A Thin Layer Chromatographic Method for the Species Identification of Grass Leaf Stains

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ABSTRACT: Botanical material like grass leaf stains are sometimes encountered as evidence in criminal investigations. They can help in linking suspect(s), victim(s), and crime scene with each other, which can lead in solving various outdoor criminal cases. Here, we reported a good solvent system i.e the mixture of toluene: ethyl acetate: formic acid: methanol (60:15:15:10) to differentiate and identify leaf stains of twenty one grass species commonly found in the state of Punjab in Northern India.

Keywords: forensic chemistry, thin layer chromatography, species identification, grass stains

Introduction

The grasses (*Poaceae*) are of considerable forensic importance as evidence because of their ubiquitous distribution [1]. The traces of their vegetative and reproductive parts in the form of stains get easily transferred to the clothing's of the victim or suspect from the scene of crime in accordance with the Locard's exchange principle. Thus, the correct identification of the grass species from their stains is mandatory for the successful utilization of this evidence i.e. to link the suspect(s) and victim(s) with the crime scene or to prove or disprove alibis. It can be done by using chromatographic techniques or DNA based analysis as morphological identification is not possible.

In order to identify grass stains, the analysis of their constituents can be done as leaves of different grass species contain chemical constituents [2] like chlorophyll a and b, carotenoids [4], flavonoids [2, 5], anthrocyanins [6], alkaloids etc. and amino acids [7, 8]. This wide composition of constituents is different from grass species to species. Therefore, it is possible to link a particular leaf stain to its grass/plant species and subsequently can be related to specific area in which the crime has been committed [3]. As a result the leaf stains of grass/plants can be very useful during forensic investigations in various outdoor crime cases.

Tswett first did the analysis on plant constituents (pigments) in 1900's by using liquid chromatography [9]. Thereafter, a number of analytical techniques have been established for the separation and identification of the constituents of the plant stains. These include thin-layer chromatography (TLC) [10-11], high performance liquid chromatography [2, 4, 11-13]; Reversed Phase High Performance Liquid Chromatography (RP-HPLC) [14]. Among the given techniques, TLC is quick, reliable and inexpensive technique to study the constituents of grass leaf stains as it can be performed in both sophisticated and small laboratories. This technique requires minimum equipment and samples as well. Since 1960s, TLC has been employed to analyze and identify the compounds present in grasses [9]. Hayashiba *et al.* (1989) identified leaf stains of thirteen common grass weed species using High Pressure Liquid Chromatography [2]. It is lament that very limited work has been done on the analysis of leaf stains from forensic perspective.

Keeping this significant aspect in view, the present study has been undertaken to standardize the experimental TLC procedure by developing a good solvent system as mobile phase for differentiation of leaf stains of different grasses on the basis of the number of spots and hRf values to study their taxonomic significance in forensics.

Materials and Method

Sampling

Five or more samples each of twenty-one grass species belonging to two subfamilies *i.e.* Panicoideae (fifteen) and Chloridoideae (six) were collected from Patiala, Ludhiana and Sangrur districts of Punjab state in Northwest India. All the grass species collected were stored separately by sandwitching between the newspapers. Species identification was done by morphological methods using the keys given by Sharma and Khosla (2001) [15]. The details of the information regarding their subfamily, location of collection and number of species collected are given in Table 1.

Sample Preparation

Four stains of each selected grass species collected from different locality were prepared by gently rubbing the leaves of respective grass species on washed white cotton cloth pieces until visible green mark was obtained. The stain samples were then dried under shade, serially marked and stored in separate paper envelope to prevent cross contamination.

Sample Extraction

The stained part of the cloth pieces (1 cm x 1 cm) was cut for extraction and placed in 3 mL test tubes separately. The extraction was performed using four solvents i.e. acetone, ethanol, methanol and mixture of acetone and ethanol (1:1). 1 mL of each solvent was added to the test tubes separately and allowed to stand overnight to find the best solvent for extraction. The results of solvents used for the extraction of various stained samples and manufacturers of solvents used in the present study were shown in Table 2 and 3 respectively. The white cotton piece was treated in the same manner as a negative control.

