Phytology

A comparative analysis of photosynthetic pigment elution efficiency by different organic solvents of varying concentrations on leaves of *Tecoma stans* (L.) Kunth

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Abstract

Quantitative analysis of photosynthetic pigments using different organic solvents and different concentrations of the same was conducted on leaves of *Tecoma stans*. The organic solvents used were Acetone, Methanol, Chloroform, Ethanol and Butyl alcohol of 40 and 80 percentage concentrations. From the study it was understood that Methanol at 40% was the best eluent of chlorophyll-a, chlorophyll-b, total chlorophylls and carotenoids. Butyl alcohol at 80% was the weakest eluent of chlorophyll-a, chlorophyll-b and total chlorophylls; where as Methanol at 80% was giving minimum elution of carotenoids.

Keywords: eluent, *Tecoma stans*, chlorophyll-a, chlorophyll-b, carotenoids, butyl alcohol

Introduction

Elution is the process of extracting one material from another by washing with a solvent as in washing of loaded ion-exchange resins to remove captured ions. The Eluent or Eluant is the mobile phase and it moves the components through the chromatograph. In liquid chromatography the eluent is the liquid solvent and in gas chromatography it is the carrier gas. The Eluate is the analytic material that emerges from the process and it specifically includes both the analytes and solutes like pigments. Many of the colours associated with higher plants are due to the presence of pigment molecules such as chlorophylls and carotenoids which are the primary major photosynthetic pigments of higher plants. Both types of pigments are bound to proteins in the thylakoids, the photo chemically active photosynthetic bio membranes. According to Lichtenthaler, intact pigments-protein complexes which are held together by weak non-covalent bonds can be isolated from chloroplast and characterized by polyacrylamide gel electrophoresis and isoelectric focusing [1].

The pigments can be released in a protein-free form by grinding plant tissue in a solvent such as acetone, methanol or hexane. Since chlorophyll and the carotenoids are readily soluble in organic solvents, they are biochemically classified as lipids. Total leaf pigments include chlorophyll-a, chlorophyll-b and carotenoids that are necessary for photosynthesis process. The content of foliar pigments varies depending on species. Several studies concerning chlorophyll pigments determination using phytoplankton and other groups of plants have been reported [2-7].

Variation in leaf pigments in different plants can be due to internal factors and environmental conditions. It was reported that chlorophyll and carotenoid content varied with microclimatic conditions in *Adiantum* species [8]. The ratio of chlorophyll-a and chlorophyll-b in terrestrial plants has been used as an indicator of response to light shade conditions [9, 10]. Chlorophyll-a and chlorophyll-b are the two most abundant chlorophylls which occur in a ratio of approximately 3:1. They are essential for the oxygenic conversion of light energy into the stored chemical energy that powers the biosphere [11].

The chlorophyll has a porphyrin ring with a co-ordinated magnesium atom at its centre, a fused five-membraned ring and a C_{20} Phytyl side chain. This non-polar hydrocarbon side chain enhances the solubility of chlorophyll in non-polar solvents. The only difference between chlorophyll a and b is the substituent on position 3 (ring 3) where chlorophyll a has a methyl group and chlorophyll b has an aldehyde functional group. They are the primary photosynthetic pigment in the living plant cell. They absorb light in the blue region (450 nm) and the red region (650-700 nm).

The second group of plant pigments, the carotenoids can be divided into two different types (1) the carotenes which contain only carbon and hydrogen and (2) the xanthophyll which contains carbon, hydrogen and oxygen atoms in the form of hydroxyl or epoxide functional group. Carotenoids, the accessory pigments, assist in photosynthetic light harvest and prevent chlorophyll and thylakoid membrane from the damage of absorbed energy by photo oxidation. Most carotenoids are yellow, red or orange. But some are green, pink, and even black. Many of the bright colours found in flower petals are due to the presence of carotenoids. The colours of fallen foliage are due primarily to preferential destruction of green chlorophylls revealing the carotenoid colour [12].

