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Phytochemical standardization of *Diploknema butyracea* (Roxb.) H.J. Lam. seeds by HPTLC technique

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Diploknema butyracea (Roxb.) H.J. Lam. (Family Sapotaceae) commonly known as Indian butter tree or *Cheura*, is native to Nepal and distributed from Garhwal Himalaya to Sikkim and up to Bhutan. Its seeds are the richest source of edible oil known as Phulwara butter which is being used by local communities for cooking purposes. The objective of the study was to examine the phytochemical constituents and development of fingerprinting profile with the aid of HPTLC technique. The qualitative and quantitative distribution of the active principles was assessed and a HPTLC method was developed for the separation of active constituents in seed extracts. Preliminary phytochemical screening of secondary metabolites was carried out by following standard methods and found to contain lipids, saponins, tannins, alkaloids, phenols, steroids and flavonoids. The study will prove useful to compare bioactive principle present in the seeds.

Keywords: *Diploknema butyracea* (Roxb.) H.J. Lam., HPTLC, Phulwara butter, Phytochemical constituents, Sapotaceae.

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Introduction

Natural phytochemicals are known to contain substance that can be used for therapeutic purposes or as precursor for the synthesis of novel useful drugs. Use of plants as a source of medicine has been inherited and is an important component of health care system[1]. In plants, phytochemicals act as a natural defense system for host plants and provide colour, aroma and flavour. More than 4000 of these compounds have been discovered to date and it is expected that scientists will discover many more. Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well being. Their role is twofold in the development of new drugs[2]. Natural products and secondary metabolites formed by living systems, notably of plant origin have shown great potential in treating human diseases such as cancer, coronary heart diseases, diabetes and infectious diseases[3]. Plants have been known to be used as remedies for human ailments as they contain components of therapeutic values. Isolation of bioactive compounds of novel or known structures had been goals of various pharmacological studies on traditional preparations[4]. Phytochemical analysis of plants which were used in folklore has yielded a number of compounds with various pharmacological activities therefore standardization of the plant material is need of the day. Several pharmacopoeia containing monographs on plants describe only the physico-chemical characters. Hence, modern methods describing the identification and quantification of active constituents in the plant material may be useful for proper standardization of herbs and its formulations[5,6].

Currently HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost-effectiveness and rapidity. HPTLC fingerprint has better resolution and estimation of active constituents is done with reasonable accuracy in a shorter time[7,8]. Chromatographic fingerprint is a rational option to meet the need for more effective and powerful quality assessment to Indian Traditional Medicine and Chinese traditional herbal medicine. The optimized chromatographic finger print is not only an alternative analytical tool for authentication but also an approach to express various patterns of chemical ingredients

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distributed in the herbal drugs. Also WHO has emphasized the need to ensure the quality of medicinal plant product using modern controlled techniques and applying suitable standards. In the recent years, advancement in chromatography and spectral fingerprints played an important role in the quality control of complex herbal medicines[9].

Diploknema butyracea (Roxb.) H.J. Lam syn. *Aesandra butyracea* (Roxb.) Baehni, *Bassia butyracea* Roxb., *Madhuca butyracea* (Roxb.) J.F.Macbr. (Family Sapotaceae) commonly known as Indian butter tree is a large deciduous tree found in the sub-Himalayan and outer Himalayas up to an altitude of about 5000 m. Its seeds are the source of commercial Phulwara fat (60-67 % on kernel basis) which is used in soaps and cosmetics. It is also used by local community for cooking purpose. The plant flowers during winter season and the fruits ripen in June–July. Fruits are ellipsoid (0.8-1.8 cm) and each encloses 1-3 black seeds (0.8 g in weight) and almond shaped kernels (70 % of the weight of seeds) contain 60-70 % oil[10]. Oil is used as external ointment to ease rheumatism, paralysis and sprains. The oil cake contains saponins and act as fertilizers, fish intoxicant, pesticides and detergents. The flowers are rich source of sugars and utilized for preparation of gur like products. The bark of the tree is used in the treatment of rheumatism, ulcers, itching and hemorrhage, inflammation of the tonsils, leprosy and diabetes[11]. Looking at the wide medicinal uses of *D. butyracea* (Roxb.) H.J. Lam., present study was undertaken to investigate the fundamental scientific bases of the plant by defining and quantifying percentage of crude phytochemical constituents present in the seeds.

