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Determination of bioanalytical parameters for the standardization of *Abroma augusta* and *Morinda tinctoria*

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Abstract

To set the analytical parameters for the simultaneous determination of different phytochemicals present in *Abroma augusta (A. augusta)* and *Morinda tinctoria (M. tinctoria).* Different phytochemical test including total phenol and total flavonoid content were carried out in the present investigation and rapid and simple high-performance thin-layer chromatographic (HPTLC) methods were developed for the qualitative and quantitative analysis**.** Preliminary phytochemical investigation revealed the presence of glycoside, alkaloid, carbohydrate, steroid, phytosterol, flavonoid, phenols, and triterpenes in the *A. augusta* and *M. tinctoria*. The total phenol and total flavonoid content were found to be 0.26% and 0.48% w/w and 0.18% and 0.32% w/w respectively. All tested microorganism in the *A. augusta and M. tinctoria* extract was found to be under limit. Chloroform: methanol: acetic acid and H_2O (172: 26: 26: 5) was used as solvent system for the qualitative and quantitative analysis through the HPTLC method. HPTLC analysis revealed 1.98% w/w of linoleic acid in the *A. augusta* extract and 1.68 % and 1.97% w/w, scopoletin and rutin in *M. tinctoria* extract respectively. Result showed that the selected parameters in the present investigation would set the new standards for the qualitative and quantitative analysis of 50% ethanolic extract of the *A. augusta* and *M.tinctoria*.

Keyword: *Abroma augusta***,** *Morinda tinctoria***,** HPTLC**,** Phytochemical analysis standardization.

1. INTRODUCTION:

Pants offer important sources of food and drugs for human being science very early age and many of the available drugs have been derived from plant and other natural sources. Several plants have been used for the treatment of various types of disorders even without knowing its side effects, proper function and phytoconstituents. About two to three decades ago, most of the drugs where obtained from natural sources [1-3]. The plants, parts of the plants and other material from the plants have been used in the practice from time immemorial for the prevention and treatment of various health aspects. More than 25% of the drugs prescribed worldwide are mainly derived from plant source. A large number of drugs in WHO essential medicine list are exclusively derived from plants origin. In India about 80% of the rural population used medicinal herbs for their primary health concerns [4-6]. In the recent years, plant derived products gain much more importance in the world, in the form of medicinal products, nutraceuticals and cosmetics. Herbals formulations have reached widespread acceptability as therapeutic agents for the treatment of various disorders. Standardization of herbal formulation is essential in order to assess the quality of drugs. Quality evaluation of herbal preparation is a fundamental requirement of industry and other organization dealing with ayurvedic and herbal products. According to WHO guideline an herbal products need to be standardized with respect to safety before releasing in to the market [7]. Standardization is a process that ensures a predefined amount of quantity, quality, and therapeutic effect of ingredients in each dose. For quality control of herbal products, high performance thin layer chromatography (HPTLC) is a popular method for the analysis of herbal medicines. HPTLC fingerprint profile is best choice for standardization followed by determination of specifics active phytoconstituents.

Abroma augusta (Family: *Sterculiaceae*) (*A. augusta*), commonly known as Devils's cotton is a popular plant, mainly used for the treatment of various types of disorder in the traditional system of medicine. *A. augusta* is one of the widely found plants all over in India and Australia [8, 9]. The whole plant contain several alkaloids and secondary metabolites including steroids, triterpenes, flavonoids, megastigmanes, benzohydrofurans and their glycosidesand phenylethanoid glycosides[10]. The leaves of *A. augusta* contain octacosanol, taraxerol, β-sitosterol acetate, Lupeol, an aliphatic alcohol ($C_{32}H_{66}O$) and mixture of long chain fatty diols. Abromine, the active constituent of the *A. augusta* identified as betaine is mainly responsible for antihyperglycemic activity [11]. Different parts of *A. augusta* are useful in treating diabetes, stomachache, dermatitis, leucorrhoea, scabies, gonorrhea, cough, leukoderma, jaundice, nerve stimulant, weakness, hypertension, uterine disorders, dermatitis, inflammation, rheumatic pain of joints and headache with sinusitis [10]. The plant is reported to have Hypolipidemic effect, however root bark is reported to contain antifertility agent [8, 9].

