ISSN 1996-0700

Asian Journal of Biotechnology

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OPEN ACCESS Asian Journal of Biotechnology

ISSN 1996-0700
DOI: 10.3923/ajbkr.2017.50.70

Research Article
Phytochemical Screening of *in vitro* Raised Clones and Mother Plant of *Celastrus paniculatus*-Willd, an Endangered Medicinal Plant

Anusha Tharayil Koonapra Sasidharan, Megha Kizhakkepurakkal Balachandran, Joseph Madassery and Kothanam Kuzhiyil Elyas

Department of Biotechnology, University of Calicut, Kerala, India

Abstract

Background and Objective: *Celastrus paniculatus*-Willd belongs to the family *Celastraceae* is an endangered medicinal plant in India. It has potential role in primary health care system and various herbal drug formulations. The curative property of this medicinal plant is mainly due to the presence of various bioactive compounds. The present investigation was focused on the phytochemical screening of *in vitro* raised clones and mother plant in order to ensure the qualitative chemical similarity. **Materials and Methods:** The preliminary phytochemical screening such as fluorescence analysis of the leaf powder, fluorescence analysis of leaf extracts and various physiochemical properties of both *in vitro* raised clones and mother plant were done. To ensure the presence of various secondary metabolites TLC analysis was performed. The developed plates were sprayed with Dragendroff reagent for alkaloids, fast blue salt reagent for detection of phenolic group, ninhydrin for aminoacids and biogenic amines, vanillin phosphoric acid for detection of terpenoids. In order to confirm the qualitative chemical similarity of *in vitro* raised clones high performance liquid chromatography was performed. The spectral comparison of extracts was recorded by WIN CATS (1.3.4 version) software. The results were recorded and expressed as the Mean±SD for all the experiments. **Results:** The preliminary phytochemical screening of leaf extracts of both *in vitro* raised clones and mother plant exhibited the same result and there was no remarkable difference. TLC based identification of bioactive constituents revealed the presence of alkaloids, flavanoids, steroids, saponins, tannins, phenols, glycosides and reducing sugars. The HPTLC profile of the methanolic leaf extracts revealed the true to type nature of *in vitro* raised clones. **Conclusion:** The study revealed the presence of various bioactive constituents in both *in vitro* raised clones and mother plant of *Celastrus paniculatus.* The qualitative chemical comparison was done by TLC and HPTLC analysis.

Key words: *Celastrus paniculatus*, *Celastraceae*, *in vitro* propagation, bioactive constituents, phytochemistry, TLC, HPTLC

Citation: Anusha Tharayil Koonapra Sasidharan, Megha Kizhakkepurakkal Balachandran, Joseph Madassery and Kothanam Kuzhiyil Elyas, 2017. Phytochemical screening of *in vitro* raised clones and mother plant of *Celastrus paniculatus*-willd, an endangered medicinal plant. Asian J. Biotechnol., 9: 50-70.

Corresponding Author: Anusha T.S., Department of Biotechnology, University of Calicut, Malappuram, 673635 Kerala, India Tel:+91-9744815492

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Natural products are secondary metabolites of plants and microbes. Many secondary metabolites are highly potent and selective as a result of evolutionary selection. Some of them are interpreted to be signal molecules or defense mechanisms against competitors, herbivores or pathogens. They assist in maintaining survival and reproductive fitness of the producing organisms. Compounds derived from natural products may also serve as biochemical tools that demonstrate the role of specific pathways in diseases[1] and proved to be a prolific source of bioactive agents. These substances are often so complex that they would never be prepared synthetically as drug candidates, so isolation from natural sources is the only feasible way to access them[2].

Medicinal plants are the potential source of various bioactive compounds and they are being used in modern medicine for the treatment of various human ailments. Bioactive compounds derived from the plants remain the basis of a large proportion of the commercial medication, but most of them are not scientifically evaluated[3]. The scientific evaluation and standardization of a developed crude drug is essential for their therapeutic application. *Celastrus paniculatus*-Willd belonging to the family *Celastraceae* is an endangered medicinal plant, distributed in tropical and subtropical regions of India. It is a large, woody climbing shrub commonly known as 'Jyotishmati', 'Intellect tree' or 'Bitter sweet' is an important medicinal plant in India. The broad range of biological activities of this plant is mainly due to the presence of secondary metabolites such as malkanguniol, malkangunin, celapanine, celapanigine, celapanin, celapagin, celastrine, paniculatine, celastrol, pristimerin, zeylasteron, zeylasteral etc[4,5]. The isolation and characterization of bioactive constituents from the whole plant or from its different parts have always been a challenging task. The studies confirmed that the majority of the isolated bioactive compounds from the genus 'Celastrus includes sesquiterpenes (β-agarofurans), diterpenes, triterpenes, alkaloids and flavonoids and among them β-dihydroagarofuran, sesquiterpenes and triterpenes are the important active components[6]. The immunomodulatory property of petroleum ether extract of seeds of *Celastrus paniculatus* on immunological, hematological and oxidative stress parameters using pyrogallol induced immune suppression model in rats[7,8]. The earlier studies reported the antiproliferative, antioxidant, anti-fungal, anti-psychiatric, anti-inflammatory and anti-convulsant effects of seed extracts of this plant[9-11]. Keeping this in view, this study describes the detection or screening of bioactive constituents, especially from the leaf extracts which were prepared by using organic solvents of different polarity.

MATERIALS AND METHODS

The experiment was done at the laboratory of Department of Biotechnology, University of Calicut on June-September, 2016. The chemicals used for the experiments were procured from Himedia Mumbai, Merck Mumbai, SRL Mumbai, Sigma-Aldrich Germany and Qualigens Mumbai. The chemicals used were of analytical grade. The experimental designs were completely randomized. The results were recorded and expressed as the Mean±SD for all the experiments.

Plant materials: An approximately 10 year old plant of *Celastrus paniculatus* was collected from the Botanical garden, University of Calicut was used for the present study. The collected plant was identified and authenticated from Department of Botany, University of Calicut. A voucher specimen with Accession No 6890 has been deposited in the hebarium of the same department.

Fluorescence analysis of crude leaf powder: The leaf powder was subjected to fluorescence analysis by using different reagents. The color of the solution was observed in visible light and under UV light for their characteristic color reaction[12].

Physiochemical properties: The leaf powder was subjected to various physiochemical parameters. Organic solvents such as petroleum ether, chloroform, diethyl ether and methanol were subjected to loss on drying, ash value, total ash, acid-insoluble ash, pH value. Various soluble extractive values were also noted.

Fluorescence analysis of leaf extracts: Fresh leaves were collected and shade dried for 2-3 weeks and ground into fine powder. The extract was prepared by single solvent extraction method. About 10 g of powder was mixed with 100 mL of petroleum ether, chloroform, ethyl acetate and methanol in 250 mL of conical flask was kept at room temperature for 24 h. The suspension was filtered through whatmann's filter paper and collected in large petri plates and allowed to dry completely in water bath set at 40±0.2°C for 30 min. Dried extracts were scraped out by using scalpels and was collected in pre weighed vials separately for further analysis. The color of the extracts in daylight and under short (254 nm) and long (365 nm) wavelength UV was noted.

Preliminary phytochemical screening: To detect the pharmacological activities, plant materials were used in their crude form as well as extracted with suitable solvents to take out the desired components. As the herbal drugs contain so many chemical compounds, it is necessary to isolate the active constituents responsible for therapeutic application. So it is pre requisite to evaluate the nature of the extracts before evaluating its biological activity[13]. The preliminary qualitative analysis was done to identify the bioconstituents by following the standard methods[14-16].

Test for carbohydrates and glycosides: About 0.1 g of each extract was dissolved separately in 4 mL of distilled water and filtered. The filtrate was subjected to the following tests to detect the presence of carbohydrates and glycosides.

Molisch's test: The filtrate was treated with 2-3 drops of 1% alcoholic alpha-naphthol and 2 mL of conc. H_2SO_4 was added along the sides of the test tube. Appearance of the brown ring at junction of two liquids shows the presence of carbohydrates.

Fehling's test: The filtrate was treated with 1 mL of Fehling's solution and heated on a water bath. A reddish precipitate obtained shows the presence of carbohydrates.

Another portion of extracts were hydrolysed with diluted HCl for few hours on a water bath and the hydrolysate was subjected to the following tests to detect the presence of glycosides.

Borntrager's test: Hydrolysate was treated with chloroform and the chloroform layer was separated. To this equal volume of dilute ammonia solution was added. Ammonia layer acquiring pink color shows the presence of glycosides.

Detection of fixed oils

Filter paper test: A small quantity of extract was pressed separately between the filter paper. Appearance of oil stain on the paper indicated the presence of fixed oils.

Detection of proteins and free amino acids: About 0.5 g extract was dissolved in few milliliters of distilled water and they were subjected to the following test.

Biuret test: To the above prepared extracts equal volume of 5% NaOH and 1% $CuSO_4$ solution were added. Violet color indicates the presence of proteins and free amino acids.

Detection of tannins and phenolic compounds: Extracts were subjected to the following reagents for the detection of tannins and phenolics.

- 5% $FeCl_3$ solution→violet colour
- 1% solution of gelatine containing 10% NaCl→white precipitate
- 10% lead acetate solution→white precipitate

Detection of phytosterol: Approximately 0.2 g of extract was dissolved in 1 mL of chloroform separately and then subjected to the following test.