Materials and Methods

Collection of plant material

Seeds of *D. butyracea* (Roxb.) H.J. Lam. were collected from Pithoragarh, Uttarakhand, India during June-July 2012. The collected seeds were identified and authenticated by Dr. Veena Chandra, Scientist-F, Systematic Branch, Botany Division, Forest Research Institute (FRI), Dehradun and a voucher specimen (No.157027) was deposited in the herbarium of the same Division, FRI.

Preparation and extraction of plant material

The collected seeds were shade dried and decorticated manually and kernels were separated and coarsely powdered. The powdered material was extracted sequentially with the solvents of elutropic series, viz. chloroform, methanol and aqueous solvent by using Soxhlet apparatus. The extraction was carried out until the extractives became colourless. The extracts were filtered through a cotton plug, followed by Whatman filter paper (no.1) and concentrated under reduced vacuum and pressure by using rotary evaporator and evaporated to dryness. Their percentages were calculated in terms of initial air dried plant material. The yield of different extracts, viz. chloroform, methanol and aqueous was found to be 2.98, 4.47 and 3.53 %, respectively.

Phytochemical screening

The extracts were subjected to various qualitative phytochemical tests for identification of chemical constituents. Major constituents were screened using standard qualitative methods as described by Sofowora method[12]. The test for tannins was carried out by filtering 3 g of each seed extract in 6 mL of distilled water to which ferric chloride reagent was added. For cardiac glycosides, Killer-Kiliani test[13] was adopted (0.5 g of extract was added to 2 mL of each acetic acid and H_2SO_4). Aqueous extract (0.5 g) was stirred with 5 mL of 1 % HCl and filtered. The filtrate was tested carefully with Mayer's reagent. The reddish brown precipitate indicates the presence of alkaloids[13,14]. The extract was subjected to frothing test for the identification of saponin. Haemolysis test was further performed on the frothed extracts in water to remove false positive results. Extract was also tested for free glycoside bound anthraquinones. Extract (5.0 g) was heated with 10 mL of ethyl acetate over steam bath for 3 min. The mixture was filtered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution. The appearance of yellow colour shows the presence of flavonoids[12] (Table 1).

Quantitative Analysis

Quantitative determination of total flavonoids, saponins and terpenoids content was determined by following standard methods[14-17](Table 2).

Thin Layer Chromatography (TLC)

Glass plates (20 cm×20 cm) were coated with silica gel (Qualigen fine chemicals) (0.5 mm) and samples of each extract dissolved in methanol (5 mg/mL) were applied. Chromatography was performed in 100 % chloroform according to the method described by Harborne[14]. The spots were visualized using concentrated sulphuric acid as a spray reagent

followed by heating of plates at 100 °C for 10 min. The spots were identified based on the colour, produced on reacting with a spray reagent.

High performance thin layer chromatography (HPTLC)

A highly sensitive and accurate HPTLC method was developed and used for processing different extracts of *D. butyracea* (Roxb.) H.J. Lam. on the automated HPTLC system.

Instrumentation

A CAMAG HPTLC system comprising LINOMAT 5_110922 sample applicator controlled by WINCATS software v 1.3.4 was used for sample application.

Preparation of sample for HPTLC

The powdered seeds (100 g) was taken in a stopperd conical flask separately and macerated with the particular solvent. Afterwards the contents were filtered using Whatman filter paper no 42 and evaporated to dryness by rotary evaporator. From each extract, 100 mg was taken and dissolved in the corresponding solvent from which the extract was prepared and made up to 20 mL and solution obtained was applied on the TLC plate as sample solution.