Morinda tinctoria belong to the (Family: *Rubiaceae*) (*M. tinctoria*) grow wildly and distributed throughout Southeast Asia commercially known as "nunaa" and locally known as "Togaru" is a small tree with immense medicinal properties. It is indigenous to tropical countries and is considered as an important folklore medicine. In traditional system of medicine *Morinda tinctoria* used as astringent and relive pain in gout [4;12].There is greater demand for fruit extract of *Morinda* species in treatment of different kind of illness such as arthritis, cancer, gastric ulcer and other heart diseases [5;13]. Anticonvulsant, analgesic, anti-inflammatory anti-oxidant activity and cytoprotective effect of *Morinda tinctoria* leaves has also been reported [12, 14-8; 16]. The ashes of *M.tinctoria* leaves

are also reported to act as biosorbents in controlling ammonia pollution in waste water [9; 17]. Not much work has been carried out on the Hepatoprotective activity against Non alcoholic fatty liver disease of the leaves/stem of *M.tinctoria*. The current study is undertaken to identify the phytoconstituents responsible for the biological activity against NAFLD of ethanolic extract of *M.tinctoria*.

2. MATERIAL AND METHOD:

The plants *Abroma augusta linn (leaves) was* collected from Kundari rakabganj Lucknow, India and *Morinda tinctoria (leaves and stem)* was collected from Ranan Nagar, Madurai, Tamil Nadu, India in *May* 2019. The plant materials were authenticated by **Dr. Navin K. Ambasht,** Head and Associate Professor, Botany Department, Christ Church College, Kanpur and voucher specimens were deposited in the departmental herbarium of Botany, Christ Church College, Kanpur, India for future reference.

2.1 Crude extraction:

Abroma augusta:

The dried crushed leaves 2kg powder material was soaked in 50% ethanol for 72 hours at room temperature. Ethanol extract was filtered and concentrated under pressure in rotary evaporator at 60 °C and dried to a constant weight in an oven set at 40 °C. The dried extract gave a yield of 18.15% (w/w) and was stored in an air-tight container at about 20°C until used.

Morinda tinctoria:

The dried plant material of 1kg powder material soaked in 50% ethanol for 72 hours at room temperature. Ethanol extract was filtered and concentrated under pressure in rotary evaporator at 50^0C and dried to a constant weight in an oven at 35^0C. The total yield of the extracts obtained after removing the solvents 32.2% (w/w) and was stored in an air tight container about 20^0C until used.

2.2 Phytochemical analysis:

In the present investigation in order to know the different phytoconstituents in *A. augusta* and *M.tinctoria* extract, phytochemical analysis was performed as per the standard method [18]. The presence of different phytochemicals in the *A. augusta* and *M.tinctoria* extract was confirmed through the thin layer chromatography (TLC) analysis [19]. Further the crude extract of *A. augusta* and *M. tinctoria* was also subjected to the total phenol and flavonoid content determination [20, 21]. Screening of the extracts was performed using the following chemicals and reagents: Alkaloid with Mayer's, Wagner's and Hager's and Dragendroff's reagent; carbohydrates with Molish's, Fehling's, Barfoerd's and Benedict's reagents; Glycosides with modified Borntrager's tests and legal's tests; Saponins were tested with forth's test and foam test; Fixed oils and fats with stains test and acetone-water test. Tannins with gelatin test and lead acetate test; Flavonoid with lead acetate test, alkaline reagent test, shinoda test and zinc hydrochloric reduction test; Protein and amino acid with Xanthoproteic test, Biuret test, Ninhydrin test;. They were identified by characteristic color change and precipitation reactions using standard procedure [22, 23]. Phytochemical evaluation of the 50% ethanolic extract of *A. augusta* and *M. tinctoria* were done for the presence of Alkaloids, Carbohydrates, Saponins, Tannins, Flavonoid, Proteins, Fats and oils and Glycosides and result are present in Table 1 for *A. augusta* and Table 2 for *M. tinctoria*.