Salkowski test: To 1 mL of the above extract, few drops of conc. H_2SO_4 were added. Brown colour produced showed the presence of phytosterols.

Detection of alkaloids: Solution (a): 0.425 g basic bismuth nitrate in 5 mL glacial acetic acid and 20 mL water under heating. Solution (b): 4 g potassium iodide in 15 mL water. Stock solution: (a)+(b) were mixed 1:1. To about 3 mL of extract, a few drops of Dragendorff's reagent were added. Orange brown precipitate indicated the presence of alkaloid.

Detection of flavonoids: Extract was dissolved separately in aqueous NaOH. Appearance of yellow colour indicated the presence of flavonoids. To small portion of each extract, conc. H_2SO_4 was added. Yellow orange color showed the presence of flavonoids.

Shinoda's test: The extract was dissolved in alcohol and a piece of magnesium metal and conc. HCl was added drop wise and heated. Appearance of magenta color showed the presence of flavonoids.

Small quantity of extract was dissolved separately in aqueous NaOH. Appearance of yellow color indicated the presence of flavanoids.

To the small portion of each extracts, Conc. H_2SO_4 was added. Yellow orange color indicated the presence of flavanoids.

Thin layer chromatography: The identification of secondary metabolites from leaf extracts was evaluated using thin layer chromatography (TLC). The TLC plates were prepared manually and activated in an oven for 1 h at 110°C prior to use. The samples were applied at a concentration of 10 µL. Different compositions of the mobile phase were tested in

order to obtain high resolution and reproducible peaks. They are ethyl acetate: methanol: water (1:2:1), toluene: ethyl acetate (3:7), methanol: chloroform (9:1). The proportions of each solvent were varied in the solvent system to determine the best one. The developed chromatographic plates were air dried and sprayed with various reagents such as Dragendroff reagent for alkaloids, fast blue salt reagent for detection of phenolic group, ninhydrin for aminoacids and biogenic amines, vanillin phosphoric acid for detection of terpenoids and finally with anisaldehyde-sulphuric acid.

HPTLC analysis: The HPTLC analysis of the methanolic leaf extract was carried out by following the standard methods[17-19]. The plates were developed in a solvent system of toluene: ethyl acetate in the ratio 3:7.

Sample preparation: About 50 g of shade dried leaves of 8th month old field grown *in vitro* raised plants (after 20th, 40th and 60th days of culture period) and mother plant of *Celastrus paniculatus* was powdered and 5g of the powdered material was refluxed with methanol at 60°C for 4 h over a water bath. The extract was filtered and concentrated under reduced pressure in a rotary evaporator below 60°C. The concentrated extracts were dissolved in methanol used for HPTLC analysis.

Solvent system: The plates were developed in a solvent system of toluene: ethyl acetate (3:7), in order to obtain high resolution and reproducible peaks.

Sample application: A CAMAG (Switzerland) HPTLC system equipped with a sample applicator was used for the present study. About 2 µL of the sample was loaded as 5 mm band length separately on precoated silica gel $60F_{254}$ aluminium sheets (3×10 cm) using a Hamilton syringe with the help of Linomat 5 applicator attached to the CAMAG HPTLC system, which was programmed through WIN CATS software[20,21].

Detection of spot: The developed chromatogram was air dried and the plates were kept in a photo documentation chamber (CAMAG REPROSTAR 3) and captured the images in UV 254 and 366 nm. The chromatogram was scanned by the densitometer at 405 nm. The peak areas with its height, Rf values of the fingerprint were noted and spectral comparison for the four types of extracts were recorded by WIN CATS (1.3.4 version) software.

RESULTS

Fluorescence analysis of crude leaf powder: The fluorescent characteristics of the plant powder as such and after treating them with different chemical reagents were observed in day light and under UV radiation. The leaf powder of both *in vitro* propagated and mother plant of *Celastrus paniculatus* was respond to various chemical reagents and exhibited the same result. The results were shown in the Table 1.

Physiochemical properties: The leaf extracts of both *in vitro* propagated and mother plant of *Celastrus paniculatus* was subjected to measurements of various physiochemical parameters. The total ash value, acid soluble ash value, water soluble ash value and the extractive values were determined. The results indicate that the loss on drying in the sample was 6.26±0.30% w/w for mother plant and 6.12±0.32% w/w for *in vitro* propagated plant, which shows that the value of moisture content is lower in the sample. The total ash value was 14.32±0.13% w/w for mother plant and 14.24±0.11% w/w for *in vitro* propagated plant, indicating presence of inorganic content in it. The aqueous extractive value is higher (11.53±0.42% w/w for mother plant and 11.53±0.41% w/w for *in vitro* propagated plant), which indicates that the solubility of the sample drug in water is higher. In the water extract, fat or resin content is very less, whereas, compounds like tannin, amino acid, sugar, glycosides and carbohydrate

Table 1: Fluorescence analysis of leaf powder of both *in vitro* propagated and mother plant of *Celastrus paniculatus*

	Mother plant			*In vitro* propagated plant		
Particular of treatments	Under visible light (565 nm)	Under UV light (254 nm)	Under UV light (365 nm)	Under visible light (565 nm)	Under UV light (254 nm)	Under UV light (365 nm)
Powder alone	Green	Green	Green	Green	Green	Green
Powder+1N aqueous NaOH	Green	Green	Green	Green	Green	Green
Powder+1N alcoholic NaOH	Green	Light green	Light green	Green	Light green	Light green
Powder+1N HCL	Dark green	Orange	Orange	Dark green	Orange	Orange
Powder+NH_3	Green	Yellow	Green	Green	Yellow	Green
Powder+Iodine	Light green	Pink	Pink	Light green	Pink	Pink
Powder+5% $FeCl_3$	Green	Orange	Light green	Green	Orange	Light green
Powder+acetic acid	Green	Green	Green	Green	Green	Green
Powder+HNO_3	Brown	Yellow	Yellow	Brown	Yellow	Yellow
Powder+H_2SO_4	Black	Dark black	Dark black	Black	Dark black	Dark black

Table 2: Physiochemical properties of leaf extract of both *in vitro* propagated and mother plant of *Celastrus paniculatus*

Physiochemical parameters	Percentage yield from mother plant (w/w)	Percentage yield from *in vitro* propagated plant (w/w)
Loss on drying	6.26±0.30	6.12±0.32
Total ash value	14.32±0.13	14.24±0.11
Acid soluble ash	0.531±0.41	0.531±0.43
Water soluble ash	1.64±0.24	1.56±0.28
Water soluble extract	11.53±0.42	11.53±0.41
Methanol extract	13.26±0.63	12.32±0.62
Chloroform extract	4.54±0.53	4.54±0.52
Pet ether extract	3.33±0.44	3.43±0.41
Ethyl acetate extract	6.32±0.53	6.32±0.54
pH	5.5	5.5

The values are expressed as the Mean±SD for all the experiments

Table 3: Fluorescence analysis of various leaf extracts of both *in vitro* propagated and micropropagated *Celastrus paniculatus*

	Mother plant			*In vitro* propagated plant		
Extracts	Under visible light (565 nm)	Under UV light (254 nm)	Nature of the extract	Under visible light (565 nm)	Under UV light (254 nm)	Nature of the extract
Petroleum ether	Light green	Light green	Sticky	Light green	Light green	Sticky
Methanol	Dark green	Dark green	Sticky	Dark green	Dark green	Sticky
Chloroform	Dark green	Dark green	Non sticky	Dark green	Dark green	Non sticky
Ethyl acetate	Dark green	Dark green	Non sticky	Dark green	Dark green	Non sticky

are higher, pH of 5% aqueous solution shows slightly acidic nature of the leaf. The percentage yield of the solvent extracts is ranged from 3.33-13.26. The yield of the petroleum ether extract and chloroform extract was too low for the plant under study. The extractive value for the methanolic extract was comparatively very high, which can be attributed to the more amounts of methanol soluble constituents in the leaves (Table 2).

Fluorescence analysis of leaf extracts: For the detection of the fluorescent compounds, fluorescence analysis of the leaf extracts was done which can serve as valuable information. The color of the different extracts of *in vitro* propagated and mother plant of *Celastrus paniculatus* under visible light and long wavelength UV (365 nm) were noted (Table 3). These color characteristics are specific for the plant under study. Any deviation from these can result from the addition of impurities or by the substitution by another plant. Thus these observations are important to check the quality, purity and adulteration of the crude leaf powder. Hence, the study of fluorescence analysis can be used as a diagnostic tool for correct identification of the crude herbal drug. The petroleum ether extract and methanol extracts were sticky in consistency while chloroform and ethyl acetate extract were non sticky. Various extracts of both *in vitro* propagated and field grown *Celastrus paniculatus* didn't show significant variation .

Preliminary phytochemical screening of the extract: The preliminary phytochemical studies are done for detection of various constituents such as alkaloids, glycosides, carbohydrates present in plant extract, which is responsible for the pharmacological activity. The *in vitro* propagated and the mother plant was subjected to various phytochemical screening by following the standard methods[22,23] and there was no significant variation. The results were tabulated in Table 4 and 5. The qualitative tests for most of the extracts showed significant indication about the presence of secondary metabolites.

