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Comparative Spectrophotometric Analysis of Photosynthetic Pigments in Plants Using Different Solvents

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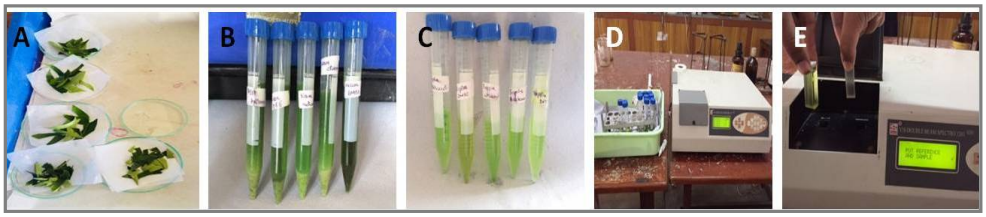
ABSTRACT

Chlorophyll-a is recognized as the main pigment which converts light energy into chemical energy. Chlorophyll-b is an accessory pigment which acts indirectly in photosynthesis by transferring the light it absorbs. The extraction of photosynthetic pigments (chlorophyll-a, chlorophyll-b) by different solvents depends on chemical nature of bio-molecules. For this purpose, Azadirachta indica (neem) and Manilkara zapota (sapota) leaves were selected and analysed for the determination of chlorophylls (Chl-a and Chl-b). Investigation reveals that ethanol is an optimum extractant for both chlorophyll a and b in the plants under study. The solvents DEE and acetone also performed well as good extractants of chlorophylls, while methanol and DMSO have extracted chlorophylls in least concentrations. Spectrophotometers are revolutionizing farming and extraction techniques. Portability, durability, and rapid speed of evaluation are all valuable characteristics of how color technology is making its mark in this field. Spectrophotometric analysis is important as it can help in further investigations regarding different photosynthetic pigments which play a significant role in plant metabolism.

Graphical Abstract



Extraction of chlorophylls from leaves (A) collection and weighing of leaf samples, (B) homogenization in extraction solvents, (C) supernatant extraction in different solvents, (D) and (E) spectrophotometric quantitation of chlorophyll a and chlorophyll b.

Keywords: Solvent extraction, *Azadirachta indica*, *Manilkara zapota*, Spectrophotometric analysis, Chlorophylls.

INTRODUCTION

Photosynthetic pigments are present in most plants, algae and cyanobacteria. They are present in the form of porphyrin pigments (chlorophyll *a*, *b* and *c*), carotenoids, anthocyanins and flavones [**1-3**]. The total leaf pigment includes chlorophyll *a*, chlorophyll *b* and carotenoids that are necessary for photosynthesis process. Leaf chlorophyll concentration is an important parameter that is regularly measured as an indicator of chloroplast content, photosynthetic mechanism and of plant metabolism. Chlorophyll is an antioxidant compounds which are present and stored in the chloroplast of green leaf plants and mainly it is present in the green area of leaves, stems, flowers and roots. The chlorophyll production is mainly depended on penetration of sun light and it is the main source of energy for plant. Chlorophyll *a* and Chlorophyll *b* are essential pigments of the plant photosystems [**3**]. Chlorophyll a is the primary photosynthetic pigment in plants which helps to produce energy in plant, it is believed that concentration of chlorophyll *a* is higher than chlorophyll b [**4**]. The chlorophyll content has medicinal qualities, and also plays important role in plant physiology and it can act as nutrition in decline blood sugar conditions, detoxification, digestion, excretion and decreasing allergens [**4**].

The spectrophotometric definition of photosynthetic pigments that cause light energy to turn into chemical energy in all photosynthetic organisms was first determined by Stokes in 1864 [**5**]. The absorbance properties of pigments facilitate the qualitative and quantitative analysis of them. There is a trade-off between choosing the best solvent for efficient quantitative extraction of chlorophylls and use of a solvent best suited for spectrophotometric assay. In the laboratory it is determined by using pestle and mortar to extract the pigments using an organic solvent such as acetone or dimethyl formamide [**6**]. But by using modern technique like satellite remote sensing technology leaf chlorophyll concentration can also be measured [**7**]. Variation in leaf chlorophyll content can provide information about the physiological condition of a leaf or plant.

