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Evaluation of phytochemical, physicochemical parameters and HPTLC fingerprints of different extracts of *Nil Aweriya* (*Indigofera tinctoria* L.) leaves grown in Sri Lanka.

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Abstract: The present study was aimed at developing several tools in proper identification, authentication and maintenance of quality control standards of raw materials of *I. tinctoria*. Leaf extracts were obtained following cold maceration with methanol, acetone and hot soxhlet extraction with distilled water. Each extract was subjected to phytochemical, physicochemical tests and High Performance Thin Layer Chromatography (HPTLC). Saponins were detected only in the aqueous extract while carbohydrates and reducing sugars were detected only in the methanol extract. With regard to physicochemical parameters; ash values, loss on drying, extractability and florescence analysis were determined. Values of total ash, acid insoluble ash, water soluble ash contents and loss on drying were 10.683±0.104% w/w, 0.7±0.14 % w/w, 0.925±0.035 % w/w, 3.17±0.422% w/w respectively. Extractability in each solvent; methanol, acetone and hot water were 29.536±10.29 % w/w, 12.93±0.622 % w/w and12.789±2.84 % w/w accordingly. According to florescence analysis; methanol extract was yellowish brown and pale yellow, acetone extract was brownish green and brownish yellow while aqueous extract was greenish black and intense green under day light and UV light respectively. HPTLC was run at wavelength 254 nm for each extract which was considered the preliminary tool in ascertaining the authenticity of *I. tinctoria*. Normal phase HPTLC profile of methanol extract showed 10 peaks with the R_f values;-0.06, -0.03, 0.00, 0.03, 0.09, 0.33, 0.63, 0.73, 0.84 and 0.86 for the solvent system; ethyl acetate : toluene : n-hexane : chloroform in 2 : 2 : 4 : 2 proportion while 10 peaks were obtained for the acetone extract with the solvent system; ethyl acetate : toluene : n-hexane : chloroform in 2.5 : 2.5 : 4 : 1 proportion in which the R_f values were -0.19, -0.05, 0.04, 0.10, 0.19, 0.39, 0.52, 0.58, 0.61 and 0.81. Reverse phase HPTLC was run for the aqueous extract with the solvent system; methanol : distilled water: ethanol in 3 : 3 : 5 proportion which showed 13 peaks with the R_f values; 0.06, 0.12, 0.14, 0.16, 0.22, 0.30, 0.37, 0.39, 0.47, 0.50, 0.75, 0.84 and 0.87. Since, many Ayurveda formulations contain *I. tinctoria* as an ingredient, above parameters and HPTLC fingerprints can be used for the proper identification and authentication of the raw materials excluding the counterfeit or sub standard materials leading to safety, quality and efficacy of the manufactured Ayurveda preparations.

Keywords: *Indigofera tinctoria* L.; fingerprints; physico-chemical; phytochemical; high performance thin layer chromatography

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INTRODUCTION

Traditional and complementary medicine holds a long therapeutic history serving worldwide (WHO, 2019). Amongst, Ayurveda which is based on two great pillars; disease prevention and health preservation is a common practice based on herbal medicine (Gupta et al., 2012). Herbal medicine comprises of herbs, herbal materials and finished herbal products according to World Health Organization (WHO). Further, herbal medicine is prepared with individual herbs or in combinations. WHO had also claimed that around 80 % of people rely on herbal medicine due to their therapeutic efficacy and lack of side effects (Kumar *et al* 2016; Gupta and Keshari, 2013). People who are suffering from chronic illnesses and due to the side effects of allopathic medicine; traditional medicine is being promoted nowadays (Anuradha et al., 2017). But the traditional medicine system carries many legislative issues since it is difficult to establish quality control, quality assurance and documentation procedures (Kumar *et al* 2016). Attention should be drawn towards the development of national regulatory framework and safety monitoring systems for Ayurveda (Baragi *et al* 2011). Out of the challenges faced by Ayurveda; lack of knowledge or information sharing about the herbal medicine, lack of safety monitoring methods and analytical techniques to determine the identity, purity, safety, quality and efficacy of herbal medicine, difficulties in standardization of herbal medicine and lack of pharmacovigilance studies are the commonest issues (Kumar *et al* 2016). Hence, this study was aimed at developing several tools in proper identification, authentication and maintenance of quality control standards of raw materials of *Nil Aweriya* (*Indigofera tinctoria*) belonging to the family *Fabaceae* which is considered a well-known medicinally important herb in which leaves, stems and roots are widely used in Traditional medicine treatments in Sri Lanka. Furthermore, the therapeutic indications of *I. tinctoria* plant include; chronic asthma, chronic bronchitis, heart diseases, gout, digestive disorders, wounds, dermatological disorders, cancer, epilepsy, diabetes mellitus, neuropathy and liver diseases. Its dry leaf powder is used in the treatment of asthma. A decoction is prepared with its leaves to be used as an antidote for sting venoms and to treat blennorrhagia. Whole plant extract is used as a prophylactic agent for hydrophobia and treatment for epilepsy, bronchitis, asthma, whooping cough, nervous, heart and lung disorders. Ointments prepared with the leaves are used to cure ulcers and hemorrhoids. Roots are used in the treatment of hepatitis, urinary tract disorders and bites. Whole plant or leaf extract mixed with honey is used to treat liver and spleen enlargement, epilepsy and nervous disorders. Ethanol leaf extract has a hypoglycemic effect (Chandra *et al* 2014). In addition, it also can improve the hair growth and is used as a natural colorant. This plant is well-known to have laxative, expectorant, diuretic, anthelmintic and thermogenic activity (Venkatachalam, 2018; Felicia and Muthulingam, 2012). In this study, preliminary phytochemical screening, analysis of physicochemical parameters, florescence analysis and development of HPTLC fingerprints were conducted.