Chlorophylls and Carotenoids can be isolated from green leaves using organic solvents. Each plant pigment has a unique visible spectrum which can provide a positive identification. Chlorophyll-a and b have absorption maxima in the 600-675 nm range and in the 400-475 nm range respectively. The absorption maximum for each peak is very dependent upon

solvent polarity. Different chlorophylls and carotenoids have a characteristics absorption spectrum absorbing certain wavelength of light more efficiently than the others. The absorbance properties of pigments facilitate the qualitative and quantitative analysis of them [13-16].

There is confusion in choosing the best solvent for effective quantitative extraction of photosynthetic pigments and their spectrophotometric assay. Acetone gives very sharp chlorophyll absorption peaks but is volatile, inflammable, skin irritant and narcotic in high concentration. Besides plastic spectrophotometer cuvettes cannot be used for acetone based chlorophyll assays. Methanol is a very good extractant for chlorophylls, particularly from recalcitrant vascular plant and algae. It is less volatile and flammable than acetone but is notoriously toxic as it is readily absorbed by inhalation and through the skin and hence should not be used in a teaching laboratory. Ethanol is considered as much safer solvent than either acetone or methanol but is not used very often for the assay of chlorophylls although equation for chlorophyll-a and chlorophyll-b are available [17-19]. Diethyl ether is a popular solvent for chlorophylls for research purposes, particularly for preparing pure pigments but is volatile, flammable, explosive and narcotic and explosion hazard in particular restricts its use [20-22]. Butyl alcohol is highily refractive, flammable and colourless liquid with banana odour which burns with a luminous flame. It is miscible with alcohol, ether and many other organic solvents. Chloroform is a clear, colourless, volatile liquid with an ethereal scent that is non-flammable and does not form explosive mixtures at atmospheric temperature and pressure. It is miscible with most organic solvents and is slightly soluble in water. It evaporates quickly and in its concentrated gaseous form, it will tend to settle to the ground before dispersing.

Many studies have been reported on the effect of type of the solvents to extract pigments in different plants. For vegetables crops the best extraction solvents for simultaneous determination of chlorophyll a and b and carotene were methanol and acetone [23]. Spectrometric quantification of chlorophylls and carotenoids from ferns like *Adiantum sp.*, *Crystiella sp. and Dryopteris sp.* using different types of organic solvents were worked out [16]. Quantification of photosynthetic pigments by using Acetone, Methanol and Diethyl ether was done in Bean and Cow pea and its further comparison by using SPAD chlorophyll meter, a convenient, non destructive light weight device used to calculate the amount of chlorophyll present in plant leaves was reported [24]. There are different reports of Photosynthetic pigments quantification in Lettuce (*Lactuca sativa*) and Nettle leaves (*Urtica dioica*) by using methanol and acetone [25]. Further studies using different types of organic solvents on different types of plant species are seldom reported. Hence the present research work has been carried out.

Materials and methods

In this study, *Tecoma stans* (L.) Kunth. was selected as the plant of interest. *Tecoma stans* is a species of flowering perennial shrub in the trumpet vine family, *Bignoniaceae.* It is an attractive plant cultivated as an ornamental and as an avenue tree. It is drought-tolerant and grows well in warm climates. The plant was collected from ponniam region of Kannur District in Kerala, and its identity was confirmed by using standard taxonomic literature.