Five solvents have been found prospective for estimating chlorophylls viz, Acetone, Methanol, Ethanol, Diethyl ether (DEE) and Dimethyl sulphoxide (DMSO) [**8**]. Although volatile and highly inflammable, acetone gives very sharp chlorophyll absorption peaks and has great merit as the solvent for assay of chlorophylls. Methanol is a very good extractant for chlorophylls, particularly from recalcitrant vascular plant. It is less volatile and flammable than acetone but is notoriously toxic. Ethanol is considered as much safer solvent than either acetone or methanol. There are considerable practical, safety and economic advantages in using ethanol as the solvent for chlorophyll extract and assay. Diethyl ether (DEE) is a very popular solvent for chlorophylls for research purposes, particularly for preparing pure pigments [**8**]. Many of the diagnostic spectra of chlorophyll pigments are for diethyl ether as a solvent. Except for freeze dried material, it cannot be directly used as a chlorophyll extractant because it is not miscible in water. The merits of dimethyl sulphoxide (DMSO) used for chlorophyll extraction and assay, and reported as efficient when pigments concentrations are low [**9, 10**]. The present study compares the use of five different solvents viz. acetone, methanol, ethanol, Diethyl ether and dimethyl sulphoxide (DMSO) for determining extraction capabilities of chlorophyll *a* and chlorophyll *b* in neem and sapota leaves.

MATERIALS AND METHODS

Collection of plant samples: In this study, we selected *Azadirachta indica* (neem) and *Manilka razapota* (sapota) plants for experiment. Leaves from the shoot tips of both the healthy plants were collected. Fresh leaf samples were washed thoroughly first in tap water followed by distilled water in the laboratory, kept to dry in room temperature and analyzed for the determination of chlorophylls (Chl *a* and Chl *b*) content.

Analytical procedure: About 0.5g of fresh plant leaf samples were weighed, and homogenized with 10 mL of five different extractant solvents, viz. Acetone, Methanol, Ethanol, Diethyl ether (DEE) and

Dimethyl sulphoxide (DMSO). Homogenized sample mixture was centrifuged for 10,000 rpm for 15 min. The supernatant was separated and 0.5 mL of it was mixed with 4.5 mL of the respective different solvents. The solution mixture was analyzed for Chlorophyll *a* and Chlorophyll *b* content on spectrophotometer (Perkin) (Figure 1). The equation used for the quantification of Chlorophyll *a* and Chlorophyll *b*, by different extractant solvents are given below.

Calculation: Chlorophyll *a* content= (Abs 663nm ×12.7) - (Abs 645nm ×2.69) × (V×W)/1000
Chlorophyll *b* content= (Abs 645×22.9) - (Abs 663×4.68) × (V×W)/1000

Quality control: Every procedure (for each plant sample and extracting solvent) was triplicated for maintaining the precision of analytical results.

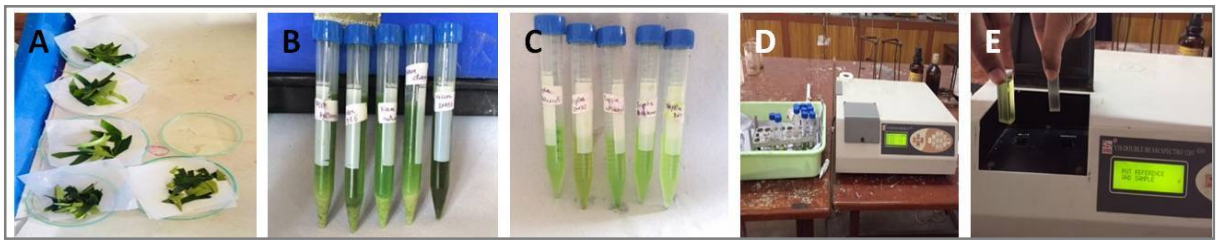


Figure 1. Extraction of chlorophylls from leaves (A) collection and weighing of leaf samples, (B) homogenization in extraction solvents, (C) supernatant extraction in different solvents, (D) and (E) spectrophotometric quantitation of chlorophyll a and chlorophyll b.

