Article

Comparative Extraction Efficacy of Different Organic Solvents for Leaf Chlorophylls and Carotenoids in (*Portulaca oleracea* L.) Varying with Growth Behavior and Stress Type

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Abstract: The chlorophyll is one of the most important natural pigments used extensively in the food industry. Two important factors for the production of chlorophyll are the use of plants rich in chlorophyll and efficiency of extraction method. Present investigation was performed to compare the extraction of photosynthetic pigments by using solvents of different chemical nature. The purslane plants with different growth behavior viz. Scrollable and standing were grown under shade and sunshine stress condition. Different solvents including diethyl ether, 5% ethanol, pure acetone, 20% acetone, pure methanol and 10% methanol were used to extract chlorophyll and carotenoids from the purslane plant. The results indicated that stress, growth type and different solvents had a significant effect on the extraction of chlorophyll and carotenoids. Different trend was observed in extraction rate for chlorophylls and carotenoids. Among the solvents, pure methanol was the best for extraction of chl a. Methanol and acetone were appropriate solvents to achieve the highest amount of chlorophyll from plant tissues. Among different solvents, pure methanol for chl a, pure acetone and methanol for carotenoids were best solvent for purslane plant with a growing type scrollable of under shade.

Keywords: chlorophyll; carotenoids; methanol; photo-protection; phytoextraction; *Portulaca oleracea*

1. Introduction

Chlorophyll is the green pigment responsible for the color of leaves and have been used in dyeing food, drinks, soap, and cosmetics [1,2]. However, the excessive use of synthetic dyes may `destroy the organs and cause poisoning. Natural colorants from plants or algae, e.g., chlorophyll are safe and could provide health benefits, due to their anti-mutagenic and anti-oxidant contents that play positive roles in avoiding chronic diseases [3,4]. Some organic solvents, e.g., methanol, ethanol, acetone and N, N-dimethyl form amide (DMF), among others, are used for the extraction of chlorophyll and carotenoid. Acetone, ethanol and methanol are more effective solvents compared to acetone [5]. Acetone is volatile, highly inflammable, narcotic in high concentrations and a skin irritant

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(erythema). Acetone attacks polystyrene and polymethylacrylates (PMMA) and therefore, plastic spectrophotometer cuvettes cannot be used for acetone based chlorophyll assays. Methanol is also very good extractant for chlorophylls, particularly from recalcitrant vascular plant and algae [6].

The complete extraction was obtained with DMF [7]. However, DMF is expensive and also toxic; thus its potential use as a solvent has decreased [5]. The extraction rate can be enhanced by increasing the solubility and mass transfer process [8]. The agitation by stirring does not essentially affect the solubility of solute. In particular, the use of aqueous solutions of acetone has been recommended, where the proportion of water must not exceed 10%. Filtration or centrifugation is used to remove solids from the solvent. After solvent elimination, the yield of extraction is around 20% in which chlorophylls, pheophytins, and other degradation products are included [1].

Purslane (*Portulaca oleracea*) is succulent, robust medicinal plant, distributed in temperate and tropical regions world-wide. It thrives not only in gardens and cultivated fields, but in waste spaces and sidewalk cracks. It has been used extensively in folk medicine since antiquity, and also as a nutritious pot herb and salad ingredient [9]. Purslane has been investigated extensively for bioactive compounds [10]. The most noteworthy are the ω-3 fatty acids, and in particular, α-linolenic acid (ALA). This class of compounds and their metabolites have been identified as inhibitors of tumorigenesis11 and promoters of good cardiovascular health [11,12]. Purslane is also rich in antioxidant compounds and it activates enzymes that quench oxidants [13]. Like most members of the order Caryophylallales, purslane synthesizes betalain pigments rather than anthocyanins [14,15]. These pigments are antioxidants and of great interest as natural food colorants and nutritional supplements [16] and have been shown to protect low density lipoprotein particles [17,18].