MATERIALS AND METHODS

The matured leaves without any insect or microbial attacks of *I. tinctoria* were collected from the Herbal Garden of Institute of Indigenous Medicine, University of Colombo, Rajagiriya (6º 55' 54.98" N x 79º 50' 52.01" E) Western Province, Sri Lanka in August to September 2020. The plant was authenticated and the voucher specimen was deposited at the National Herbarium, Botanical Garden, Peradeniya, Sri Lanka. Fresh leaves were collected, washed under running tap water to remove dust and dirt, rinsed with distilled water, air dried, weighed, oven dried until a constant weight was obtained at a temperature below 40 °C and powdered (Keo *et al* 2017; Zakaria *et al* 2011). Extracts were obtained by cold maceration with methanol, acetone and hot soxhlet extraction with distilled water according to WHO Guidelines (WHO, 1998). Each extract was subjected to preliminary phytochemical screening to screen for the phytoconstituents; alkaloids, saponins, tannins, phenols, carbohydrates, reducing sugars, anthranol glycosides, cardiac

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glycosides, flavonoids, anthraquinones, diterpenes, triterpenes, terpenoids, steroids, proteins and amino acids (Keo *et al* 2017; Visweswari *et al* 2013). Analysis of physicochemical parameters was conducted following WHO Guidelines including; total ash, acid insoluble ash and water-soluble ash. Loss on drying was calculated for *I. tinctoria* air dried leaves according to Gravimetric determination method according to WHO Guidelines (WHO, 1998). Extractability was determined for each solvent; methanol, acetone and hot distilled water (Oeung *et al* 2017). Each test was done in triplicate and the values were expressed as results ± standard deviation. Significant differences within the parameters were analyzed using one sample t test in SPPS 20 software at 95 % confidence interval. Florescence analysis was done for each extract under day light and UV light. Thin Layer Chromatography (TLC) was done to determine the best solvent system for each extract and HPTLC fingerprints were developed accordingly. Normal phase Thin Layer Chromatography (TLC) was run for methanol and acetone extracts on TLC silica gel 60 f_{254} plates (precoated sheets ALUGRAM Xtra SIL, 2.0cm × 10.0cm, 0.20mm thickness) while reverse phase TLC was run for aqueous extract on reverse phase TLC silica gel 60 f_{254} plates (precoated sheets ALUGRAM Xtra SIL, 2.0cm × 10.0cm, 0.20mm thickness). The TLC plate was kept in the photo documentation chamber (CAMAG REPROSTAR 3) and was scanned at UV light of wavelength, 254 nm (Meng et al., 2017). Peak display, peak value table, baseline display and 3D display were obtained using the WINCATS software. Each was run in triplicate (Felicia & Muthulingam, 2012; Laitonjam & Wangkheirakpam, 2011).

