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Research Article

ANALYSIS OF CAROTENOID PIGMENTS EXTRACTED BY COLUMN CHROMATOGRAPHY FROM THE LEAVES AND FLOWERS OF *PELTOPHORUM PTEROCARPUM* BY THIN LAYER CHROMATOGRAPHY, MASS SPECTROMETRY AND NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Jean Tony Amalya J* and Judia Harriet Sumathy V

Postgraduate & Research Department of Biotechnology Women's Christian College, Chennai – 600 006

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*Corresponding Author: **Jean Tony Amalya ***
Postgraduate & Research Department of Biotechnology Women's Christian College, Chennai – 600 006, Contact Numbers: 044 – 28231676, 9710352658

ABSTRACT

Nature is our greatest medicine cabinet. It has provided mankind with numerous cures even for deadly diseases. Still there are so many cures that lie untapped in earth's ecosystem and many researches are being done in order to find them. *Peltophorum pterocarpum* is a very common deciduous tree grown in tropical countries and known by a variety of names such as Yellow Poinciana, Golden Flame, Copper pod, Rusty shield bearer and Yellow flamboyant. The plant is native to tropical southeastern Asia and northern Australia and widely grown in Sri Lanka, Thailand, Vietnam, Indonesia, Malaysia, Papua New Guinea, Philippines and the islands of the coast of Northern Territory, Australia. The present study involves the extraction of carotenoid pigments from the leaves and flowers of *Peltophorum pterocarpum* by Column chromatography and analysis of their pigments by Mass Spectrometry and Nuclear Magnetic Resonance Spectroscopy.

Keywords: Nature, *Peltophorum pterocarpum,* carotenoid pigments, Mass Spectrometry and Nuclear Magnetic Resonance Spectroscopy.

INTRODUCTION

Peltophorum pterocarpum is a very common deciduous tree grown in tropical countries. This upright, handsome, spreading, semi evergreen tree has a rounded canopy and is capable of reaching 50 feet in height with a 35 to 50-foot spread. Form can be quite variable from tree to tree, unfortunately, eliminating this plant from the palette of many architects (**Figure 1**). With proper training and pruning in the nursery and in the landscape, a more uniform crown will develop[10]. The dark green, delicate, feathery leaflets provide a softening effect for the tree's large size and create a welcoming, dappled shade[1]. From May through September, the entire tree's canopy is smothered with a yellow blanket of flowers, appearing in showy, terminal panicles and exuding a delicious, grape-like perfume. These flower clusters are followed by four-inch-long seed pods which ripen to a brilliant, dark, wine-red[2].

Figure 1: Tree, flowers and leaves of *Peltophorum pterocarpum*

Leaves are large, 30-60 cm long, with 8-10 pairs of pinnae each bearing 10-20 pairs of oblong leaflets which are 0.8-2.5 cm long with oblique bases (www.worldagroforestry.org). Flowers are orange-yellow, each about 2.5 cm in diameter, fragrant, particularly at night; inflorescence is brown-tomentose and the panicles are terminal with rust-coloured buds. Fruits are 1-4 seeded pods, flat, thin, winged, 5-10 cm long, dark red when ripe, then turning black. *Peltophorum pterocarpum* has a deep root system[11]**.** The specific epithet 'pterocarpum' alludes to its winged seed. Flowering occurs from March-May, although sporadic flowering may occur throughout the year (particularly in young trees), and a second flush of flowers may occur in September-November[6]. Under natural conditions, *P. pterocarpum* is a lowland species, rarely occurring above an altitude of 100 m. It frequently grows along beaches and in mangrove forests, especially the inner margins of mangroves (www.ncbi.nlm.nih.gov/pubmed). The species prefers open forest conditions. *Peltophorum pterocarpum* will grow in tropical climates with a dry season of 1-3 months. The tree prefers light to medium free draining alkaline soils although it tolerates clay soils[7] **(**www.worldagroforestry.org). The taxonomic classification of *Peltophorum pterocarpum* is given below in **Table 1.**