Table 1: Various grass species collected for the present study

S. No.	Name of grass species	Sub families	Location of collection (District)	Number of samples
1	*Arundinella nepalensis*	Panicoideae	Patiala, Ludhiana	8
2.	*Cenchrus ciliaris*	Panicoideae	Sangrur, Patiala	7
3.	*Dichanthium annulatum*	Panicoideae	Patiala, Sangrur	10
4.	*Eleusine indica*	Chloridoideae	Patiala, Sangrur	7
5	*Seteria tomentosum*	Panicoideae	Patiala	6
6	*Cynodon dactylon*	Chloridoideae	Patiala,Ludhiana, Sangrur	10
7	*Bothriochloa pertusa*	Panicoideae	Sangrur	6
8	*Panicum paludosum*	Panicoideae	Ludhiana, Patiala	6
9	*Paspalidium flavidum*	Panicoideae	Ludhiana, Sangrur	9
10	*Cenchrus setigerus*	Panicoideae	Sangrur	6
11	*Echinochloa colonum*	Panicoideae	Sangrur, Patiala	8
12	*Echinochloa crusgalli*	Panicoideae	Sangrur	6
13	*Panicum antidotale*	Panicoideae	Patiala, Ssngrur	7
14	*Sporobolus diander*	Chloridoideae	Patiala	7
15	*Brachiaria ramosa*	Panicoideae	Patiala	7
16	*Leptochloa panacea*	Chloridoideae	Sangrur, Patiala	8
17	*Pennisetum purpureum*	Panicoideae	Sangrur	6
18	*Dactyloctenium aegyptium*	Chloridoideae	Patiala, Sangrur, Ludhiana	10
19	*Setaria glauca*	Panicoideae	Sangrur, Patiala	7
20	*Paspalum paspaloides*	Panicoideae	Ludhiana, Patiala	7
21	*Eragrostis pilosa*	Chloridoideae	Patiala, Sangrur	6

Table 2: Results of various solvents used to prepare extract of selected grass stains

S. no.	Samples	Solvent Systems			
		Acetone	Ethanol	Methanol	Acetone : Ethanol (1:1)
1	*Arundinella nepalensis*	++	++	+++	++
2.	*Cenchrus ciliaris*	++	++	+++	++
3.	*Dichanthium annulatum*	++	++	+++	++
4.	*Eleusine indica*	++	+	+++	++
5	*Seteria tomentosum*	++	++	+++	++
6	*Cynodon dactylon*	+++	+	+++	++
7	*Bothriochloa pertusa*	++	+	+++	+
8	*Panicum paludosum*	++	+	+++	+
9	*Paspalidium flavidum*	++	++	+++	+
10	*Cenchrus setigerus*	+++	++	+++	++
11	*Echinochloa colonum*	++	++	+++	+
12	*Echinochloa crusgalli*	+++	++	+++	+
13	*Panicum antidotale*	++	+++	+++	++
14	*Sporobolus diander*	++	++	+++	+
15	*Brachiaria ramose*	++	++	+++	++
16	*Leptochloa panacea*	++	+	+++	++
17	*Pennisetum purpureum*	++	++	+++	++
18	*Dactyloctenium aegyptium*	++	++	+++	++
19	*Setaria glauca*	++	++	+++	+
20	*Paspalum paspaloides*	++	+	+++	++
21	*Eragrostis pilosa*	++	++	+++	-

Note: **-** : Not soluble (stain not dissolved); + : Sparingly soluble (some part of stain dissolved); ++: Soluble (stain dissolved but with difficulty); +++: Highly soluble (stain dissolved easily)

