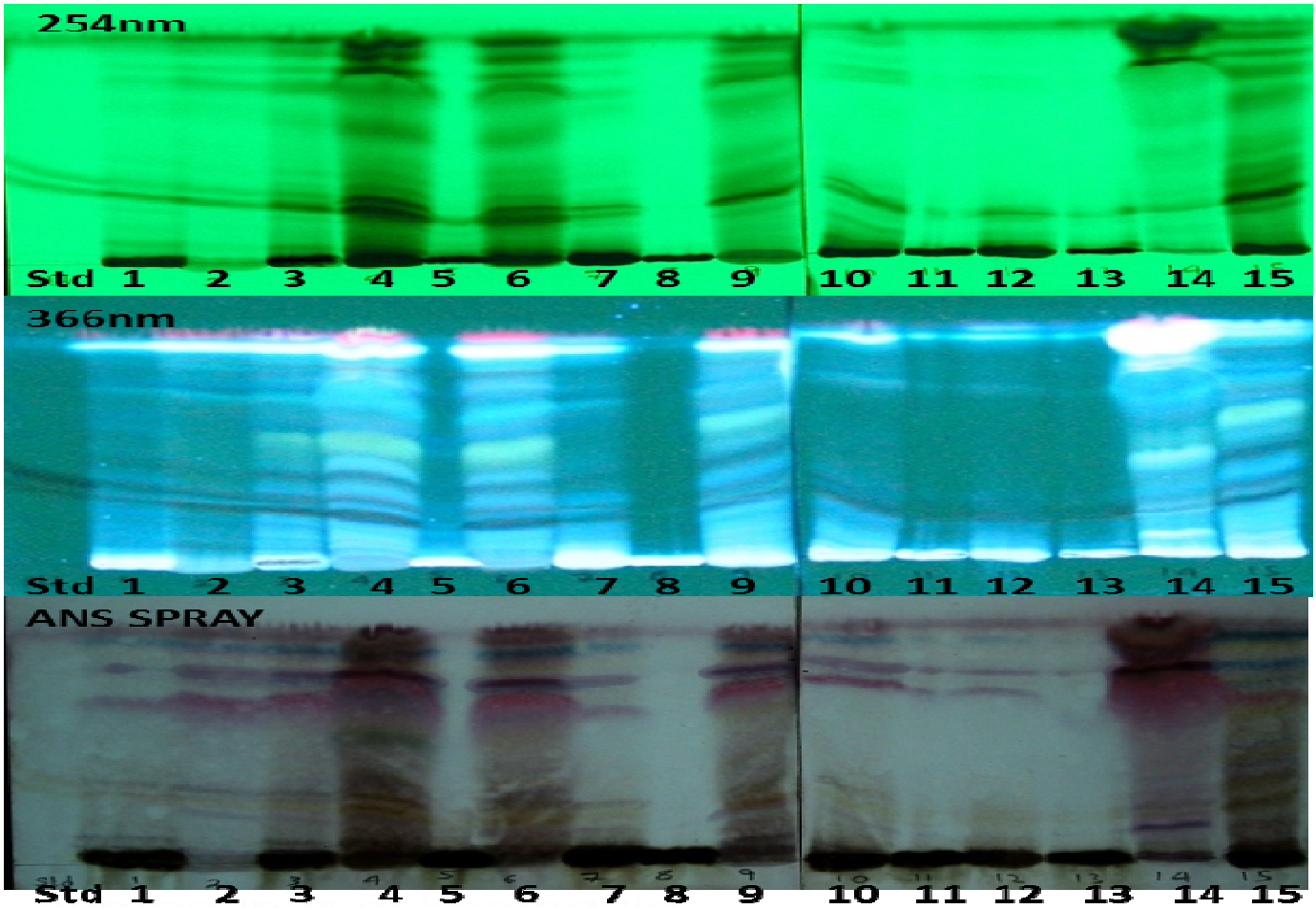

Figure-1

TLC of the Partitioned Samples[3]