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High performance thin layer chromatography fingerprinting, phytochemical and physico-chemical studies of anti-diabetic herbal extracts

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Abstract

Introduction: Herbal medicines have gained increasing popularity in the last few decades, and this global resurgence of herbal medicines increases their commercial value. However, this increasing demand has resulted in a decline in their quality, primarily due to a lack of adequate regulations pertaining to herbal medicines. **Aim:** To develop an optimized methodology for the standardization of herbal raw materials. **Materials and Methods:** The present study has been designed to examine each of the five herbal anti-diabetic drugs, *Gymnema sylvester* R. Br., *Pterocarpus marsupium* Roxburgh., *Enicostema littorale* Blume., *Syzygium cumini* (L.) Skeels. and *Emblica officinalis* Gaertn. The in-house extracts and marketed extracts were evaluated using physicochemical parameters, preliminary phytochemical screening, quantification of polyphenols (Folin–Ciocalteu colorimetric method) and high performance thin layer chromatography (HPTLC) fingerprint profiling with reference to marker compounds in plant extracts. **Results:** All the plants mainly contain polyphenolic compounds and are quantified in the range of 3.6–21.72% w/w. *E. officinalis* contain the highest and *E. littorale* contain the lowest content of polyphenol among plant extracts analyzed. HPTLC fingerprinting showed that the in-house extracts were of better quality than marketed extracts. **Conclusion:** The results obtained from the study could be utilized for setting limits for the reference phytoconstituents (biomarker) for the quality control and quality assurance of these anti-diabetic drugs.

Key words: Anti-diabetic, high performance thin layer chromatography fingerprint, physicochemical parameters, standardization

Introduction

Nature has been a source of medicinal agents for thousands of years, and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of the agents in traditional medicine.[1] According to World Health Organization (WHO), about three-quarter of the world population relies upon traditional remedies for the health care of its people. In fact, plants are the oldest friends of mankind. They not only provided food and shelter, but also served the humanity to cure different ailments.[2] The plants and their extracts are a common elements in Indian systems of medicine. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value. The plant-based, traditional medicine systems continue to play an essential role in health care.[3] The global market for herbal medicines currently stands at over $60 billion annually.[4]

In the world, diabetes is a serious disease due to irrational food habits. Most of the hypoglycemic agents used in allopathic practice to treat diabetes mellitus are reported to

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have side effects in long term use.[5] Hence, there is a need to search for effective and safe drugs for these ailments. Pharmaceutical research across the world shows that natural products are potential sources of novel molecules for drug development.

The recent global resurgence in herbal medicines has led to an increase in the demand for them. Commercialization of these medicines to meet this increasing demand has resulted in a decline in their quality, primarily due to a lack of adequate regulations pertaining to this sector of medicine. The need of the hour is to evolve a systematic approach and to develop well-designed methodologies for the standardization of herbal raw materials and their formulations. Various methods of phytochemical standardization, such as preliminary phytochemical screening, fingerprint profiling and quantification of the marker compound with reference to plant extracts and polyherbal formulations are used.[6] Standardization is necessary to make sure the availability of an uniform product in all parts of the world. It assures a consistently stronger product with guaranteed constituents.[7]

Materials and Methods

Plant material and market extracts

The five herbal anti-diabetic drugs and their extracts, chosen for this study were *Gymnema sylvestre* R. Br. - Asclepiadaceae (*Madhunashini* - leaves), *Pterocarpus marsupium* Roxb. - Fabaceae (*Vijaysara* - heart-wood), *Enicostema littorale* Blume. - Gentianaceae (*Mamejaka* - whole plant), *Syzygium cumini* (L.) Skeels. - Myrtaceae (*Jambu* - seeds) and *Emblica officinalis* Gaertn. - Euphorbiaceae (*Amalaki* - whole fruits). All the authenticated crude drugs and market extracts of *Madhunashini*, *Mamejaka*, *Jambu* were procured from Plantex Agro Products (P) Ltd., Vashi, Navi Mumbai. *Amalaki* and *Vijaysara* market extracts were procured from Kisalaya herbals, Ratlam Kothi, Indore and Amruta Herbals Private Ltd., Sanwer Road, Indore respectively.