Polysaccharides are abundant as well, and exhibit antiviral, antidiabetic and antitumor activities [19-21]. The seeds and aqueous extracts [22,23] have been found to be useful in the control of diabetes and to ameliorate some side effects of the disease. The use of purslane plant as a source of chlorophyll and carotenoid has not been investigated in the literature yet. In this study, the chlorophyll was extracted from purslane leaves using diethyl ether, 5% ethanol, pure acetone, 20% acetone, pure methanol and 10% methanol. The effects of stress conditions and growth brigade on the extraction process were also investigated and the optimal conditions were determined.

2. Results

The analysis of variance showed that the effects of stress and growth type on the relative water content (RWC) were significant (Table 1). Different experimental treatments including stress, growth type and solvents as well as their interactions significantly affected chlorophyll (*a*, *b* and total), sum of leaf carotenoids (x+c), chl *a*/*b*, chl *a*/ C(x+c) and total chlorophyll/ C(x+c) (Table 2).

Table 1. Analysis of variance the RWC under stress and growth type.

S.O.V	Stress (S)	Growth Type (G)	S*G	Error	CV(%)
Df	1	1	1	11	
RWC	87.43**	571.62**	1641.54**	6.35	3.81

** significant at 1%.

Table 2. Analysis of variance the photosynthetic pigment content of purslane under stress, growth type and solvent

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S.O.V	Df	Chl *a*	Chl *b*	Total Chl	Chl *a*/*b*	C (x+c)	*Chl a*/ C(x+c)	Total Chl /C(x+c)
Stress (S)	1	14.22**	19.24**	67.42**	4.77**	6.78**	34.29**	49.55**
Solvent type (Solv.)	5	37.11**	6.46**	36.05**	32.06**	21.96**	451.77**	5244.655**
Growth type (G)	1	13.04**	19.38**	64.22**	1.91**	5.58**	232.46**	2486.29**
S×Solv.	5	3.62**	10.93**	20.43**	5.41**	0.63**	72.07**	1003.82**
S×G	1	8**	28.29**	66.38**	3.87**	0.82**	130.66**	1816.74**
Solv.×G	5	6.05**	10.04**	22.18**	3.23**	2.15**	41.31**	639.62**
S×Solv.×G	5	4.16**	11.97**	23.92**	7.29**	0.98**	546.94**	5937.81**
Error	-	0.008	0.006	0.006	0.008	0.007	0.229	3.081
CV(%)	-	1.22	1.59	0.61	4.66	0.53	10.18	12.94

** significant at 1%.

2.1. Effects of stress and growth type on RWC

Purslane leaves with scrollable growth type had higher water content under sunshine conditions followed by standing growth type. The RWC in purslane with the standing growth type and grown under sunshine had an average 73.79, %. The purslane plant with both growth types had less RWC content under shade condition. Standing growth type had the lowest RWC of 45% (Figure 1).

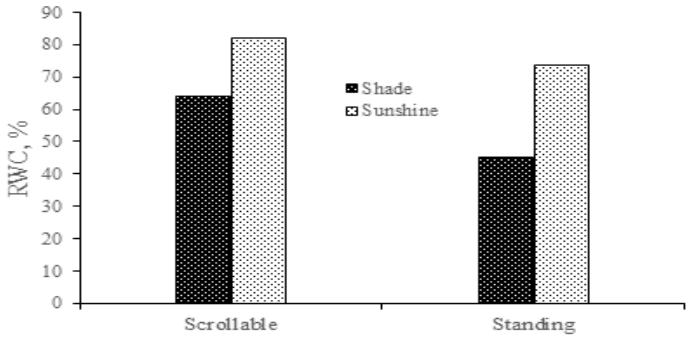

Figure 1. Mean comparison of RWC in stress × growth type interaction

2.2. Impact of stress, growth type and solvents on Chl a

The highest chl *a* content 5.56 mg m^{-2} was obtained with pure methanol solvent extraction in the purslane plant under shade conditions with scrollable growth type. Among other treatments, pure methanol had highest extraction efficiency for chl *a*. Growth type and stress were also effective in increasing the extraction of chlorophyll a. After pure methanol, the highest chlorophyll a was extracted with pure acetone, except under shade conditions and with scrollable growth type. The chl*a* was extracted with 5% ethanol and 10% methanol at all treatment levels (Figure 2).

