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## Quantitative estimation of chlorophyll and caretenoid contents in endangered medicinal plants *Gentiana kurroo* Royle and *Swertia chirayita* (Roxb.) H. Karst

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**ABSTRACT:** *Gentiana kurroo* Royle and *Swertia chirayita* (Roxb.) H. Karst., commonly found in the Indian Himalayan Region have been listed into the category of critically endangered medicinal plants. Considering the important role of chlorophyll and carotenoid contents in plant's photosynthetic ability, the present study focuses on solvent extraction methods to compare photosynthetic pigment content in *G. kurroo* Royle and *S. Chirayita* (Roxb.) H. Karst. A higher chlorophyll and carotenoid contents in *S. chirayita* (Roxb.) H. Karst. was observed than that of *G. kurroo* Royle. Extraction using DMSO and methanol showed a higher content than that with water. A variation in values was observed in both the plants as they grow at different elevations implying a difference in biochemical components of the plants and requirement of different solvents for extraction.

**Key words:** Carotenoid, chlorophyll, extraction, *Gentiana kurroo* Royle, *Swertia chirayita* (Roxb.) H. Karst.

**Abbreviations:** Chl a= Chlorophyll a, Chl b= Chlorophyll b

Human beings have been interested in medicinal herbs due to their pharmacological relevance and have been using therapeutic plants growing in their natural environments since the beginning of mankind (Shukla *et al.*, 2010). It has been incredibly common to look for natural products possessing bioactive components and, therefore, the search of therapeutic plants has led to novel medication discoveries and has well been evidenced by vast literature. The use of herbals, particularly therapeutic plants, has long served as a general gauge of ecosystem health (Singh, 2002). *Gentiana kurroo* Royle, a critically endangered medicinal plant often known as “Karu” (due to the bitter nature of its roots), grows between 1500 and 5000 meters above sea level in the mid Himalayas (Skinder *et al.*, 2017). *Swertia chirayita* (Roxb.) H. Karst. commonly known as “Chiretta”, is a critically endangered plant very well documented since ages growing at high elevations in the sub-temperate parts of the Himalayas between 1200 and 2100 metres from Kashmir to Bhutan (Clarke, 1885) in moist shady patches (Gaur, 1999). The primary bioactive elements responsible for pharmacological relevance to both these plants, include Iridoids (gentiopicroside, swertiamarin, and sweroside), Xanthones (isogentisin, bellidifolin and mangiferin)

and Flavonoids like isovitexin and isoorientin (Menkovic *et al.*, 2010; Mustafa *et al.*, 2015; Öztürk *et al.*, 2006; Schaufelberger and Hostettmann, 1988; Wu *et al.*, 2017; Zhao *et al.*, 2013).

Plants have an inherent photosynthetic capacity to convert solar energy into chemical energy in the presence of chlorophylls and accessory pigments. Chlorophylls found in higher plants, cyanobacteria, various photosynthetic algae, dinoflagellates are categorized as chlorophyll a, b, c, d, e, and f; are prenyl lipids having a porphyrin head and a phytol tail. The primary pigment chlorophyll a is bluish green (chl-a;  $C_{55}H_{72}N_4O_5Mg$ ;  $\lambda_{max} = 647$  nm), whereas the auxiliary pigment chlorophyll b is yellowish green (chl-b;  $C_{55}H_{70}N_4O_6Mg$ ;  $\lambda_{max} = 664$  nm) (Hartzler *et al.*, 2014; McCartney-Melstad *et al.*, 2018; Rebeiz, 2014). Depending on the type of plant, the habitat, the age of leaves, these two pigments are present in a ratio ranging between 2 to 5 (Parry *et al.*, 2014; Prasann *et al.*, 2018; Rebeiz, 2014). Due to its sandwiching between protein and lipid layers of the chloroplast lamellae where porphyrin is protein bound and tail is lipid bound, the chlorophyll molecule is insoluble in water but soluble in organic solvents (Palta, 1990). The

