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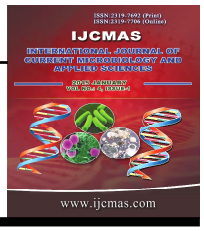


**Original Research Article**

# Sequential extraction and quantification of *Tinospora cordifolia* leaf pigments and metabolites

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**A B S T R A C T**

Plant pigments and metabolites are being routinely extracted and estimated by scientists from medicinal plants to elucidate metabolic profiles. Extraction procedures and estimation techniques involve use of plant materials like leaf, stem, root and flower as well as a range of solvents and reagents. Many a times samples obtained from single plant are insufficient for the extraction of primary and secondary metabolites for this reason, samples taken from randomly selected plants are used for extractions and estimations. Hence, if a sequential extraction method is used one can extract chlorophylls, carotenoids, sugars, starch and proteins along with secondary metabolites like phenols and flavonoids from samples taken from single plant. Hence a four step sequential extraction was adopted in present paper to extract pigments, primary metabolites and secondary metabolites from fresh leaves of *T.cordifolia.* Data obtained clearly indicate that the sequential extraction method exhibited similar values for said metabolites with insignificant deviations as compared to routine standard methods. Thus, a sequential extraction can be used for extraction of chlorophylls, carotenoids, sugars, starch, proteins, free amino acids along with total phenols and flavonoids.

**K e y w o r d s**

Sequential extraction, Chlorophylls, Carotenoids, Metabolites, Tinospora cordifolia, Leaf

## Introduction

In present era oxidative stresses induced by reactive oxygen species (ROS) have been considered the main cause of induction and progression of a number of chronic diseases. Hence, plant scientists, biochemists and pharmacists have focused their research on isolation and identification of plant metabolites having antioxidant capacities and ROS scavenging properties. Human dietary guidelines have been formulated around the world for prevention of life style diseases and disorders such as diabetes, cardiovascular diseases, osteoporosis and cancer based on the evidences in support of the relationship between diet and chronic disorders. In the guidelines it is recommended that one should take fruits and vegetables that have enough carotenoids and pharmacologically active phytochemicals (Rao and Rao 2007). Carotenoids are from family of pigmented compounds which are mainly synthesized by

plants and microorganisms but not animals. These are important dietary sources of vitamin A and are considered valuable in preventing human diseases (Paiva et al., 1999).

It is well documented that there is a direct relationship between antioxidant activity and phenolic compounds. Gupta *et al.* (2004) demonstrated antioxidant and antilipid peroxidation activity of *Ervatamia coronaria* leaves and suggested that polyphenols and flavonoids are responsible for free radical scavenging and antioxidant activities. Limaye (2010) studied the antioxidant and DNA protection potentials of *Adiantum trapeziforme* and reported substantial antioxidant potentials and DNA safeguarding activity in fronds of *Adiantum trapeziforme* and ascribed these activities to the presence of phenols and flavonoids.

Pereira *et al*. (2009) stated that, phenolic compounds are able to act as antioxidants in a number of ways. Hydroxyl groups of phenols are good hydrogen donors and can donate hydrogen to reactive oxygen and reactive nitrogen species and thus break the cycle of generating new free radicals.

Flavonoids have been reported to possess many useful properties, including anti-inflammatory, oestrogenic, enzyme inhibition, antimicrobial, antiallergic, antitumour activity along with undoubtedly antioxidant activity (Cushnie and Lamb, 2005). According to Aron and Kennedy (2008), this well established antioxidant activity of flavonoids is responsible for other biological activities, in which, prevention of oxidative stress is beneficial. For example, anti-cancerous activity of some flavonoid compounds is due to their ability to scavenge free radicals.

Some of the earlier studies indicated that chlorophyll pigments have antioxidant, anti inflammatory and wound healing properties. It has been observed that chlorophyll pigments contain chlorophyllin which is responsible for increasing the number and activity of dominant immune cells like B-cells, T- cells and macrophages essential to human health. The great antioxidant capacity of chlorophylls helps to neutralize free radicals and reduce oxidative damage and work as an anti-carcinogenic agent in our body (Rajalaksmi and Banu, 2014, Durgadevi and Banu, 2015).