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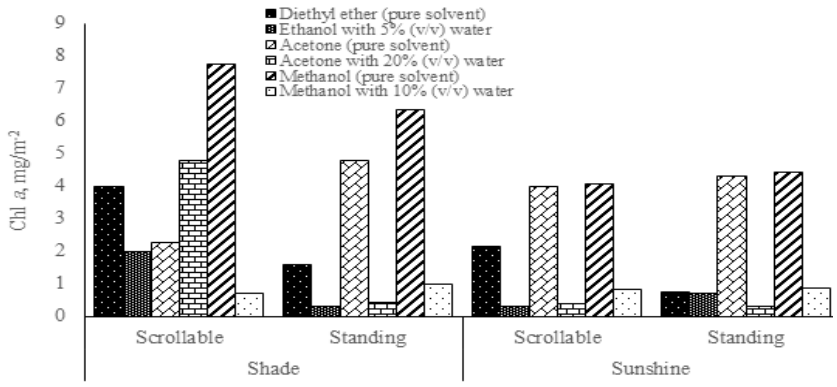

Figure 2. Mean comparison of Chl *a* in stress × growth type × solvent interaction

2.3. Impact of stress, growth type and solvents on Chl b

The highest chl *b* content 10.2 mg m^{-2} was obtained with 20% acetone solution under shade with scrollable growth type. The minimum extraction efficacy of chlorophyll *b* was observed with 20% acetone. The purslane plant with scrollable growth type showed higher chl *b* contents. The minimum chl *b* 2 mg m^{-2} extraction was observed with 10% methanol in sunshine and scrollable growth type 2 mg/m^{-2} (Figure 3).

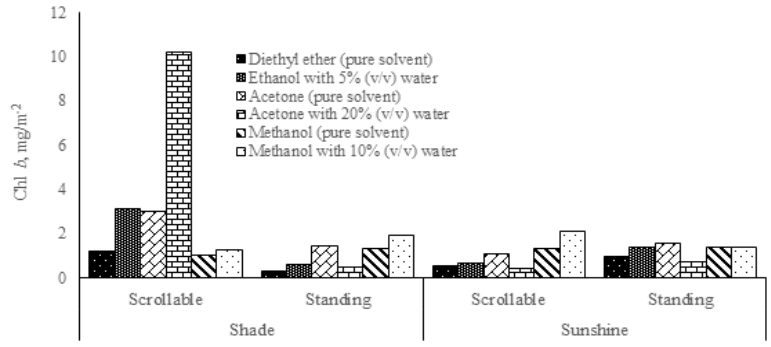

Figure 3. Mean comparison of Chl *b* in stress × growth type × solvent interaction

2.4. Impact of stress, growth type and solvents on total Chl

The highest total chlorophyll contents 15.01 mg m^{-2} was obtained using 20% acetone solvent in purslane leaf grown under shade condition with scrollable growth type. Extraction with pure methanol was 8.81 mg m^{-2} with similar growth type. Among solvents, maximum extraction efficacy was obtained with pure acetone solvent, however, the extraction of total *Chl* contents with pure methanol also showed similar efficiency (p<0.01) (Figure 4).

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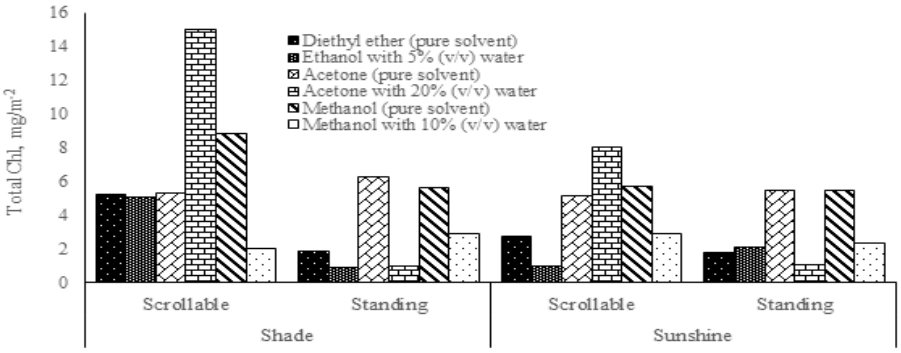