Development and optimization of the solvent system

Chromatographic separation was carried on 10 cm×10 cm aluminium plate precoated with silica gel $60F_{254}$ (Merck) as the stationary phase for different extracts prepared. 10 µL of the sample was applied and different solvent systems were selected for different extracts. The scan was performed at a wavelength of 366 nm and also at visible range. A saturation time of 25 min was allowed before chromatographic run. The sample was spotted on the TLC plate in triplicate with the help of automatic TLC applicator system (CAMAG Linomat 5 programmed by WIN CATS software). After trying various solvent systems with variable volume ratio, the suitable solvent system for chloroform extracts was observed as ethyl acetate: pet ether (15:85) while chloroform: methanol:water (13:7:2) was selected for methanol extract. The solvent system selected for aqueous extract was chloroform:methanol:water (16:6:1). The plate was developed in the twin trough chamber (CAMAG) to the maximum height (80 mm distance in a typical 10 cm×10 cm plate and slit dimension was 6.0 mm×0.45mm) of the plate so that it is able to separate all the components on the polar phase of silica gel and that of mobile phase of solvent system. The components get separated by the principle of adsorption, having differential migration rates of individual component towards the phases.

Detection of spots

After development, TLC plate was removed, dried and detected with suitable detection system as 5 % methanolic sulphuric acid or UV cabinet system for detection of spots. The chromatograms were scanned by densitometer at multiple wavelength i.e. 200, 400, 500 and 650 nm. After exhaustive scanning, the most feasible wavelength (which shows the maximum number of spots) was analyzed. A corresponding densitogram was then obtained in which peaks appeared for the corresponding spots being detected in the densitometer while scanning. The peak area under the curve corresponds to the concentration of different extracts (Table 3). The R_f values and finger print data was obtained using WIN CATS software.

Results and Discussion

D. butyracea (Roxb.) H.J. Lam. extract solution was spotted as 8-10 mm on the precoated HPTLC silica gel $60F_{254}$ plates. The R_f values of the corresponding component as obtained through the software system attached with the instrument and are being presented in Table 3. Seeds of *D. butyracea* (Roxb.) H.J. Lam. extracted separately with different solvents were subjected to HPTLC analysis by specific solvent system. The densitogram obtained upon scanning under the densitometer are shown in Fig. 1-3.

The qualitative analysis of the seed extracts showed the presence of phytochemical constituents such as alkaloid, tannin, glycosides, fixed oils, fats, flavonoids, sterols, phenol, terpenoids and saponins (Table 1). The presence of flavonoids in the plant indicates that it may have antioxidant and free-radical scavenging properties[18]. Steroidal compounds are of importance in pharmacy as they act as sex hormones. Saponins are linked to antibacterial activity and glycosides are associated in lowering blood pressure[19]. Table 2 summarizes the quantitative determination of phytochemical constituents of the plant showing the presence of higher contents of terpenoids and flavonoids. Due to the presence of these bioactive constituents *D. butyracea* (Roxb.) H.J. Lam. is considered an important medicinal plant and has been utilized in a number of ways in pharmaceutical sector. HPTLC is the advancement of