dynamic balance of photosynthetic pigments aids in the cell's ability to maintain photostasis (Huner *et al.* 1998). The chl-a to chl-b ratio in terrestrial plants has been used as a measure of their sensitivity to light or shade conditions (Porra, 1991; Vicas *et al.* 2010). Photosynthetic ability of a plant is determined by the total chlorophyll content (chl-a+chl-b) and chl-a/b ratio (Croft *et al.*, 2017). The chlorophylls are recognized as antioxidants, anti-inflammatory, and anti-cancer agents because of their positive effects on health and are also used for food colourings and in cosmetics (Feng *et al.*, 2017; Viera *et al.*, 2019). The low fraction of chl-a/b is a sensitive biomarker of pollution and environmental stress (Tripathi and Gautam, 2007). An important group of accessory pigments in photosynthesis *i.e.*, carotenoids transmit the absorbed light energy to chlorophyll pigments (Palett and Young, 1993). Carotenoids are non-polar pigments which are essential for photoprotection as they inactivate the reactive oxygen species (ROS) generated on light exposure. Structurally they belong to the terpenoid-class pigments, and have heavily conjugated polyene chains which gives them distinctive colours like red, orange, purple, or yellow. Carotenoids absorb visible light primarily between 400 and 500 nm (Britton, 2008). They can be divided into two categories: one carotenes (carotene and lycopene), which are unsaturated hydrocarbons and secondly xanthophylls (lutein, zeaxanthin, and fucoxanthin), which essentially possess at least one oxygen containing functional group on a structure similar to carotenes (Poojary *et al.*, 2016). Carotenoids, due to their antioxidant nature, also have a role in prevention of cancer and cardiovascular diseases (Sangeetha and Baskaran, 2010). The plant's photosynthetic ability is correlated with the amount of carotenoid and chlorophylls (Gamon and Surfus, 1999).

Various methods have been employed for chlorophyll extraction from plants. The extraction of pigments is routinely done using a mortar and pestle with organic solvents like acetone or dimethyl formamide and is quantified using well-established formulae (Arnon 1949; Porra *et al.*, 1989). Polar aprotic solvents like acetone, dimethyl sulfoxide (DMSO), and N, N-Dimethylformamide (DMF) as

well as polar protic solvents like ethanol and methanol have been used extensively in solvent extraction techniques (Lichtenthaler and Buschmann, 2001). Numerous researchers have thoroughly examined the chlorophylls' absorption coefficients in various organic solvents and proposed concurrent equations to estimate the chlorophyll concentrations (Cole *et al.*, 2019; Masaló and Oca, 2020; Negrão *et al.*, 2017; Özkan and Bilek, 2015; Porra and Scheer, 2019; Ritchie, 2006). Pure chl-a and chl-b absorption coefficients have been calculated for a variety of solvents, and these coefficients have been found to be solvent dependent (Prasann *et al.*, 2018; Saito *et al.*, 2018). Standardization of chlorophyll extraction becomes cumbersome due to content difference within leaves and other parts. Other factors like temperature, light solvent polarity and solution's pH make the standardization protocol more complex (Campbell *et al.*, 2019).

Considering the important role of chlorophyll and carotenoid contents in plant's photosynthetic ability, the present study focuses on solvent extraction methods to compare photosynthetic pigment content in the critically endangered multi utility therapeutic plants *G. kurroo* Royle and *S. Chirayita* (Roxb.) H.Karst.

## MATERIALS AND METHODS

**Plant Material:** Present investigation was conducted in the Department of Biochemistry, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand. The plant material of *G. kurroo* Royle and *S. chirayita* (Roxb.) H. Karst. was collected from mid Himalayan hills at Gaurikund and High-Altitude Nursery of Herbal Research and Development Institute (HRDI), Mandal, Gopeshwar, respectively, situated in Chamoli district, Uttarakhand. The herbarium specimen of the plant was submitted at the G.B. Pant University of Agriculture and Technology, Pantnagar. Dr. D.S. Rawat, Assistant Professor and Plant Taxonomist, Department of Biological Science, C.B.S.H., G.B.P.U.A.&T, helped in establishing the identity

of *Gentiana kurroo* Royle (voucher number GBPUH- 1512/28-12-2022) and *Swertia chirayita* (Roxb.) H. Karst. (voucher number GBPUH- 1511/28-12-2022).