Figure 4. Mean comparison of total *Chl* in stress × growth type × solvent interaction

2.5. Impact of stress, growth type and solvents on Chl a/b

The extraction of Chl *a/b* ratio obtained by pure methanol ranged from 7.43 and 2.92 by the stress and growth type and by pure acetone the values ranged from 0.98 to 0.48. The lowest extraction for Chl *a/b* ratio was obtained for 5% ethanol, 20% acetone and 10% methanol. Nonetheless, the higher Chl *a/b* ratio of purslane plant was extracted under shade with standing growth type (Figure 5).

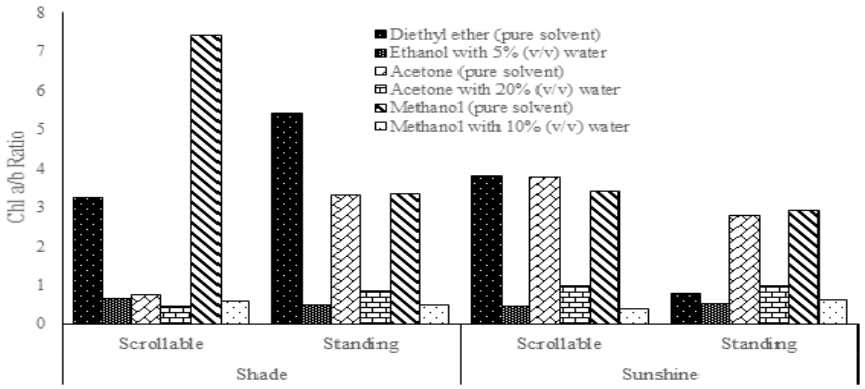

Figure 5. Mean comparison of Chl *a/b* in stress × growth type × solvent interaction

2.6. Impact of stress, growth type and solvents on Carotenoids

The higher amount of carotenoids was extracted under sunshine and standing growth type. The carotenoids content obtained by pure methanol extraction ranged from 4.04 -2.26 mg m^{-2} under sunshine and growth type treatments and by pure acetone the values ranged from 4.33 to 1.11 mg m^{-2}. The lowest content of carotenoids were extracted in 5% ethanol 20% acetone and 10% methanol solvents (Figure 6).

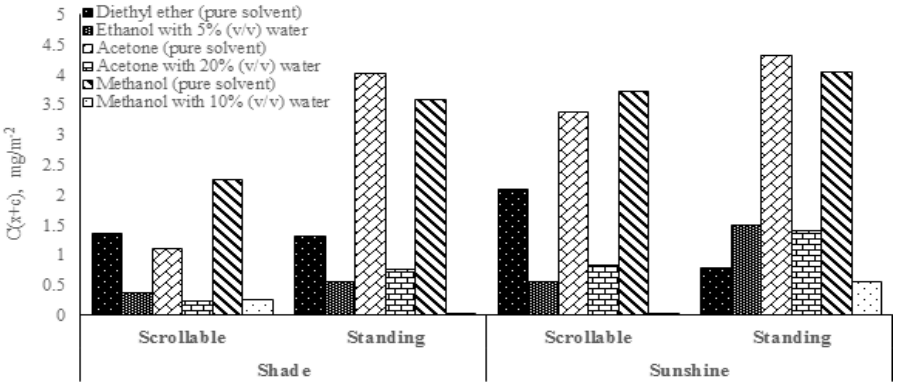

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Figure 6. Mean comparison of C(x+c) in stress × growth type × solvent interaction

2.7. Chl a/Carotenoids under effects of stress, growth type and solvents

For chl *a*/ C(x+c) ratio extraction, the best coefficients of variation were obtained with 10% methanol (11.23) under sunshine and scrollable growth type. The extraction with 20% acetone ranged between 8.54 and 0.24 for chl *a*/ C(x+c) ratio, while for pure acetone and pure methanol low extraction values were obtained (Figure 7).