RESULTS

Qualitative phytochemical analysis revealed that the flavonoids, anthraquinones, diterpenes, triterpenes, terpenoids and proteins were available in all three extracts while tannins, phenols and amino acids were available in methanol and acetone extracts. Saponins were detected only in the aqueous extract while carbohydrates and reducing sugars were detected only in the methanol extract. Total ash, acid insoluble ash and water-soluble ash values of *I. tinctoria* leaves were 10.683±0.104 % w/w, 0.7±0.141 % w/w and 0.925±0.035 % w/w respectively. It was revealed that the total ash, acid insoluble ash and water-soluble ash values were not significantly different ($p>0.05$). Loss on drying value for *I. tinctoria* leaves was 3.17±0.422 % w/w. Methanol soluble extractable matter, acetone soluble extractable matter and water-soluble extractable matter were 29.536±10.29 % w/w, 12.93±0.622% w/w and 12.789±2.84% w/w in sequence. A significant difference was found in the extractability values of acetone and aqueous extracts ($p<0.05$). According to florescence analysis; methanol extract was yellowish brown and pale yellow, acetone extract was brownish green and brownish yellow while aqueous extract was greenish black and intense green under day light and UV light respectively. Normal phase HPTLC profile of methanol extract showed 10 peaks with the R_f values; -0.06, -0.03, 0.00, 0.03, 0.09, 0.33, 0.63, 0.73, 0.84 and 0.86 for the solvent system; ethyl acetate : toluene : n-hexane : chloroform (2 : 2 : 4 : 2). Normal phase HPTLC fingerprint of acetone extract showed 10 peaks for ethyl acetate : toluene : n-hexane : chloroform (2.5 : 2.5 : 4 : 1) with the R_f values; -0.19, -0.05, 0.04, 0.10, 0.19, 0.39, 0.52, 0.58, 0.61 and 0.81. Reverse phase HPTLC fingerprint of the aqueous extract showed 13 peaks (R_f; 0.06, 0.12, 0.14, 0.16, 0.22, 0.30, 0.37, 0.39, 0.47, 0.50, 0.75, 0.84, 0.87) with methanol : distilled water: ethanol (3 : 3 : 5) (Figures 1 and 2).

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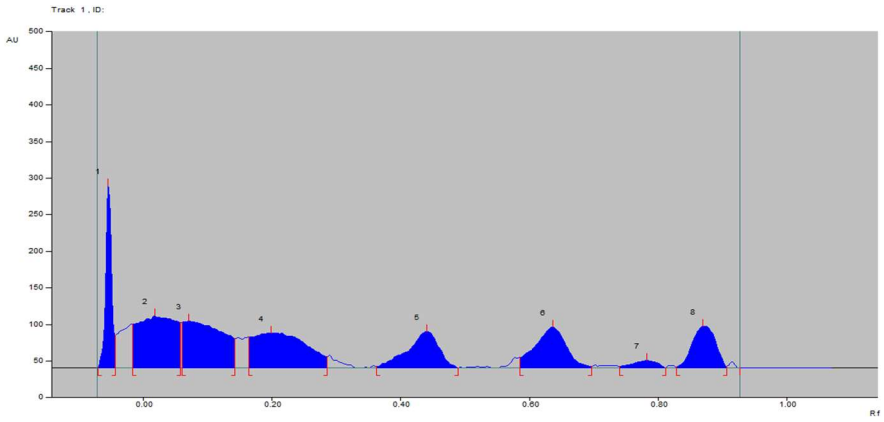
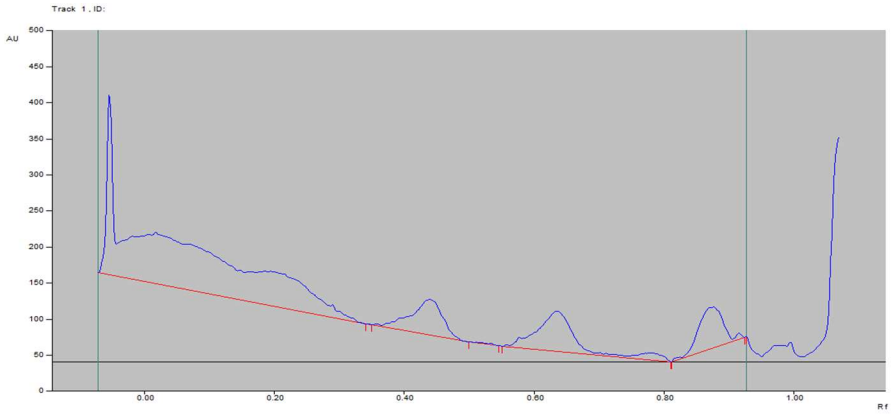


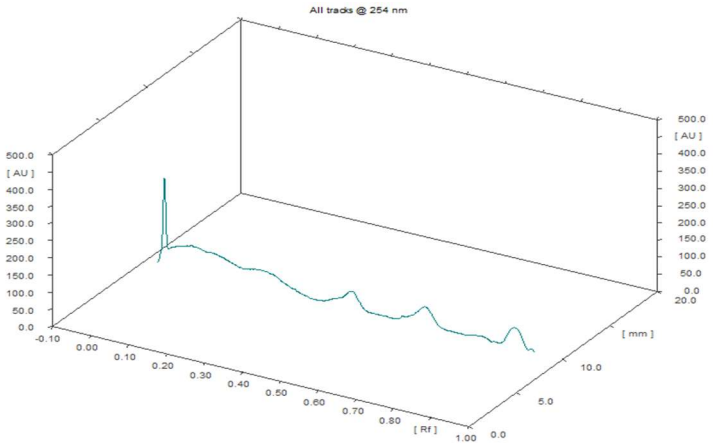
a)

