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Quality control analysis of seed of *Dolichous biflorus* Linn.

Wasim Ahmad[1]

Mohd. Mujeeb[1]

S M Arif Zaidi[2*]

[1]Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, N. Delhi-110062
[2]Department of Surgery, Faculty of Medicine (U), Jamia Hamdard, N. Delhi-110062

Corresponding Authors:
Dr. S. M. Arif Zaidi
Department of Surgery, Faculty of Medicine,
Jamia Hamdard, New Delhi-110062, India
Email: Arifzaidi05@yahoo.co.in
Tel: +91-9891392903

Abstract:
Dolichous biflorus Linn. Popularly known as kulthi belongs to family fabaceae. It is also known by its medicinal properties, it is one of the most effective ayurvedic medicines for treating kidney stones. Its diuretic, anthelminthic properties make it an ideal drug for urinary and digestive disorders. Pharmacognistical standardization of *Dolichous biflorus* seed has been carried out in the present study. The study includes macroscopic evaluation along with estimation of its physicochemical parameters, preliminary phytochemical screening, estimation of total phenolics and flavonoids as well as fingerprint profile by HPTLC, HPLC and GCMS. Determinations of contaminants such as aflatoxins and pesticides also has been analysed and found no contaminants is present in the *Dolichous biflorus* seed sample. The quality control parameters in present study reveals standardization profile of *Dolichous biflorus* which would be of immense value in botanical identification and authentication of plant drug as well as may be helpful in preventing adulteration.

Keywords: *Dolichous biflorus* Linn., GCMS, HPLC.

Introduction

The present study deals with standardization of medicinal plant i.e. *Dolichous biflorus* Linn. seed (fabaceae). It is an annual branched herb; cultivated all over India, commonly known as Kulthi (1). The seeds of *D. biflorus* contain crude proteins, pentose, water soluble gums, haemaglutinin and phytosterols such as 24-methylene, 25-methylcholesterol and 24a-ethyl-5a-cholest-9-en-3β-ol. It is the rich source of enzyme urease. A dipeptide has been isolated and characterized as pyroglutamylglutamine. Its diuretic activity is 3 times more potent than acetozolamide in albino rats (2). The standardization is based on physicochemical properties, phytochemical analysis and HPTLC, HPLC and GC-MS fingerprinting.

Materials and methods

Collection and authentication of drugs

The seed of *D. biflorus* were collected from Khari Baoli, local drug market, New Delhi, and were authenticated by Dr. H. B. Singh, Ref. NISCAIR/RHMD/1327/129, New Delhi. The *D. biflorus* seeds powder (1.0 g) was extracted separately with 15 mL of methanol by sonication for 30 min at 45°C. The process was repeated twice to ensure complete extraction. The extract obtained were pooled and dried under reduced pressure. The dried extract, was dissolved in HPLC grade methanol to get the concentration of 1.0 mg mL^{-1} and subjected to total phenolic and flavonoid content analysis, and for the fingerprint profile by HPTLC and HPLC. For HPTLC finger printing profile of chloroform and petroleum ether extract *D. biflorus* powder (1.0 g) was extracted

separately with 15 mL of chloroform and petroleum ether, respectively by sonication for 30 min at 45ºC temperature. For GCMS fingerprinting *D. biflorus* powder (1.0 g) was extracted separately with 20 mL of hexane by sonication for 30 min at room temperature. Prior to use, all samples were filtered through a 0.45 µm nylon membrane filter.

Physicochemical studies

The organoleptic characters of the powder formulation were observed. Phytochemical constituents like alkaloids, tannins, glycosides, resins, terpenes, flavonoids, carbohydrate and saponins were tested using standard chemical reagent (3-4). Other parameters determined were total ash, acid insoluble ash, water soluble ash, extractive values in ether, alcohol and water, successive extractives in petroleum ether, chloroform, acetone, methanol and water, loss on drying at 105ºC, *p*H of filtrate of 1% and 10% w/v aqueous solutions as per the AYUSH protocol (5-7).