Healthy and uninfected, medium sized leaves were collected. Fresh leaf samples were washed thoroughly first in tap water and followed by rinsing with distilled water three times in the laboratory. Leaves were taken and water was drained off and remaining droplets were cleaned with blotting paper. Leaves were deveined and petioles were chopped off. Then leaves were minced with a sterilized blade. 500 mg of chopped, fresh leaf samples were weighed in an electronic balance (Setra BL-410S) and transferred to mortar and pestle and ground thoroughly by using 5 ml of selected solvent. The solvent used were 80% and 40% concentrations of Acetone, Methanol, Ethanol, Chloroform and Butyl alcohol. After 15 minutes of grinding, the mixture was transferred to sterilized glass centrifuge tube and centrifuged for a period of 8 minutes at 7000 rpm using the laboratory centrifuge machine (Remi TBA). Later the supernatant green solution was transferred to a measuring cylinder and settled leaf mass was again transferred to mortar and ground for 15 minutes using 5 ml of the selected solvent and the supernatant was collected as in the previous procedure. The process was repeated 5 times by the end of which the leaf mass was turned colourless or brownish white in colour and the collected supernatant was made up to 50 ml by using the respective concentrations of organic solvent. The spectrophotometer (UV/VS Digital spectrophotometer, Model 371, EI) was standardized and later the absorbance or optical density of the supernatant pigment solution was recorded by using Silica cuvettes at wavelength of 661.6, 644.8 and 470 nm. The absorbance readings were recorded for supernatant solutions of all the five solvents with 80% and 40 % concentrations. Recordings were tabulated and the equation by Lichtenthaler (1987) [1] was used to quantify chlorophyll a, chlorophyll b, total chlorophyll and total carotenoids. The equations used for quantification were,

$Ca = 11.24_{A661.6} - 2.04_{A644.8}$

$Cb = 20.13_{A644.8} - 4.19_{A661.6}$

$Ca+b = 7.05_{A661.6} + 18.09_{A644.8}$

$Cx+c = 1000_{A470} - 1.90Ca - 63.14Cb \div 214$

Where,

Ca = Concentration of chlorophyll a in plant extract solution (µg/mL)

Cb = Concentration of chlorophyll b in µg/mL

Ca+b = Concentration of total chlorophyll in µg/mL

Cx+c = Concentration of total carotenoids (xanthophylls and carotenes) in µg/mL

Result and discussion

Concentration of chlorophyll-a, chlorophyll-b, total chlorophylls and total carotenoids showed much variation according to the solvents used and the varying concentrations. Based on the absorbance value, calculations were made using Lichtenthaler (1987) [1] equation and the amount of chlorophyll-a, chlorophyll-b, total chlorophyll and total carotenoids were estimated and tabulated.

Results (Table-1) showed that the organic solvent Chloroform at 80 % showed maximum elution of chlorophyll-a, chlorophyll-b and total chlorophylls in Tecoma leaves. Total carotenoids were maximum when Butyl alcohol at 80 % was used. The minimum elution of Chlorophyll-a, Chlorophyll-b and total Chlorophyll was observed when Butyl alcohol (80 %)

was the extractant and minimum elution of carotenoids was provided by Methanol at 80 %. The elution capacity of five organic solvents at 80 % can be summarized as follows.

Chlorophyll-a: Chloroform > Ethanol >Methanol > Acetone > Butyl alcohol.

Chlorophyll-b: Chloroform >Ethanol > Methanol > Acetone > Butyl alcohol.

Total Chlorophyll: Chloroform >Ethanol >Methanol > Acetone > Butyl alcohol.

Total Carotenoids: Butyl alcohol >Chloroform > Ethanol > Acetone > Methanol.

Results in Table - 2 showed that at 40 % concentrations, best solvent for elution of all photosynthetic pigments was Methanol. The 40 % solvent that showed least elution capacity was Butyl alcohol and Acetone. The Chlorophyll-a and the total Chlorophyll was least eluted by Butyl alcohol at 40 % and Chlorophyll-b and total Carotenoids was least eluted by Acetone at 40 %. The elution capacity of five organic solvents at 40 % can be summarized as follows.

Chlorophyll-a: Methanol > Chloroform > Ethanol > Acetone > Butyl alcohol

Chlorophyll-b: Methanol > Ethanol > Chloroform >Butyl alcohol >Acetone.

