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Research Article

ISOLATION AND IDENTIFICATION OF PIGMENT MOLECULES FROM LEAVES OF *PROSOPIS JULIFLORA*

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ABSTRACT
Crude pigments were extracted from fresh leaves of *Prosopis juliflora* by use of ethanol. It was subjected to repeated column chromatography for further purification, with silica as stationary phase and petroleum ether, ethyl acetate and methanol as mobile phase. At each step of separation purity was checked by thin layer chromatography and HPTLC analysis. Identification of the compound was done by UV-visible spectrometry and ^{1}H NMR technique and then comparing these data with the data present in the chemical libraries. The pigments isolated from the leaves of *P.juliflora.,* include, two carotene, three xanthophylls and two pheophytin.
KEYWORDS: pigment, chromatography, ^{1}H NMR technique, carotens, xanthophylls, pheophytin

INTRODUCTION

A pigment is a molecule that absorbs and reflects light. The broad array of colors found in plant tissues such as leaves, flowers, and fruits, can be accounted for by the presence of thousands of different kinds of plant pigments. Chlorophylls and carotenoids have been considered to be responsible for the color of green plants[1,2,3]. Other Chlorophyll derivatives such as pheophytins are not widely present in fresh green plants[4], but their content increases during processing[5]. The main cartenoids present in green plants are lutein and carotene[6,7], but other carotenes, such as neoxanthin and violaxathin, may also be present[8,9].

Chlorophylls are the pigments of photosynthesis. Chlorophylls are found in living organisms almost exclusively as chlorophyll-proteins complexes. Chlorophylls are of interest to agriculture and ecology, where they are indicators of the health status of individual plants and communities, and are often used as a quantitative reference in physiological research. They are also permitted as food colors[9]. Chlorophylls have recently attracted interest as phototherapeutic drugs[10].

Carotenoids are tetraterpenoid organic pigments that are naturally occurring in the chloroplasts and chromoplasts of plants[11] They are split into two classes, xanthophylls (which contain oxygen) and carotenes (which are purely hydrocarbons, and contain no oxygen). They serve two key roles in plants, they absorb light energy for use in photosynthesis, and they protect chlorophyll from photo damage. Currently there is a substantial research effort in place to explore the potential health benefits of carotenoids to humans. They are known to have antioxidant properties and many lines of research suggest that consuming a diet rich in plant pigments may slow the process of cellular aging and reduce the risks of some types of disease, such as cancer, heart disease, and stroke[12].

Thus in view of importance of these plant pigments, a plant species, *Prosopis juliflora*, found in semi arid regions of rajasthan was subjected for isolation and identification of the pigments present in leaf.

MATERIALS AND METHODS

Plant material: Plant leaf was collected from their natural habitat in the Shekhawati regions of Rajasthan, India. The plant was identified with the help of "Flora of Rajasthan"[13]. Leaves were washed thoroughly under tap water and then air dried under shade for two weeks and oven dried for 24 hrs at 40°C. The dried plant material was grounded to form fine powder and filtered through sieve of 345 micron pore size.

Extraction of pigments: Five hundred grams of dry powder was taken in a beaker and ethanol was added to it so that the plant material gets totally immersed in the solvent. This whole setup was kept for 48 hours with frequent shaking. It was first filtered with a muslin cloth, then with whatman filter paper (No.1) and finally centrifuged at 5000 rpm for 5 mins. Whole process was repeated 3 times and supernatant were collected and pooled together. The supernatant collected were concentrated to 1/20th of their initial volume with the help of rotary evaporator (Buchi Rotavapor R-200/205).

The crude ethanol extract of leaf was further subjected to purification. Separation of the extract was achieved by repeated column chromatography using silica (60:120) as stationary phase and petroleum ether (PE)/ethyl acetate (EA) /methanol (M) as mobile phase. Following gradient of solvent system was used for the initial separation, PE (100%), PE:EA (3:1), PE:ET (1:3), EA:M (3:1), M (100%). At each step of separation purity was checked by thin layer chromatography and HPTLC analysis. Identification of the compound was done by UV-visible spectrometry and ^{1}H NMR techniques and then comparing these data with the data present in the chemical libraries.

Identification techniques: The UV–visible spectra were recorded on a Jasco V-630 spectrophotometer with 1 cm matched quartz cuvettes. The absorbances were read at 200-700 nm against the blank containing respective solvents. The ^{1}H (300 MHz) NMR (Nuclear magnetic resonance) spectra was recorded on a Varian XL-300 NMR spectrometer. For ^{1}H NMR, 4 mg of sample was dissolved in an inert solvent deuterochloroform (CDCl3), or deuterated acetone The different chemical shifts of the proton according to their molecular environments within the molecule were measured in the NMR apparatus relative to a standard, tetramethyl silane (TMS).