Chemicals and standards

Gallic acid, gymnemagenin (Sigma-Aldrich Co. LLC.), pterostilbine (Wuxi Cima Science Co. Ltd.), vanillin, Folin–Ciocalteu phenol reagent, ortho-phosphoric acid, sodium acetate, formic acid (Merck chemicals Ltd.), anisaldehyde, acetic acid, anhydrous sodium carbonate, sulfuric acid, methanol, ethanol, acetone, ethyl acetate, acetonitrile (SD Fine-Chem. Ltd.).

Preparation of in-house plant extracts

About 100 g of well-dried crude powder of *Amalaki* and *Jambu* were macerated with water while *Madhunashini*, *Vijaysara* and *Mamejaka* were macerated with hydro-ethanolic (1:1 v/v) solvents respectively. Each extract was then concentrated using rotary vacuum evaporator (25°C) and finally lyophilized (Maro Scientific Works, 10A/UA).

Physicochemical properties

Physical characteristics such as extractive values, moisture content, ash values, were determined according to the standard test procedures.[8-11]

Preliminary phytochemical screening of extracts

All plant extracts were subjected to phytochemical screening for checking presence of chemical constituents such as alkaloids, tannins, flavonoids, saponins, sterols, proteins and carbohydrates.[12]

Total polyphenol estimation of extracts

Total polyphenol content was measured using Folin–Ciocalteu colorimetric method, in which 2 ml of test and standard sample was taken in 25 ml volumetric flask, 10 ml of demineralized water was added, then mixed with 2 ml of diluted Folin–Ciocalteu's phenol reagent (1:5 with distill water; 0.4 N). Then, volume was adjusted to 25 ml with 29% sodium carbonate solution. The reaction was kept in dark for 30 min and the absorbance was read at 760 nm (Shimadzu UV–VIS spectrophotometer 1600) against the corresponding test and standard blanks prepared in the same way without the extract. Gallic acid was used as a reference standard.

Standard solution was prepared by accurately weighing about 50 mg of gallic acid and adding it to 100 ml of volumetric flask. Made up the volume with demineralized water. Diluted 2 ml of resulting solution to 25 ml with demineralized water. Used the resulting solution as a standard solution.[13,14]

Test solution was prepared by accurately weighing extract and adding to 100 ml volumetric flask. Added 80 ml of demineralized water and heated for 60 min on a water bath at 100°C. Cooled and made up the volume with demineralized water. Filtered the content of volumetric flask and diluted 5 ml of resulting the solution to 25 ml with demineralized water. Used the resultant solution as the test solution.

$$\text{Total polyphenol as gallic acid (\% w/w)} = \text{AT/AS} \times \text{WS/DS} \times \text{DT/WT} \times \text{P/100} \times 100$$

Where, AT = Absorbance of test solution at 760 nm; AS = Absorbance of standard solution at 760 nm; WS = Weight of standard; DS = Dilution of standard solution; DT = Dilution of test solution; WT = Weight of test extract; P = Potency of standard.

Development of high performance thin-layer chromatography (HPTLC) methods for extracts

Qualitative estimation of biologically active compounds like gallic acid from *Amalaki* and *Jambu*, gymnemagenin from *Madhunashini*, and pterostilbine from *Vijaysara* extract was performed by using HPTLC (CAMAG Linomat V applicator and CAMAG TLC SCANNER-III equipped with Win-CAT software).[15,16]

Preparation of test solution

Accurately weighed 100 mg extracts were dissolved in 15 ml methanol and sonicated for 10 min. It was then diluted with methanol up to 20 ml (5 mg/ml). The solution was filtered through Whatman filter paper no. 1.

Preparation of standard solution

Accurately weighed 5 mg of standard was dissolved in 5 ml methanol, sonicated and diluted 1 ml of this solution to 10 ml with methanol (100 μg/ml).