### Chlorophyll estimation

#### a) Chlorophyll estimation using dimethyl sulphoxide (Hiscox and Israelsham, 1979)

The fresh leaves (50mg) of *G. kurroo* Royle and *S. chirayita* (Roxb.) H. Karst. after cutting into small pieces were suspended in 10 mL DMSO and kept in water-bath for 3h at 60°C. This mixture after keeping at room temperature for 1h was decanted and absorbance of the solution (leaf extract) was taken at 663 and 645nm against DMSO blank. The carotenoid content was estimated using the same leaf extract by measuring the absorbance at 480nm and quantified as per formulas in Table 1. All the quantifications were carried out in triplicate.

#### b) Chlorophyll estimation using methanol and water (Longjam *et al.*, 2018)

The fresh leaves (0.5g) of *G. kurroo* Royle and *S. chirayita* (Roxb.) H. Karst. were cut into small pieces and ground in mortar pestle with 10 mL of different

solvents (methanol and water). The samples were kept in the freezer for 2h. The samples were again ground and then kept for centrifugation at 10,000 rpm for 20min at 4°C. The supernatant was separated and 0.5mL of it was mixed with 4.5mL of the respective solvent. The absorbance of this mixture was measured at different wavelengths for estimation of Chl a, Chl b and carotenoids using the formula as given in Table 2. All the quantifications were carried out in triplicate.

### RESULTS AND DISCUSSION

It was observed in the present study that the leaves of *S. chirayita* (Roxb.) H. Karst. possessed higher contents of Chl a, b and carotenoid in comparison to those of *G. kurroo* Royle in all the three solvents (Table 3). Li *et al.* (2018) reported that latitude, climate, soil, and phylogeny were some factors that caused a variation in chlorophyll content in plant species.

The efficacy of extraction of Chl a, which was more non-polar than other pigments followed the order of DMSO>methanol>water for both species. The extraction efficacy for Chl b in *S. chirayita* (Roxb.)