Total Chlorophyll: Methanol > Chloroform > Ethanol > Acetone > Butyl alcohol.

Total Carotenoids: Methanol > Chloroform > Butyl alcohol > Ethanol >Acetone.

On analyzing the total results, it was found that Methanol at 40 % was the most promising eluent of photosynthetic pigments. It recorded 2.34 µg/mL of chlorophyll-a, 3.193 µg/ml of chlorophyll-b, 5.53 µg/mL of total chlorophylls and 0.079 µg/mL of total carotenoids which was maximum in all 10 organic eluents. Similarly, Butyl alcohol at 80 % performed as weakest eluent in of Chlorophyll-a (0.319 µg/mL), Chlorophyll-b (0.482 µg/mL) and total chlorophyll (0.799 µg/mL). The solvent that was least eluting carotenoids was Methanol at 80 % which yielded only 0.0017 µg/mL. Overall results of both 80 % and 40 % concentrations of five selected organic solvents can be summarized as follows.

Chlorophyll-a: Methanol (40%) > Chloroform (40%) > Ethanol (40%) > Chloroform (80%) > Acetone (40%) > Ethanol (80%) > Methanol (80%) > Butyl alcohol (40%) > Acetone (80%) > Butyl alcohol (80%)

Chlorophyll-b: Methanol (40%) > Ethanol (40%) > Chloroform (40%) > Butyl alcohol (40%) > Acetone (40%) > Chloroform (80%) > Ethanol (80%) > Methanol (80%) > Acetone (80%) > Butyl alcohol (80%)

Total Chlorophyll: Methanol (40%) > Chloroform (40%) > Ethanol (40%) > Acetone (40%) > Butyl alcohol (40%) > Chloroform (80%) > Ethanol (80%) > Methanol (80%) > Acetone (80%) > Butyl alcohol (80%)

Total Carotenoids: Methanol (40%) > Butyl alcohol (80%) > Chloroform (40%) > Chloroform (80%) > Ethanol (80%) > Acetone (80%) > Butyl alcohol (40%) > Ethanol (40%) > Acetone (40%) > Methanol (80%)

In fact, Lichtenthaler analytical method was designed for photosynthetic pigment quantification in 100 % of acetone as the organic solvent. The present study confirmed that same analytical method can be used even for other organic solvents with varying concentrations. Some organic solvents like Methanol and Chloroform showed profound elution capability than the Acetone. So, use of these organic solvents for the elution and quantification of photosynthetic pigments can be tried in future experimental designs.

Chlorophyll-a is the most prominent photosynthetic pigment that converts light energy into chemical energy during photosynthesis. Chlorophyll-b and carotenoids act as accessory pigments which indirectly help photosynthesis by transferring the light to chlorophyll-a by resonance transfer. The chlorophyll molecule has Mg^{++} at its reaction centre by which it becomes ionic and hydrophilic and a ring that is hydrophobic in nature with a tail which bears a carbonyl group. This carbonyl group makes it polar in nature. It is held in plant cell within water soluble chlorophyll binding protein (WSCP). Chlorophyll-b differ from Chorophyll-a in –CHO functional group which is bound to the porphyrin ring and is soluble than Chlorophyll-a in polar solvents because of its carbonyl group. In present study quantity of Chlorophyll-b eluted is more than Chlorophyll-a, which is due to the presence of carbonyl group bound to the porphyrin ring which makes it more soluble in polar solvents.