Assay of total phenolics and flavonoids

Total flavonoid content

Aluminum chloride colorimetric method was used for flavonoids determination (8). The calibration curve of rutin was prepared by preparing different dilutions in the concentrations range of 10-100 µg mL^{-1} in methanol.

Total phenolic content

Total phenols were determined by Folin Ciocalteu method (8). The standard curve was prepared using 25, 50, 100, 150, 200, 250 and 300 µg mL^{-1} solutions of gallic acid in methanol.

Fingerprinting profile by HPTLC, HPLC and GC-MS

HPTLC instrumentation and chromatographic conditions

High Performance Thin Layer Chromatography was carried out on 5 × 10 cm aluminum plates coated with 0.2 µm layers of silica gel $60F_{-254}$.(E-Merck) benzene: ethyl acetate (9:1, v/v), toluene: ethyl acetate: formic acid (6: 3: 1, v/v/v) and ethyl acetate: methanol : water (77: 13.5: 8, v/v/v) as mobile phase for petroleum ether, chloroform and methanol extract, respectively, After the development, plates were dried. Densitometric scanning was performed for petroleum ether, chloroform and methanol extracts, respectively at 550, 600 and 530 nm using CAMAG TLC scanner III operated by win Cats software.

HPLC instrumentation and chromatographic conditions

High Performance Liquid Chromatography was carried out on a Waters Alliance e2695 separating module (Waters Co., MA, USA) using photo diode array detector (Waters 2998) with autosampler and column oven. The mobile phase consisted of acetonitrile and water in the ratio of 50:50 (v/v). The flow rate was 1.0 mL min^{-1}; and column was maintained at room temperature. Analysis was performed at a wavelength of 296 nm using 10 µL of injection volume.

GC-MS instrumentation and chromatographic conditions

GC analysis was carried out using Agilent gas chromatograph equipped with a split/splitless injector (230°C) and a Mass Spectrometer Detector (230°C). The carrier gas used was helium (1 mL min^{-1}), and HP-5MS 5% Phenyl Methyl Silox (325°C: 30 m x 250 µm x 0.25 µm) was used as capillary col-umn. 2 µL sample was injected in splitless mode and programmed as follows: 170°C for 1 min, 8°C min^{-1} up to 250°C hold for 2 min, finally 3°C min^{-1} up to 310°C hold for 2 min. The MSD was operated under 70 eV, with a scan range of 70–600 amu. Total run time was 63 min. Identification of individual components was achieved using the Wiley and NIST Library.

Determination of contaminants

Aflatoxin determination

The official method of AOAC (Association of Official Analytical Chemists) of analysis was followed for the determination of aflatoxins (AOAC official method 991.31and 970.52) (9).

The analysis was carried out on a Waters Alliance e2695 separating module (Waters, USA). The derivatised samples (Both extract and standards) were injected into HPLC column (C18; 15 cm x 4.6 mm) and analysed using fluorescent detector. The peaks of aflatoxin in drug samples were compared with peak of standards (B1, G1, B2 and G2) (10).

Pesticides determination

Pesticide determination was done by (Agilent 7890A GC system, USA) using established method (AOAC official method 991.31and 970.52) (9).

RESULTS AND DISCUSSION

Phytochemical screening

The seed of *D. biflorus* was subjected to qualitative estimation of phytochemicals using standard methods, which revealed the presence of various bioactive components of like carbohydrates, proteins, flavonoids, phenolics, and terpenoids. All the physicochemical parameters were carried out in triplicate using standards methods of WHO, AYUSH are summarized in Table 1.