Table: 1- Phytochemical screening of the 50% ethanolic extract of *Abroma augusta.*

S. No.	Chemical Constitute	Tests	AAE
1.	Carbohydrate	Molish`s test	+
		Fehlings`s test	+
2.	Amino acids	Million`s test	-
		Ninhydrin test	+
		Biuret test	+
3.	Tanin	Ferric chloride test	+
4.	Alkaloids	Dragendroff`s test	-
		Mayer`s test	+
		Wanger`s test	+
		Hanger`s test	-
5.	Flavonoids	Aqueous NaOH test	+
		Con.H_2SO_4 test	+
		Shinoda`s test	+
6.	Saponins	Foam test	-
7.	Glycoside	Legal`s test	-
		Borntrager`s test	-

Table: 2- Phytochemical screening of the 50% ethanolic extract of *Morinda tinctoria.*

S. No.	Chemical Constitute	Tests	MTE
1.	Carbohydrate	Molish`s test	-
		Fehlings`s test	+
		Benedict test	+
2.	Protein and Amino acids	Million`s test	-
		Ninhydrin test	+
		Biuret test	+
3.	Tanin	Ferric chloride test	-
		Potassium dichromate($K_2Cr_2O_7$)	-
		Lead acetate	-
4.	Alkaloids	Dragendroff`s test	+
		Wanger`s test	+
		Hanger`s test	+
5.	Flavonoids	Aqueous NaOH test	+
		Lead acetate test	+
6.	Saponins	Foam test	+
7.	Glycoside	Killer-Killani test	-

2.3: Determination of total phenol and flavonoid content.

2.3.1. Preparation of standard solution

10 mg of standard chemical (Gallic acid, rutin) were dissolved in 10ml of distilled methanol to get 1000µg/ml solution. Further it was diluted with methanol to obtained required concentration of the solution.

2.3.2. Preparation of test solution

100 mg of extract were dissolved in 10 ml of methanol to get 1000µg/ml solution. Then solution was diluted with methanol to obtained lower dilution.

2.3.3. Total phenol determination

The total phenol content was determined in the *A. augusta* extract by Folin- Ciocalteu method. Test is based on the oxidation of phenolic group with phosphomolybdic and phosphotungstic acid. After oxidation a green blue complex is measurable at 750nm. 0.4 ml of extract separately mixed with 2ml of Folin reagent and 1.6 ml of Na_2CO_3. After shaking it kept for 2 hours (Reaction time) and the absorbance was measured at 750nm using Gallic acid standard curve. The total phenol content was expressed as Gallic acid equivalent in % w/w of extract.

2.3.4. Total flavonoid determination

Aluminum chloride colorimetric method was used for the total flavonoids content determination. Plant extract 0.5ml in methanol was separately mixed with 105ml of methanol, 0.1ml of 10% aluminum chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. Then it remained at room temperature for 45 minute; the absorbance of the reaction mixture was measured at 415nm. The calibration curve was prepared by preparing quercetin solution at concentration 15.5 to 100µg/ml in methanol.

2.4. Development of analytical parameter for HPTLC standardization

2.4.1. Preparation of standard

For the preparation of calibration curve in the quantitative analysis different concentration of the standard stock solution were prepared in the HPLC grade methanol.

2.4.2 Preparation of sample

For the preparation of the sample solution weight 1000 mg of extract and dissolve in methanol and then sonicated for 15 minute and the final volume of solution was made up to 7ml to get stock solution. All the concentration of the sample was prepared in the stock solution by suitable dilution.

2.4.3. Chromatographic conditions

For the chromatographic analysis precoated silica gel 60 F_{254} HPTLC plate are used. All the sample and standard was dissolved in the HPLC grade methanol and filter through microne filter before application. Sample and standard compound was applied in a 5.5 mm band for qualitative analysis and 7.5 mm band for quantitative analysis, 14 mm from the bottom of the plate using an automated TLC applicator linomat V (Camag, Switzerland) with nitrogen flow. This critical parameter for the HPTLC analysis was maintained for all analysis performed. The respective analytical parameter for the fingerprint and quantitative analysis was presented in the table 3 and table 4.

