Research Journal of Chemical Sciences _____ ISSN 2231-606X
Vol. **4(9),** 63-69, September **(2014)** *Res. J. Chem. Sci.*

Spectrophotometric Analysis of Chlorophylls and Carotenoids from Commonly Grown Fern Species by Using Various Extracting Solvents

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Available online at: www.isca.in, www.isca.me
Received 29th August 2014, revised 15th September 2014, accepted 18th September 2014

Abstract

Present investigation is performed on the comparative extraction of photosynthetic pigments (chlorophyll-a, chlorophyll-b and carotenoids) by using solvents of different chemical nature. The study is also concern on the extraction ratio of biomolecules with respect to time duration/variation. Different trend is observed in extraction rate for chlorophylls and carotenoids. Highest extraction of chlorophylls (Ch-a and Ch-b) is noted for DEE (except chlorophyll b in Adiantum sp.). Whereas maximum extraction of caroteniods is performed by acetone in Adiantum sp., and for Crystiella sp. and Drypteris sp DMSO execute best extraction of carotenoids. Significant variations ($p<0.01$) in pigment concentrations are also noted for sampled species exposed to different chemical extractant solvents for different time duration.

Keywords: Solvent extraction, chlorophylls, carotenoids, fern species, spectrophotometric analysis.

Introduction

Photosynthetic Pigments are the substances with very different chemical structure; they are present in the form of porphyrin pigments (chlorophyll a, b and c), carotenoids, anthocyanins and flavones[1-3]. Total leaf pigment includes chlorophyll-a, chlorophyll-b and carotenoids that are necessary for photosynthesis process. The content of foliar pigments varies depending on species. Variation in leaf pigments (chlorophylls and carotenoids) and its relation can be due to internal factors and environmental conditions. Shaikh and Dongare[4] reported that chlorophyll and carotenoids content varied with microclimatic conditions in *Adiantum* species. The ratio of chlorophyll-a and chlorophyll-b in terrestrial plants has been used as an indicator of response to light shade conditions[5,6]. The small proportion of chlorophyll a/b is considered as sensitive biomarker of pollution and environmental stress[7]. The absorbance properties of pigments facilitate the qualitative and quantitative analysis of them[8]. 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.

Acetone gives very sharp chlorophyll absorption peaks and has great merit as the solvent for assay of chlorophylls[9]. But acetone is not the ideal solvent for extraction; and sometimes a poor extractant of chlorophyll from many vascular plants and some algae such as *Scenedesmus*, *Chlorella* and *Nannochloris*[10,11]. Acetone is volatile, highly inflammable, is narcotic in high concentrations and is a skin irritant (erythema). Acetone attacks polystyrene and polymethylacrylates (PMMA) and therefore, 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[5,12,13]. It is less volatile and flammable than acetone but is notoriously toxic. It is an insidious poison because it is readily absorbed by inhalation and through the skin and so should not be used in a teaching laboratory. Methanol slowly fogs polystyrene spectrophotometer cuvettes leading to false readings and cannot be used at all with polystyrene and polymethylacrylates (PMMA) cuvettes. Ethanol is considered as much safer solvent than either acetone or methanol but is not used very often for the assay of chlorophylls although equations for chlorophyll-a and chlorophyll-b are available[14-16]. Although flammable it is not very toxic and is suitable for use in a teaching laboratory. Ethanol does not attack polystyrene and so polystyrene plastic spectrophotometer cuvettes can be used. 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[5,12,17]. Many of the diagnostic spectra of chlorophyll pigments are for diethyl ether as solvent[15]. Except for freeze dried material, it cannot be directly used as a chlorophyll extractant because it is not miscible in water. It is not a solvent of choice for routine and class laboratory work because it is extremely volatile, flammable, explosive and narcotic. The explosion hazard in particular restricts its use. Ether also attacks plastic cuvettes and most plastic laboratory ware.

Porra et al.[13] and Wright et al.[15] have discussed the merits of dimethyl sulphoxide (DMSO) used for chlorophyll extraction and assay, and reported as efficient when pigments concentrations are low. Traditional methods for analysis of photosynthetic pigments employed spectroscopy and extinction coefficient, and have been calculated for a range of solvents[9,18-20]. But most of these studies concerning the higher plants (angiosperms) or phytoplankton (algae). 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, chlorophyll-b and carotenoids form fern leaves.