Selection of mobile phase

It is a fact that mobile phase optimization is among the important steps that affect the quality of a separation in TLC method development. The results show the optimized solvent system for each extract.

Chromatographic condition

The samples were spotted in the form of bands, width 6 mm with a Camag 100 µl sample syringe (Hamilton, Bonaduz, Switzerland) on silica gel precoated aluminum plate 60 F_{254} plates, (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany) using a Camag Linomat V (Switzerland) sample applicator. The plates were developed using optimized mobile phase in twin trough development chamber. The plates were removed, dried at 105°C and sprayed with spraying reagents (Anisaldehyde: Sulphuric acid solution for Madhunashini; vanillin: Sulphuric acid solution for *Vijaysara* and 5% ferric chloride solution for *Amalaki*, *Jambu* and *Mamejaka*. *Madhunashini* (500 nm), *Vijaysara* plates (550 nm) were scanned at visible light and *Amalaki*, *Jambu* and *Mamejaka* were scanned under UV-254 nm.

Results and Discussion

Physicochemical properties

Physicochemical properties play an important role in the evaluation of crude drugs. The less extractive value indicates addition of exhausted material, adulteration or incorrect processing during drying or storage. The extractive value also signifies the presence of chemical constituents extracted by a specific solvent. All the individual drugs were found to have water-soluble extractive and alcohol-soluble extractive in the range 18.90–23.04% and 12.20–23.18% w/w, respectively. Moisture is one of the major factors responsible for the deterioration of the crude drugs and formulations. Low moisture content is always desirable for higher stability of drugs. Moisture contents of the individual drug were found in the range of 1.35–5.39% w/w. A high ash value is indicative of contamination or carelessness in preparing the drug or drug combinations for marketing. All the individual drugs were found to have total ash and acid insoluble ash values in the range of 3.06–12.59% and 0.85–2.04% w/w, respectively [Table 1].

Percent yield of in-house plant extract

The extraction of crude material was carried out with appropriate solvent. Aqueous extracts of *Amalaki* and *Jambu* were prepared and hydro-alcoholic extracts of *Madhunashini*, *Vijaysara* and *Mamejaka* were prepared [Table 2].

Preliminary phytochemical screening of extracts

In the preliminary phytochemical screening, *Madhunashini*, *Mamejaka* and *Jambu* extracts were found to contain alkaloids, tannins, flavonoids, saponins, sterols, proteins and carbohydrates. The *Amalaki* and *Vijaysara* contain tannins, flavonoids, saponins, sterols, proteins and carbohydrates. All the plants mainly found to contain polyphenolic compounds like tannins and flavonoids in their extracts.

Total polyphenol estimation of extracts

All the in-house extracts and market extracts were subjected to total polyphenol content estimation. It was found that polyphenol content of the extract was in the range of 3.6–21.72%. *Amalaki* contains the highest and *Mamejaka* contain the lowest content of polyphenol among the five plant extracts [Table 3].

Development of HPTLC methods for extract

The standards like, gymnemagenin for *Madhunashini* [Figure 1a and Table 4], pterostilbine for *Vijaysara* [Figure 2a and Table 5], gallic acid for *Amalaki* and *Jambu* [Figures 3a and 4a] [Tables 6 and 7] were used for checking quality of extracts. As per the HPTLC observations, the market extract of *Madhunashini* 9 peaks [Figure 1c and Table 4] and *Mamejaka* 11 peaks [Figure 5b and Table 8] shown more number of peaks but as per the height and area in comparison with marker compounds [Figures 1b-4b] the in-house extracts of all the plants [Figures 1d-4d, 5c] were found to be of better quality than market extracts [Figures 1c-5c]. Typical HPTLC fingerprinting of particular plant species will not only help in the identification and quality control of a particular species, but also provide basic information useful for the isolation, purification, characterization, and identification of marker chemical compounds of the species and also employed for taxonomic categorization. Thus, the present study will provide sufficient information about therapeutic efficacy of the extracts and also in the identification, standardization, and quality control of medicinal plant.