RESULTS AND DISCUSSION

The chlorophylls, Chl *a* and Chl *b*, are the most important photosynthetic pigments, and are thus virtually essential for the oxygenic conversion of light energy to the stored chemical energy that powers the biosphere. From a physiological perspective, leaf chlorophyll content is therefore a parameter of significant interest in its own right. Traditionally, wet chemical methods have required chlorophyll extraction in a solvent, followed by the spectrophotometric determination of absorbance by the chlorophyll solution, and conversion from absorbance to concentration using standard published equations [6] and modifications thereof. The absorbance maximum for chlorophyll *a* was determined to be 663 nm and for chlorophyll *b*, 646 nm (Figure 2). The absorbance values are tabulated in tables 1 and 2 for neem and sapota respectively.

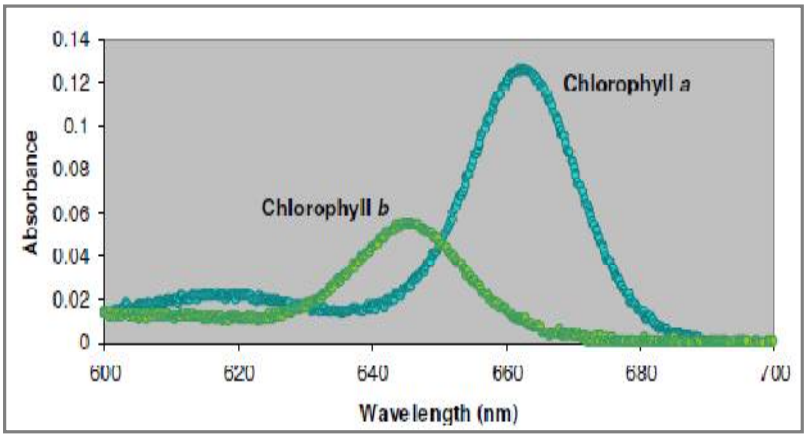


Figure 2. Absorbance spectra of chlorophyll a and b.

It was determined that the solvents used were important in the pigment extraction. Highest and stable extraction of chlorophylls (Chl *a* and Chl *b*) is noted by using ethanol. In neem, methanol has shown a

higher peak for extraction of chlorophyll b and DMSO has proven to be a good extractant of chlorophyll a. As both the graphs show different rates of extraction, we can consider ethanol to be a stable solvent for extraction of chlorophyll components. In sapota, although solvents like DEE and

Table 1. Spectrophotometric analysis of chlorophyll a and chlorophyll b in *Azadirachta indica* using various chemical solvents with respect to different time duration

Solvent	Absorbance at 663 nm (24h)	Absorbance at 646 nm (24h)	Absorbance at 663 nm (48h)	Absorbance at 646 nm (48h)
Acetone	1.076	0.897	0.660	0.372
DMSO	0.732	0.533	0.152	0.004
Methanol	0.441	0.244	0.543	0.349
Diethyl Ether	0.548	0.389	0.272	0.052
Ethanol	0.993	0.831	0.510	0.344

(The analysis was done in Systronics, Vis Double Beam Spectro 1203.The samples were kept at 4ºC for incubation. Triplicate results were obtained to maintain precision. Average values are tabulated)

Table 2. Spectrophotometric analysis of chlorophyll a and chlorophyll b in *Manilkara zapota* using various chemical solvents with respect to different time duration