Table 3: Chemicals used in the analysis

Chemicals	Manufacturer
Acetic acid	E. Merck Ltd. Worli Mumbai 18.
Acetone	LOBA CHEMIE Pvt. Ltd. Mumbai 05.
Butanol	Qualigens fine chemicals, glaxo smith Kline Pharmaceutical Ltd. Dr. Annie Besant Road Mumbai 30.
Ethanol	Bengal Chemicals and Pharmaceuticals 164- Maniktala Road, Kolkata 54.
Ethyl acetate	LOBA CHEMIE Pvt. Ltd. Mumbai 05.
Formic acid	LOBA CHEMIE Pvt. Ltd. Mumbai 05.
Isopropanol	Merck specialist Pvt. Ltd., Shiv Sagar Estate 'A' Dr. Annie Besant Road, Worli Mumbai 18.
Methanol	Merck specialist Pvt. Ltd., Shiv Sagar Estate 'A' Dr. Annie Besant Road, Worli Mumbai 18.
Phenol	S.D. fine chemical Ltd. Mumbai 25.
Pyridine	S.D. fine chemical Ltd. Mumbai 25.
Tetrahydrofuran	LOBA CHEMIE Pvt. Ltd. Mumbai 05.
Toluene	Merck specialist Pvt. Ltd., Shiv Sagar Estate 'A' Dr. Annie Besant Road, Worli Mumbai 18.

Thin Layer Chromatography

TLC analysis was performed using 20 cm x 20 cm silica gel G plates. The slurry of silica gel G was prepared by mixing the silica gel G with twice the amount of water. The slurry was spread on glass plates using applicator forming a thin layer having a thickness of 0.25 mm followed by their activation for an hour at a temperature of 110°C in an oven. The aliquot of respective samples were spotted manually 1 cm from the bottom on activated plates using a Hamilton syringe (2 µL). The solvent chambers were saturated using various solvent systems as mobile phase as given in Table 4 and the spotted TLC plates were put at an angle of 45° in it and covered properly with a lid. The solvent front was allowed to migrate to a distance of 10 cm above the origin. After the run has been completed the developed plates were air dried at room temperature. The separated spots were visualized under strong daylight (visible light) and with iodine fuming method. The developed plates were photographed using a camera (Sony DSC-W35). The colour of spots and their respective hR_f values were calculated using following equation:

$$\mathrm{hR}f = \frac{Distance\ travelled\ by\ solute\ from\ origin}{Distance\ travelled\ by\ solvent\ from\ origin} \times 100$$

Table 4: Various solvent systems attempted for TLC development

No	Composition(v/v)	Saturation Time (Min)	Development Time (Min)	Temperature (°C)
1	Butanol:Methanol:Water (50:25:25)	25 min	30 min	22 °C
2	Butanol:Isopropanol:Water (80:15:5)	25 min	25 min	22 °C
3	Absolute Butanol (100)	25 min	15 min	23 °C
4	Butanol:Water (80:20)	25 min	20 min	23 °C
5	Ethyl acetate:Pyridine:Water (15:7:5)	25 min	25 min	22.5 °C
6	Formic acid:Ethyl acetate:Water (1:6:1)	25 min	25 min	23 °C
7	Toluene:Ethyl Acetate:Formic acid:Methanol (60:15:15:10)	25 min	20 min	22 °C
8	Ethyl acetate:Formic Acid:Acetic acid:water (70:0.01:0.01:30)	25 min	20 min	22°C
9	Phenol:Formic acid:water (75:1:25)	25 min	25 min	23°C

Results and discussion

In the present study, the extraction was performed with four solvents and results were given in Table 2. The obtained results revealed that immersion of stained cloth piece into the methanol results in complete extraction of the constituents as no spot of leaf stain was left on cloth piece. The negative controlled extract was transparent. The earlier studies revealed the use of acetone [14, 16-17], petroleum ether [16], ethyl acetate [18], Ethanol [17], chloroform: methanol (1:1) [19] for the extraction of pigments and other constituents from the leaves of plants.