Table 1: Physicochemical parameters of crude drugs

Name of plant	Percent (w/w)				
	WSEV	ASEV	LOD	TA	AIA
Amalaki	23.04	12.20	5.39	3.5	1.04
Jambu	20.63	13.40	1.35	3.21	0.85
Vijaysara	18.90	15.14	4.19	3.06	0.95
Mamejaka	19.23	23.18	5.08	12.59	2.04
Madhunashini	20.14	15.14	4.19	8.07	1.03

WSEV: Water-soluble extractive, ASEV: Alcohol-soluble extractive, LOD: Loss on drying, TA: Total ash, AIA: Acid insoluble ash

Table 2: Extraction details and percent yield of in-house plant extract

Name of crude material	Part used	Solvent for extraction	Yield (%)
Amalaki	Whole fruit	Aqueous	24.80
Jambu	Seeds	Aqueous	22.63
Vijaysara	Heart wood	Hydro-alcoholic	16.49
Mamejaka	Whole plant	Hydro-alcoholic	25.14
Madhunashini	Leaves	Hydro-alcoholic	25.17

Table 3: Total polyphenol content of extracts

Name of plant	Polyphenol assay (% w/w)	
	Market extract	In-house extract
Amalaki	16.42	21.72
Jambu	7.92	15.54
Vijaysara	8.84	20.97
Madhunashini	4.95	5.03
Mamejaka	3.6	3.42

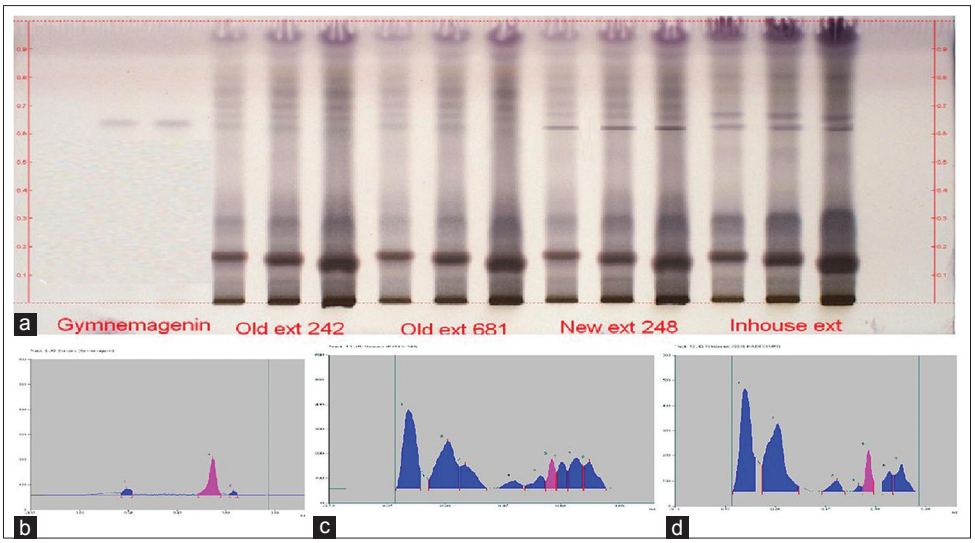

Figure 1: High performance thin layer chromatography study of *Madhunashini* (*Gymnema sylvestre*); (a-d) represent as *Madhunashini* plate at visible light, fingerprint of standard gymnemagenin, *Madhunashini* market extracts (new extract) and *Madhunashini* in-house extract at 500 nm respectively