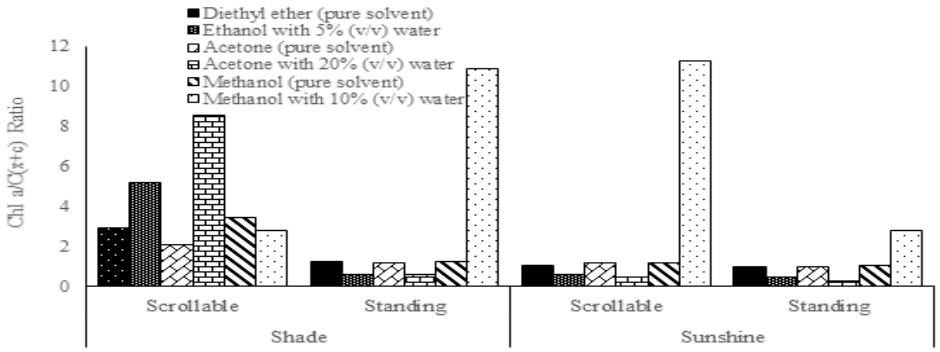

Figure 7. Mean comparison of Chl *a*/ C(x+c) in stress × growth type × solvent interaction

2.8. Impact of stress, growth type and solvents on total Chl / Carotenoid

The total chl/ C(x+c) ratio under different solvents and plant treatments showed that 10% methanol had the highest total chl/ C(x+c) ratios in two different positions of purslane 20.64 and standing stable 18.62 (Figure 8). In this trait, the highest total chl/ C(x+c) ratio was observed with methanol 10% solvents used under non-stress shade and scrollable growth type.

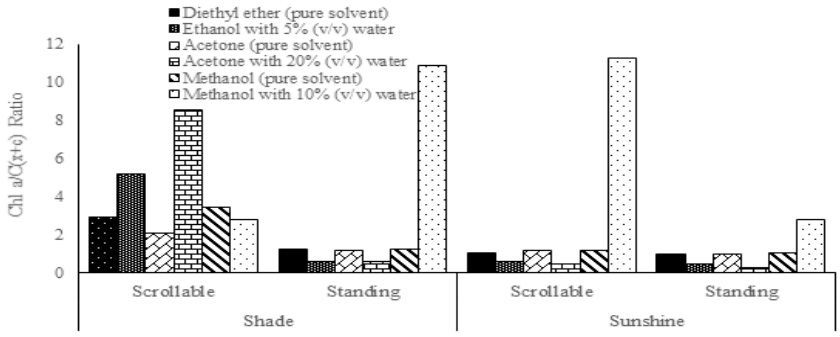

Figure 8. Mean comparison of total chl / C(x+c) in stress × growth type × solvent interaction

3. Discussion

Chlorophyll is the main pigment involved in photosynthesis and two other pigments and carotenoids, play supporting roles in photosynthesis [26]. Methanol and ethanol compare well as extraction solvents used in chlorophyll and carotenoids evaluation from plants. Comparing the values of the extraction variants of Chl a, best result was obtained with methanol. Most of the research methods use acetone as the extraction solvent, and report it as good method to evaluate the trophic level of waters [27]. However, Pepe et al [28] suggested that for the quantification of Chl a in *Nannochloropsis gaditana* (Eustigmatophyceae), the best solvent is methanol. A spectrophotometric method to quantify Chl uses methanol even in the absence of other disruption processes, has as main advantage over the use of ethanol due to short evaluation time (20 min).

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However, it was suggested that yields in Chl extraction with methanol can be improved either by increasing the extraction t, by applying 15 min of ultrasounds or by using the freezing/unfreezing method with liquid nitrogen [29]. In present study, Chl and carotenoids increased in purslane plants under non-stress shade and scrollable conditions, and their concentration decreased in stress sunshine and standing conditions. Chlorophylls molecule adapt in different environmental conditions to improve the maximum photon absorption in different conditions [30].