Solvent	Absorbance at 663nm(24h)	Absorbance at 646nm(24h)	Absorbance at 663nm(48h)	Absorbance at 646nm(48h)
Acetone	1.268	0.911	0.833	0.458
DMSO	0.720	0.454	0.601	0.305
Methanol	0.757	0.467	0.779	0.457
Diethyl Ether	0.791	0.479	1.257	0.918
Ethanol	1.389	0.992	0.783	0.393

(The analysis was done in Systronics Vis Double Beam Spectro 1203. The samples were kept at 4ºC for incubation. Triplicate results were obtained to maintain precision. Average values are tabulated)

acetone have shown good extraction abilities, ethanol can still be considered as a better solvent for extraction of chlorophyll a and b. The chlorophyll extractions of the two plant leaves under study by using different solvents are in the sequence of–**Neem-*Chlorophyll-a***: DMSO > Methanol > Ethanol > DEE > Acetone. ***Chlorophyll-b***: Acetone > Methanol > Ethanol > DEE > DMSO. **Sapota–*Chlorophyll-a***: Ethanol >Acetone > DEE >Methanol>DMSO. ***Chlorophyll-b:*** Ethanol > DEE > Acetone > Methanol > DMSO.

Our studies show variable results as compared to a similar studies, wherein acetone, chloroform, diethyl ether, dimethyl formamide and methanol were used with high plant leaves, and it was determined that the extraction rate was various in every solvent [**11**]. Several solvents, such as acetone, N,N-dimethylformamide (DMF), methanol, ethanol, and dimethylsulfoxide (DMSO) have been used to extract chlorophyll pigments from a variety of plant tissues [**3, 12-17**]. DMSO, which has also been recommended by others [**18**], has the advantage of being faster and more stable over other extractions (e.g. methanol, ethanol, or acetone). It was reported that DMSO extracts are stable for up to 5 days, whereas with acetone extracts the measured level of chlorophylls begins to fall off immediately [**19**].

Chlorophyll extraction capabilities of solvents have been reported to be very much time dependent [**8**]. In neem, the highest extractions were observed in methanol and ethanol. Results also indicate the variation in 24 h and 48 h to be contrastingly different with acetone and DMSO solvents. In sapota, highest extraction of chlorophylls (Chlorophyll *a* and *b*) is observed in ethanol. The solvents DEE and acetone also performed well as good extractants of chlorophylls. Methanol and DMSO have extracted chlorophyll (*a* and *b*) in least concentrations. In neem, methanol and ethanol have shown considerably

higher peaks as compared to other solvents; while in sapota ethanol has proved to be the best extractant for chlorophylls. The average concentrations of chlorophylls are shown in figures 3 and 4.

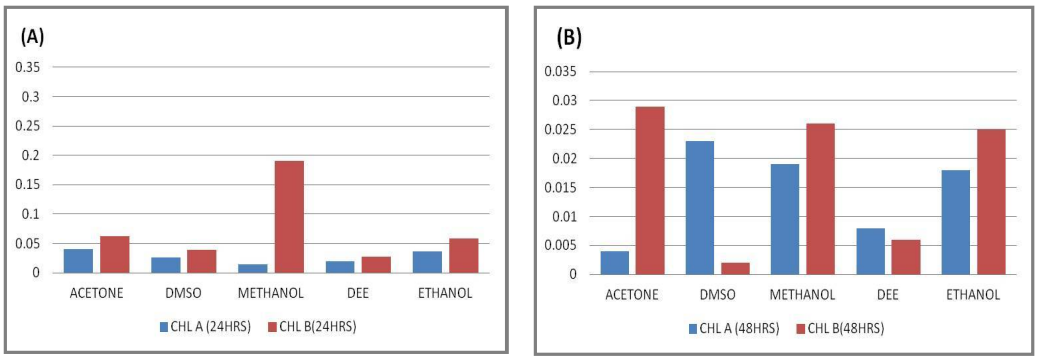


Figure 3. The average concentrations of chlorophyll *a* and chlorophyll *b* in *Azadirachta indica* (neem) (A) after 24 h and (B) after 48 h.