Table 1: Summary of physicochemical parameters of *D. biflorus* (n=3)

S. No	Parameters	% w/w(Mean ± SD*)	Limits (UP#-2007)
1	**Foreign matter**	1.91 ± 0.37	< 2.0%
2	**LOD**	9.72 ± 0.06	-
3	**Ash value**		-
	Total ash	3.08 ± 0.07	<5.0%
	Acid insoluble ash	0.49 ± 0.05	< 1.0.%
	Water soluble ash	1.58 ± 0.03	-
4	**Ph**		
	10 % solution	6.77 ± 0.03	-
	1 % solution	7.43 ± 0.05	-
5	**Successive extraction values**		
	Petroleum ether	0.70 ± 0.03	-
	Chloroform	1.58 ± 0.07	-
	Acetone	0.48 ± 0.02	-
	Methanol	8.56 ± 0.07	-
	Water	9.63 ± 0.02	-
6	**Extractive values**		
	Ether soluble extractives	00.97 ± 0.05	-
	Water soluble extractives	16.78 ± 0.07	> 12%
	Alcohol soluble extractives	10.42 ± 0.04	> 03%
7	**Total Phenolic and flavonoid**		
	Phenolic content	0.723 ± 0.022	
	Flavonoid content	0.426 ± 0.010	
8	**HPTLC fingerprinting**	**No. of spots**	**After spray**
	Methanol extract (Ethyl acetate: methanol: water (77:13.5:8, v/v/v)	**(7)** 0.15, 0.25, 0.41, 0.65, 0.78, 0.81, 0.87	
	Chloroform extract (Toluene: ethyl acetate: formic acid 6:3:1, v/v/v)	**(10)** 0.11, 0.13, 0.20, 0.24, 0.35, 0.41, 0.55, 0.79, 0.83, 0.91	
	Petroleum ether extract (Benzene: ethyl acetate 9:1, v/v)	**(6)** 0.14, 0.30, 0.43, 0.47, 0.65, 0.95	
9	**HPLC fingerprinting**	**296 nm**	
	Methanol extract (Water: Acetonitrile, 50:50, v/v)	**(10)** 0.9, 1.3, 1.5, 2.0, 2.3, 3.1, 4.0, 4.7, 8.6, 18.0	

* Standard deviation#Unani Pharmacopoeia

Assay of total phenolic and flavonoids

The quantitative estimation of total phenolics and flavonoid content showed 0.723% w/w and 0.426% w/w, respectively. These are the important antioxidant constituents of plants. Phenolics are the most wide spread secondary metabolite in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator.

HPTLC, HPLC and GC-MS fingerprinting profile

The HPTLC fingerprint profile of extracts showed (Fig 1) presence of 6, 10 and 7 compounds for petroleum ether, chloroform and methanol extracts, respectively in developed chromatogram at different Rf values (Table 1). However, HPLC fingerprinting profile showed (Fig 2) presence of 10 well separated compounds (Table 1). The GC-MS fingerprinting profile of *D. biflorus* hexane extract led to separation and identification of 28 components showed in chromatogram (Fig 3), which were identified as per the NIST and Wiley library using M/Z as given in Table 2. However, respective concentration of palmitin was highest (25.08%) followed by n-hexadecanoic acid (9.52), 9, 12-octadecadienoic acid (8.37) and tetradecanoic acid (6.67%).

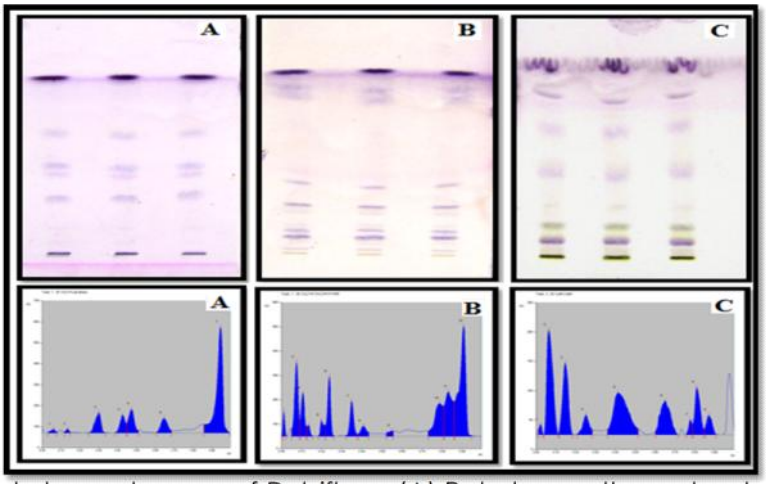

Fig 1: HPTLC plate and chromatogram of *D. biflorus* (A) Petroleum ether extract showing 6 spots at 550 nm. (B) Chloroform extract showing 10 spots at 600 nm. (C) Methanolic extract showing 7 spots at 530 nm.