The methanole and ethyl acetate extract of the *in vitro* propagated as well as the mother plant showed majority of the bioactive compounds such as alkaloids, flavanoids, steroids, saponins, tannins, phenols, glycosides and reducing sugars. Of these the phenolic content was detected to be prominent as the intensity of the colored reaction was high. The petroleum ether extract and chloroform extract showed the presence of only few bioactive compounds. The petroleum ether extract showed the presence of steroids, terpenoids, glycosides and phlobotanins while chloroform extract showed the presence bioactive compounds such as the flavanoids, steroids, glycoside and reducing sugars. These findings of phytochemicals were good enough to reflect its importance. There are yet many other phytoconstituents, for which the tests are not performed in this study. Hence, to detect them, thin layer chromatography was carried out.

Thin layer chromatogram: The preliminary phytochemical analysis of the solvent extracts was further evaluated by thin layer chromatoghaphy. After TLC separation of four solvent

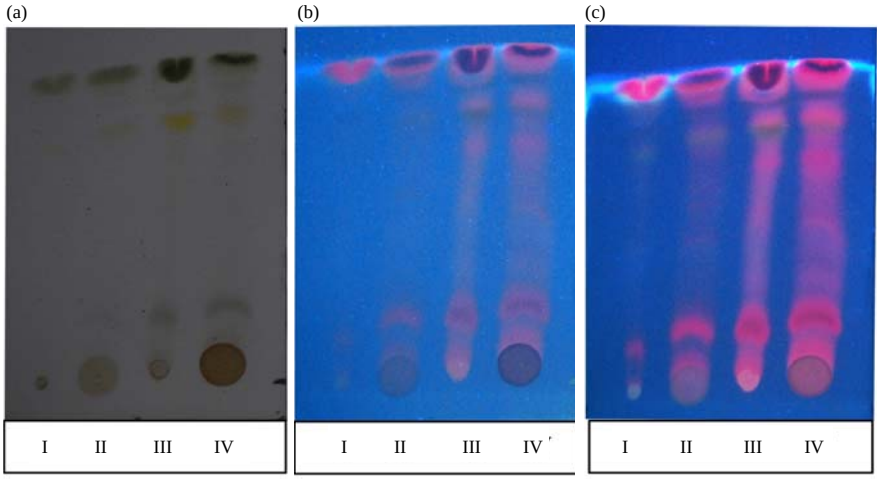


Fig. 1(a-c): Thin layer chromatogram of leaf extracts of mother plant of *Celastrus paniculatus*. (a) Observed in daylight, (b) Under UV 254 nm and (c) Under 366 nm. Lane I: Petroleum ether extract, Lane II: Chloroform extract, Lane III: Ethyl acetate extract and Lane IV: Methanol extract

Table 4: Preliminary phytochemical evaluation of leaf extracts of mother plant of *Celastrus paniculatus*

Chemical constituents	Petroleum ether extract	Chloroform extract	Ethyl acetate extract	Methanol extract
Alkaloids	-	-	+	+
Flavanoids	-	+	+	+
Steroids	+	+	+	+
Terpenoids	+	-	-	-
Saponins	-	-	+	+
Tannins	-	-	+	+
Phenols	-	-	+	+
Glycosides	+	+	+	+
Anthraquinones	-	-	-	-
Phlobotanins	+	-	-	-
Reducing sugar	-	+	-	+
Volatile oil	-	-	-	-

+: Present, -: Absent

Table 5: Preliminary phytochemical evaluation of leaf extracts of *in vitro* propagated *Celastrus paniculatus*

Chemical constituents	Petroleum ether extract	Chloroform extract	Ethyl acetate extract	Methanol extract
Alkaloids	-	-	+	+
Flavanoids	-	+	+	+
Steroids	+	+	+	+
Terpenoids	+	-	-	-
Saponins	-	-	+	+
Tannins	-	-	+	+
Phenols	-	-	+	+
Glycosides	+	+	+	+
Anthraquinones	-	-	-	-
Phlobotanins	+	-	-	-
Reducing sugar	-	+	-	+
Volatile oil	-	-	-	-

+: Present, -: Absent

extracts, the plates were observed in visible light as well as under UV light (254 and 365 nm). The chromatogram of all the four extracts of both mother plant and *in vitro* plant showed the presence of a yellow and green colored compound, both separating from other constituents at an R_f value corresponding to 0.52 and 0.81, respectively (Fig. 1, 2). These two are the only colored constituents seen in visible light and showed a red fluorescence when observed under UV

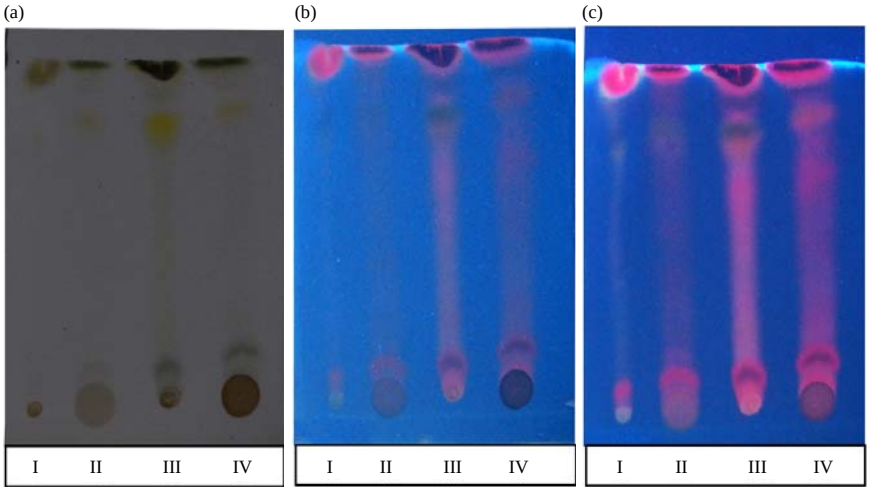


Fig. 2(a-c): Thin layer chromatogram of leaf extracts of *in vitro* progenies of *Celastrus paniculatus*. (a) Observed in daylight, (b) Under UV 254 nm and (c) Under 366 nm. Lane I: Petroleum ether extract, Lane II: Chloroform extract, Lane III: Ethyl acetate extract and Lane IV: Methanol extract

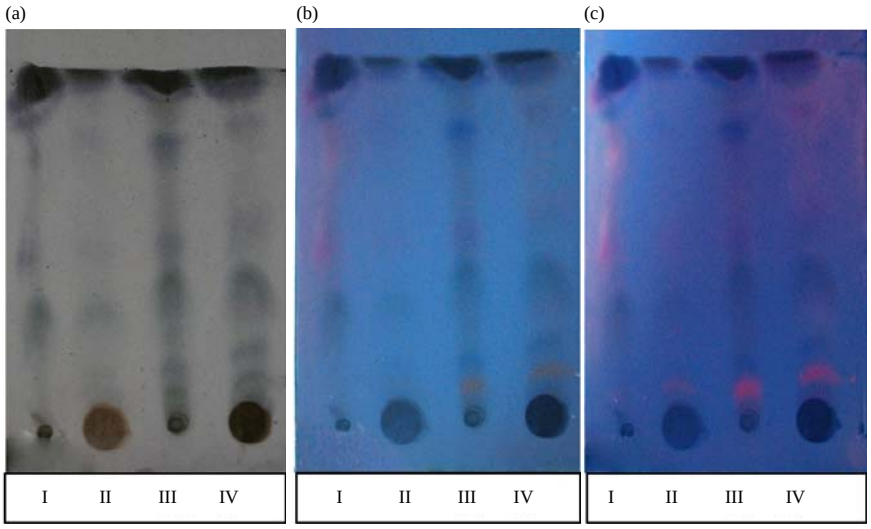


Fig. 3(a-c): Thin layer chromatogram of leaf extracts of mother plant of *Celastrus paniculatus* sprayed with anisaldehyde reagent. (a) Observed in daylight, (b) Under UV 254 nm and (c) Under 366 nm. Lane I: Petroleum ether extract, Lane II: Chloroform extract, Lane III: Ethyl acetate extract and Lane IV: methanol extract

(365 and 254 nm), as it is clear from the Table 6 and 7. The chromatogram of chroroform extract showed the presence of 4 compounds with Rf value 0.02, 0.14, 0.57 and 0.70. In addition to the compounds seen in chloroform extract, the methanolic and ethyl acetate extract showed the presence another one compound showing red fluorescence under UV. The chromatogram of petroleum ether extract revealed the presence of only 3 compounds (Rf = 0.01, 0.10 and 0.70) when visualized under longer and shorter wavelength UV. The developed plates were observed under UV at 254 and 365nm wavelengths and at visible light.

For better observation the developed plates were sprayed with about 10 mL of anisaldehyde-sulphuric acid reagent[24]. The sprayed plates were heated at 100°C for 5-10 min (Fig. 3, 4). The post chromatographic derivitisation by using 2% ethanolic $FeCl_3$ of various leaf extracts of both *in vitro* propagated and mother plant ensure the presence of phenolic compound (Fig. 5, 6).