Extraction of metabolites is the first step in biochemical profiling of herbs, which makes downstream process easier for isolation, characterization, identification and estimation of bioactive components from medicinal plants. Such extraction procedures in particular are labour intensive, time consuming and require large volume of solvents and reagents. Many a times, samples obtained from single plant are insufficient for extraction of all the metabolites and thus samples taken from randomly selected plants are used for extractions and estimations. Data obtained from such samples are unclear and may lead to an erroneous elucidation.

Recently, a simple four step method for sequential extraction of chlorophylls, carotenods, primary and secondary metabolites in peanut and okra has been proposed (Laware, 2015). However, it is not yet applied for extraction of pigments and metabolites in medicinal plants. Therefore, in present investigation *Tinospora cordifolia*, a model medicinal plant was considered for sequential extraction of pigments and metabolites. *Tinospora cordifolia* (Wild) Hok F. & Thomson, commonly known as Guduchi belongs to family Menispermaceae. It is distributed throughout tropical Indian subcontinent, Shri-Lanka and China. Leaf, stem, root and flower of *T. cordifolia* possess anti-

spasmodic, anti-inflammatory, anti-allergic and antioxidant properties; hence it is widely used in ayurvedic system of medicine for the treatment of diabetes, urinary diseases, cancer and asthma. Considering the medicinal importance of *T. cordifolia*, a sequential extraction method proposed by Laware (2015) was used with slight modification to extract primary and secondary metabolites from a single plant leaf samples. The results of sequential extraction method and routine extraction methods are compared and discussed.

## Materials and methods

*Tinospora cordifolia* plants were authenticated and marked in the Fergusson College campus, Pune (MS). Fully mature physiologically active leaves were collected randomly in the morning and used for extraction of leaf bio-chemicals.

### Step-1: Extraction of chlorophylls and carotenoids.

Fresh leaves were chopped in small pieces and exactly 0.1 g material was weighed and macerated in mortar and pastel with 2 ml of 90% ethyl alcohol. The content was centrifuged at 10000 g for 10 minutes. The residue was re-extracted in 1 ml of 90% ethyl alcohol for two times and centrifuged at 10000 g for 10 minutes. The supernatants were pooled and made to 10 ml with ethyl alcohol.

Appropriately diluted extracts were read on spectrophotometer for estimation of pigments. After estimation samples were saved and put back in original sample.

### Step-2: Extraction of soluble sugars, phenols and amino acids.

The residue obtained in step-1 was extracted in 2 ml of 80% ethyl alcohol in boiling water bath for 30 minutes. The content was cooled and centrifuged at 10000 g for 10 minutes and the residue obtained was re-extracted with fresh 2 ml of 80% alcohol. The supernatant obtained in step-1(chlorophyll extract) and step-2 were pooled and condensed in water bath to 1-2 ml and diluted to 10 ml with distilled water and centrifuged at 10000 g for 10 minutes. Supernatant obtained was used for estimation of reducing sugars, total sugars, total phenols, total flavonoids and free amino acids.

### Step-3: Extraction of starch

The residue obtained after step-2 was re-suspended in digestion mixture (0.65 ml of 52% perchloric acid + 0.5ml distilled water) and subjected to digestion in cold condition at 0 $^{0}$C in refrigerator for 30 minutes. After cold incubation the content was centrifuged at 10000 g for 10 minutes and supernatant was collected as source of starch.

The pellet was further extracted with same volume of (52 % PCA and water) and supernatants were pooled. The extracted starch solution was neutralized with sodium carbonates. Final volume was made to 2.5 ml with distilled water.

### Step-4: Extraction of proteins

The residue obtained in step-3 was used for extraction of proteins. The residue was treated with 2.0 N solution of NaOH (1 ml) for 30 minutes and centrifuged at 10000 g for 10 minutes and supernatant was collected as a source of proteins.

The extraction was repeated with fresh NaOH solution (1 ml) and centrifuged. The supernatants were pooled and final volume was made to 2.0 ml with distilled water. The pooled supernatant was saved as source of proteins.