After extraction, different solvent systems as mobile phase were employed for thin layer chromatographic development. Nine solvent systems (mobile phase) were tried for the separation of various constituents of grass leaf samples using TLC (Table 4). The developed thin layer chromatographic plates were examined under the strong day light (Figure 1) and using iodine fuming technique (Figure 2). The results obtained in terms of hR_f, number of spots and respective color by the TLC analysis of selected grass leaf stains were different with different mobile phases. The mobile phase 2,3,4,6 and 9 failed to separate the constituents of selected samples and gave no spots. The mobile phase 1, 5 and 8 gave insufficient number of spots (One or two spots for each sample) for different samples when viewed under strong day light and using iodine fuming. The above mobile phase did not produce result of analytical significance. The mobile phase 7 comprising Toluene : Ethyl acetate : Formic acid : Methanol in the ratio 60:15:15:10 (v/v/v/v) was found to be the suitable mobile phase as it gave best results in terms and separation and differentiation among selected grass stain samples. The spots were visualized under strong day light and after treating with iodine fuming.

The results obtained under strong day light are given in Figure 1 and Table 5. The sample 1, 10 and 20 gave nine different colored separated spots at different hR_f values. The sample 1 showed two characteristic spots at hR_f value 33 and 82 respectively. The total of four different colored spots at different hR_f values was found for sample 2 and 21 with one spot in common at hR_f 49. The sample 3, 13 and 16 gave seven separated spots with some at different or some at same hR_f values and colors. The sample 4, 8 and 14 showed five different colored separated spots at different hR_f values. The sample 4 and 8 showed four spots at same hR_f value and one different spot at hR_f value 51 and 95 for sample 4 and 8 respectively. The total of six different colored spots at different hR_f values was found for sample 5, 6, 7, 17, 18 and 19. The sample 9 and 15 showed ten different colored spots at different or same hRf value with six common spots at hRf value 15,46,49, 55, 63 and 73. The sample 11 and 12 showed 3 and 8 separated spots of different hRf value respectively.