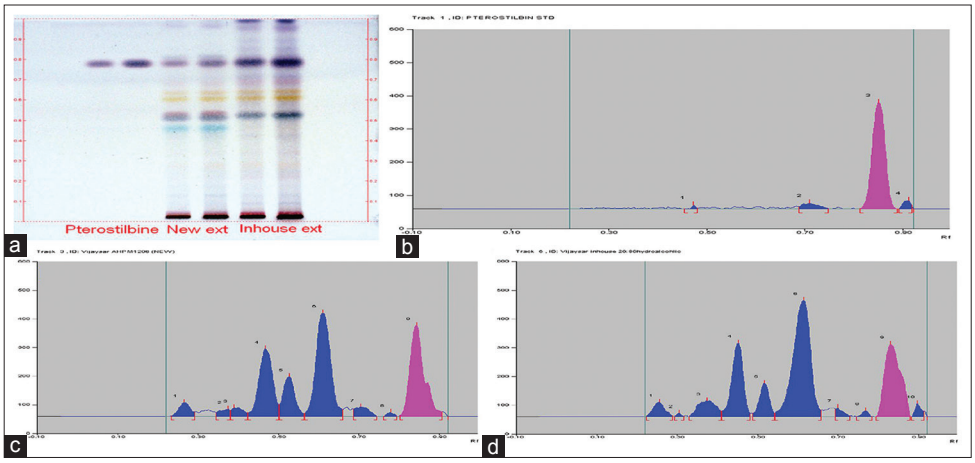

Figure 2: High performance thin layer chromatography study of *Vijaysara* (*Pterocarpus marsupium*); (a-d) represent as *Vijaysara* plate at visible light, fingerprint of standard pterostilbine, *Vijaysara* market extracts (new extract) and *Vijaysara* in-house extract at 550 nm respectively

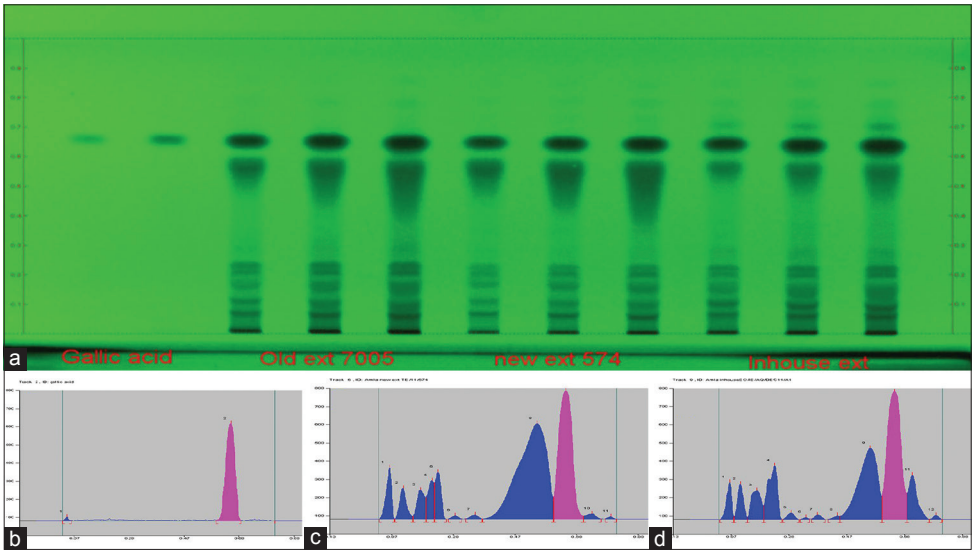

Figure 3: High performance thin layer chromatography study of *Amalaki* (*Emblica officinalis*); (a-d) represent as *Amalaki* plate, fingerprint of standard gallic acid, *Amalaki* Market extracts (new extract) and *Amalaki* in-house extract at 254 nm respectively