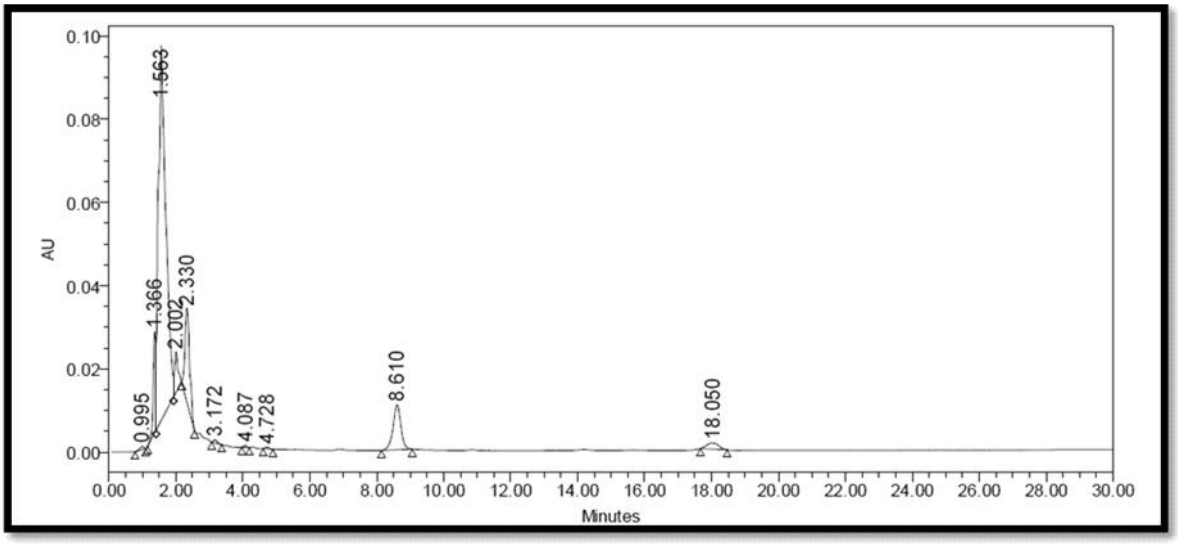

Fig 2: HPLC chromatogram of *D. biflorus* methanol extract showing 10 peaks at 296 nm.