Material and Methods

Collection of plant samples: In this study, we select three commonly grown fern species of low altitude (viz. *Adiantum* sp., *Crystiella* sp. and *Draypteris* sp.) for experimental purpose. These species are mostly preferred to grow in moist condition under the shade in plane land areas. Healthy and uninfected fern species were collected at their stage of maturity; and care was also taken during sampling of fern leaves/fonds to avoid mechanical injuries. Fresh leaf samples were wash thoroughly first in tap water followed by distilled water in the laboratory, kept to dry in room temperature (18^0C) and analyzed for the determination of chlorophylls (Ch-a and Ch-b) and carotenoids content.

Analytical procedure: Accurately weighted 0.5g of fresh plant leaf sample was taken, and homogenized in tissue homogenizer with 10 ml of different extractant solvent. Homoginized sample mixture was centrifuge for 10,000 rpm for 15min at 4^0C. The supernatant were separated and 0.5ml of it is mixed with 4.5ml of the respective solvent. The solution mixture was analyzed for Chlorophyll-a, Chlorophyll-b and carotenoids content in spectrophotometer (Parkin). The equation used for the quantification of Chlorophyll-a, Chlorophyll-b, and carteniods by different extractant solvents are given in table 1; and spectral absorbance for Chlorophyll-a, Chlorophyll-b, and carteniods for various solvents are represented in table 2.

Table-1
Equations to determine concentrations (µg/ml) of chlorophyll a (Ch-a), chlorophyll b (Ch-b) and total carotenoids (C x+c) by different extractant solvents in spectrophotometer [12,14,18]

Solvents	Equations/Formula
80% Acetone	Ch-a=$12.25A_{663.2} - 279A_{646.8}$ Ch-b=$21.5A_{646.8} - 5.1A_{663.2}$ C x+c=$(1000A_{470} - 1.82C_a - 85.02C_b)/198$
95% Ethanol	Ch-a=$13.36A_{664} - 5.19\ A_{649}$ Ch-b=$27.43A_{649} - 8.12\ A_{664}$ C x+c=$(1000A_{470} - 2.13C_a - 97.63C_b)/209$
Diethyl-ether (DEE)	Ch-a=$10.05A_{660.6} - 0.97A_{642.2}$ Ch-b=$16.36A_{642.2} - 2.43A_{660.6}$ C x+c=$(1000A_{470} - 1.43C_a - 35.87C_b)/205$
Dimethyl-sulphoxide (DMSO)	Ch-a=$12.47A_{665.1} - 3.62A_{649.1}$ Ch-b=$25.06A_{649.1} - 6.5A_{665.1}$ C x+c=$(1000A_{480} - 1.29C_a - 53.78C_b)/220$
Methanol	Ch-a=$16.72A_{665.2} - 9.16A_{652.4}$ Ch-b=$34.09A_{652.4} - 15.28A_{665.2}$ C x+c=$(1000A_{470} - 1.63C_a - 104.96C_b)/221$

A = Absorbance, Ch-a = Chlorophyll a, Ch-b = Chlorophyll b, C x+c = Carotenoids

Table-2
Spectrophotometric determination of absorbance for Chlorophyll a, Chlorophyll b and Carotenoids with various extracting solvents

ExtractantSolvent	*Adiantum sp.*			*Crystiella sp.*			*Draypteris sp.*		
	A_{663nm} Ch-a	A_{645nm} Ch-b	A_{470nm} C x+c	A_{663nm} Ch-a	A_{645nm} Ch-b	A_{470nm} C x+c	A_{663nm} Ch- a	A_{645nm} Ch-b	A_{470nm} C x+c
Acetone	0.338	0.135	0.326	0.211	0.085	0.309	0.429	0.177	0.438
Methanol	0.242	0.107	0.317	0.261	0.107	0.317	0.359	0.146	0.466
Ethanol	0.286	0.117	0.331	0.430	0.142	0.487	0.406	0.126	0.429
DEE	0.228	0.1.69	0.179	0.312	0.171	0.239	0.397	0.161	0.330
DMSO	0.251	0.065	0.236	0.251	0.103	0.273	0.294	0.114	0.343