Table 4: R_f values by densitometric scan of *Madhunashini* at 500 nm

R_f values	B	Gm-5	Gm-10	OE 242-05	OE 242-10	OE 242-15	OE 681-05	OE 681-10	OE 681-15	NE 248-05	NE 248-10	NE 248-15	IE-05	IE-10	IE-15
0.08				+	+	+			+			+			+
0.09							+	+		+	+		+	+	
0.27				+	+	+							+	+	+
0.28							+	+	+	+	+	+			
0.32				+	+	+	+	+	+	+	+	+			
0.51				+	+	+							+	+	+
0.52							+	+	+	+	+	+			
0.61				+	+	+							+	+	+
0.62							+	+	+						
0.63										+	+	+			
0.64		Gm	Gm	Gm	Gm	Gm	Gm	Gm	Gm	Gm	Gm	Gm	Gm	Gm	Gm
0.69				+	+	+	+	+	+	+	+	+			
0.80							+	+	+	+	+	+			
0.81				+	+	+							+	+	+
0.88							+	+	+						
0.89				+	+	+				+	+	+	+	+	+
Total	Nil	01	01	09	09	09	09	09	09	09	09	09	07	07	07

B represents blank, Gm – 5 and 10: Gymnemagenin 5 µl and 10 µl, OE: Old extract, NE: New extract procured from Plantex Agro Products with batch number 242 (P9020242); 681 (P9060681); 248 (P1112248) respectively, IE: In-house extract loaded 5 µl; 10 µl and 15 µl using mobile phase – Toluene: ethyl acetate: formic acid – 5:5:2

Table 5: R_f values by densitometric scan of *Vijaysara* at 550 nm

R_f values	Blank	Pterostilbene-05 µl	Pterostilbene-10 µl	New extract-10 µl	New extract-15 µl	In-house extract-10 µl	In-house extract-15 µl
0.13				+	+	+	+
0.19						+	+
0.27				+	+	+	+
0.31						+	+
0.33				+	+		
0.40				+	+	+	+
0.51				+	+	+	+
0.61				+	+	+	+
0.69				+	+	+	+
0.76				+	+	+	+
0.78		Pterostilbene	Pterostilbene	Pterostilbene	Pterostilbene	Pterostilbene	Pterostilbene
0.80			+			+	+
Total	Nil	01	01	09	09	10	10

New extract procured from Amruta Herbals-AHPM 1206. Mobile phase – Toluene: ethyl acetate: formic acid-7:3:0.5

Table 6: R_f values by densitometric scan of *Amalaki* at 254 nm

R_f values	GA-05	GA-10	OE 7005-05	OE 7005-10	OE 7005-15	NE 574-05	NE 574-10	NE 574-15	IE-05	IE-10	IE-15
0.08			+	+	+						
0.09						+	+	+	+	+	+
0.11			+	+	+	+	+	+	+	+	+
0.22			+	+	+						
0.23						+	+	+	+	+	+
0.28			+	+	+	+	+	+			
0.30						+	+	+	+	+	+
0.31			+	+	+						
0.39			+	+	+						
0.40						+	+	+	+	+	+
0.43									+	+	+

Contd...

Table 6: Contd...

R_f values	GA-05	GA-10	OE 7005-05	OE 7005-10	OE 7005-15	NE 574-05	NE 574-10	NE 574-15	IE-05	IE-10	IE-15
0.48			+	+	+	+	+	+	+	+	+
0.51									+	+	+
0.58			+	+	+						
0.59						+	+	+	+	+	+
0.67	GA	GA	GA	GA	GA	GA	GA	GA	GA	GA	GA
0.71			+	+	+	+	+	+	+	+	+
0.80			+	+	+						
0.81						+	+	+	+	+	+
Total	01	01	11	11	11	11	11	11	12	12	12

GA – 5 and 10 represent: Gallic acid 5 µl and 10 µl, OE: Old extract, NE: New extract procured from Kisalaya herbals with batch number 7005 (TE/07005); 574 (TE/11/574) respectively, IE: In-house extract loaded 5 µl; 10 µl and 15 µl using mobile phase – Toluene: ethyl acetate: methanol: formic acid-9:9:4:1