Table 1: Taxonomic Classification

Kingdom	Plantae
Sub-kingdom	Tracheobionta
Super-division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Sub-class	Rosidae
Order	Fabales
Family	Fabaceae
Sub-family	Caesalpinioidae
Genus	Peltophorum
Species	*P. pterocarpum*
Binomial Name	*Peltophorum pterocarpum* (DC.) Baker ex K. Heyne

CAROTENOIDS

Carotenoids are an abundant group of naturally occurring pigments. They occur ubiquitously in all organisms capable of conducting photosynthesis[3]**.** Carotenoids are important constituents of photosynthetic organelles of all higher plants, mosses, ferns and algae (**Figure 2**). They are also found in photosynthetic membranes of phototropic bacteria and cyanobacteria. More than 600 different carotenoids from natural sources have been isolated and characterized (www.upb.pitt.edu). Carotenoids consist of 40 carbon atoms (tetraterpenes) with conjugated double bonds[12]**.** They consist of 8 isoprenoid units joined in such a manner that the rearrangement of isoprenoid units is reversed at the centre of the molecule so that the two central methyl groups are in a 1, 6 position and the remaining non terminal methyl groups are in a 1,5-position relationship[5]**.** Carotenoid hydrocarbons are called carotenes and their derivatives containing oxygen are called xanthophylls. Because of the extensive double bond system in the carotenoid molecule, a carotenoid can exist in a large number of geometric isomers (cis/trans isomers). Most carotenoids are, in fact, found to be in the all-trans form, but cis isomers do exist (www.nature.com). The most obvious structural feature of a carotenoid molecule is the chromophore of conjugates double bonds which, in carotenoids of plant tissues, varies from three in the colourless phytoene to 13 in canthaxanthin, which is red. This double bond system also renders them susceptible to isomerization and oxidative degradation[4]**.** Leaf Carotenoids accumulate in the photosynthetic tissues of all higher plants. Both carotenes and xanthophylls are found in leaves with the same four major carotenoids , β- carotene, lutein, violaxanthin and neoxanthin[9]. Minor components include α-carotene, β-cryptoxanthin, zeaxanthin, antheraxanthin and lutein 5,6 epoxide. During leaf senescence the chloroplast disintegrates and esterification of the xanthophylls occurs. The carotenoids of flower petals can be divided into three main groups: a)highly oxygenated carotenoids such as auroxanthin and flavoxanthin b) carotenes, sometimes in high concentrations, ex: β-carotene in Narcissus and c) species specific, ex: Crocetin from Crocus. Flower carotenoids are frequently esterified (www.encyclopedia.com). Some of the structures of the typical carotenoids present in higher plants are given in **Figure 2.**

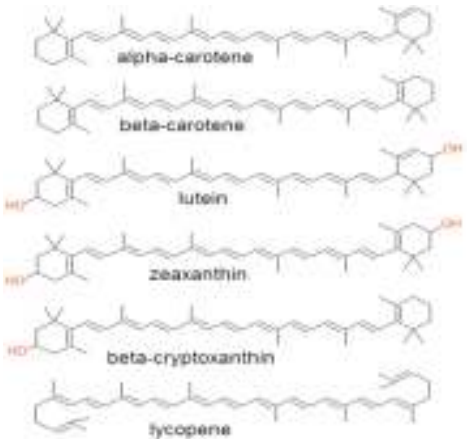

Figure 2: Structure of some typical carotenoids present in higher plants

MATERIALS AND METHODS

Isolation of Carotenoid Pigments From *Peltophorum Pterocarpum* By Column Chromatography