A = Absorbance, Ch-a = Chlorophyll a, Ch-b = Chlorophyll b, C x+c = Carotenoids

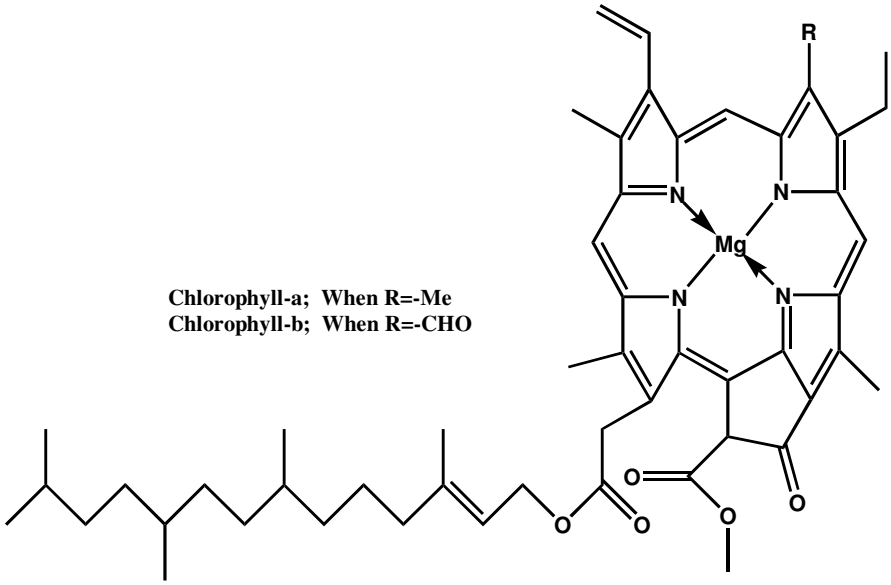
Quality control: Analytical reagents used during the extraction process were of AR grade (Marck). Milli Q water was used for preparation of intermediate solution and for dilution purpose (wherever needed). Quartz cuvette ($1cm^2$) was used and corresponding solvent was taken as reference during spectrophotometric observation. Every procedure (for each plant sample and extracting solvent) was triplicated for maintaining the precision of analytical results.

Statistical interpretations: Analysis of variance (Two way ANOVA) has been performed for sampled plant species to analyze the significance of enhancement of decline in pigment concentrations (chlorophyll a and b, and carotenoids) among themselves, and with respect to time duration (24hr, 48hr and 72hr).

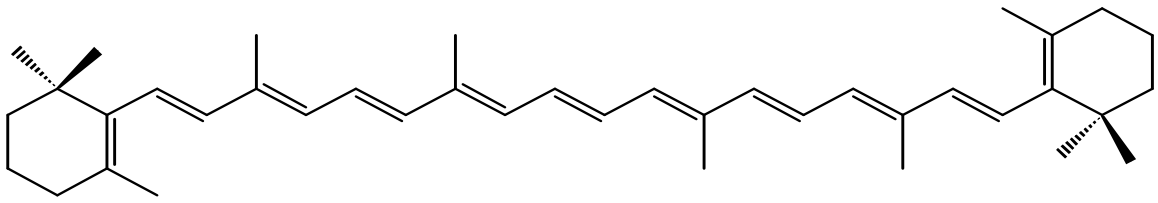
Results and Discussion

Chlorophyll-a is recognized as the main pigments which convert light energy into chemical energy. Chlorophyll-b as accessory pigments acts indirectly in photosynthesis by transferring the light it absorbs to chlorophyll-a[3]. The chlorophyll molecule has Mg^{2+} at its center which makes it ionic and hydrophilic, and a ring that is hydrophobic in nature with a carbonyl group at its tail which makes it polar. It is held in place in the plant cell within a water-soluble chlorophyll-binding protein (WSCP). Chlorophyll-b differ from chlorophyll-a only in one functional group (i.e -CHO) bounded to the porphyrin ring, and is more soluble than chlorophyll-a in polar solvents because of its carbonyl group[18].

Highest extraction of chlorophylls (Chlorophyll a and b) is noted by using DEE (except chlorophyll b in *Adiantum* sp.). This observation can be supported and explained by the fact that chlorophyll molecule is polar in nature, and therefore more soluble in non polar solvent like DEE. Results also indicate methanol as good extractant of chlorophylls after DEE. For the studied species, lowest extraction is observed mostly for ethanol, whereas chlorophyll-a in *Crystiella* sp. and chlorophyll-b in *Adiantum* sp. exhibit their least concentrations for acetone (figure-1 and figure-2).