Table 7: R_f values by densitometric scan of *Jambu* at 254 nm

R_f values	GA-5	GA-10	GA-15	OE-05	OE-10	OE-15	NE-05	NE-10	NE-15	IE-05	IE-10	IE-15
0.11							+	+	+	+	+	+
0.12				+	+	+						
0.15				+	+	+	+	+	+	+	+	+
0.20							+	+	+			
0.21				+	+	+						
0.28				+	+	+	+	+	+	+	+	+
0.32							+	+	+			
0.33				+	+	+						
0.34										+	+	+
0.39				+	+	+	+	+	+			
0.49	GA	GA	GA	GA	GA	GA	GA	GA	GA	GA	GA	GA
0.52										+	+	+
0.66							+	+	+	+	+	+
0.68				+	+	+						
0.79										+	+	+
Total	01	01	01	08	08	08	08	08	08	08	08	08

GA – 5; 10 and 15 represent: Gallic acid 5 µl; 10 µl and 15 µl, OE: Old extract, NE: New extract procured from Plantex Agro Products with batch number P9020241; P1112295 respectively, IE: In-house extract loaded 5 µl; 10 µl and 15 µl using mobile phase – Toluene: ethyl acetate: methanol: formic acid-5:2:2:1

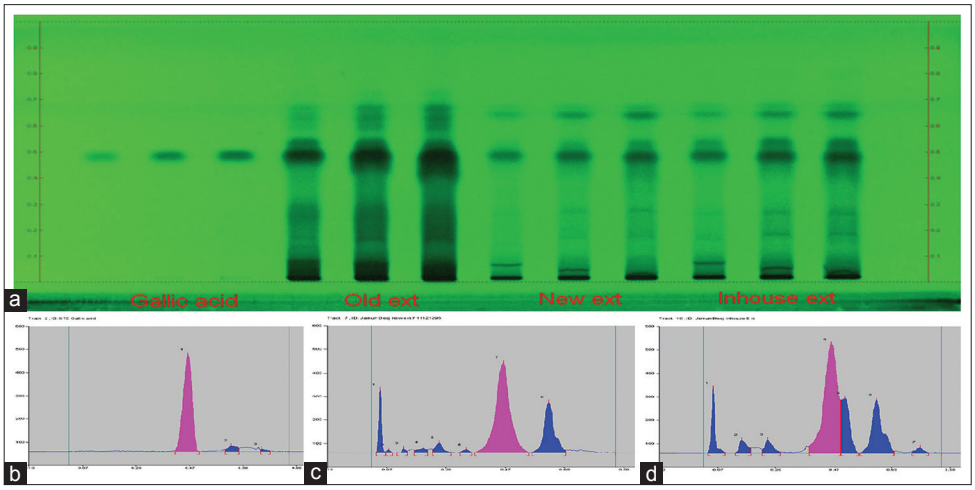

Figure 4: High performance thin layer chromatography study of *Jambu* (*Syzygium cumini*); (a-d) represent as *Jambu* plate, fingerprint of standard gallic acid, *Jambu* market extracts (new extract) and *Jambu* in-house extract at 254 nm respectively

Table 8: R_f values by densitometric scan of *Mamejaka* at 254 nm

R_f values	OE-05	OE-10	NE-05	NE-10	IE-05	IE-10
0.09	+	+				
0.10			+	+	+	+
0.17	+	+				
0.18			+	+	+	+
0.25	+	+	+	+		
0.36			+	+	+	+
0.37	+	+				
0.41	+	+				
0.42			+	+	+	+
0.50	+	+				
0.51			+	+	+	+
0.58					+	+
0.63			+	+		
0.64	+	+				
0.68			+	+	+	+
0.69	+	+				
0.73	+	+	+	+		
0.79	+	+	+	+	+	+
0.83					+	+
0.86	+	+				
0.87			+	+	+	+
Total	11	11	11	11	10	10

OE – 05 and 10 represents: Old extract 5 µl and 10 µl, NE – 5 and 10: New extract 5 µl and 10 µl; procured from Plantex Agro Products with batch number P9060682; P11121249 respectively, IE: In-house extract loaded 5 µl and 10 µl using mobile phase – ethyl acetate: acetic acid: formic acid: water - 10:1:0.5:1