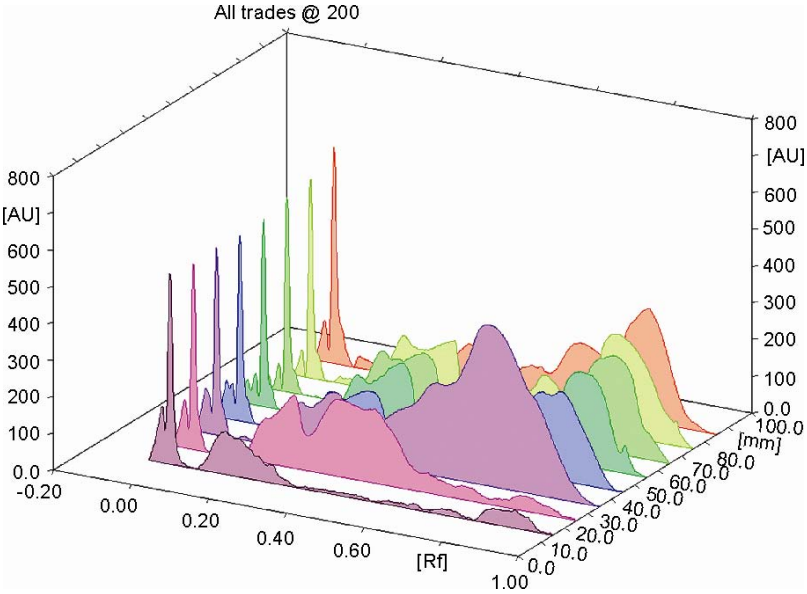

Fig. 1—Densitogram for chloroform extract of kernels at 200 nm

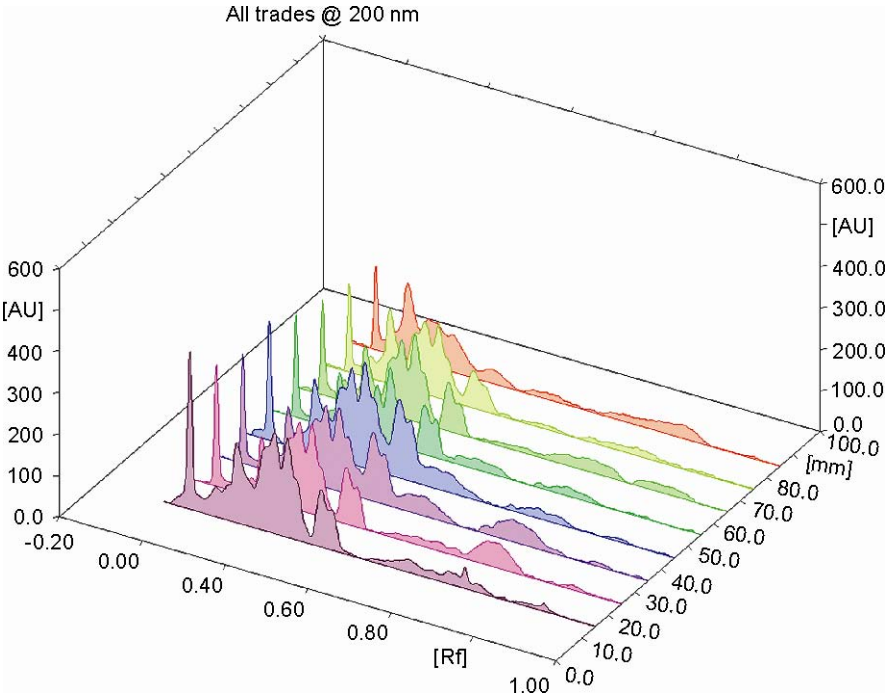

Fig. 2—Densitogram for methanol extract of kernels at 200 nm

conventional TLC and has been used for profiling of different extracts (Table 3). The main advantage in this technique is the use of relatively smaller particle size of silica gel (5-6 µm) in the preparation of TLC plates which reduces the developing time and led to better resolution. Analysis time is reduced and less mobile phase is required and developing distances could also be reduced. The HPTLC fingerprinting profiling of chloroform, methanol and water extract of kernels showed 7, 11 and 8 peaks. The phytochemical studies provide valuable information which may help in authenticating the genuine specimen along with the nature of phytoconstituents present in it. Preliminary qualitative phytochemical screening is reported in this paper. In plants, flavonoids can function as attractants to pollinators and seed