Scheme-1
Chemdraw structure of Chlorophyll-a and Chlorophyll-b



Scheme-2
Chemdraw structure of Carotenoids

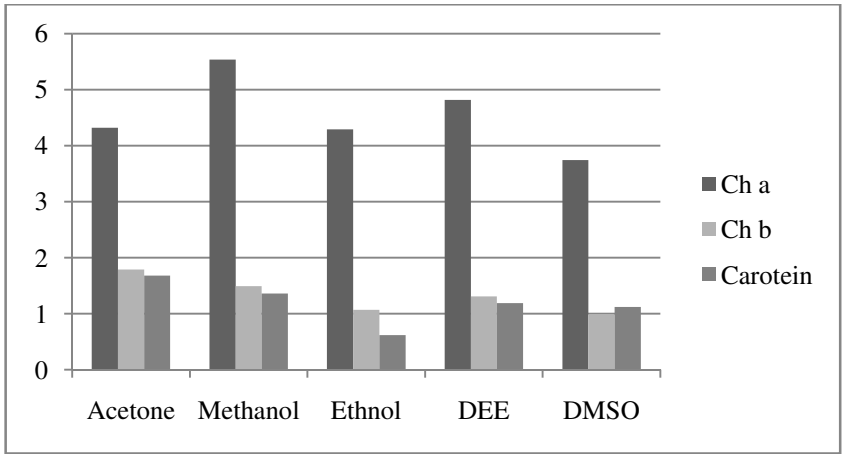


Figure-1
The average concentrations (µg/ ml) of Chlorophyll a, Chlorophyll b and Carotenoids in *Adiantum* Species

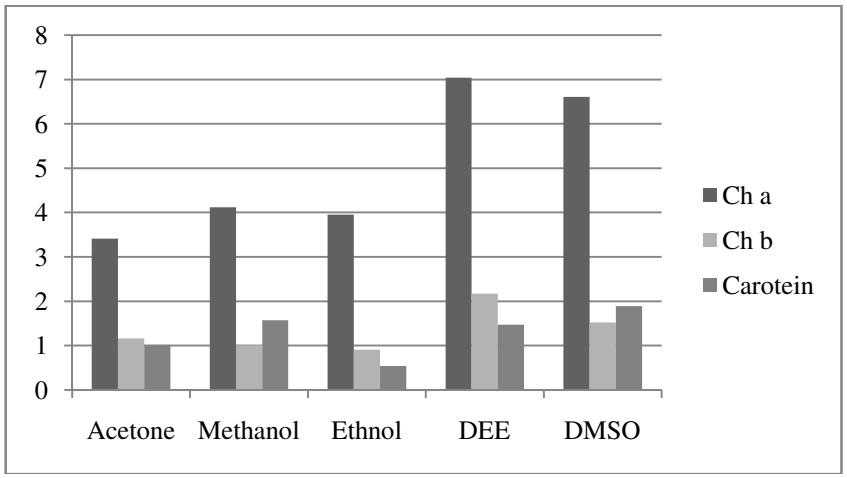


Figure-2
The average concentrations (µg/ ml) of Chlorophyll a, Chlorophyll b and Carotenoids in *Crystiella* species

The extractions of chlorophylls by different solvent for examined species are in the sequence of - *Adiantum* sp.– **Chlorophyll-a**: Methanol > DEE > Acetone > Ethanol > DMSO. **Chlorophyll-b**: DEE > Acetone > Methanol > Ethanol > DMSO. *Crystiella* sp.– **Chlorophyll-a**: DEE > DMSO > Methanol > Ethanol > Acetone. **Chlorophyll-b**: DEE > DMSO > Acetone > Ethanol > Methanol. *Drypteris* sp.– **Chlorophyll-a**: Methanol > DEE > DMSO > Acetone > Ethanol. **Chlorophyll-b**: DEE > Methanol > Acetone > DMSO > Ethanol

Chlorophyll extraction capabilities of solvents are very much time dependent. The observation reveals rapid extraction of chlorophylls by acetone showing sharp peaks for chlorophyll-a and chlorophyll-b on 24hr, and gradual decrease in chlorophyll content on 48hr followed by 72hr results. For *Crystiella* sp., no variation in concentrations of chlorophyll-b is noted for 24hr and 48hr duration, although sharp decline is observed at 72hr. Methanol and ethanol shows maximum extractions of chlorophylls (a and b) after 48hr, and decreases at 72hr duration; and decline is more in case of chlorophylls in comparison to carotenoids. DEE and DMSO execute slow extraction of chlorophylls and carotenoids at initial stage (24hr), gradual increase after 48hr and highest extraction of chlorophylls after 72hr duration (table 3).