Column chromatography involves the separation of compounds based on the differences in partitioning between mobile and stationary phases. The stationary phase is placed in a support through which the mobile phase is passed. The stationary phase serves as an adsorbent. Several types of interactions can aid in developing the desired separation[8]**.** Plant material (leaves and flowers of *P. pterocarpum*), mortar and pestle, acetone, centrifuge tubes, hexane, distilled water, burette, cotton, silica gel are the following materials required. About 10g of leaves are weighed, washed of impurities and dried in air. The leaves are ground in a mortar and pestle by adding acetone little by little. The leaves are ground until a fine paste is obtained. Now the mixture along with acetone is transferred to the centrifuge tube. The mortar and pestle is rinsed with acetone and again the mixture is transferred to the centrifuge tube. 5 ml of hexane is added to the centrifuge tube and capped and the mixture is shaken thoroughly. Then 5 ml of distilled water is added and shaken thoroughly with occasional venting. The mixture is centrifuged to break the emulsion. The pigment layer is the top hexane layer, which should be dark green. Most of the acetone will dissolve in the water. The pigment layer is carefully pipetted out into a clean test tube. The dark green hexane solution of the leaf pigments in the test tube may contain traces of water that must be removed before separating the components through column chromatography. To dry the solution 0.5g of anhydrous sodium sulphate is added to the hexane solution. Sodium sulphate is allowed to contact all parts of the hexane solution. After standing for 5 minutes, the liquid is transferred to another clean test tube. This extract is used for column chromatography.

COLUMN CHROMATOGRAPHY

A dry and clean burette is clamped onto a burette stand and a very small plug of cotton is pushed to the bottom of the burette. 15g of silica gel is taken and mixed well with 100% hexane in a slurry like consistency and the mixture is poured into the burette carefully. The silica gel must be kept wet with solvent all the time. Now the set up is allowed to stand for an hour without disturbance so that the silica gel gets packed well. After an hour, the solvent is drained till it reaches a little above the silica gel. Then the flower/leaves extract is poured gently into the burette. As the extract drains into the silica gel, the pigments begin to separate into a yellow colour carotene band and a green colour chlorophyll band. The pigment of interest here is the yellow carotenes. So, the yellow carotene band is eluted with 100% hexane and the eluant is collected separately. The carotenes are light sensitive and so care must be taken to protect the pigments from light. The fractions collected are evaporated and the thick concentrated carotene fractions is used for TLC.

QUANTIFICATION OF CAROTENOIDS

The total carotenoid content was calculated and the absorbance of the extracted carotenoids was measured at 450 nm. The extracted carotenoids was quantified by the following formula:

Total carotenoid content (µg/g) = A x V (ml) x 10^4 / $A^{1\%}$cm x W (g)

Where A is the absorbance of the carotenoid pigment at 450 nm, V is the total extract volume, $A^{1\%}$cm is the absorption coefficient of β carotene in hexane (2600), W is the sample weight.

THIN LAYER CHROMATOGRAPHY

Silica gel TLC plates are cut as per need. A horizontal pencil line about 1cm from the bottom of the TLC plate is drawn gently. The crude extract, the yellow carotene pigment and the standard are spotted in the horizontal line with the help of a capillary tube. The spots in the plate are dried and the plate is then carefully placed in a chromatography jar containing the developing solvent (hexane and acetone in the ratio of 6:4). The solvent is allowed to rise until it reaches 75% of the plate. The plate is taken out and the solvent front (the farthest reach of the solvent) is marked immediately. The spots that are yellow in colour are also marked. The Rf values are calculated using the formula below:

$$\text{Rf Value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

The purified carotenoid extracts are further analysed by Mass Spectrometry and Nuclear Magnetic Resonance Spectroscopy inorder to determine the structure and molecular weight of the carotenoids and also help in the identification of the carotenoid pigments.

MASS SPECTROMETRY (MS)

MS consists of degrading trace amounts of an organic compound and recording the fragmentation pattern according to mass. The sample vapour diffuses into the low pressure system of the mass spectrometer where it is ionized with sufficient energy to cause fragmentation of the chemical bonds. The resulting positively charged ions are accelerated in a magnetic field which disperse and permits relative abundance measurements of ions of given mass to charge ratio. The resulting record of ion abundance versus mass constitutes the mass spectral graph, which thus consists of a series of lines of varying intensity at different mass units[4]**.** The technique works successfully with almost every type of molecular weight plant constituent. The compounds which are too involatile to vaporize in the MS instrument are converted to trimethylsilyl esters, methyl esters or similar derivatives.