In severe and direct sunlight, Chl is easily damaged [31]. The highest Chl content has been reported in shoots grown in incomplete senescent 76% [32]. The plants grown under sunlight have more Chl than the plants under shade because of less light absorption for photosynthesis. As a result, the leaf area and the amount of pigments is increase in their leaves, in order to compensate for this light deficiency, as a consequent, the amount of Chl increases under such conditions [33]. In severe sunlight, the process of Chl depletion is very active. Under shading, the concentration of Chl increases and subjected to relatively high relative radiation intensity [34].

The intense light outside the plant decrease the tolerance in the light sensor, and thus, the granuloma becomes smaller in the photosystem II and part of the chlorophyll b [35]. Like chlorophylls, the carotenoids also decreased under shade conditions and scrollable growth type, and increased under sunshine and standing conditions in present study. Optimum sunlight increases the content of carotenoids more than chlorophylls and the accumulation of precursors responsible for absorbing light in the range of 350-400 nm [31]. Although, light is an essential signal for the synthesis of carotenoids in citrus fruits in a certain stage of fruit development, on the other, it will prevent intense light from the synthesis of the components of carotenoids. This suggests that an optimal level of light is needed for synthesis of carotenoids.

On the other, biosynthesis of carotenoid pigments can occur in the dark, meaning some carotenoids are synthesized in the presence of light, and others in the dark [36]. It seems that the most important reason for the increase in purslane leaf carotenoids under sunshine conditions is that the purslane has certain carotenoids for which synthesis is stimulated in brightness. According Lichtenthaler [25] chlorophylls possess a phytol chain bound to a porphyrin ring system and is esterified to the carboxyl group of the ring giving lipid character to the chlorophylls molecule.

Moreover, chlorophylls are fat-soluble compounds that can be extracted from water-containing living plant tissue by organic solvents such as acetone, methanol, or ethanol. Though aqueous solutions of these solvents are also suitable and may sometimes be preferable for extraction, their water content should not exceed 5 or 10%. The widely used method to extract isolated chloroplasts by 80% aqueous acetone does not fully extract the less polar pigment chl a. An additional step of extraction with 100% acetone is needed to guarantee complete extraction.

4. Materials and Methods

4.1. Plant materials

The experiment was carried out under natural conditions in Research Farm of Medicinal Plants, Shahed University of Science and Technology with the air temperature of 24–28 °C during the day and 11–15°C during the night. The experiment was performed in the northern latitude of 35° and 34', and the eastern longitude of 51° and 8', 1190 m above the sea level in the southern part of Tehran province, Iran. Purslane samples were prepared in both stress (Shade and Sunshine) and two growth type (Scrollable and Standing). The full sun treatment was exposed to sunlight for 12 hours and

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special thickness nets were used for shade treatment. In this study 100% shade of 4 × 4 (four layers) of tarpaulin was used.

4.2. Raw materials

Prior to be dried overnight at 50 °C, purslane leaves were cleaned and cut into small pieces of about 0.5 cm2. The dried material was then ground into smaller pieces and sieved at a mesh size of 710 µm.

4.3. Measurement of Relative Water Content (RWC)

The RWC were determined for the each stress (shade and sunshine) and growth type (scrollable and standing) treatments [24].

$$RWC = \frac{(freshweight\text{-}dryweight)}{(turgid\ weight\text{-}dry\ weight)} \times 100, \quad (1)$$

4.4. Extraction process

The solvents with different concentration were prepared and were used as pure diethyl ether, 5% ethanol, pure acetone, 20% acetone, pure methanol, and 10% methanol individually poured into a 100 mL three-neck flask equipped with a thermometer and a stirrer. The leaves' powder was added after the flask was heated up to the system temperature and stirred at the desired agitation speed. The extracted leaves powder was separated from the solutions by decantation. The filtrate was then analyzed to measure the chlorophyll content. Various temperatures and agitation speeds were investigated in the range of 40–70 °C and 100–400 rpm, respectively.