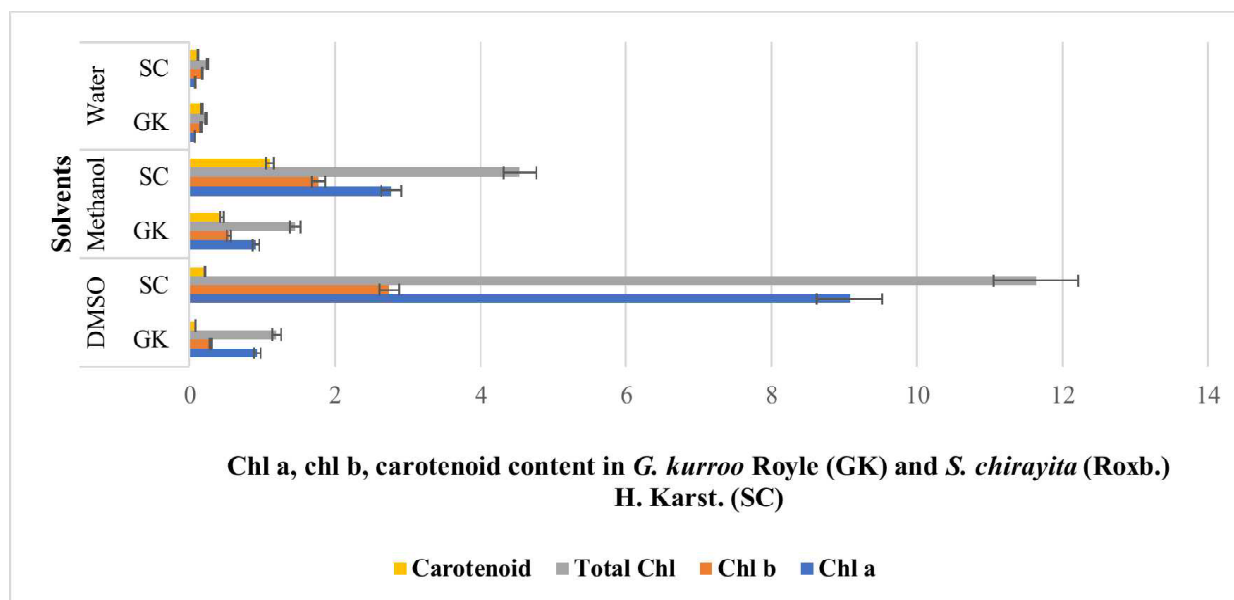


Fig. 1: Chl a, Chl b and carotenoid content in different leaf extracts of *G. kurroo* Royle and *S. chirayita* (Roxb.) H. Karst. [Units: DMSO: (mg g<sup>-1</sup> fresh wt.); methanol and water: (µg ml<sup>-1</sup>)]

**Table 1: Formula to quantify Chl a, Chl b and carotenoid (mg g<sup>-1</sup> fresh wt.) by DMSO**

Chl a (mg g <sup>-1</sup> fresh wt.)	$(12.7 \times A_{663} - 2.69 \times A_{645}) V \div 1000 \times W$
Chl b (mg g <sup>-1</sup> fresh wt.)	$(22.9 \times A_{645} - 4.48 \times A_{663}) V \div 1000 \times W$
Total chl (mg g <sup>-1</sup> fresh wt.)	$(20.2 \times A_{645} + 8.02 \times A_{663}) V \div 1000 \times W$
Carotenoid (mg g <sup>-1</sup> )	$(A_{480} + 0.11 \times A_{663} - 0.638 \times A_{645}) V \div 1000 \times W$

where, A = Absorbance of chlorophyll extract at specific indicated wavelength; V = Final volume of the sample; W = Weight of tissue extracted on fresh weight basis

**Table 2: Formula to quantify Chl a, Chl b and carotenoid (µg mL<sup>-1</sup>) by different extraction solvents**

Extraction in methanol	Extraction in water
Chlorophyll a = $15.65 \times A_{666} - 7.340 \times A_{653}$	Chlorophyll a = $12.7 \times A_{663} - 2.69 \times A_{645}$
Chlorophyll b = $27.05 \times A_{653} - 11.21 \times A_{666}$	Chlorophyll b = $22.9 \times A_{645} - 4.68 \times A_{663}$
Carotene = $(1000 \times A_{470} - 2.860 \text{ Chl a} - 129.2 \text{ Chl b}) \div 245$	Carotene = $(1000 \times A_{470} - 1.82 \text{ Chl a} - 85.02 \text{ Chl b}) \div 198$

where, A = Absorbance of chlorophyll extract at specific indicated at wavelength; Chl a = Chlorophyll a, Chl b = Chlorophyll b

**Table 3: Chl a, Chl b and carotenoid content in different leaf extracts of *G. kurroo* Royle and *S. chirayita* (Roxb.) H. Karst.**

Solvents/	DMSO (mg g <sup>-1</sup> fresh wt.)		Methanol (µg mL <sup>-1</sup> )		Water (µg mL <sup>-1</sup> )	
Contents	<i>G. kurroo</i> Royle	<i>S. chirayita</i>	<i>G. kurroo</i> Royle	<i>S. chirayita</i>	<i>G. kurroo</i> Royle	<i>S. chirayita</i>
Chl a	0.9275	9.0722	0.9133	2.7727	0.0707	0.0782
Chl b	0.288	2.7395	0.537	1.7694	0.1565	0.1686
Total Chl	1.1978	11.6388	1.4503	4.5421	0.2272	0.2468
Carotenoid	0.0823	0.2088	0.4394	1.1039	0.1611	0.112