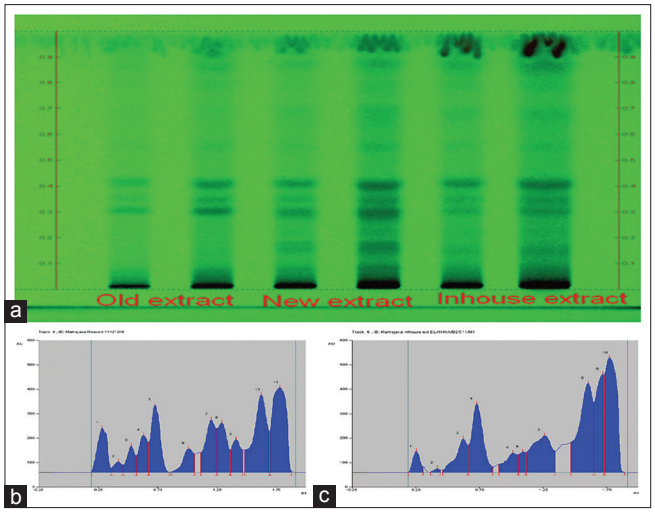

Figure 5: High performance thin layer chromatography study of *Mamejaka* (*Enicostema littorale*); (a-c) represent as *Mamejaka* plate, fingerprint of *Mamejaka* market extract and *Mamejaka* in-house extract at 254 nm respectively

Conclusion

The results obtained from the study could be utilized for the preliminary quality control and quality assurance of these anti-diabetic drugs. This component of research may also be useful in estimating the precise shelf life of the respective extract or formulations having these crude drugs as constituents.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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हिन्दी सारांश

मधुमेह विरोधी हर्बल दवाओं के अर्क का एचपीटीएलसी फिंगरप्रिंटिग, फायटोकेमिकल एवं फिजीकोकेमिकल मूल्यांकन

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हर्बल दवाओं ने पिछले कुछ दशकों में बढ़ती लोकप्रियता प्राप्त की है और हर्बल दवाओं के इस वैश्विक पुनरुत्थान से उनका वाणिज्यिक मूल्य बढ़ जाता है। हालांकि इस बढ़ती मांग के कारण मुख्य रूप से हर्बल दवाओं से संबंधित पर्याप्त नियमों की कमी के कारण उनकी गुणवत्ता में गिरावट, हुई है। इसको ध्यान में रखकर हर्बल कच्चे माल के मानकीकरण के लिए एक अनुकूलित पद्धति को विकसित करने हेतु रखा। वर्तमान अध्ययन पांच (मधुनाशिनी, विजयसार, मामेजक, जम्बु, और आमलकी) मधुमेह विरोधी दवाओं में से प्रत्येक की जांच करने के लिए डिजाइन किया गया है। उपरोक्त औषधियों का घर निर्मित अर्क और विपणन अर्क में मार्कर यौगिकों के संदर्भ में भौतिक मापदंडों, प्रारंभिक फायटोकेमिकल स्क्रीनिंग, पॉलीफिनोल के मात्रा का ठहराव और एचपीटीएलसी फिंगरप्रिंट की रूपरेखा का उपयोग कर मूल्यांकन किया गया। सभी पौधों में मुख्य रूप से पॉलीफिनोलिक यौगक होते हैं और ३.६ से २१.७२% डब्ल्यू./डब्ल्यू. की सीमा में मात्रा उपलब्ध हैं। पौधों के अर्क के बीच पॉलीफिनोल की मात्रा का विश्लेषण करने पर आमलकी में अधिक और मामेजक में कम मात्रा पायी गयी। एचपीटीएलसी फिंगरप्रिंटिंग में घर निर्मित अर्क, विपणन अर्क की तुलना में बेहतर गुणवत्ता का पता चला है। अध्ययन से प्राप्त परिणामों का इन मधुमेह विरोधी दवाओं की गुणवत्ता नियंत्रण और गुणवत्ता आश्वासन के लिए संदर्भ फायटोकन्स्टीट्युण्ट (बायोमार्कर) की सीमा निर्धारित करने के लिए उपयोग किया जा सकता है।