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	-0.07 Rf	0.4 AU	-0.05 Rf	249.4 AU	41.21 %	-0.04 Rf	44.9 AU	1625.4 AU	10.17 %	unknown *
2	-0.02 Rf	59.6 AU	0.02 Rf	71.1 AU	11.75 %	0.06 Rf	62.0 AU	3477.5 AU	19.37 %	unknown *
3	0.06 Rf	62.1 AU	0.07 Rf	63.8 AU	10.54 %	0.14 Rf	39.8 AU	3252.7 AU	18.12 %	unknown *
4	0.16 Rf	41.9 AU	0.26 Rf	48.0 AU	7.94 %	0.29 Rf	15.2 AU	3274.0 AU	18.24 %	unknown *
5	0.36 Rf	1.8 AU	0.44 Rf	49.6 AU	8.19 %	0.49 Rf	0.1 AU	1957.1 AU	10.90 %	unknown *
6	0.59 Rf	14.0 AU	0.64 Rf	55.8 AU	9.21 %	0.70 Rf	2.2 AU	2170.6 AU	12.09 %	unknown *
7	0.74 Rf	1.8 AU	0.78 Rf	10.3 AU	1.70 %	0.81 Rf	0.6 AU	313.1 AU	1.74 %	unknown *
8	0.83 Rf	1.3 AU	0.87 Rf	57.2 AU	9.45 %	0.91 Rf	2.4 AU	1683.2 AU	9.38 %	unknown *

b)



c)



d)

Figure 1: a) Peak value display, b) peak value table, c) baseline display, d) 3D display of HPTLC fingerprint of methanol extract of *I. tinctoria* (solvent system; ethyl acetate : toluene : n-hexane = 2 : 2 : 6)

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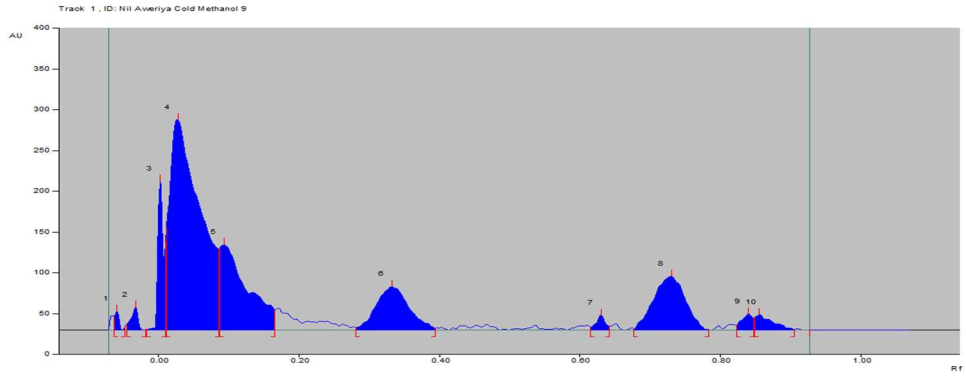
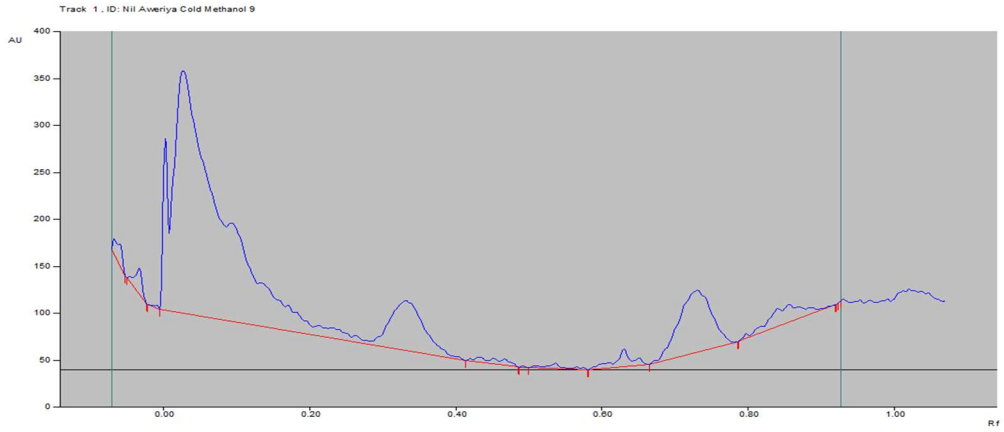


a)