4.5. Measurement of chlorophyll and carotenoid contents

Chlorophyll and carotenoid contents were evaluated using the method [25]. In this way, 0.2 g of fresh leaf tissue was extracted with 20 mL of each solvent with its concentration, and the content of chlorophyll and carotenoid was analysed using spectrophotometer UV–Vis (Lambda 25; Perkin Elmer, America).

Diethyl ether (pure solvent):

$$Ca, \mu g\ ml = 10.05\ A660.6 - 0.97\ A642.2, \quad (2)$$

$$Cb, \mu g\ ml = 16.36\ A642.2 - 0.97\ A660.6, \quad (3)$$

$$C_{(X+C)}, \mu g\ ml = \frac{(100\ A470 - 1.43\ Ca - 35.87\ Cb)}{205}, \quad (4)$$

Ethanol with 5% (v/v) water:

$$Ca, \mu g\ ml = 13.36\ A664.1 - 5.19\ A648.6, \quad (5)$$

$$Cb, \mu g\ ml = 27.43\ A648.6 - 8.12\ A664.1, \quad (6)$$

$$C_{(X+C)}, \mu g\ ml = \frac{(100\ A470 - 2.13\ Ca - 97.64\ Cb)}{209}, \quad (7)$$

Acetone (pure solvent):

$$Ca, \mu g\ ml = 11.26\ A661.6 - 2.04\ A644.8, \quad (8)$$

$$Cb, \mu g\ ml = 20.13\ A644.8 - 4.19\ A661.6, \quad (9)$$

 doi:10.20944/preprints201909.0152.v1

$$C_{(X+C)}, \mu g\ ml = \frac{(100\ A470 - 1.90\ Ca - 63.14\ Cb)}{214}, \quad (10)$$

Acetone with 20% (v/v) water:

$$Ca, \mu g\ ml = 12.25\ A663.2 - 2.79\ A646.8, \quad (11)$$

$$Cb, \mu g\ ml = 21.50\ A646.8 - 5.10\ A663.2, \quad (12)$$

$$C_{(X+C)}, \mu g\ ml = \frac{(100\ A470 - 1.82\ Ca - 85.02\ Cb)}{198}, \quad (13)$$

Methanol (pure solvent):

$$Ca, \mu g\ ml = 16.72\ A665.2 - 9.16\ A652.2, \quad (14)$$

$$Cb, \mu g\ ml = 34.09\ A652.4 - 15.28\ A665.2, \quad (15)$$

$$C_{(X+C)}, \mu g\ ml = \frac{(100\ A470 - 1.63Ca - 104.96\ Cb)}{221}, \quad (16)$$

Methanol with 10% (v/v) water (equation):

$$Ca, \mu g\ ml = 16.82\ A665.2 - 9.28\ A652.4, \quad (17)$$

$$Cb, \mu g\ ml = 36.92\ A652.4 - 16.54\ A665.2, \quad (18)$$

$$C_{(X+C)}, \mu g\ ml = \frac{(100\ A470 - 1.91Ca - 95.15\ Cb)}{225}, \quad (19)$$

The concentrations for Chl a (Ca), Chl b (Cb), and the sum of leaf carotenoids (x+c) was calculated with the above equations given for different solvents, where the pigment concentrations are given in µg/ml extract solution.

Statistical analysis of data

A factorial experiment based on a completely randomized design was used with three replications. Differences among treatments were analyzed by SAS 9.1, taking $p< 0.05$ as significant according to Duncan's multiple range test.

5. Conclusions

In conclusion, using an appropriate solvent could help to increase the extraction of chlorophyll and C(x+c) from plant tissues. The best extraction solvents for simultaneously determination of Chl a, b and C(x+c) were methanol and acetone. For purslane plant with a growing type under shade, the best extraction solvent was pure methanol for Chl a, pure acetone and methanol for C(x+c).

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