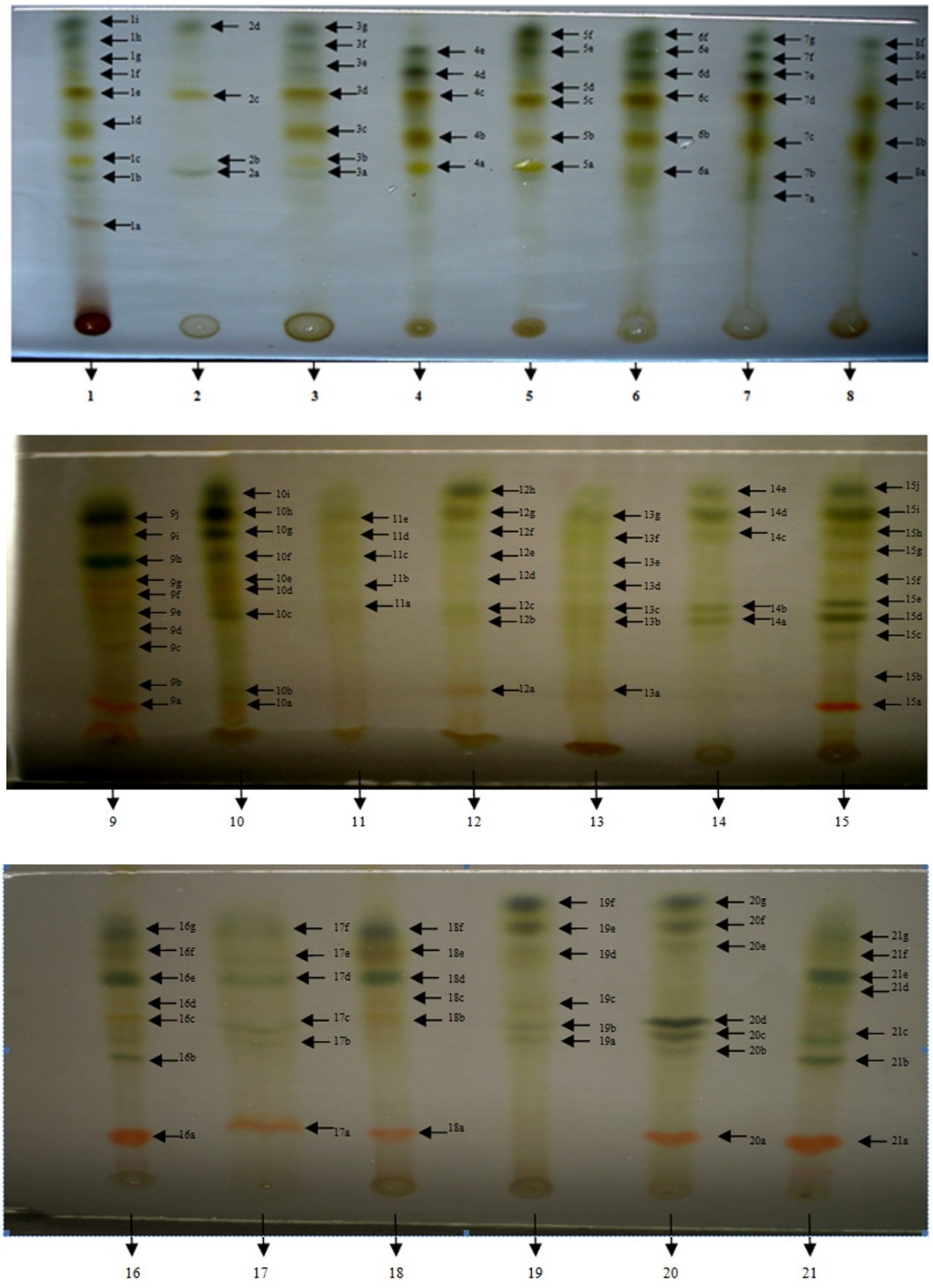

Figure 1: TLC chromatograms of selected grass leaf stains developed by solvent system (Toluene: Ethyl Acetate: Formic acid: Methanol 60:15:15:10 v/v/v/v) under strong day light (1.*Arundinella nepalensis*; 2.*Cenchrus ciliaris*; 3.*Dichanthium annulatum*; 4.*Eleusine indica*; 5.*Seteria tomentosum*; 6.*Cynodon dactylon*; 7.*Bothriochloa pertusa*; 8.*Panicum paludosum*; 9.*Paspalidium flavidum*; 10.*Cenchrus setigerus*; 11.*Echinochloa colonum*; 12.*Echinochloa crusgalli*; 13.*Panicum antidotale*; 14.*Sporobolus diander*; 15.*Brachiaria remosa*; 16.*Leptochloa panicea*; 17.*Pennisetum purpureum*; 18.*Dactyloctenium aegyptium*; 19.*Seteria glauca*; 20.*Paspalum paspaloides*; 21.*Eragrostis pilosa*)

Table 5: Thin layer chromatographic analysis of selected grass leaf stains using solvent system Toluene: Ethyl Acetate: Formic acid: Methanol (60:15:15:10 v/v/v/v) under strong day light