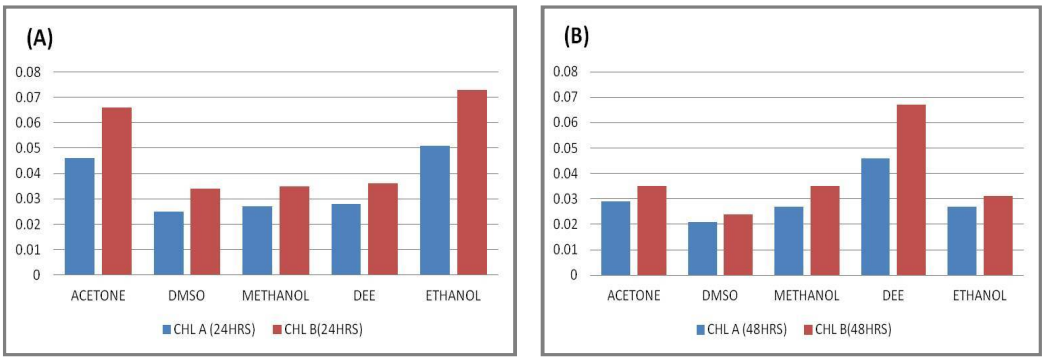


Figure 4. The average concentrations of chlorophyll *a* and chlorophyll *b* in *Manilkara zapota* (sapota) (A) after 24 h and (B) after 48 h.

The variations in chlorophyll extractions may be attributed to various reasons. First, the amount of solar radiation absorbed by a leaf is largely a function of the foliar concentrations of photosynthetic pigments, and therefore low concentrations of chlorophyll can directly limit photosynthetic potential and hence primary production [**20, 21**]. Second, much of leaf nitrogen is incorporated in chlorophyll, so quantifying Chl content gives an indirect measure of nutrient status [**21, 22**]. Third, pigmentation can be directly related to stress physiology, as concentrations of carotenoids increase and chlorophylls generally decrease under stress and during senescence [**23**]. Fourth, the relative concentrations of pigments are known to change with abiotic factors such as light (e.g. sun leaves have a higher Chl *a*: Chl *b* ratio; [**24**] and so quantifying these proportions can provide important information about relationships between plants and their environment.

APPLICATION

Chlorophyll is a very important macromolecule which indicates performance of photosynthesis and energy utilization rate in plants. Chlorophyll estimation is done to know the content of different types of chlorophyll present in the leaf. Each component of chlorophyll (*a*, *b*, carotenoids) is important in maintaining the physiological conditions in plants and helps in various processes in plants like detoxification, digestion, excretion and metabolism. It bears antioxidant properties which can be used in medicinal drug discovery. Therefore, extraction of chlorophyll helps in studying its important properties and can be used in enhancement of plant-derived pharmaceuticals.

CONCLUSION

Our study is indicative of the fact that the extraction of chlorophylls from leaves using different solvents totally depends on the chemical nature of the photosynthetic pigments (chlorophyll *a* and chlorophyll *b*). The calculated chlorophyll levels reveal that ethanol serves as a stable and better extraction solvent for chlorophyll *a* and chlorophyll *b* in both the plant species, while DEE and acetone also performed fairly well as extraction solvents. With time duration, more of the chlorophylls were seen to be extracted after 48 h than after 24 h in neem, while in sapota contrastingly, declining levels of chlorophylls were recorded at 48 h using all solvents except DEE. Slight variations may persist among the experimented plants due to various factors like inherent physiological characteristics, temporal and seasonal changes etc. due to which there might be variations in pigment concentrations in plants. However, further studies are recommended to ascertain the importance of solvents in pigment extractions.

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