Track	Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	1	-0.06 Rf	16.8 AU	-0.06 Rf	23.3 AU	3.01 %	-0.05 Rf	4.1 AU	138.0 AU	0.72 %	unknown *
1	2	-0.05 Rf	7.0 AU	-0.03 Rf	28.5 AU	3.69 %	-0.02 Rf	0.5 AU	223.7 AU	1.16 %	unknown *
1	3	-0.02 Rf	0.6 AU	0.00 Rf	182.3 AU	23.59 %	0.01 Rf	08.1 AU	1157.9 AU	6.03 %	unknown *
1	4	0.01 Rf	125.5 AU	0.03 Rf	257.8 AU	33.37 %	0.09 Rf	99.3 AU	9096.9 AU	47.33 %	unknown *
1	5	0.09 Rf	100.0 AU	0.09 Rf	104.7 AU	13.55 %	0.17 Rf	25.1 AU	3330.6 AU	17.33 %	unknown *
1	6	0.28 Rf	3.3 AU	0.33 Rf	53.4 AU	6.91 %	0.39 Rf	2.7 AU	2027.2 AU	10.55 %	unknown *
1	7	0.62 Rf	3.6 AU	0.63 Rf	18.5 AU	2.39 %	0.64 Rf	5.0 AU	193.2 AU	1.01 %	unknown *
1	8	0.68 Rf	1.8 AU	0.73 Rf	65.8 AU	8.52 %	0.78 Rf	0.4 AU	2411.2 AU	12.55 %	unknown *
1	9	0.82 Rf	5.6 AU	0.84 Rf	19.6 AU	2.53 %	0.85 Rf	15.0 AU	252.1 AU	1.31 %	unknown *
1	10	0.85 Rf	15.6 AU	0.86 Rf	18.7 AU	2.43 %	0.91 Rf	1.1 AU	387.4 AU	2.02 %	unknown *

b)



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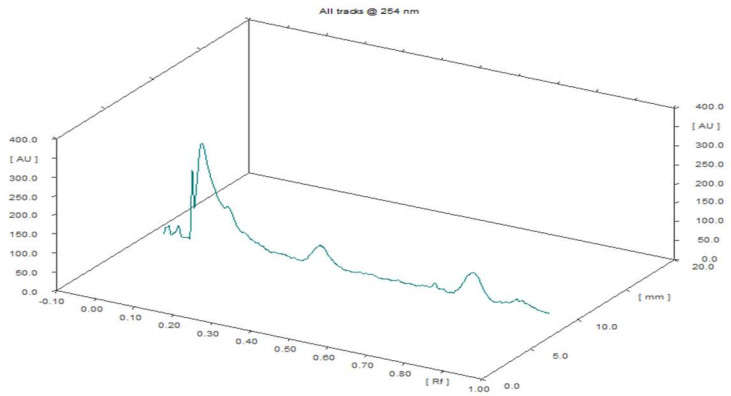

d)

Figure 2: a) Peak value display, b) peak value table, c) baseline display, d) 3D display of HPTLC fingerprint of methanol extract of *I. tinctoria* leaves (solvent system; ethyl acetate : toluene : chloroform : n-hexane = 2 : 2 : 2 : 4)