HPTLC profile: Qualitative chemical similarity of *in vitro* regenerated clones and mother plant was compared using high performance thin layer chromatography (HPTLC)

Table 6: Thin layer chromatogram of leaf extracts of mother plant of *Celastrus paniculatus*

	Visible (565 nm)		UV (365 nm)		UV (254 nm)	
Extracts	Color	R_f	Color	R_f	Color	R_f
Petroleum ether	Yellow	0.52	Red	0.01	Red	0.02
	Yellowish green	0.81	Red	0.10	Red	0.01
			Red	0.70	Red	0.71
Methanol	Yellow	0.57	Red	0.02	Red	0.02
	Green	0.76	Red	0.06	Red	0.09
			Red	0.14	Red	0.13
			Red	0.57	Red	0.59
			Blue	0.71	Blue	0.70
Chloroform	Yellow	0.57	Red	0.02	Red	0.02
	Dark green	0.76	Red	0.14	Red	0.06
			Green	0.57	Green	0.58
			Blue	0.70	Blue	0.70
Ethyl acetate	Yellow	0.57	Red	0.02	Red	0.02
	Dark green	0.76	Red	0.14	Red	0.11
			Red	0.59	Red	0.65
			Red	0.67	Red	0.69
			Blue	0.70	Blue	0.73

Table 7: Thin layer chromatogram of leaf extracts of *in vitro* propagated plants of *Celastrus paniculatus*

	Visible (565 nm)		UV (365 nm)		UV (254 nm)	
Extracts	Color	R_f	Color	R_f	Color	R_f
Petroleum ether	Yellow	0.50	Red	0.02	Red	0.02
	Yellowish green	0.77	Red	0.12	Red	0.01
			Red	0.69	Red	0.72
Methanol	Yellow	0.60	Red	0.03	Red	0.04
	Green	0.77	Red	0.08	Red	0.09
			Red	0.13	Red	0.14
			Red	0.60	Red	0.60
			Blue	0.70	Blue	0.71
Chloroform	Yellow	0.58	Red	0.02	Red	0.04
	Dark green	0.78	Red	0.12	Red	0.06
			Green	0.57	Green	0.58
			Blue	0.70	Blue	0.69
Ethyl acetate	Yellow	0.59	Red	0.02	Red	0.04
	Dark green	0.77	Red	0.6	Red	0.13
			Red	0.6	Red	0.64
			Red	0.67	Red	0.70
			Blue	0.70	Blue	0.72

Table 8: HPTLC profile of *in vitro* raised clones and mother plant of *Celastrus paniculatus*

	Under UV (254 nm)		Under (UV 366 nm)	
Samples	No. of spot	Rf value	No. of spot	Rf value
Mother plant	13	0.01, 0.03, 0.08, 0.13, 0.16, 0.26, 0.33, 0.41, 0.47, 0.65, 0.70, 0.82, 0.96	13	0.01, 0.03, 0.06, 0.08, 0.14, 0.25, 0.40, 0.47, 0.54, 0.68, 0.82, 0.93, 0.96
In vitro propagated plant after 20 days	10	0.02, 0.08, 0.13, 0.27, 0.47, 0.59, 0.70, 0.82, 0.90, 0.98.	9	0.02, 0.08, 0.14, 0.26, 0.37, 0.47, 0.67, 0.81, 0.90
In vitro propagated plant after 40 days	12	0.01, 0.09, 0.16, 0.29, 0.35, 0.49, 0.56, 0.61, 0.69, 0.72, 0.84, 0.96	10	0.02, 0.06, 0.09, 0.15, 0.28, 0.40, 0.48, 0.70, 0.83, 0.96
In vitro propagated plant after 60 days	12	0.02, 0.08, 0.16, 0.28, 0.40, 0.50, 0.60, 0.64, 0.68, 0.72, 0.83, 0.90.	10	0.02, 0.06, 0.08, 0.14, 0.26, 0.39, 0.48, 0.67, 0.82, 0.90

profiling[11]. It can also be used as a reference for the proper authentication of bioconstituents. The methanolic leaf extract of both *in vitro* propagated and mother plant was subjected to HPTLC, for this 8th month old field grown tissue culture raised plants after 20, 40 and 60 days of incubation was randomly selected. The parameters such as the number of the compounds and their corresponding peaks under both short wavelength (254 nm) and long (366 nm) wavelength UV were tabulated (Table 8).

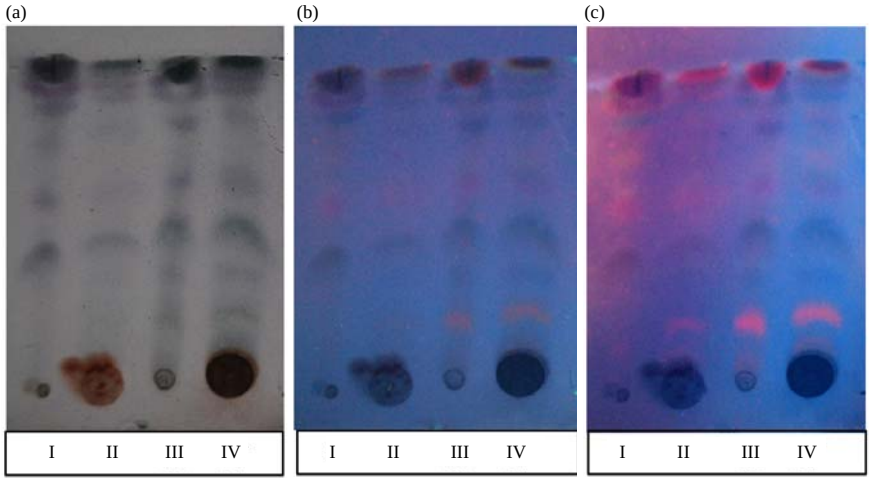


Fig. 4(a-c): Thin layer chromatogram of leaf extracts of *in vitro* progenies of *Celastrus paniculatus* sprayed with anisaldehyde reagent. (a) Observed in daylight, (b) Under UV 254 nm and (c) Under 366 nm. Lane I: Petroleum ether extract, Lane II: Chlorofom extract, Lane III: Ethyl acetate extract and Lane IV: Methanol extract

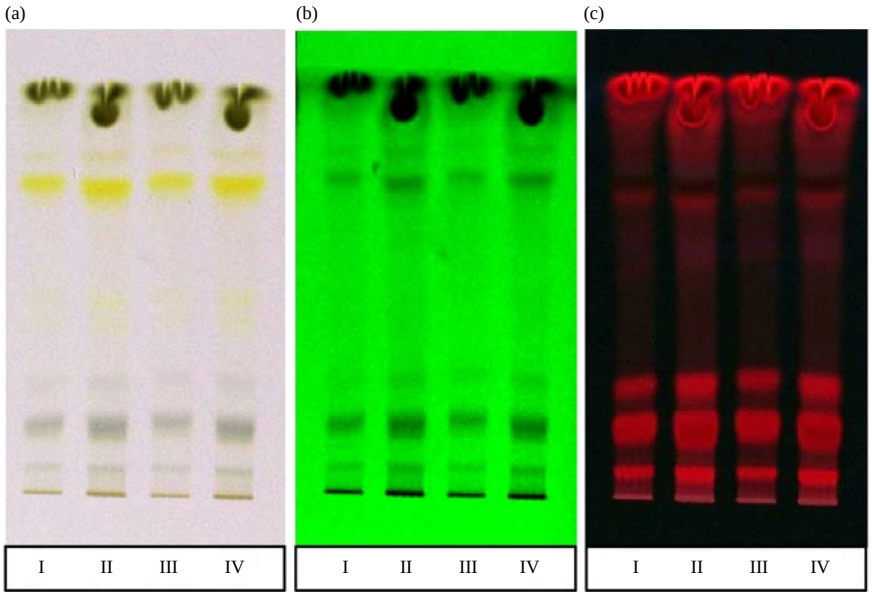


Fig. 5(a-c): Post chromatographic derivatization of methanolic leaf extract of mother plant. (a) Observed in daylight, (b) Under UV 254 nm and (c) Under 366 nm. Lane I-IV: Methanol extract

The HPTLC profile of methanolic leaf extract of mother plant showed 13 peaks when visualized under UV at 254 and 366 nm and the major peak was found at Rf value 0.96 (Fig. 7, 8). The *in vitro* propagated plants after 20 days of hormonal treatment exhibited 10 peaks and major peak was found at Rf value 0.98 under short wavelength UV, whereas, in long wavelength UV the total number of peaks were 9 and major peak was found at Rf value 0.90 (Fig. 9, 10).

In the case of *in vitro* propagated plants after 40 and 60 days under short UV showed 12 bands with highest Rf value 0.96 and 0.90, respectively (Fig. 11, 12). But under long UV it showed a total number of 10 spots with highest Rf value 0.96 and 0.82, respectively (Fig. 13, 14).