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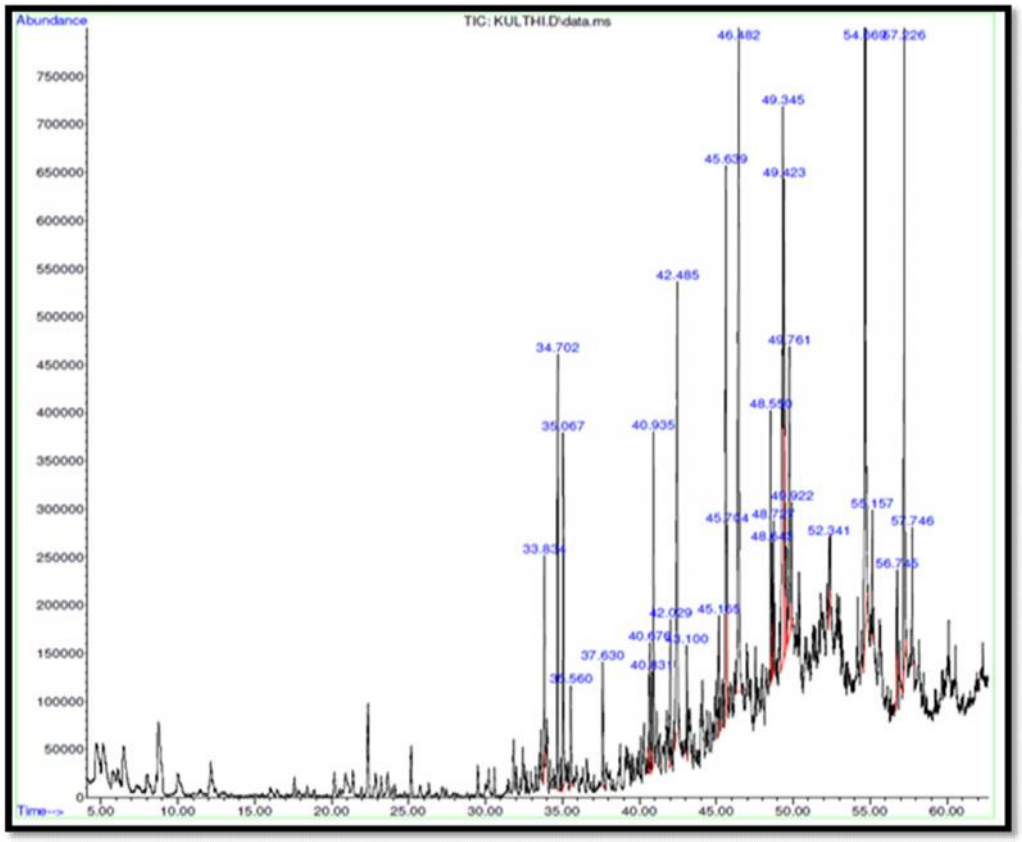

Fig 3: GC-MS chromatogram of hexane extract of *D. biflorus* showed 28 compounds

Table 2: Result of GCMS finger print profile of hexane extract of *D. biflorus*

S. No.	Compound	Retention time	% components
1	Docosane	33.836	1.76
2	Phenol, 2,4-bis(1,1-dimethylethyl)	34.702	4.05
3	Benzoic acid, 4-ethoxy-, ethyl ester	35.065	3.64
4	Eicosane	35.559	1.14
5	Hexadecane	37.631	1.19
6	Heptadecane	40.678	1.31
7	Heneicosane	40.833	0.87
8	2-Bromo dodecane	40.937	2.56
9	Octadecane	42.029	1.50
10	Tetradecanoic acid	42.486	6.67
11	Hexatriacontane	43.098	0.72
12	Nonadecane	45.165	1.07
13	79-Di-tert-butyl-1-oxaspiro(45)deca-69-diene-28-dione	45.640	5.17
14	Hexadecanoic acid	45.706	1.16
15	n-Hexadecanoic acid	46.483	9.52
16	Octadecanoic acid, methyl ester	48.550	1.76
17	1-Cyclopropyl-6-undecen-1-ol	48.645	1.41
18	Heneicosane	48.729	0.99
19	9,12-Octadecadienoic acid (Z,Z)-	49.346	8.37
20	9-Octadecenoic acid (Z)-	49.422	5.89
21	Vanicol	49.671	3.43
22	n-Tricosane	49.921	1.11
23	Octadecane	52.341	0.60
24	Palmitin	54.672	25.08
25	Pentacosane	55.157	1.02
26	Nonadecane	56.743	0.39
27	Stearin	57.224	6.51
28	Eicosane	57.746	1.12
		Total	**100.00**

Determination of contaminants

After the comparison of GC-MS chromatogram of sample with retention time of 31 standard pesticides, this was observed that SPP was not containing any pesticide. The aflatoxins (B1, B2, G1 and G2) analysis of SPP was also carried out by HPLC method and found freed from any type of aflatoxins (Limit NMT 4ppb for all B1, B2, G1 and G2).

It can be concluded from present investigation that, physicochemical parameters, qualitative and quantitative analysis, HPTLC, HPLC and GCMS finger print profile together with determination of contaminants like aflatoxin and pesticides may be used for quality evaluation and standardization of *D. biflorus*.

Conclusion

The present study may be useful to supplement the information with regards to standardization and identification of *D. biflorus* and in carrying out further research and its use in Unani/Ayurvedic system of medicine.

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