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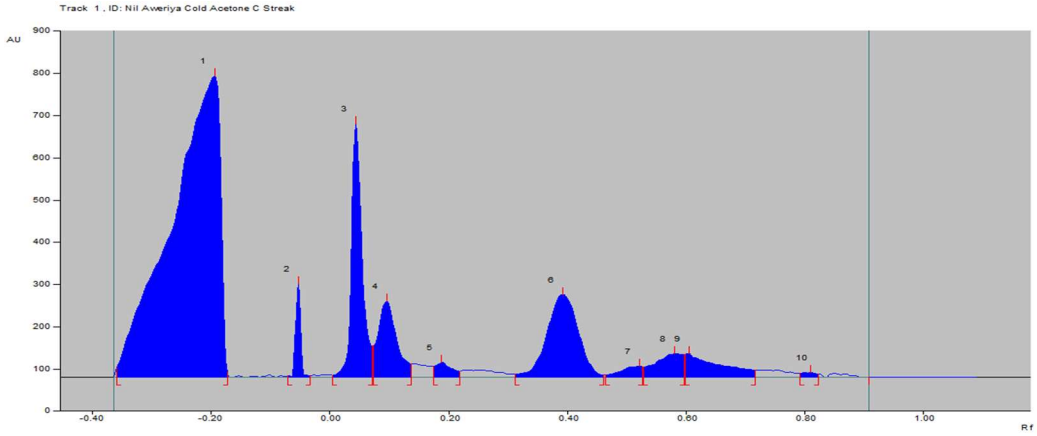
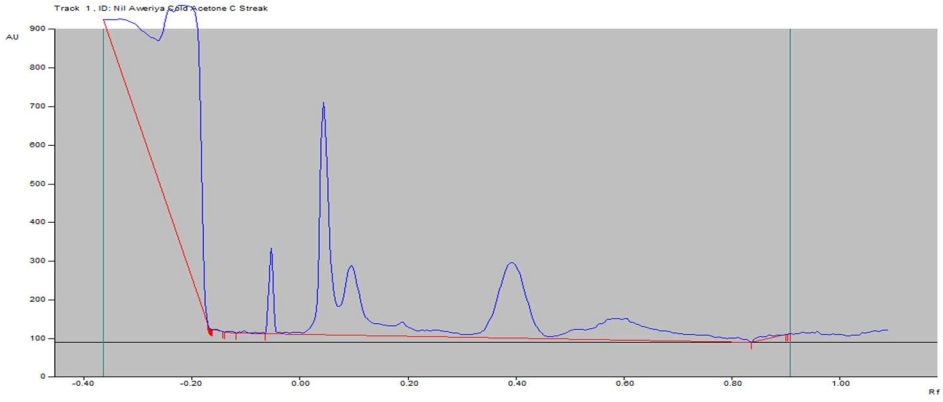


a)

Track	Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	1	-0.36 Rf	21.7 AU	-0.19 Rf	714.3 AU	34.04 %	-0.17 Rf	11.0 AU	36905.8 AU	61.33 %	unknown *
1	2	-0.07 Rf	2.8 AU	-0.05 Rf	222.0 AU	10.58 %	-0.03 Rf	3.1 AU	1179.2 AU	1.96 %	unknown *
1	3	0.01 Rf	4.4 AU	0.04 Rf	601.2 AU	28.65 %	0.07 Rf	73.9 AU	6788.4 AU	11.28 %	unknown *
1	4	0.07 Rf	74.9 AU	0.10 Rf	180.3 AU	8.59 %	0.14 Rf	32.1 AU	3579.9 AU	5.95 %	unknown *
1	5	0.17 Rf	25.9 AU	0.19 Rf	35.6 AU	1.70 %	0.22 Rf	13.8 AU	648.4 AU	1.08 %	unknown *
1	6	0.31 Rf	7.4 AU	0.39 Rf	196.1 AU	9.35 %	0.46 Rf	5.5 AU	6566.4 AU	10.91 %	unknown *
1	7	0.47 Rf	5.6 AU	0.52 Rf	25.9 AU	1.23 %	0.53 Rf	24.7 AU	597.6 AU	0.99 %	unknown *
1	8	0.53 Rf	24.1 AU	0.58 Rf	55.3 AU	2.64 %	0.60 Rf	53.6 AU	1664.1 AU	2.77 %	unknown *
1	9	0.60 Rf	53.9 AU	0.61 Rf	56.4 AU	2.69 %	0.72 Rf	17.2 AU	2076.5 AU	3.45 %	unknown *
1	10	0.79 Rf	8.5 AU	0.81 Rf	11.1 AU	0.53 %	0.82 Rf	6.6 AU	172.6 AU	0.29 %	unknown *

b)



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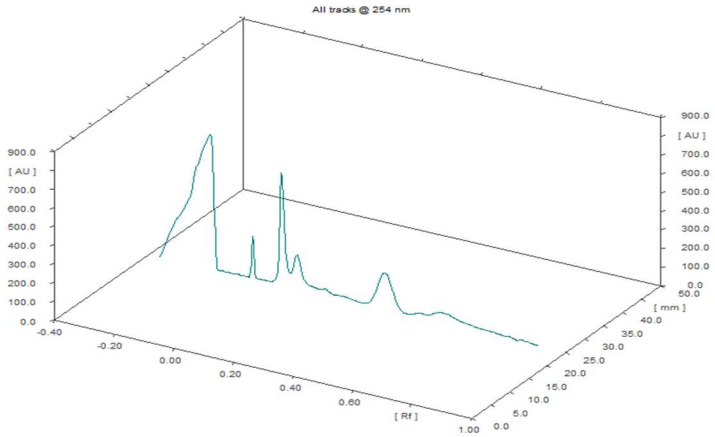

d)