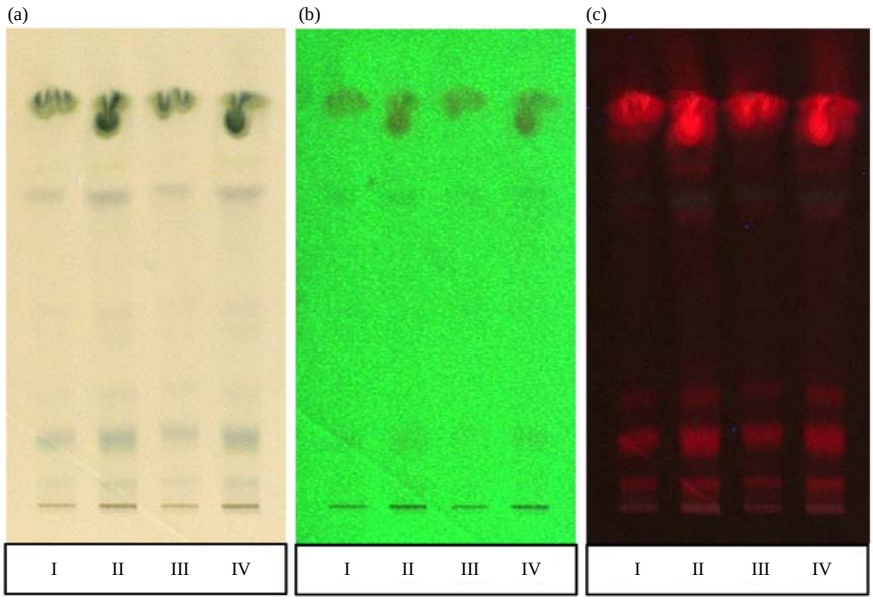


Fig. 6(a-c): Post chromatographic derivatization of methanolic leaf extract of *in vitro* progenies. (a) Observed in daylight, (b) Under UV 254 nm and (c) Under 366 nm. Lane I-IV: Methanol extract,

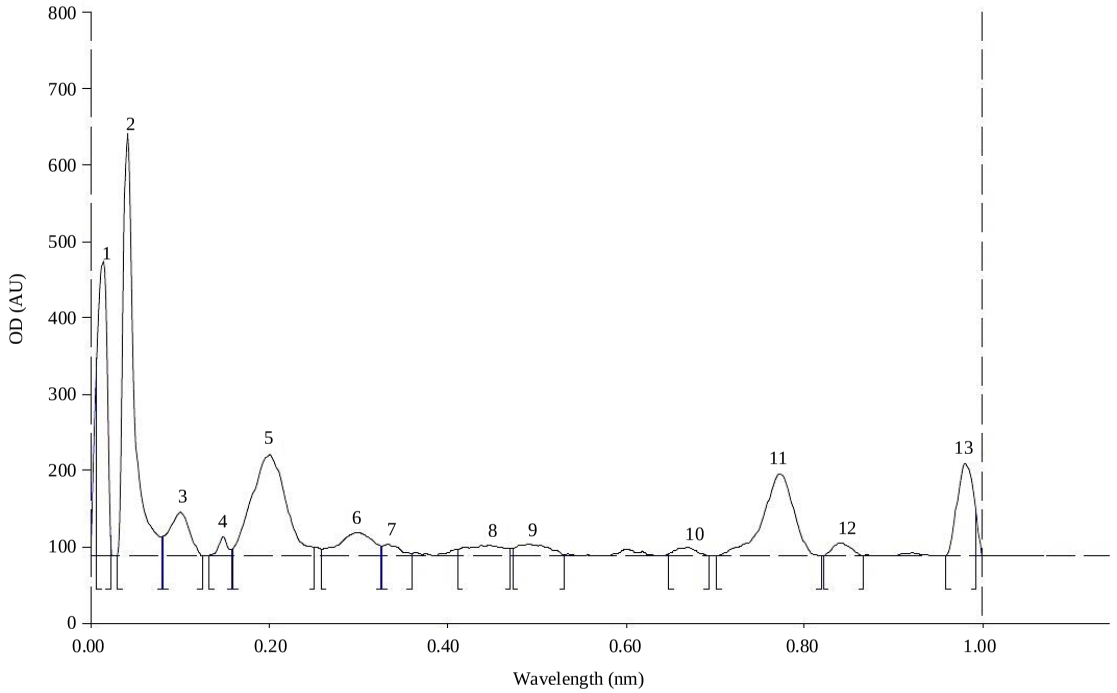
DISCUSSION

The qualitative chemical comparison of *in vitro* raised clones is an essential step in the application of biotechnology for micropropagation of true to type nature. Because there are many factors like length of culture periods, genotype and nature of explant, which could influence the stability of the tissue cultured plants. Even though *in vitro* germplasm conservation is highly influences on micropropagation methods, phenotypic and genetic variations are reported to occur during *in vitro* regeneration, originating somaclonal variants.

Fluorescence is the phenomenon exhibited by various chemical constituents present in plant material. The ultra violet light produces fluorescence in many natural compounds which do not visibly fluoresce in daylight. If the substances themselves are not fluorescent, they may often be converted into fluorescent derivatives by applying different reagents and this is considered as an important parameter of phytochemical evaluation[25]. The fluorescence analysis of the leaf powder was carried out according to the standard methods[26,27]. The color formation with respect to the particular reagents was noted and is aid in the determination of quality and purity of the leaf powder. Fluorescent studies of the leaf powder showed different coloration under visible light and UV light and the results were supportive with earlier fluorescent studies[28,29]. The fluorescent studies of *Cajanus cajan* (L) leaf powder reported that various solvents like acetone, ethanol and methanol did not show any fluorescence while nitric acid, chloroform and HCl showed characteristic coloration[30,31].

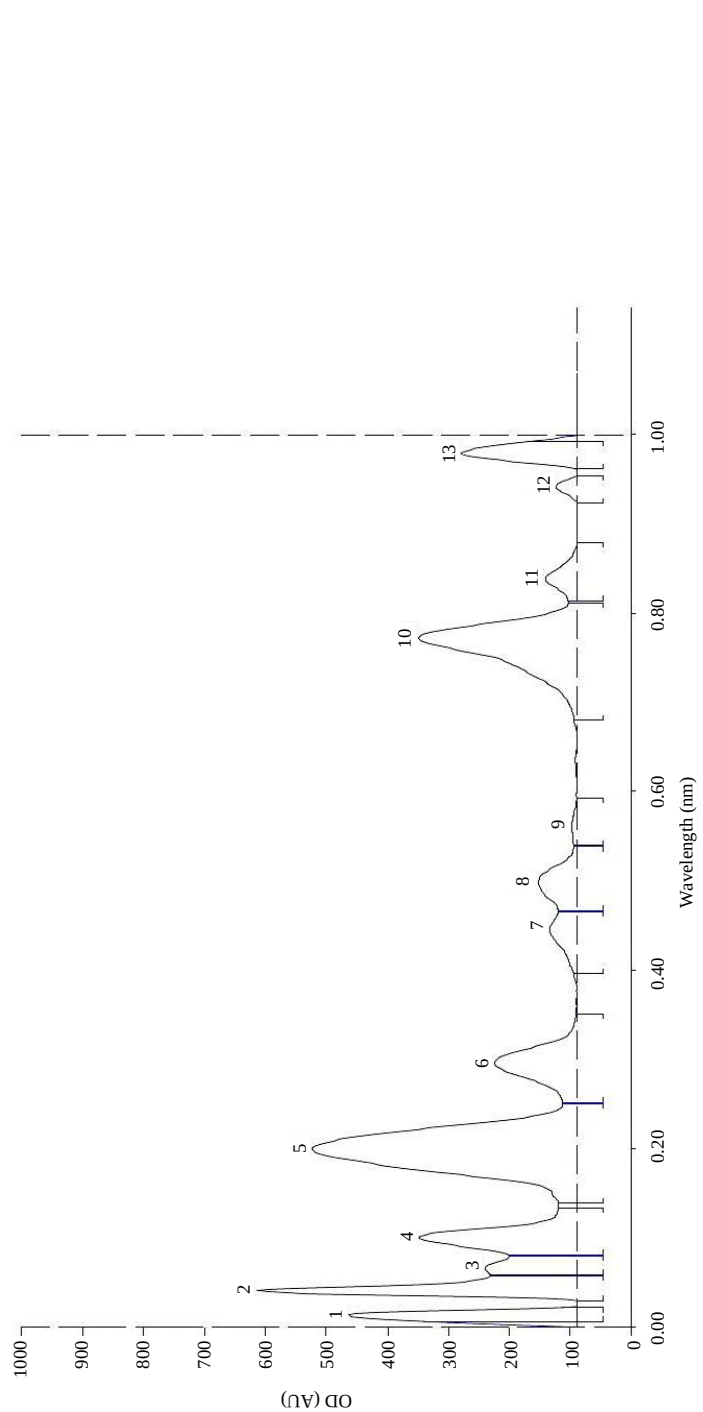
The physicochemical standards are important to check the quality, purity and adulteration of the given crude drug[32]. The physicochemical parameters were determined by following the standard methods[12,33]. The ash values of a drug determine quality and purity of crude drug which indicates the presence of various impurities like carbonate, oxalate and silicate along with the drug. The water soluble ash estimate the amount of inorganic compound present in drugs. The acid insoluble ash consist mainly silica and indicate contamination with earthy material. Moisture content of drugs should be at minimal level to prevent the growth of bacteria, yeast or fungi during storage. The physiochemical parameters of the *Celastrus paniculatus* revealed that water soluble ash is higher than acid insoluble ash. The similar results were reported in the leaf extracts of *Syzygium cumini* L. which showed the highest water soluble ash than the acid insoluble ash[34] .