Carotenoids are located in chromoplast, contribution colour to vegetables/fruits; and also in chlorophylls, where together with chlorophylls involved in the two photosystems. Carotenoids group and their derivatives consist of about 70 compounds that are present in most vegetables and fruits[3]. Vechetel and Ruppel reported that carotene pigments were the most important photosynthetic pigments, and they prevented chlorophyll and thylakoid membrane from the damage of absorbed energy by peroxidation[21]. Carotenoids extraction by various solvents from studied species represents very much similar trend as noted in case of chlorophylls. Rapid extraction is observed for acetone (highest value at 24hr), followed by methanol and ethanol (maximum extraction after 48hr); while DEE and DMSO exhibits peak absorption at 72hr duration (table 3).

Table-3
Quantification of Chlorophyll a, Chlorophyll b and Carotenoids (µg/ ml) in various chemical solvents with respect to different time duration

Plants	*Adiantum species*			*Crystiella species*			*Drypteris species*		
Time	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Acetone extraction									
Ch-a	4.63 ±1.06	4.40 ±0.26	3.95 ±0.09	3.60 ±0.80	3.02 ±0.31	3.61 ±0.75	5.68 ±0.49	5.41 ±0.96	5.04 ±0.65
Ch-b	1.92 ±1.26	1.90 ±0.76	1.55 ±0.14	0.92 ±0.38	1.21 ±0.19	1.36 ±0.37	1.38 ±0.22	1.48 ±0.35	1.48 ±0.12
C x+c	1.75 ±0.86	1.60 ±0.70	1.70 ±1.05	1.33 ±0.69	0.70 ±0.09	1.03 ±0.30	1.80 ±0.21	1.66 ±0.55	1.37 ±0.04
Methanol extraction									
Ch-a	5.07 ±1.02	6.64 ±1.72	4.90 ±1.87	2.06 ±1.73	4.16 ±2.93	4.13 ±0.91	7.56 ±5.23	6.09 ±0.38	10.36 ±0.36
Ch-b	2.04 ±0.67	1.51 ±1.48	0.94 ±1.38	1.23 ±0.26	0.96 ±0.27	0.83 ±0.39	2.24 ±2.11	2.99 ±0.64	2.77 ±0.13
C x+c	0.93 ±0.26	1.59 ±1.17	1.56 ±0.83	0.99 ±0.05	2.28 ±0.12	1.45 ±0.18	2.23 ±0.06	1.65 ±1.43	1.99 ±1.39
Ethanol extraction									
Ch-a	4.24 ±0.69	3.99 ±0.44	4.65 ±0.07	4.17 ±0.38	4.20 ±0.12	3.48 ±0.09	6.27 ±0.72	5.05 ±1.45	4.87 ±0.19
Ch-b	0.89 ±0.51	1.02 ±0.24	1.29 ±0.16	0.87 ±0.14	1.13 ±0.16	0.73 ±0.42	1.47 ±0.42	1.10 ±0.37	0.93 ±0.09
C x+c	0.61 ±0.09	0.54 ±0.07	0.71 ±0.08	0.57 ±0.14	0.55 ±0.04	0.49 ±0.05	0.94 ±0.11	0.67 ±0.20	0.67 ±0.05
DEE extraction									
Ch-a	3.85 ±0.20	3.98 ±1.53	6.64 ±1.03	4.86 ±4.20	5.68 ±4.70	10.58 ±0.79	4.59 ±3.59	8.06 ±0.31	10.72 ±2.09
Ch-b	1.30 ±0.28	1.50 ±0.43	2.64 ±1.00	1.55 ±1.47	2.04 ±1.61	6.92 ±4.49	1.41 ±1.17	2.83 ±0.21	6.97 ±4.88
C x+c	1.08 ±0.06	1.17 ±1.91	1.33 ±0.04	1.32 ±1.14	1.53 ±1.30	1.56 ±1.50	1.44 ±1.10	1.82 ±0.09	2.31 ±0.11
DMSO extraction									
Ch-a	2.82 ±0.50	3.45 ±0.38	4.94 ±1.44	6.41 ±2.84	6.43 ±3.49	6.98 ±4.38	5.85 ±0.18	5.93 ±0.27	6.46 ±0.09
Ch-b	0.72 ±0.26	1.15 ±0.03	1.10 ±0.03	1.31 ±1.07	1.64 ±1.46	1.61 ±1.06	1.08 ±0.15	1.16 ±0.43	1.65 ±0.20
C x+c	1.06 ±0.16	1.21 ±0.01	1.38 ±0.19	1.84 ±0.46	1.87 ±0.72	1.97 ±0.97	1.93 ±0.06	1.92 ±0.17	2.36 ±0.11