Figure 3: a) Peak value display, b) peak value table, c) baseline display, d) 3D display of HPTLC fingerprint of acetone extract of *I. tinctoria* leaves (solvent system; ethyl acetate : toluene : chloroform : n-hexane = 2.5 : 2.5 : 1:4

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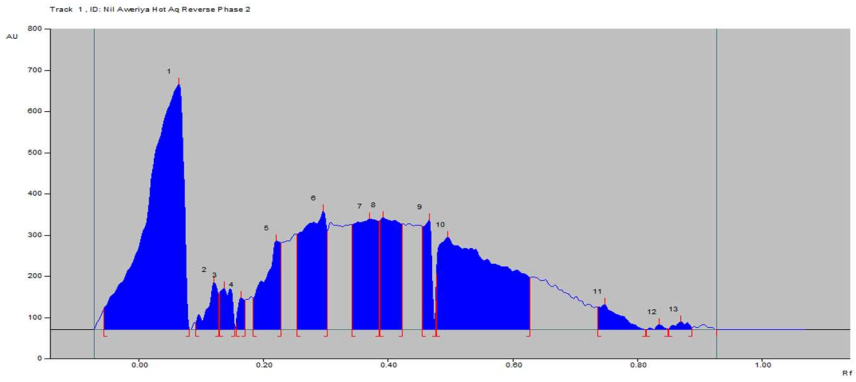
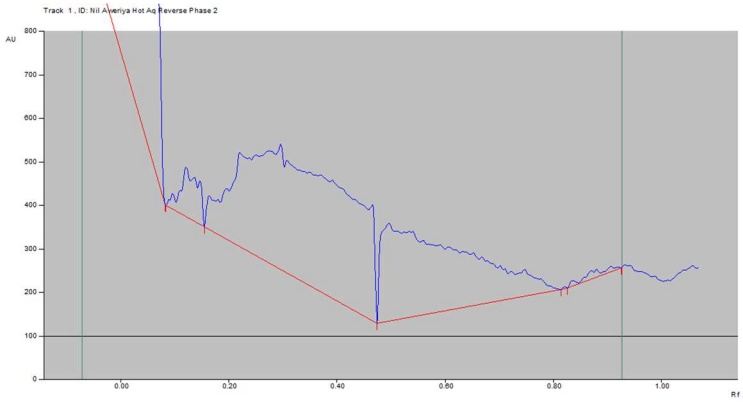


a)

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	-0.06 Rf	48.9 AU	0.06 Rf	594.2 AU	23.69 %	0.08 Rf	1.5 AU	27630.7 AU	33.13 %	unknown *
2	0.09 Rf	16.2 AU	0.12 Rf	113.5 AU	4.53 %	0.13 Rf	86.5 AU	1555.8 AU	1.87 %	unknown *
3	0.13 Rf	88.1 AU	0.14 Rf	100.7 AU	4.02 %	0.15 Rf	3.7 AU	1457.0 AU	1.75 %	unknown *
4	0.16 Rf	14.3 AU	0.16 Rf	77.2 AU	3.08 %	0.17 Rf	71.4 AU	702.0 AU	0.84 %	unknown *
5	0.18 Rf	76.0 AU	0.22 Rf	215.0 AU	8.57 %	0.23 Rf	10.7 AU	4621.0 AU	5.54 %	unknown *
6	0.25 Rf	231.2 AU	0.30 Rf	287.4 AU	11.45 %	0.30 Rf	38.6 AU	8895.9 AU	10.67 %	unknown *
7	0.34 Rf	255.2 AU	0.37 Rf	268.3 AU	10.69 %	0.39 Rf	63.3 AU	8132.4 AU	9.75 %	unknown *
8	0.39 Rf	264.1 AU	0.39 Rf	271.4 AU	10.82 %	0.42 Rf	56.6 AU	6869.3 AU	8.24 %	unknown *
9	0.46 Rf	250.9 AU	0.47 Rf	266.5 AU	10.62 %	0.48 Rf	69.0 AU	3051.1 AU	3.66 %	unknown *
10	0.48 Rf	135.8 AU	0.50 Rf	223.6 AU	8.91 %	0.63 Rf	26.3 AU	18416.8 AU	22.08 %	unknown *
11	0.74 Rf	55.0 AU	0.75 Rf	60.8 AU	2.43 %	0.81 Rf	0.1 AU	1654.6 AU	1.98 %	unknown *
12	0.82 Rf	0.0 AU	0.84 Rf	11.5 AU	0.46 %	0.85 Rf	1.5 AU	113.4 AU	0.14 %	unknown *
13	0.85 Rf	2.4 AU	0.87 Rf	18.6 AU	0.74 %	0.89 Rf	5.5 AU	311.8 AU	0.37 %	unknown *

b)