Estimation of extractive values determines the amount of the active constituents in a given amount of plant material when extracted with a particular solvent and it is very helpful for determination of exhausted or adulterated drug[35-37]. The extractions of any crude drug with a particular solvent yield a solution containing different phytoconstituents. The compositions of these phytoconstituents depend upon the nature of the drug and the solvent used[38]. In the present study



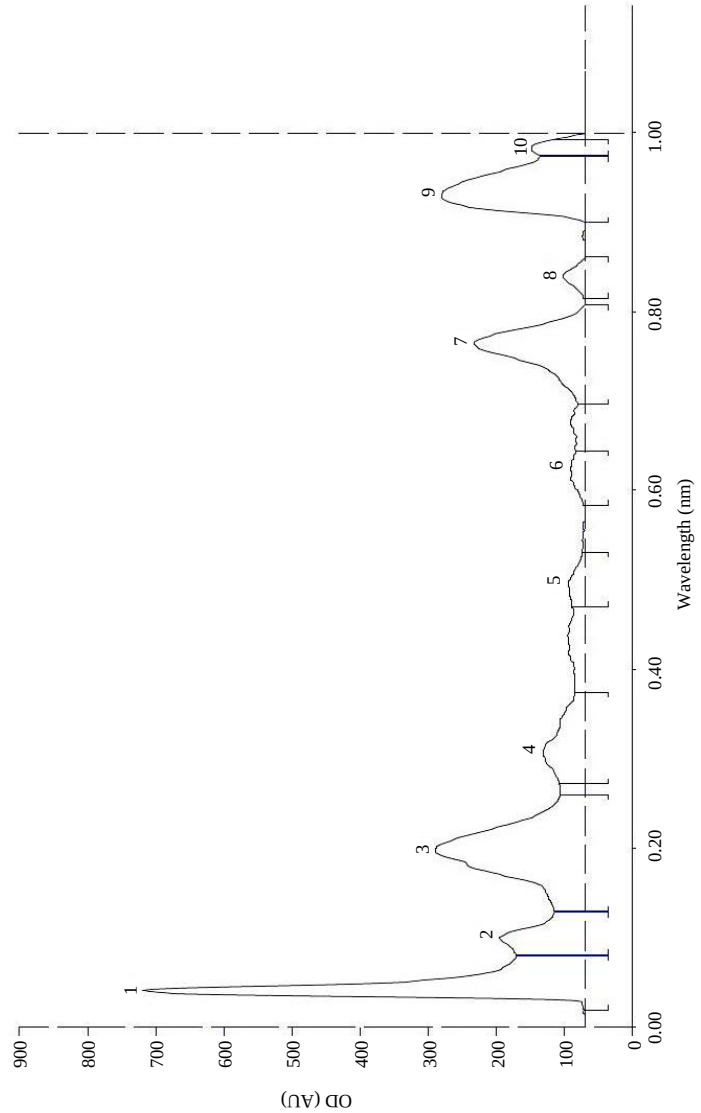
Peak	Start Rf	Start height	Max Rf	Max height	Max (%)	End Rf	End height	Area (%)
1	0.01	259.0	0.01	385.4	25.92	0.02	26.4	15.05
2	0.03	0.5	0.04	552.9	37.18	0.08	25.3	25.63
3	0.08	25.9	0.10	57.2	3.84	0.13	0.3	4.84
4	0.13	1.5	0.15	25.1	1.69	0.16	8.6	0.99
5	0.16	10.1	0.20	132.6	8.92	0.25	11.7	19.14
6	0.26	8.7	0.30	30.5	2.05	0.33	13.5	4.42
7	0.33	13.5	0.33	15.5	1.04	0.36	3.1	1.09
8	0.41	8.0	0.45	14.5	0.97	0.47	9.2	2.27
9	0.47	9.5	0.49	16.1	1.08	0.53	2.2	2.15
10	0.65	3.5	0.67	11.1	0.74	0.70	0.0	1.00
11	0.70	0.8	0.78	107.3	7.21	0.82	0.0	13.95
12	0.82	0.3	0.84	17.9	1.21	0.87	1.3	1.37
13	0.96	0.2	0.98	121.2	8.15	1.00	59.0	8.11

Fig. 7: HPTLC profile of mother plant at 254 nm



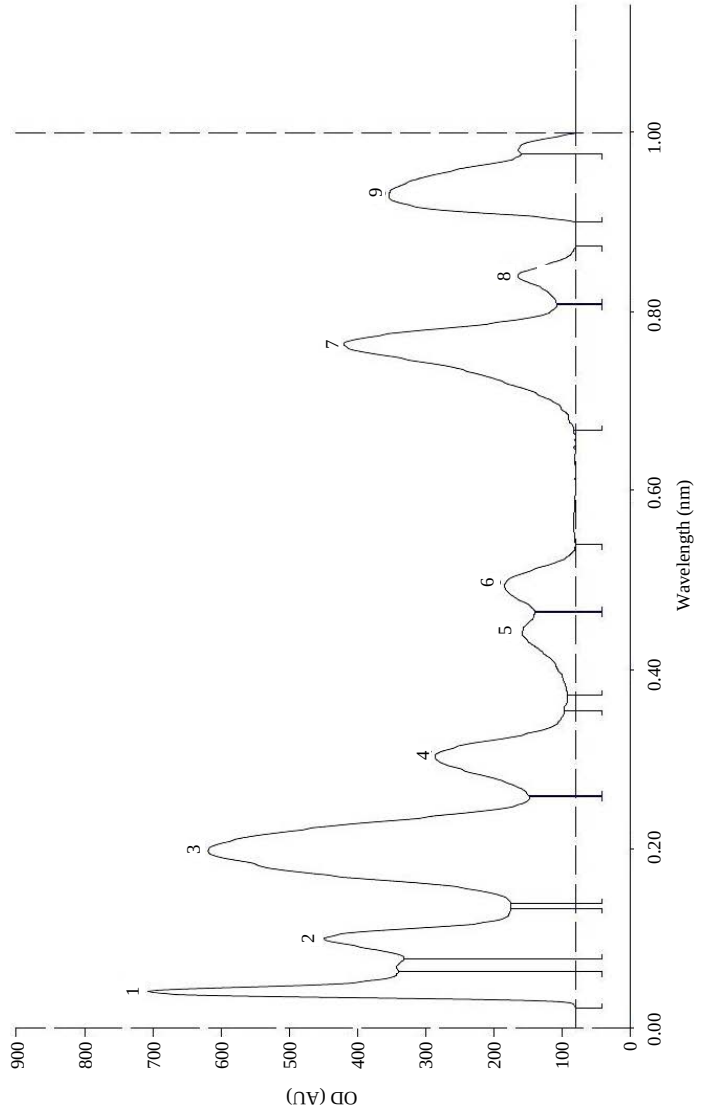
Peak	Start Rf	Start height	Max Rf	Max height	Max (%)	End Rf	End height	Area (%)
1	0.01	253.4	0.01	375.9	14.78	0.02	25.6	5.95
2	0.03	1.2	0.04	525.1	20.64	0.06	142.7	9.90
3	0.06	143.5	0.07	150.8	5.93	0.08	111.8	3.85
4	0.08	113.5	0.10	261.1	10.26	0.13	31.3	9.77
5	0.14	30.3	0.20	435.0	17.10	0.25	24.6	30.69
6	0.25	24.9	0.30	136.8	5.38	0.35	2.8	7.78
7	0.40	7.0	0.45	46.5	1.83	0.47	31.1	2.67
8	0.47	31.2	0.50	64.4	2.53	0.54	6.9	3.84
9	0.54	7.1	0.56	10.9	0.43	0.59	1.3	0.51
10	0.68	6.1	0.77	260.5	10.24	0.81	14.9	16.95
11	0.82	15.2	0.84	51.4	2.02	0.88	0.1	2.18
12	0.93	0.4	0.94	34.9	1.37	0.96	1.5	0.78
13	0.96	3.6	0.98	190.2	7.48	1.00	77.9	5.15

Fig. 8: HPTLC profile of mother plant at 366 nm



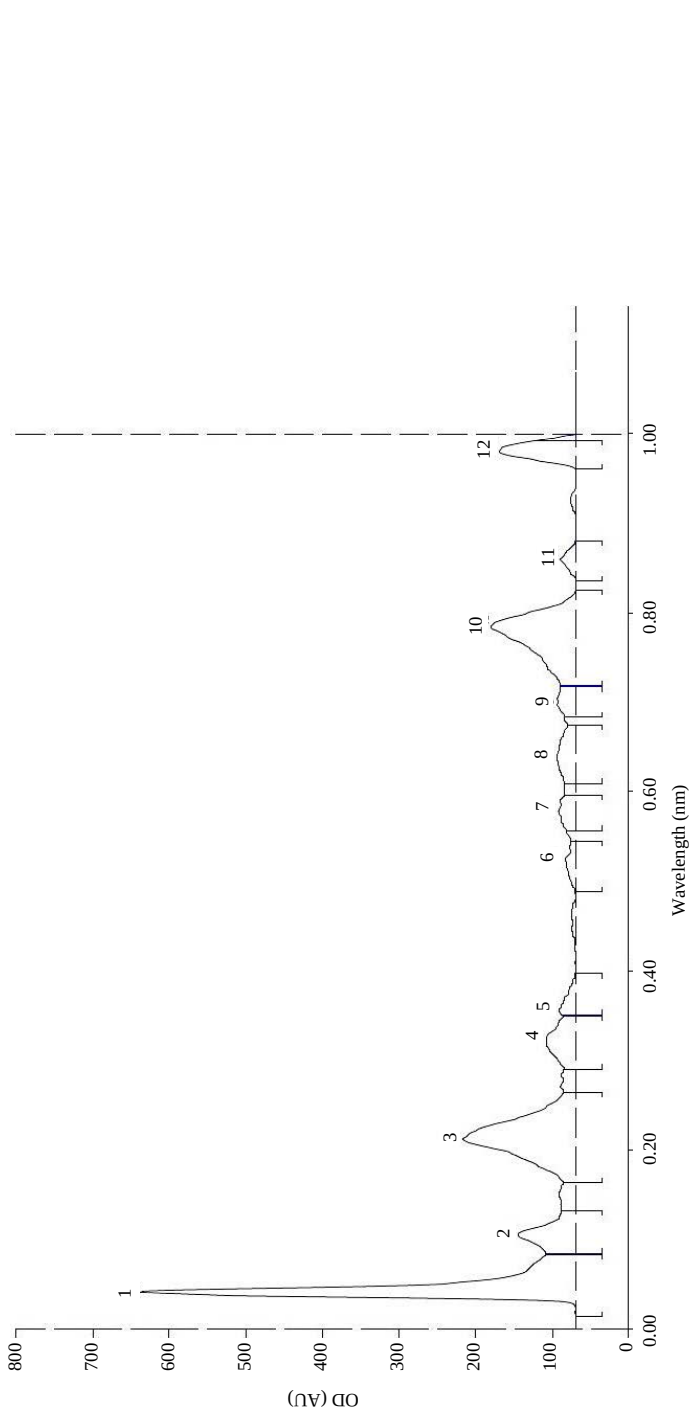
Peak	Start Rf	Start height	Max Rf	Max height	Max (%)	End Rf	End height	Area (%)
1	0.02	1.5	0.04	650.7	40.90	0.08	101.9	22.41
2	0.08	102.2	0.10	125.7	7.90	0.13	45.70	7.70
3	0.13	46.1	0.20	220.7	13.87	0.26	36.4	25.31
4	0.27	38.1	0.31	62.0	3.90	0.38	15.3	7.25
5	0.47	18.6	0.50	25.1	1.58	0.53	3.5	1.78
6	0.59	2.2	0.63	21.1	1.33	0.65	13.9	1.56
7	0.70	11.1	0.77	163.7	10.29	0.81	0.7	13.04
8	0.82	1.9	0.84	32.2	2.03	0.86	0.1	1.30
9	0.90	0.6	0.93	210.6	13.24	0.98	67.0	17.27
10	0.98	67.4	0.99	79.1	4.97	1.00	46.7	2.39