No.	Name of grass species	Colour and hR_f																								
		G 15	G 18	G 20	G 25	G 33	Y 36	G 38	G 40	G 46	Y 49	G 51	Y 52	Y 55	Y 60	G 63	Y 65	G 68	G 70	G 73	G 78	Y 80	G 82	G 85	G 90	G 95
1	*Arundinella nepalensis*					1a					1b		1c			1d				1e		1f	1g		1h	1i
2.	*Cenchrus ciliaris*										2a		2b							2c						2d
3.	*Dicanthium annulatum*										3a		3b			3c				3d		3e			3f	3g
4.	*Eleucine indica*											4a				4b				4c		4d			4e	
5	*Seteria tomentosum*										5a		5b			5c				5d					5e	5f
6	*Cynodon dactylon*										6a					6b				6c		6d			6e	6f
7	*Brothriochloa pertusa*						7a	7b								7c				7d		7e			7f	7g
8	*Panicum paludosum*							8a								8b				8c		8d			8e	8f
9	*Paspalidium flavidum*	9a	9b				9c			9d	9e		9f	9g		9h				9i	9j					
10	*Cenchrus setigerus*	10a		10b							10c	10d		10e			10f			10g		10h		10i		
11	*Echinochloa colonum*										11a		11b		11c				11d		11e					
12	*Echinochloa crusgalli*			12a						12b	12c		12d	12e						12f		12g		12h		
13	*Panicum antidotale*			13a						13b	13c			13d	13e			13f				13g				
14	*Sporobolus diander*									14a	14b							14c			14d			14e		
15	*Bracharia remosa*	15a			15b			15c		15d	15e			15f		15g				15h		15i		15j		
16	*Leptochloa panacea*	16a							16b		16c		16d				16e			16f		16g				
17	*Pennisetum purpureum*		17a								17b	17c					17d		17e			17f				
18	*Dactyloctenium aegyptium*	18a											18b		18c		18d			18e		18f				
19	*Seteria glauca*										19a	19b			19c					19d		19e			19f	
20	*Paspalum paspaloides*	20a								20b	20c	20d								20e		20f			20g	
21	*Eragrostis pilosa*	21a							21b		21c						21d									

(Note: G-Green, Y-Yellow)