Ch-a = Chlorophyll a, Ch-b = Chlorophyll b, C x+c = Carotenoids

The carotenoids extraction by various solvents in examined species following the order of –
Adiantum sp.: Acetone > Methanol > DEE > DMSO > Ethanol
Crystiella sp.: DMSO > Methanol > DEE > Acetone > Ethanol
Drypteris sp.: DMSO > Methanol > DEE > Acetone > Ethanol

Caroteniods are non polar in chemical nature, and therefore shows their higher affinity towards polar solvents (viz., methanol and acetone) which is well documented by several earlier findings[6, 8,14]. Similar observation has also been noted for *Adiantum* sp. showing highest extraction of caroteniods by acetone and then methanol; while for the remaining species (i.e *Crystiella* sp. and *Drypteris* sp.) DMSO executes maximum extraction of carotenoids followed by methanol (figure-3).

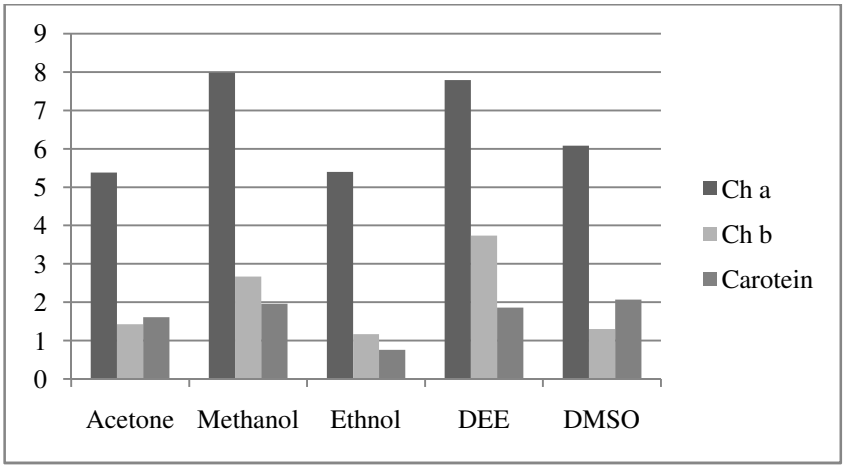


Figure-3
The average concentrations (µg/ ml) of Chlorophyll a, Chlorophyll b and Carotenoid in *Drypteris* species

The experimental results shows positive trend of increased pigment concentrations with time duration (except acetone and methanol). For most of the studied species significant enhancement in pigment concentration ($p<0.01$) is observed in between 48-72 hr duration, while for acetone and methanol significant decline ($p<0.01$) in chlorophyll content is noted during that time duration. Variation in pigment concentrations are significantly evident ($p<0.1$) for different solvent for every studied species, which can be explained by difference in solubility of pigments (or affinity of bio-molecules) towards various chemical solvents. Variation in chlorophyll (Ch-a and Ch-b) concentrations for *Crystiella* sp. and *Drypteris* sp. are noted within the significance level of $p<0.01$.

Conclusion

Results from this experiment clearly indicate that extraction of photosynthetic pigments by different solvents are depends on chemical nature of bio-molecules (cholorophyll-a, chlorophyll-b and carotenoids). Investigation reveals DEE as best extractant solvent for chlorophyll-a and chlorophyll-b for most of sampled species; while DMSO is recognized for the highest extractions of caroteniods form experimented fern species. No significant differences observed in the trend for pigment extraction as reported earlier form phytoplankton and also from higher plants with the present study. Though slight variations persists among the experimented plants/species even for same extractant solvent which can be attribute to inherent physiological characteristics of individual species. Temporal and seasonal changes and local geological condition can also be the reason for variations in pigment concentrations in plants, therefore further study in this context is recommended.

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