NMR SPECTROSCOPY

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical chemistry technique used in research for determining the content and purity of a sample as well as its molecular structure. The principle behind NMR is that many nuclei have spin and all nuclei are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy level (generally a single energy gap). The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy is emitted at the same frequency. The signal that matches this transfer is measured in many ways and processed in order to yield an NMR spectrum for the nucleus concerned. The spectra of the leaf and flower carotenoid pigments were recorded on a Bruker AVANCE III 500 MHz (AV 500) multi nuclei solution NMR Spectrometer. The samples were dissolved in an inert solvent – deuterochloroform. The different chemical shifts according to their molecular environments within the molecule

were measured in the NMR apparatus relative to a standard, tetramethylsilane (TMS).

RESULTS AND DISCUSSION

Isolation of Carotenoid Pigments by Column Chromatography

Figure 3: Isolation of pigments by Column Chromatography

Carotenoid pigments were effectively separated from the leaves and flower extracts separately in a silica gel column with 100 % hexane. The yellow band that separates when eluted with 100% hexane is identified to be the carotenoid pigments. Thus the carotenoid pigments with hexane was collected and stored in glass vials at -20^{o}C (**Figure 3).**

QUANTIFICATION OF CAROTENOIDS

The absorbance of the extracted carotenoids was measured at 450nm. The extracted carotenoids was quantified by the following formula:

Total carotenoid content (µg/g) = A x V (ml) x 10^4 / $A^{1\%}$cm x W (g)

Where A is the absorbance of the carotenoid pigment at 450 nm, V is the total extract volume, $A^{1\%}$cm is the absorption coefficient of β carotene in hexane (2600), W is the sample weight.

Thus,

Total carotenoid content in leaves = $0.231 \times 10 \times 10^4 / 2600 \times 10$

$= 0.88 \mu g/g$

Total carotenoid content in flowers = $0.145 \times 10 \times 10^4 / 2600 \times 10$

$= 0.56 \mu g/g$

THIN LAYER CHROMATOGRAPHY

Figures 4 & 5: Thin Layer Chromatography for Leaf and Flower Extracts

The crude leaf and flower extracts, the purified carotenoid pigments and the standard are subjected to thin layer chromatography (**Figures 4 & 5).** The standard used was beta carotene. The mobile phase used was hexane and acetone in the ratio of 6:4. Their respective Rf values are calculated as below:

$$\text{Rf value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Rf value of the leaf carotenoid pigment = 4.8/5.0 = 0.96
Rf value of the flower carotenoid pigment =3.1/3.4 = 0.91
From the thin layer chromatography it was confirmed that beta carotene is the carotenoid pigment present in both leaves and flowers of *Peltophorum pterocarpum*.

MASS SPECTROMETRY

Mass spectrometry can provide the molecular weight of the compound of interest. When the vaporised organic sample passes into the ionisation chamber of a mass spectrometer, it is bombarded by a stream of electrons. These electrons have a high enough energy to knock an electron off an organic molecule to form a positive ion. This ion is called the molecular ion. The molecular ions tend to be unstable and some of them break into smaller fragments. These fragments produce the familiar stick diagram. In the mass spectrum, the heaviest ion (the one with the greatest m/z value) is likely to be the molecular ion. A few compounds have mass spectra which don't contain a molecular ion peak, because all the molecular ions break into fragments (http://www.chemguide.co.uk/analysis/masspec/mplus.html).
The mass spectrum of the carotenoid pigments are given below.
In the mass spectrum of the Leaf Carotenoid pigment (**Figure 6)**, the heaviest ion has an m/z value of 536.87. Because the largest m/z value is 536.87, that represents the largest ion going through the mass spectrometer - and it is reasonably assume that this is the molecular ion. The relative formula mass of the compound is therefore 536.87**.**
In the mass spectrum of the Flower Carotenoid pigment (**Figure 7)**, the heaviest ion has an m/z value of 536.87. The relative formula mass of the compound is therefore 536.87. The molecular weight of beta carotene is 536.90. Thus, the carotenoid pigment isolated from the leaves and flowers of *Peltophorum pterocarpum* is found to be beta carotene**.**