Note: Polarity of solvents: DMSO=0.444; Methanol=0.762; Water=1.0

H. Karst. was similar to that of Chl a but for *G. kurroo* Royle it was methanol>DMSO>water. Highest extraction efficacy was observed in methanol for carotenoid in both the plants (Fig. 1). Earlier studies on several plant species reported that different solvents would extract different amounts of chlorophyll due to biotic factors (variation in leaf shapes, plant age, physiological status), abiotic factors (light, temperature, growth season) and different equations or formulae used for content calculation. Besides the efficacy of solvents, the pigment quantification would also depend on technical factors like spectrophotometric resolution, range, and absorbance parameters (Almohamdi and Örmeci, 2018; Lichtenthaler, 1988; Martín-Tornero *et al.*, 2020; Masaló and Oca, 2020).

Evidently, DMSO being most non-polar amongst the three solvents (Table 3) was the best solvent for Chl a extraction. Castle *et al.* (2011) compared different solvents for chlorophyll extraction from soil crusts and found ethanol and DMSO being the best solvents for extractions. Martín-Tornero *et al.* (2020) also reported DMSO being used for increased Chl a and b extraction in a variety of plant species. It was

interesting to note that the ratio of Chl a: Chl b contents extracted in DMSO was 3.22 and 3.31 in case of *G. kurroo* Royle and *S. chirayita* (Roxb.) H. Karst., respectively, which was very similar. The ratio of Chl a: Chl b was fairly comparable in methanol extraction *i.e.*, 1.70 and 1.56 in case of *G. kurroo* Royle and *S. chirayita* (Roxb.) H. Karst., respectively. In the case of water, the ratio of Chl a: Chl b was 0.45 and 0.46 in *Gentiana* and *Swertia*, respectively which clearly indicated that water did not prove to be a good solvent.

*G. kurroo* Royle and *S. chirayita* (Roxb.) H. Karst. show an altitudinal variation in their growth *i.e.*, *Gentiana* grows between 1500 and 5000 meters while *Swertia* grows between 1200 and 2100 metres. Several reports proclaimed that photosynthetic pigments involved in light absorption and transformation would directly impact photosynthetic capacity and were also vulnerable to environmental stresses. Chlorophyll content would decrease due to slow down in its synthesis, increased decomposition, or damaged chloroplast structures during water, light, and temperature stresses (Ashraf and Harris, 2013; Hazrati *et al.*, 2016). In present



study also, the pigment content in *G. kurroo* Royle decreased with increasing elevation and was an adaptive response to prevent oxidative damage by reducing light absorption and thereby preventing ROS production. Armstrong *et al.* (1996) and Demmig-Adams *et al.* (1992) reported that carotenoids were multifunctional complex playing an important role in light-harvesting complexes (LHCs) construction, affecting photosynthesis as well as efficient removal of accumulated ROS thus preventing photooxidative damage to chloroplast (Armstrong *et al.*, 1996; Demmig-Adams *et al.*, 1992). Ahmad *et al.* (2016) also reported a decrement in carotenoid content with increasing elevation. In the present study, the chlorophyll and carotenoid contents depicted a large variation in their values for leaves of both the plants (Table1).

## CONCLUSION

Chlorophyll and carotenoid from *G. kurroo* Royle and *S. chirayita* (Roxb.) H. Karst. were extracted and estimated using different solvents. A higher chlorophyll and carotenoid content in *S. chirayita* (Roxb.) H. Karst. was observed than that of *G. kurroo* Royle. Extraction using DMSO and methanol showed a higher content than that with water. A variation in values was observed in both the plants as they grew at different elevations implying a difference in biochemical components of the plants and requirement of different solvent for extraction. This led to the conclusion that photosynthetic pigment requirements varied between plant species and were directly related to the activity and rate of photosynthesis. Further investigations regarding the other biochemical parameters like total soluble protein and sugar content along with chromatographic techniques would be suggestive to have a greater understanding to examine the valuable attributes of bioactive pigments.

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