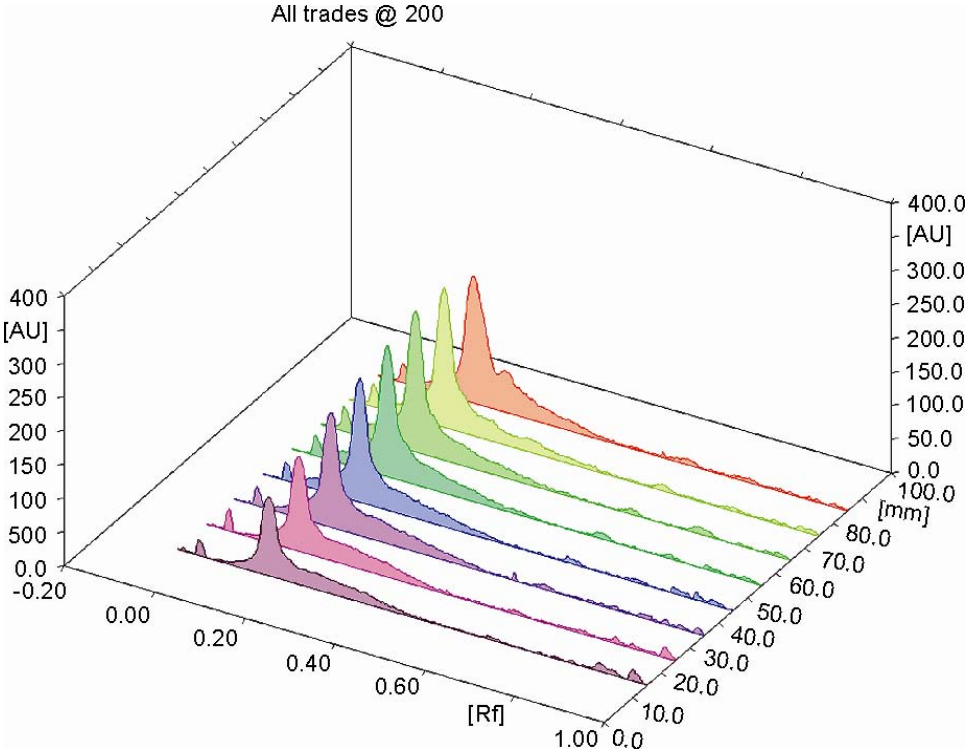

Fig. 3—Densitogram for aqueous extract of kernels at 400 nm

Table 1—Qualitative analysis of *Diploknema butyracea* (Roxb.) H.J. Lam. seed extracts.

Chemical components	Chloroform extract	Water extract	Methanol extract
Alkaloids	-	-	-
Carbohydrates	-	+	+
Glycosides	-	-	+
Proteins and amino acids	-	-	-
Saponins	-	+	+
Phenolics and tannins	-	-	-
Fixed oils and fats	+	-	-
Flavonoids	-	+	+
Sterols	-	+	+
Terpenoids	+	+	+

+ = Present, – = Absent

Table 2—Quantitative analysis of *Diploknema butyracea* (Roxb.) H.J. Lam. seed extracts

S. No.	Components	% yield
1.	Flavonoids	23.15 mg/g
2.	Terpenoids	92 mg/g
3.	Saponins	20 mg/g

Table 3—R_f values of the chromatogram of chloroform, methanol and aqueous extract

Peaks	R_f values		
	Chloroform extract*	Methanol extract**	Aqueous extract***
1	0.02	0.02	0.02
2	0.01	0.07	0.14
3	0.08	0.12	0.21
4	0.23	0.21	0.32
5	0.31	0.24	0.39
6	0.58	0.33	0.50
7	0.72	0.35	0.68
8		0.61	0.81
9		0.68	
10		0.81	

Solvent System- *Ethyl acetate: Pet ether (15:85), **Chloroform: Methanol: Water (13:7:2) and ***Chloroform: Methanol: Water (16:6:1)

dispersers, as antioxidants to protect plants against UV radiation, as insect feeding attractants in host species recognition, as signal molecules to facilitate nitrogen fixation, in inducible defense against bacterial and fungal attack[20]. Tannins are widely distributed in almost all plant foods and are found effective in protecting the kidneys and show potential

antiviral, antibacterial and anti-parasitic effects[20,21]. Alkaloids are the most important class of natural products responsible for the antiplasmodial activity of many plant species used in traditional practices for the treatment of malaria[22].

Conclusion

The study will prove useful in comparison of the amount of bioactive principle present in the plant with other species. The HPTLC-Fingerprinting profiling is an important tool for herbal drug standardization to identify medicinal plants properly. If adulteration is suspected in some specified plant material, this parameter would be helpful for its detection. Moreover this profile will help in determining the quality and purity of the plant material in future studies. Further research on this plant may help in the isolation of therapeutically potent compounds which can finally be subjected to pharmacological activities and clinical trials, thus leading to opening up a new path for the use of this plant.

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