**Extraction of metabolites by routine methods**

The leaf material (0.1 g) was also extracted with 80 % acetone for estimation of pigments. Reducing sugars, soluble sugars, total phenols, total flavonoids and free amino acids were extracted with 80 % methyl alcohol from 0.1 g leaf material. Starch was extracted from 0.1 g material with 52 % perchloric acid at 0 $^{o}$C after extracting and washing out soluble sugars with methanol. Proteins were extracted in 0.2 N NaOH from 0.1 g leaf materials after treating materials with 80% acetone, 80 % methanol and 52% perchloric acid in a sequence for washing out pigments, phenols, sugars, free amino acids, and starch.

1. **Estimation of pigments:** Chlorophyll pigments and Carotenoids were estimated by Lichtenthaler and Welburn (1983) method. After estimation samples were saved and replaced in original sample.

Chlorophyll **a** (µg/ml) = 12.21 (A663) - 2.81 (A646)
Chlorophyll **b** (µg/ml) = 20.13 (A646) - 5.03 (A663)
Carotenoids (µg/ml) = (1000$A_{470}$ - 3.27[chl a] - 104[chl b])/227

2. **Estimation of soluble carbohydrates:** Soluble carbohydrates were estimated by Anthrone reagent as per the method given by Hansen and Moller (1975). D-Glucose at the concentration of 20 to 100µg ml$^{-1}$ was used to prepare the standard curve.

3. **Estimation of reducing sugars:** Reducing sugars were estimated with DNSA reagent according to Miller (1959) method. Maltose at the concentration of 20 to 100µg ml$^{-1}$ was used to prepare the standard curve.

4. **Estimation of starch:** Starch was estimated with Anthrone reagent as per the method given by Hansen and Moller (1975). D-Glucose at the concentration of 20 to 100µg ml$^{-1}$ was used to prepare the standard curve.

5. **Estimation of free amino acids:** Total free amino acid content was estimated according to the Ninhydrin method (Moore and Stein, 1948). L-lysine was used as standard amino acid to prepare standard curve.

6. **Estimation of phenols:** Total phenols were estimated as per the method given by Farkas and Kiraly (1962). Catechol at the concentration of 20 to 100µg ml$^{-1}$ was used to prepare the standard curve.

7. **Estimation of flavonoids:** Aluminium chloride method was used for flavonoid determination (Chang *et al*., 2002). Calibration curve was prepared with quercetin at concentrations 12.5 to 100mg ml$^{-1}$ in methanol.

8. **Estimation of proteins:** Protein content was estimated by Lowry *et al*. (1951) method. Bovine serum albumin-fraction V (BSA) was used at the concentration of 0.2 to 1.0 mg ml$^{-1}$ as a standard protein to prepare the standard curve.

Experiments were carried out in four replications. All reactions were read at

respective wavelengths on UV-Vis spectrophotometer (Shimadzu). Data recorded from four replications were subjected to single way analysis of variance (ANOVA) and critical differences were calculated at p=0.05 level.

## Result and Discussion

Results related to methods of extraction of chlorophyll pigments, carotenoids carbohydrates, proteins and amino acids, phenols and flavonoids are given in table-1and 2. Estimated values clearly indicate that the sequential extraction method used in present investigation, exhibited estimate with insignificant deviations as compared to routine standard methods. Results on carbohydrate content i.e. soluble sugar, reducing sugar, and starch profiles are fairly close, irrespective of the extraction methods.

Generally, the selection of an extraction method depends on working requirements and available laboratory facilities. Any metabolite extraction includes extraction instruments, labour cost, operational cost and cost of consumables as well as the extraction time. Generally reducing sugars, amino acids, phenols and flavonoids are extracted with 80% methyl alcohol and during these extractions, the residue is generally discarded, which otherwise contains valuable insoluble components like starch, proteins and dietary fibers.

On the other hand during protein extraction plant material is first treated with alcohol for removal of chlorophyll pigments and free sugars and then with perchloric acid for digestion of starch. In this process, supernatants containing sugars, amino acids, phenols, flavonoids, and starch obtained after each step are discarded and proteins are extracted with NaOH solution. Thus, in protein extraction method huge quantities of organic solvents are utilized and discarded. However, the present sequential extraction method, if followed for extraction one can save organic solvents and utilize least amount sample for extraction.