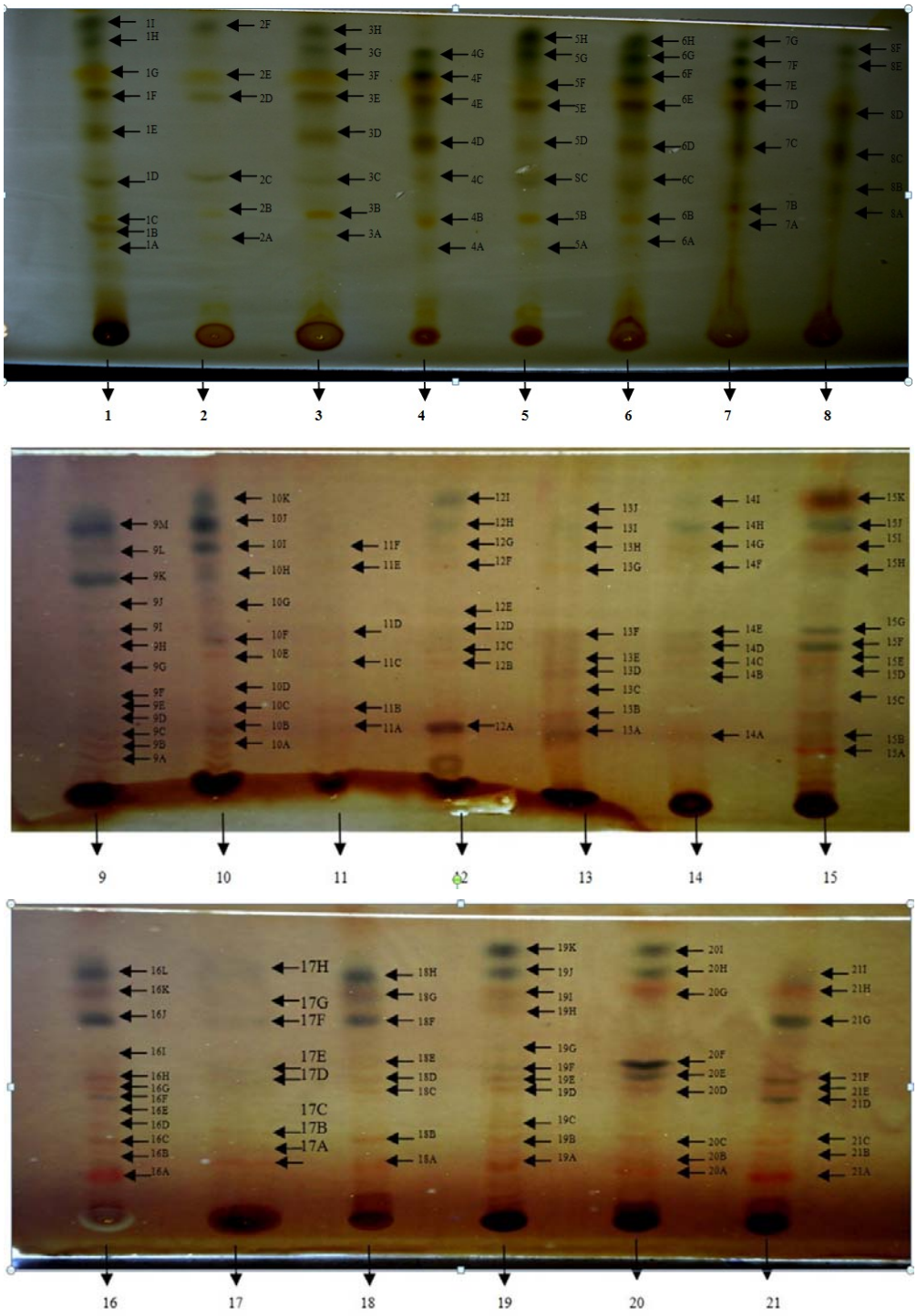

Figure 2: TLC chromatograms of selected grass leaf stains developed by solvent (Toluene: Ethyl Acetate: Formic acid: Methanol 60:15:15:10 v/v/v/v) after treating with iodine fuming (1. *Arundinella nepalensis*; 2. *Cenchrus ciliaris*; 3. *Dichanthium annulatum*; 4. *Eleusine indica*; 5. *Seteria tomentosum*; 6. *Cynodon dactylon*; 7. *Bothriochloa pertusa*; 8. *Panicum paludosum*; 9. *Paspalidium flavidum*; 10. *Cenchrus setigerus*; 11. *Echinochloa colonum*; 12. *Echinochloa crusgalli*; 13. *Panicum antidotale*; 14. *Sporobolus diander*; 15. *Brachiaria remosa*; 16. *Leptochloa panicea*; 17. *Pennisetum purpureum*; 18. *Dactyloctenium aegyptium*; 19. *Seteria glauca*; 20. *Paspalum paspaloides*; 21. *Eragrostis pilosa*

Table 6: Thin layer chromatographic analysis of selected grass leaf stain samples using solvent system Toluene: Ethyl Acetate: Formic acid: Methanol (60:15:15:10 v/v/v/v) after treating with iodine fuming