2.4.4. HPTLC plate development

For the HPTLC analysis plate was developed in AMD2 development chamber using chloroform: methanol: acetic acid: H_2O (172: 26: 26: 5) as solvent system. The composition of the mobile phase was selected based on testing of different solvent composition of varying polarities. HPTLC analysis was performed at $(20\pm5)^0$C and 50% relative humidity. After development of the plate of separated constituent, compound was quantified using a TLC scanner (Camag, model 3) equipped with CATS software (Camag). Slit width 8mm x 0.4mm; wavelength 256nm; absorption reflection scan mode was used in the present analysis.

Table: 3. Analytical and chromatographic conditions for HPTLC fingerprint analysis of *A. augusta.*

Parameter	Analytical conditions
Analysis	HPTLC fingerprint analysis of *A. augusta*
Plate material	HPTLC precoated plate silica gel Merck $60F_{254}$
Solvent system	Chloroform: Methanol: Acetic acid: H_2O, 172: 26: 26: 5
Application mode	CAMAG automatic sampler III
Development mode	Ascending

Table: 4 Chromatographic parameter for HPTLC quantification of linoleic acid in *A. augusta.*

Parameter	Analytical conditions
Analysis	Estimation of linoleic acid in *A. augusta*
Plate material	HPTLC precoated plate silica gel Merck $60F_{254}$
Solvent system	Chloroform: Methanol: Acetic acid: H_2O, 172: 26: 26: 5
Application mode	CAMAG automatic sampler III
Development mode	Ascending

Table: 5 – Qualitative phytochemical constituents of *Morinda tinctoria*

S.No.	Phytochemicals	Mg/g
1.	Phenol	0.18 ± 0.017
2.	Flavonoid	0.32 ± 0.016
3.	Alkaloid	14 ± 0.035
4.	Protein	0.35 ± 0.073
5.	Free radical scavenging%	63.14 ± 0.264

3. RESULT:

For the development of analytical parameters, different phytochemical analyses were performed in the present investigation. Glycoside, Alkaloid, carbohydrate and steroid were found to be present were other phytoconstituents such tannin, triterpenoids, saponins, amino acids and protein were found to be absent in the *Abroma augusta* extract. TLC analyses were also

performed for *A. augusta* extract which show two spot in chloroform: methanol: H_2O (63:52:98) solvent system with 0.82 and 0.88 R_f value and three spot in toluene: glacial acetic acid: acetone (4:3:3) solvent system with 0.83: 0.92: 0.71 R_f value respectively. HPTLC fingerprint analysis revealed the presences of five prominent spot in Chloroform: Methanol: Acetic acid: H_2O, (172: 26: 26: 5) solvent system (Table-6). Total phenol and flavonoid content were determined as per the standard official method and were found to be 0.26% and 0.48% w/w in the *A. augusta* extract. HPLC techniques were used in the present investigation to quantify the linoleic acid content in the *A. augusta*. Chloroform: methanol: acetic acid: H_2O (172: 26: 26: 5) solvent system was found to be most suitable solvent system for the quantification of linoleic acid in the *A. augusta* and for fingerprint analysis. The content of linoleic acid in *A. augusta* was found to be 1.98% w/w. Preliminary phytochemical screening result of 50 % ethanolic extract of *M. tinctoria* showed the presence of many phytochemicals such as alkaloid, flavonoid, saponins, and tannins. Flavonoid and phenol compound exhibited wide range of biological activity like antioxidant and lipid peroxidation inhibitor properties. As a tabulated in table 5, the total flavonoids is 0.32 ± 0.016 mg/g, total phenol was 0.18 ± 0.017 mg/g and alkaloid were 14 ± 0.035 mg/g and protein 0.32 ± 0.073 mg/g was present in ethanolic extract. The flavonoid and phenolic compounds are responsible for broad range of biological activity like antioxidant and lipid peroxidation inhibitor properties.

Table: 6 – HPTLC fingerprint analysis of *A. augusta* extract.