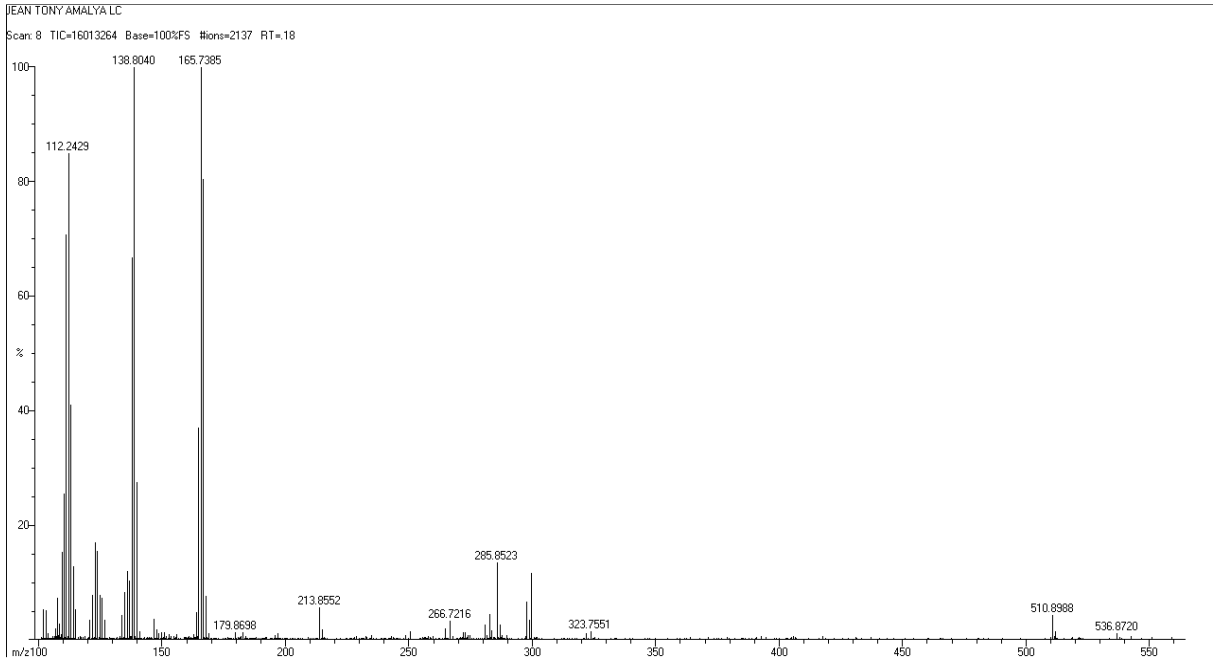

Figure 6: Mass Spectrum of the Leaf Carotenoid pigment

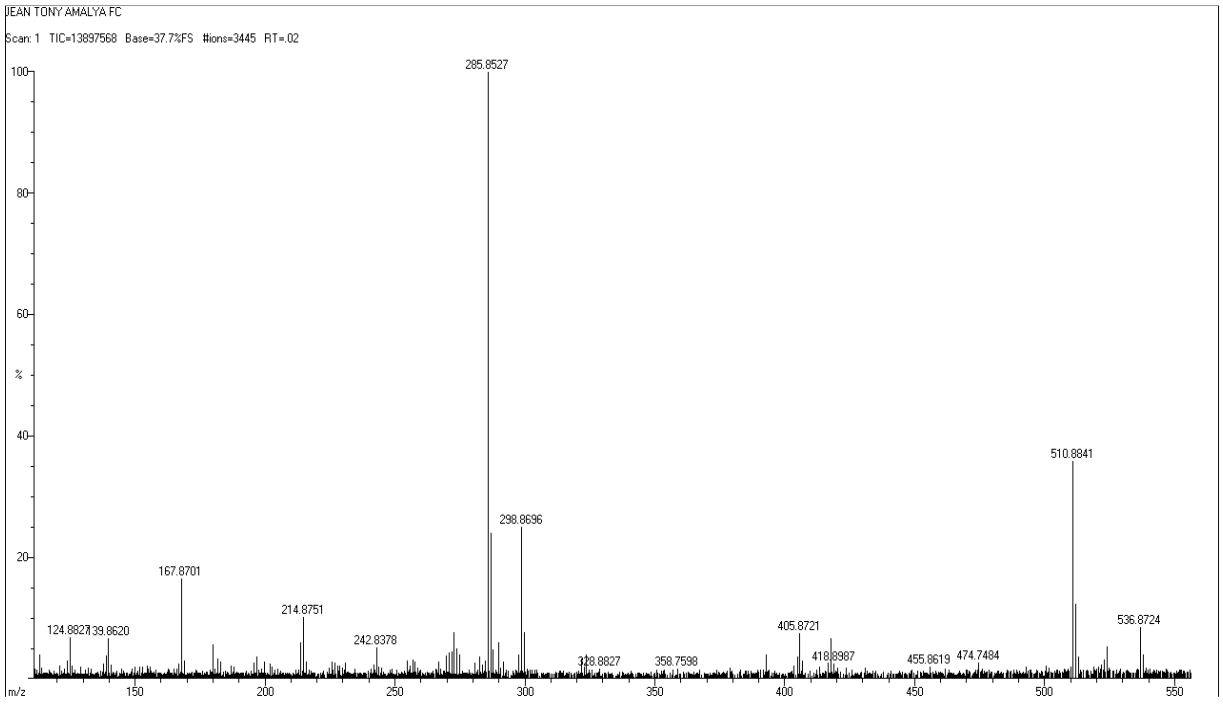

Figure 7: Mass spectrum of the flower carotenoid pigment

NMR ANALYSIS

The spectra of the leaf and flower carotenoid pigments were recorded on a Bruker AVANCE III 500 MHz (AV 500) multi nuclei solution NMR Spectrometer (**Figure 8**). The ^{13}C NMR Spectra of the Leaf carotenoid **(Figure 9)** and the flower carotenoids were found in correlation with the ^{13}C NMR Spectra of β – carotene.