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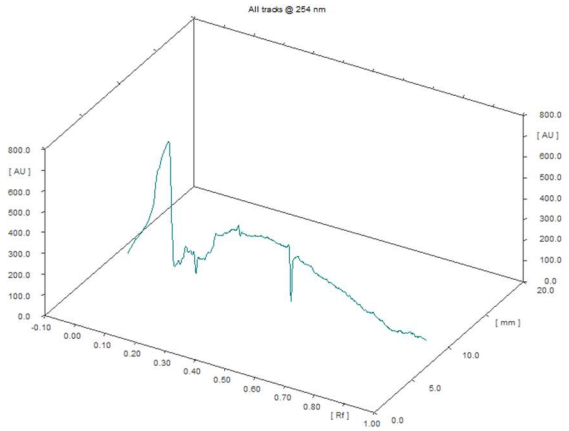

d)

Figure 4: a) Peak value display, b) peak value table, c) baseline display, d) 3D display of HPTLC fingerprint of aqueous leaf extract of *I. tinctoria* (solvent system; methanol : ethanol : distilled water = 3 : 3 : 5)

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CONCLUSION

It was revealed that many of the phytochemicals were available in all three extracts but the methanol extract was rich in most of the phytochemicals than that of acetone and hot water extracts. Plants are rich in biologically active secondary metabolites known as; phytochemicals which are responsible for therapeutic activity against many disorders (Forni et al., 2019). Since, the methanol extract is rich in phytochemical composition than acetone and aqueous extracts, further studies should be conducted for the quantitative determination and separation of phytoconstituents in methanol extract. Furthermore, this plant rich in phytoconstituents can be used as a resource for drug development. Under the evaluation of physicochemical parameters; ash values were determined including; total ash, acid insoluble ash and water-soluble ash. Ash values determine the mineral or inorganic content, earthy matter or other impurities of a raw material. They depend upon age or maturity of the plant, plant part used, time of collection, seasonal variations, organ to organ and treatment or how samples were prepared. Quality of a raw material differs according to its mineral composition (type and amount). Hence, the ash values are an important quality control parameter which can be used for the detection of adulterated, exhausted or substandard raw materials. In the present study, acid insoluble ash and water soluble ash values were comparatively lower which might be due to low contamination by inorganic impurities such as, sand or earth during plant collection, storage and drying (Kadam et al., 2013). Total ash value was higher than the acid insoluble and water-soluble ash values might be due to the availability of adhered inorganic salts occurring naturally hence, further studies are required in the analysis of constituent composition (Kadam et al., 2013). Loss on drying determines both the moisture and volatile matter content available in *I. tinctoria* leaves. They show a low level of moisture and volatile matter content, so that low level of moisture reduces microbial growth, presence of fungi or insects and deterioration due to hydrolysis. Hence, storage conditions and shelf life can be determined. The highest extractability value was obtained with methanol but was not significantly different with the other two solvents; acetone and water. But extractability in acetone and hot distilled water were significantly different. Though Ayurveda most often uses water as the solvent of extraction, methodology should be modified to obtain extracts out of other solvents which are efficient in extractability than that of water. HPTLC is a qualitative parameter in which HPTLC fingerprints are considered as an important tool in quality assessment of *I. tinctoria*. HPTLC fingerprints are identification standards so that, a species can be properly identified amongst other closely related species (Felicia & Muthulingam, 2012). HPTLC is also considered the preliminary tool in ascertaining the authenticity of a plant species. Each extract obtained different HPTLC fingerprints with different R_f values. Further studies are required in order to determine the constituents available in each extract and to evaluate their mode of action with the therapeutic indication which ultimately can be used as potential drug candidates or new drug entities. Much number of peaks was obtained with the aqueous extract than methanol and acetone extract which ensures the need of developing a methodology to find the best solvent for extraction. Since, there is a growing trend nowadays towards the use of natural herbs and are more preferred than the synthetic entities; establishment of standardization parameters in identification and authentication is advantageous for Ayurveda drug discovery and further development (Gupta *et al* 2012; Srinivasan *et al* 2016). Hence, the above parameters can be used as quality control standards in detection and authentication of leaves of *I. tinctoria* as raw materials excluding the counterfeit or substandard materials leading to safety, quality and efficacy of the manufactured Ayurveda preparations.

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DECLARATION OF CONFLICT OF INTEREST

We hereby declare that the study does not encompass any conflict of interest.

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