No.	Name of grass species	Colour and hR_f																										
		G 12	G 15	G 18	G 20	G 23	G 25	Y 28	G 30	G 33	Y 36	G 38	G 40	G 43	G 46	G 49	Y 52	Y 55	Y 60	Y 65	G 68	G 70	G 73	G 75	G 78	Y 80	G 85	G 90	G 95
1	*Arundinella nepalensis*							1A		1B	1C				1D				1E					1F		1G		1H	1I
2.	*Cenchrus ciliaris*								2A			2B					2C							2D		2E			2F
3.	*Dicanthium annulatum*								3A			3B					3C		3D					3E		3F		3G	3H
4.	*Eleucine indica*							4A			4B						4C		4D					4E		4F		4G	
5	*Seteria tomentosum*										5A						5B		5C					5D		5E		5F	5G
6	*Cynodon dactylon*								6A		6B				6C				6D					6E		6F		6G	6H
7	*Brothriochloa pertusa*										7A	7B							7C			7D				7E		7F	7G
8	*Panicum paludosum*											8A							8B			8C						8D	8E
9	*Paspalidium flavidum*	9A	9B	9C			9D				9E		9F	9G		9H		9I		9J			9K		9L	9M			
10	*Cenchrus setigerus*	10A	10B		10C		10D							10E	10F			10G		10H			10I			10J	10K		
11	*Echinochloa colonum*						11A						11B									11C			11D				
12	*Echinochloa crusgalli*	12A			12B								12C	12D				12E			12F		12G			12H	12I		
13	*Panicum antidotale*				13A		13B				13C	13D		13E			13F				13G					13H			
14	*Sporobolus diander*				14A								14B	14C		14D					14E		14F		14G	14H	14I		
15	*Bracharia remosa*		15A		15B		15C		15D			15E	15F		15G	15H								15I			15J	15K		
16	*Leptochloa panacea*		16A		16B	16C	16D		16E		16F		16G	16H	16I					16J			16K			16L			
17	*Pennisetum purpureum*				17A		17B		17C					17D		17E				17F		17G				17H			
18	*Dactyloctenium aegyptium*				18A		18B							18C		18D	18E			18F			18G			18H			
19	*Seteria glauca*		19A			19B	19C				19D			19E	19F		19G					19H		19I		19J		19K	
20	*Paspalum paspaloides*		20A	20B			20C							20D	20E		20F							20G		20H		20I	
21	*Eragrostis pilosa*	21A				21B			21C				21D	21E	21F					21G				21H		21I			

(Note: G-Green, Y-Yellow)

The results obtained after treating with iodine fuming are given in Figure 2 and Table 6. The sample 1, 12, 14 and 20 showed nine separated spots of different color at same or different or same hRf value. The sample 1 showed two characteristics yellow and green colored spots at hRf value 28 and 33 respectively. The six spots were found for sample 2 and 4. The sample 3,6,13,17,18 and 21 showed eight different colored separated spots at different hRf values. The sample 5 and 7 showed seven separated spots at different or same hRf value. The sample 8, 9, 11, 16 and 19 showed five, thirteen, four, twelve and ten different colored spots respectively at same or different hRf value. The total of eleven different colored spots at different hRf values was found for sample 10 and 15 with five common spots at hRf value 15, 20, 25, 46, 73, 80 and 85. The sample 12 and 14 showed total of 9 spots from which from which seven spots were found common at hRf values 20, 40, 43, 68, 73, 80.

The results indicated that the solvent system comprising toluene : ethyl acetate : formic acid : methanol in the ratio 60:15:15:10 (v/v/v/v) is the suitable mobile phase for thin layer chromatographic analysis as it showed more number of separated spots with different hR_f values and colour. The chromatographic profile of each grass species was different *w.r.t* other samples studied herein under strong day light and iodine fuming. The iodine fuming method was found to be the best in comparison to strong day light as the former showed more number of spots. No spot was observed for negative controlled extract. In respect to the discrimination between leaf stain of two grasses of same species collected from different locality, no difference in their chromatogram was observed. The aliquot of leaf of grass samples were analyzed five times under same set of experimental conditions to check the reproducibility of results and they were in concordance with each other. Thus, the results obtained showed that TLC with appropriate solvent system as mobile phase permits the separation of constituents of leaf stained samples of selected grass species.

Conclusion

The methanol was found to be the best solvent for the extraction of leaf stains. The TLC using solvent system toluene: ethyl acetate: formic acid: methanol (60:15:15:10 v/v/v/v) was found to be the best mobile phase as it can separate the constituents of leaf stains of selected grass species and possess the potential to differentiate them from each other which can be used for species identification. In addition this solvent system (mobile phase) is very quick and takes only 20 minutes to complete the chromatographic run. The iodine fuming has been found to be best visualization aid among the various visualization method used.

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