Fig. 9: HPTLC profile of *in vitro* raised plant after 20 days at 254 nm



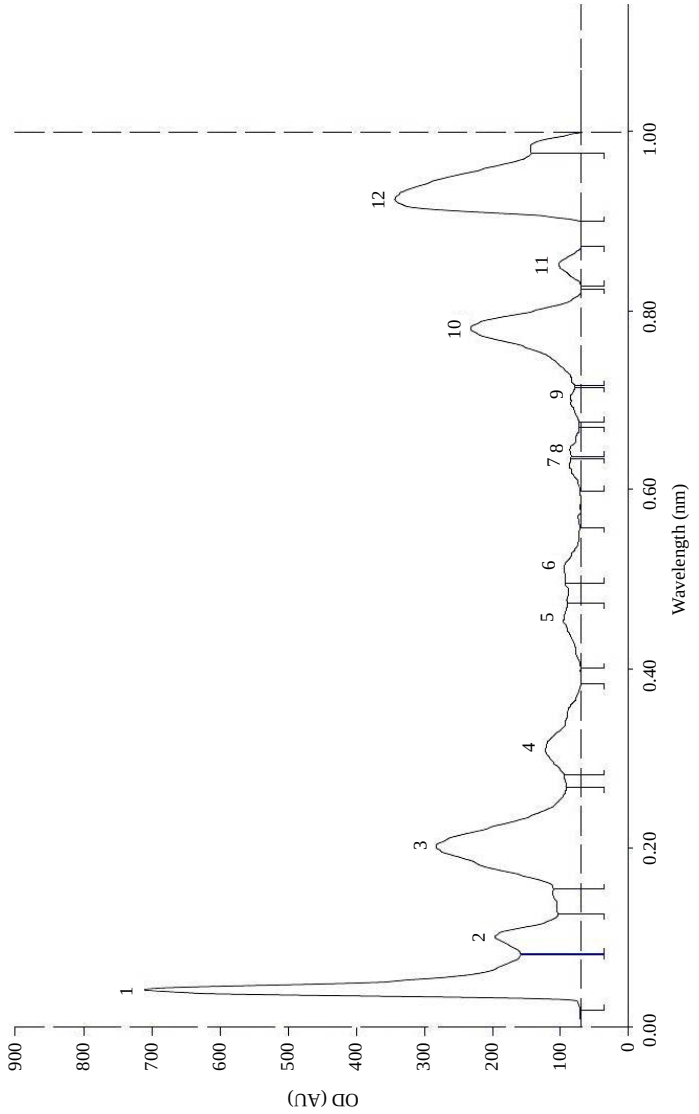
Peak	Start Rf	Start height	Max Rf	Max height	Max (%)	End Rf	End height	Area (%)
1	0.02	1.3	0.04	627.8	23.86	0.06	261.5	10.78
2	0.08	252.1	0.10	370.0	14.06	0.13	95.9	11.36
3	0.14	95.7	0.20	539.7	20.51	0.26	68.9	31.49
4	0.26	69.7	0.30	207.5	7.88	0.36	17.0	9.00
5	0.37	12.4	0.44	78.4	2.98	0.47	59.6	3.61
6	0.47	59.9	0.50	105.7	4.02	0.54	1.0	3.92
7	0.67	3.8	0.77	341.6	12.98	0.81	27.8	15.55
8	0.81	28.0	0.84	85.3	3.24	0.88	0.4	2.32
9	0.90	2.4	0.93	275.1	10.45	0.98	81.1	11.97

Fig. 10: HPTLC profile of *in vitro* raised plant after 20 days at 366 nm



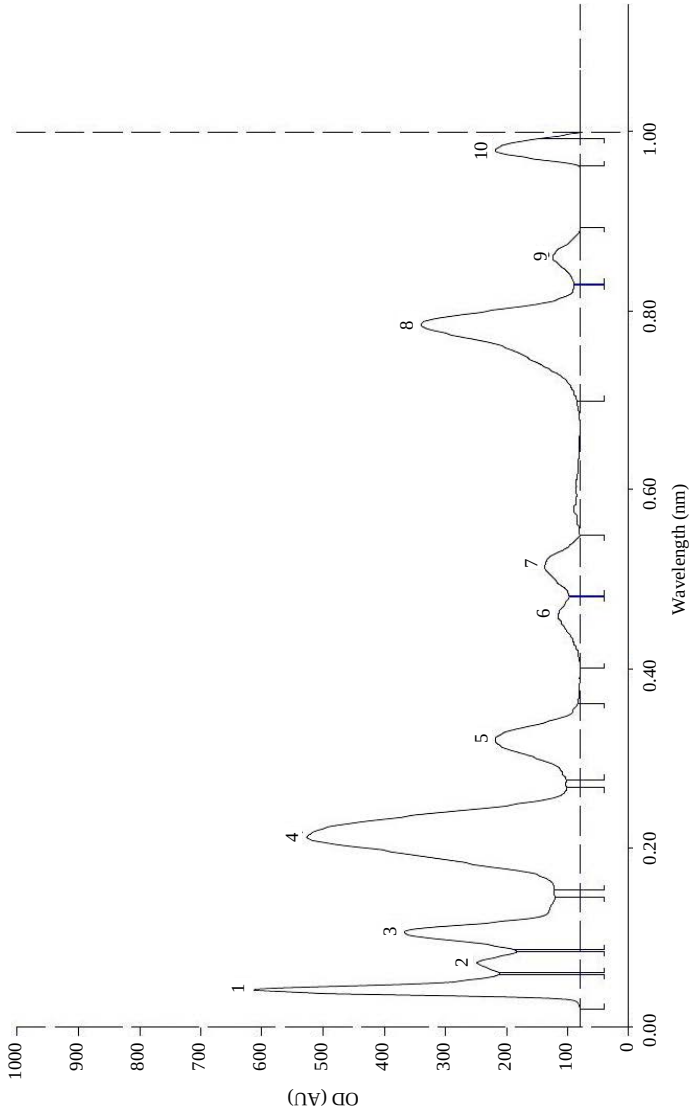
Peak	Start Rf	Start height	Max Rf	Max height	Max (%)	End Rf	End height	Area (%)
1	0.01	0.2	0.04	569.8	48.63	0.08	39.4	28.31
2	0.09	39.4	0.11	75.9	6.48	0.13	18.2	6.88
3	0.16	17.6	0.21	148.0	12.63	0.27	17.0	22.52
4	0.29	16.4	0.33	38.2	3.26	0.35	17.2	5.30
5	0.35	18.1	0.36	22.4	1.92	0.40	1.7	1.68
6	0.49	1.7	0.53	13.7	1.17	0.55	6.4	1.49
7	0.56	12.5	0.58	22.8	1.94	0.60	16.2	2.36
8	0.61	15.8	0.64	24.8	2.12	0.68	10.7	4.01
9	0.69	15.0	0.70	24.4	2.08	0.72	19.9	2.33
10	0.72	20.0	0.79	111.1	9.48	0.83	0.9	17.30
11	0.84	0.7	0.86	20.3	1.73	0.88	1.4	1.37
12	0.96	0.1	0.98	100.3	8.56	1.00	52.6	6.45