No. of spots	Solvent system	R_f Value	Maximum peak height	Peak area %
5	Chloroform	0.32	29.5	5.48
	Methanol	0.43	20.3	1.82
	Acetic acid	0.69	35.8	8.85
	H_2O	0.89	215.4	48.73
	172: 26: 26: 5	1.02	247.8	38.86

3.1. HPTLC fingerprint analysis of *Morinda tinctoria* extract:

The continuous trial by using different mobile phase for the separation of ethanolic extract of Morinda tinctoria by HPTLC, the desired resolution of Scopoletin and Rutin with reproducible peak were succeed by using chloroform: methanol (9.6:0.7) as the mobile phase using 6 µl and 9 µl volume of ethanolic extract of *Morinda tinctoria*. The regression data have shown a good linear relationship over the concentration range of 2-23 µl/spot. The linearity of calibration graphs and adherence to the system to beer's law is validated by high volume of correlation coefficient and the SD for intercept value noticed to be less than 7%. No significant difference is observed in the slopes of standard curves was observed.

4. DISCUSSION:

There for the optimization of suitable solvent system for HPTLC analysis, different composition of mobile phase was used in the TLC analysis before quantitative analysis. The solvent system optimizes the entire sample and standard was developed in the HPTLC plates in order to know the suitability of the chosen solvent system. In the present study Chloroform: methanol: acetic acid: H_2O (172: 26: 26: 5) was found to be the most suitable solvent system for determination of linoleic acid in the *A. augusta* extract and fingerprint analysis and chloroform: methanol (9.6: 0.7) was found in *M. tinctoria* extract. Data of HPTLC fingerprint analysis was presented in table 6. HPTLC analyses were performed as per the method optimized above and the respective quantities of linoleic acid and Scopoletin and Rutin were calculated as per the standard method. From the data analysis it was found that *A. augusta* contained good amount of linoleic acid M. tinctoria contained excellent amount of Scopoletin and Rutin. The presences of linoleic acid and Scopoletin and Rutin were confirmed by comparing the R_f value and spectra of the sample spot with that of the standard. The peak purity of the standard compound and samples was determined by comparing the spectra and three different concentration using peak area, peak height, and R_f value of the spot. This phytochemical analysis revealed the presence of various phytoconstituents in *A. augusta* and *M. tinctoria* such as glycoside, alkaloid, carbohydrate, etc. these phytoconstituents have been reported to have multiple biological effect such as anti-inflammatory, antiallergic, antioxidant, antidiabetic, aldose reducing inhibitory potential, antiviral, anticancer activities [24]. Several medicinal plants have been used as dietary supplements and for the treatment of various disorders without proper knowledge about their mode of action. Therefore there is a need for more documented standard, clinical trials and laboratory work to justify their pharmacological action and toxicity for safe and effective treatment [25]. Alkaloid, flavonoids, tannins, phenol, saponins and several other compounds play an important role in the defence mechanism against microorganisms, insects and other herbivores in the plant [26]. Phytochemicals standard are generally used for the determining identity, purity, and strength of the drug sources. These parameter are also used to check the nature of the crude drug, and it help to play important role in preventing the possible role of adulteration [7, 27, 28]. Phytochemical standardization includes preliminary phytochemical analysis and quantification of different phytoconstituents present in the extracts [29]. Standardization of the plant materials HPTLC play an important role due to analysis of several samples simultaneously in less time, TLC and HPTLC techniques solve many qualitative and quantitative analytical problems in a wide range of fields including medicine, pharmaceuticals, chemistry, biochemistry, food analysis, toxicology and environmental analysis [30]. Therefore from the above mentioned analytical parameters, it was found that *A. augusta* contain significant amount of flavonoid, linoleic acid and phenol and *M. tinctoria* contained

scopoletin, rutin, phenol and triterpenes. The above mentioned analytical parameter could be useful to the researcher to quantify the phytoconstituents present in *A. augusta and M. tinctoria*, and these parameter will also be helpful for the standardization of the *A. augusta and M. tinctoria* in the future.

Conflict of Interest:

The author declares that there are no conflicts of interest.

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