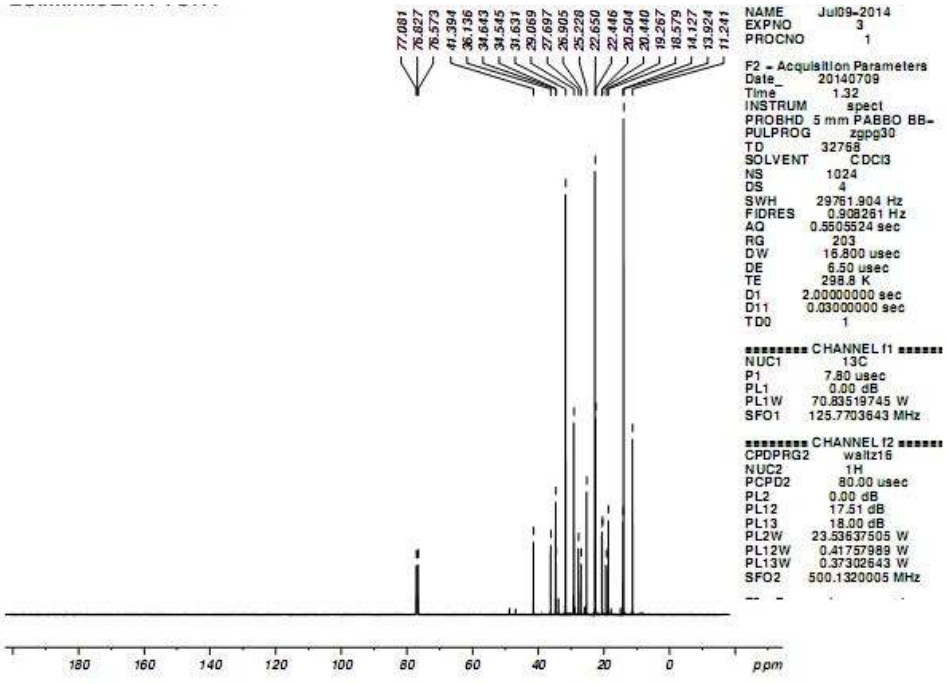

Figure 8: ^{13} C NMR of Leaf Carotenoid

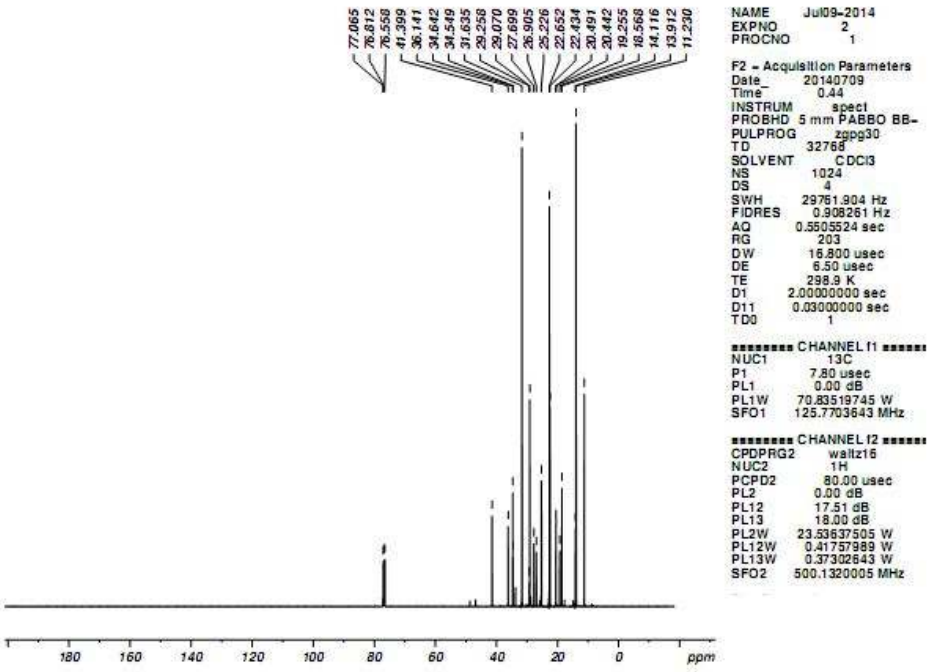

Figure 9: ^{13} C NMR of Flower Carotenoid

CONCLUSION

Carotenoids were extracted from the leaves and flowers by Column chromatography and were subjected to TLC. The pigments were analysed by Mass Spectrometry and Nuclear Magnetic Resonance Spectroscopy to analyse the carotenoid pigment **β- carotene** present in the leaves and flowers. It is interesting to note Carotenoids also play a very important role in human health. They are known to be very efficient physical and chemical quenchers of singlet oxygen (O_2), as well as potent scavengers of other reactive oxygen species (ROS), thus acting as very important natural antioxidants. This is of special significance, because the uncontrolled generation and concomitant increase of ROS level in the body results in "oxidative stress", an essential contributor to the pathogenic processes of many diseases. They are suggested to play a protective role in a number of ROS-mediated disorders, such as, *i.e.*, cardiovascular diseases, several types of cancer

or neurological, as well as photosensitive or eye-related disorders. Carotenoids are also suggested to participate in the stimulation of the immune system, the modulation of intracellular signaling pathways (gap junction communication), the regulation of the cell cycle and apoptosis, the modulation of growth factors, cell differentiation and the modulation of various types of receptors or adhesion molecules. Thus future studies can be undertaken to study the multi-medicinal value of these carotenoids pigments.

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