The present study indicated that extraction of photosynthetic pigments by different solvents is dependent on chemical nature of pigments [24]. Scientists had observed the Methanol and Acetone extractions giving maximum elution of Chlorophyll-a & b and carotenes in leaves of vegetables like tomato, pepper and cucumber [23]. It is reported that Methanol has the ability to elute total chlorophyll pigments in algae like *Cladophora glomerata* and *Ulva rigita* [26]. The present study was true in the case of Methanol but Acetone produced a negative trend. This may be due to the presence of other bio chemicals of secondary metabolites like phytosterols, alkaloids, quinines, aminoacids, monoterpenes, triterpene, glycosides, phenols, tannins, saponins and flavonoids in the leaf extract of Tecoma. Carotenoids are chemically non-polar in nature and therefore show higher affinity towards polar solvents like Methanol as described by Nayek *et al*. (2014) [16] in selected fern species. In this study the Methanol at 40 % produced maximum carotenoids which supported the findings by Nayek. This study was a useful prelude for further experimental procedures in this field.

Table 1: The average quantity (µg/ml) of Chlorophyll-a, Chlorophyll-b, total Chlorophylls and Carotenoids in Tecoma leaf extracted by different solvents of 80 % concentration

Extractant Solvents (80%)	Leaves of Tecoma stans			
	Ch-a (µg/mL)	Ch-b (µg/mL)	C a+b (µg/mL)	C x+c (µg/mL)
Acetone	0.418	0.677	1.094	0.020
Methanol	0.707	1.034	1.74	.0017
Ethanol	0.79	1.062	1.851	0.030
Butyl alcohol	0.319	0.482	0.799	0.055
Chloroform	0.906	1.309	2.213	0.040

Table 2: The average quantity (µg/ml) of Chlorophyll-a, Chlorophyll-b, total Chlorophylls and Carotenoids in Tecoma leaf extracted by different solvents of 40 % concentration

Extractant Solvents (40%)	Leaves of Tecoma stans			
	Ch-a (µg/mL)	Ch-b (µg/mL)	C-a+b (µg/mL)	C-x+c (µg/mL)
Acetone	0.888	1.49	2.376	0.010
Methanol	2.348	3.193	5.539	0.079
Ethanol	1.172	2.314	3.484	0.012
Butyl alcohol	0.659	1.614	2.271	0.017
Chloroform	1.708	2.249	3.956	0.054

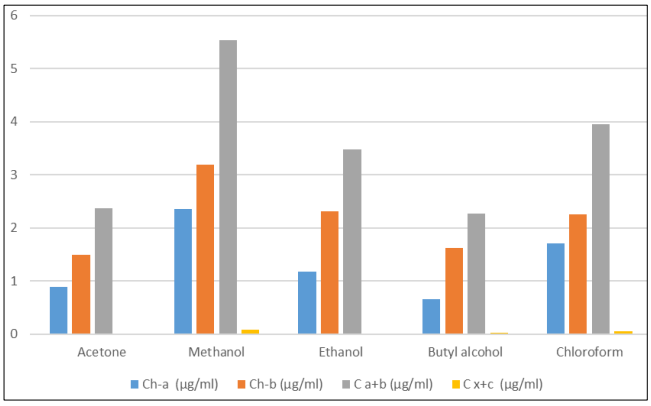

Fig 1: Elution Solvents at 40 % Vs Quantity of Pigments extracted

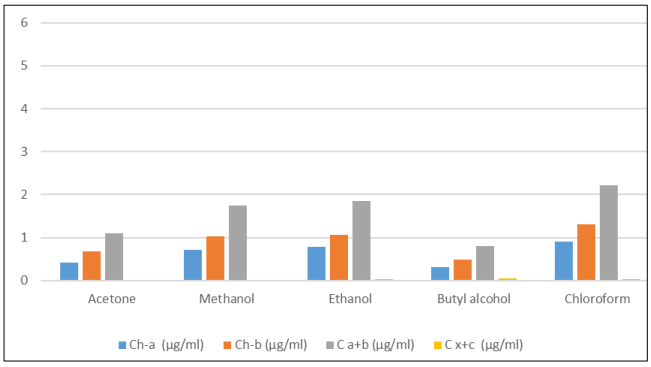

Fig 2: Elution Solvents at 80 % Vs Quantity of Pigments extracted

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