Fig. 11: HPTLC profile of *in vitro* raised plant after 40 days at 254 nm



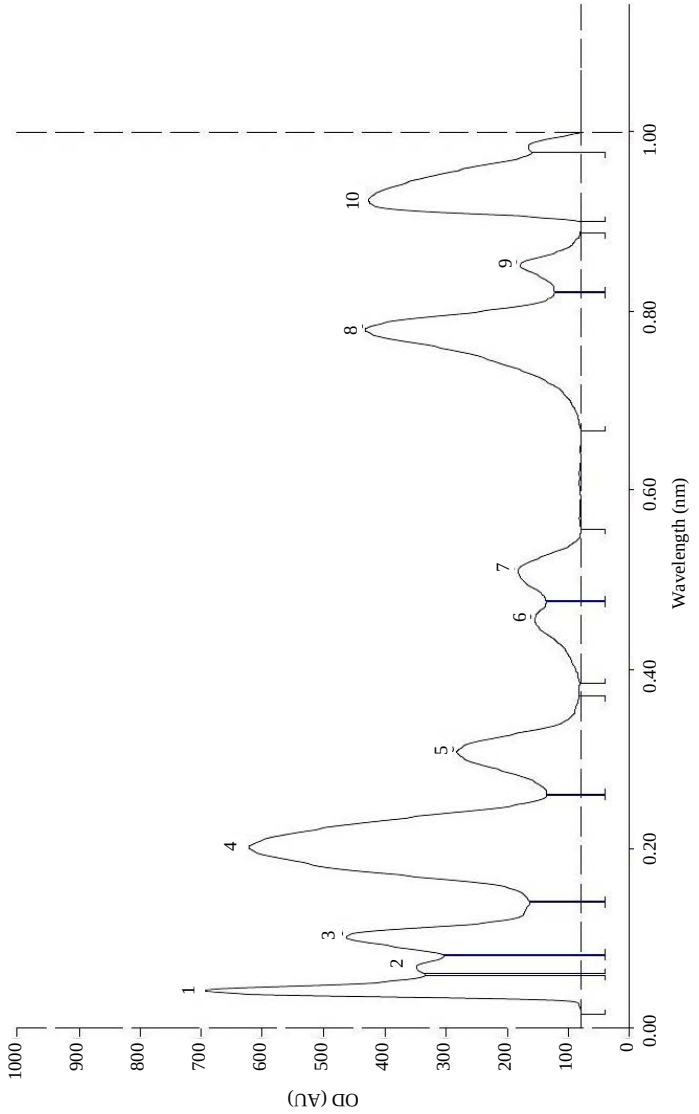
Peak	Start Rf	Start height	Max Rf	Max height	Max (%)	End Rf	End height	Area (%)
1	0.02	2.0	0.04	641.0	39.78	0.08	89.3	22.69
2	0.08	89.8	0.10	127.1	7.89	0.13	35.0	7.06
3	0.16	42.1	0.20	214.3	13.30	0.27	23.1	21.85
4	0.28	26.2	0.31	52.5	3.26	0.38	0.9	5.15
5	0.40	0.9	0.46	27.2	1.69	0.47	20.5	1.97
6	0.50	24.1	0.51	26.7	1.65	0.56	2.0	1.63
7	0.60	1.8	0.63	18.0	1.12	0.64	15.7	0.72
8	0.64	15.0	0.65	17.0	1.05	0.67	3.6	0.64
9	0.68	3.5	0.71	16.3	1.01	0.72	10.5	0.83
10	0.72	10.5	0.78	163.6	10.15	0.83	0.1	12.68
11	0.83	0.3	0.85	33.2	2.06	0.87	0.0	1.30
12	0.90	2.3	0.93	274.3	17.03	0.98	74.3	23.50

Fig. 12: HPTLC profile of *in vitro* propagated plant after 60 days at 254 nm



Peak	Start Rf	Start height	Max Rf	Max height	Max (%)	End Rf	End height	Area (%)
1	0.02	1.6	0.04	534.8	25.17	0.06	132.8	11.22
2	0.06	133.4	0.07	169.4	7.98	0.09	103.9	5.11
3	0.09	107.2	0.11	289.0	13.60	0.15	42.9	11.66
4	0.15	43.1	0.21	448.6	21.11	0.27	23.4	34.38
5	0.28	24.2	0.32	140.3	6.60	0.36	5.6	8.31
6	0.40	0.0	0.46	36.4	1.71	0.48	18.8	2.08
7	0.48	19.5	0.52	58.8	2.77	0.55	1.9	3.37
8	0.70	6.9	0.79	261.0	12.28	0.83	9.4	17.68
9	0.83	9.8	0.86	46.7	2.20	0.90	0.1	2.08
10	0.96	2.1	0.98	139.6	6.57	1.00	65.8	4.13

Fig. 13: HPTLC profile of *in vitro* propagated plant after 40 days at 366 nm



Peak	Start Rf	Start height	Max Rf	Max height	Max (%)	End Rf	End height	Area (%)
1	0.02	0.5	0.04	615.8	20.53	0.06	254.8	8.92
2	0.06	256.2	0.07	270.5	9.02	0.08	225.2	4.27
3	0.08	229.1	0.10	385.0	12.83	0.14	85.1	10.53
4	0.14	85.7	0.20	542.7	18.09	0.26	56.6	29.05
5	0.26	56.9	0.31	203.2	6.77	0.37	5.0	8.26
6	0.39	3.4	0.46	76.7	2.56	0.48	58.0	2.93
7	0.48	58.6	0.51	104.2	3.47	0.56	0.3	3.82
8	0.67	1.6	0.78	353.0	11.77	0.82	44.9	15.49
9	0.82	44.9	0.85	100.3	3.35	0.89	2.3	2.70
10	0.90	3.1	0.93	348.4	11.61	0.98	82.4	14.03

Fig. 14: HPTLC profile of *in vitro* propagated plant after 60 days at 366 nm

solvent extracts are giving clear evidence that the active compound is present in methanol and ethyl acetate. As it is more soluble in methanol, the purity of the compound can be ensured and can be easily extracted from the mixture. The shelf life of the drug can be increased as methanol soluble components will carry a very less moisture content. The various phytoconstituents can be extracted depending on the polarity and solubility of the solvent which is used for extraction. In the case of *Celastrus panicultus*-willd it was found that the extractive values of methanol were higher followed by ethyl acetate, chloroform and petroleum ether. But in the case of *Syzygium cumini* L. it was found that the extractive value of water was higher followed by ethanol and methanol[39].

Phytochemicals are chemicals derived from plants, presence or absence of these compounds in the extract is determined by the specific color reaction with appropriate chemicals. It is necessary to perform the preliminary phytochemical screening of a crude extract to get an idea regarding the type of bioactive compounds present in the mixture of extract. The qualitative phytochemical analysis confirmed the presence of rich variety of bioactive compounds and providing information which justify the medicinal usage of this species. The systematic phytochemical investigations not only help in revealing the active components but also help in the synthesis of better and newer analogues and congeners of higher therapeutics activities or the various active principals isolated from plants. Present study showed the presence of various phytoconstituents such as alkaloids, flavanoids, steroids, saponins, tannins, phenols, glycosides and reducing sugars. These results are supportive with earlier reports performed with leaf extracts of *Cajanus cajan* (L) and *Syzygium cumini* (L) where the same secondary metabolites were found[40-42]. Studies suggested the utilization of biologically active compound from plant resources by extraction, pharmacological screening, isolation and characterization of bioactive compounds[43-48].

The TLC based identification of the secondary metabolites confirmed the presence of flavanoids, phenolics and terpenoid. The thin layer chromatogram of *in vitro* propagated and field grown plants were showing no variation in any of the parameters under study, such as number and color of bands and their respective R_f values. These results depict the true to type nature of the *in vitro* propagated plant. Various phytochemicals gave different R_f values in different solvent system[49]. This variation in Rf values of the phytoconstituents provided a very important clue in understanding the polarity and also helped in selection of appropriate solvent system for separation of pure compounds.

The HPTLC analysis is mainly applicable for the comparison of samples on scanning densitometry or video technology and has become time and cost effective alternative to HPLC[50-54]. The HPTLC fingerprint profile of *in vitro* regenerated and mother plant was identical in terms of parameters such as number of bands and corresponding Rf values. The post chromatographic dervitization revealed the presence of phenolic compounds. Phenolic phytochemicals are most abundant secondary metabolites present in plants and most of them exhibit antioxidant properties. The major categories of phenolic compounds like simple phenol, poly phenol, flavonoids, coumarins, lignans, phenolic acids, xanthones, etc., which helps to enhance our health benefits[55-57]. Comparing the results of phytochemical analysis, thin layer chromatogram and HPTLC profile of the extract revealed the true to type nature of *in vitro* raised clones of *Celastrus paniculatus*. Further studies are required to isolate the novel bioactive compounds from this species.

CONCLUSION

The current study depicts the true to type nature of *in vitro* raised clones of *Celastrus paniculatus*-willd. The TLC based identification of various leaf extracts of both mother plant and *in vitro* propagated plant ensure the presence of rich variety of phytoconstituents. The qualitative chemical similarity is confirmed with the help of HPTLC analysis.

SIGNIFICANCE STATEMENTS

This study ensured the qualitative chemical similarity of *in vitro* raised clones of *Celastrus paniculatus*-willd, because there are many factors like length of culture periods, genotype and nature of explant, which could influence the stability of the *in vitro* cultured plants which is highly significant for further studies. For this leaves were solvent extracted by using petroleum ether, chloroform, ethyl acetate and methanol sequentially. The extracts were subjected to phytochemical screening. The TLC and HPTLC based identification of secondary metabolites were performed in order to ensure the presence of various secondary metabolites.

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