Data with respect to chlorophyll pigments and carotenoids extracted with sequential extraction method and routine method given in table-1 indicate that *T. cordifolia* leaf has substantial amounts of these pigments. It has been stated that regular intake of chlorophyll keeps digestive and circulatory system healthier. Chlorophylls are mainly used in food industry as natural colorants as well as to give green colour to alcoholic drinks. Rajalaksmi and Banu (2014) reported the antioxidant activity of chlorophyll derivative i.e. chlorophyllin extracted from *Mimosa pudica* leaves. Recently Durgadevi and Banu (2015) studied antioxidant activity of chlorophylls isolated from some plants and reported that *Phyllanthus emblica* chlorophyll has better activity than ascorbic acid.

Data regarding total phenols and flavonoids extracted with sequential extraction method and routine method given in table-2 indicate that *T. cordifolia* leaf has considerable quantities of secondary metabolites. Dushing and Laware (2012) studied the phyto-chemicals and antioxidant potential of Ashokarishta (a fermented wine) and ascribed antioxidant capacity of this ayurvedic wine to the presence of phenols and flavonoids. It was proposed that self generated ethyl alcohol in such ayurvedic wines, extracts and preserves active compounds for longer time for human consumption. Thus, extraction of chlorophylls, carotenoids, sugars, free amino acids along with phenols and flavonoids in ethyl alcohol can be safer way in utilization of these bio-molecules in human diet.

**Table.1** Methods of extraction and estimates of chlorophyll pigments and carotenoids

| **Leaf Pigments** | Routine extraction methods | Sequential extraction method | CD 5% |
|---|---|---|---|
| Chlorophyll a (mg $g^{-1}$) | 1.41 ±0.52 | 1.39 ±0.48 | 0.22 |
| Chlorophyll b (mg $g^{-1}$) | 0.36 ±0.08 | 0.38 ±0.04 | 0.02 |
| Chlorophyll a+b (mg $g^{-1}$) | 1.76 ±0.54 | 1.75 ±0.42 | 0.16 |
| Carotenoids (mg $g^{-1}$) | 0.34 ±0.06 | 0.35 ±0.08 | 0.04 |

Values with ± indicate standard deviation of mean; CD= critical difference

**Table.2** Methods of extraction and estimates of primary and secondary metabolites

| **Phyto-constituents** | Routine extraction methods | Sequential extraction method | CD 5% |
|---|---|---|---|
| Reducing sugars (mg $g^{-1}$) | 4.65 ±0.62 | 4.42 ±0.52 | 0.24 |
| Soluble sugars (mg $g^{-1}$) | 44.12±1.44 | 43.56 ±1.52 | 1.56 |
| Starch (mg $g^{-1}$) | 8.23 ± 0.76 | 8.14 ±0.82 | 0.84 |
| Proteins (mg $g^{-1}$) | 101.89 ± 3.12 | 102.82 ±4.13 | 2.26 |
| Free amino acids (mg $g^{-1}$) | 6.04 ± 0.82 | 5.98 ±0.66 | 0.84 |
| Total Phenols(mg $g^{-1}$) | 5.34± 0.44 | 5.36 ±0.65 | 0.72 |
| Total flavonoids (mg $g^{-1}$) | 2.58 ±0.18 | 2.36 ±0.19 | 0.28 |

Values with ± indicate standard deviation of mean; CD= critical difference

Data regarding sugars, starch, proteins and free amino acids, given in table-2 point out that *T. cordifolia* leaf has substantial quantity of proteins and amino acids along with carbohydrates. According to WHO technical report series no.935 (2007) efficient dietary provision of protein is must along with energy providing bio-molecules for normal cellular and tissue function. A diet with adequate amino acid is essential to fulfil the demand for protein synthesis and other metabolic pathways in a healthy individual.

The metabolic profile carried out in present investigation by extracting the sample with conventional methods and by sequential extraction method is at par. This indicates that one can save solvents and material, which can be used for multiple extractions and same quality of results. Therefore, we conclude that the sequential extraction method can be suitable for extraction of chlorophyll pigments, carotenoids, primary metabolites as well as some secondary metabolites like phenols and flavonoids to compare metabolite profile of medicinal plants and elucidate pharmacologically important components.

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