RESULTS AND DISUSSION

Pigments are non polar compounds and hence are extracted better by non polar solvents[14]. The ethanol extract was first separated by PE, the fraction eluted first was an orange yellow color extract which contained 3 compounds as

determined by TLC. The orange fraction was further subjected to silica gel purification using PE:EA (9:1) as eluting solvent which resulted in the isolation of 2 compounds (designated as compound 1and 2).

When the solvent system was changed to PE:EA (3:1), 12 fractions were eluted, which were again repeatedly subjected to silica gel purification. Fractions were analyzed by TLC and those containing common compounds were pooled together and eluted with a gradient of solvent system containing PE and EA. This separation resulted in isolation of various compounds of different colours , 2 yellow colored compounds (designated as compound 3 and 4), light yellow grey (designated as compound 5), one dark gray compound (designated as compound 6) and dark brown (designated as compound 7). Purity of all the compounds were checked by TLC and HPTLC analysis.

Compound 1 – was obtained as red – orange color compound, purity of the compound was checked by HPTLC analysis, which showed a single spot. It was found to be soluble in more non polar solvents, such as ether and chloroform. UV-visible absorption spectra showed maximum absorption at 425, 450, 477 nm and a small peak at 675, characteristic of beta carotene[15].

^{1}H NMR spectra, showed signals for the olefinic region of beta carotene between 6.0–7.0 ppm, however the signals in this region were very weak. Strong signals of CH_3 and CH_2 of acyl chains between 0.9 and 3.0 ppm were found. Three methyl signals were observed at δ 1.25, 2.03 and 2.29. Protons of CH_2 group of cyclohexene was shown as a broad area between 1.59-1.97 ppm.

Compound 2 - was also an orange-red colour compound that appeared as a single spot on TLC plate, HPTLC analysis confirmed the purity of this compound. UV-Visible spectra showed maximum absorbtion between 400-450 nm. Based on the above characteristics, this compound was identified as a carotene.

It was subjected to ^{1}H NMR analysis for further identification. Proton signals were found to be identical with beta carotene, with some minor variations. Characteristic olefinic protons signals were observed in the region between 6-7 ppm. Signal for a hydroxyl proton were observed at 5.36 ppm, which differs from beta carotene, indicating the presence of a new compound. Three signals for CH_3 protons were seen at δ 1.25, 2.03 and 2.29. Signal for CH_2 groups present in cyclohexene ring was spread between 1.9-1.4 ppm, with a strong signal at 1.59 ppm.

Compound 3–was obtained as yellow coloured compound and appeared as a separate spot on TLC plate. The maximum UV-visible absorption was found at 414, 435, 470,664 nm, which is characteristic of lutein[15,16].

Proton NMR showed oleifinic region between 5.8-7 ppm, whereas signal for CH_3 protons were observed at δ 0.99, 1.29 and 2.22. Signal at 3.5 ppm corresponds to hydrogen present in CH group. Signal for CH_2 group of cyclohexene was present at 1.84 ppm and between 1.60-1.68 ppm. Strong signal of OH group was observed at 4.09 ppm.

Compound 4 - was a yellow grey coloured pigment, and showed absorbtion at 418, 435, 470,664 nm similar to xanthophylls. Proton NMR confirmed the presence of xanthophylls, as signal of hydroxyl group was observed at 4.14 ppm. Signal of oleifinic protons were very weak, probably due to the presence of deuterium in solvent chloroform, which might have replaced the proton. Signal for CH_3 protons were observed at δ 0.88, 1.26 and 2.23, whereas. Signal for CH_2 group of cyclohexene were found between 1.58-1.65 ppm. Two other strong signals at 2.17 and 2.05 ppm were observed, which indicates the presence of a different form of xanthophylls.

Compound 5- was yellow in colour with almost 95% purity, as confirmed by HPTLC analysis and showed similar absorption spectra, as that of lutein. Olefinic signals in proton NMR were observed between 6-7 ppm and signal for CH_3 protons were observed at δ 0.99, 1.24, 2.23 and signal at 3.5 ppm corresponds to hydrogen present in CH group. Signal for CH_2 group of cyclohexene at 1.84ppm and between 1.60-1.68 ppm. Strong signal of OH group were observed at 4.09 ppm. Hexadiene signal was observed at 5.04 and 5.35 ppm.

This compound was identified as anhydroleutin. Main difference between them is, lutein contains 2 hydroxyl group, whereas anhydrolutein contains one and this difference was evident while observing its proton signals.

Compound 6 and 7- Compound 6 was obtained as dark brownish-green compound, whereas compound 7 was obtained as grey colour coumpound. The HPTLC analysis, showed that both the compounds are present as single spot with almost 98% purity. Compound 6 showed major diphasic peaks at 410 and 667 nm together with minor peaks at 505, 534 and 610 nm indicating it to be a chlorophyll derivative and compound 7 exhibited major peak at 435 nm with a shoulder of 415 nm and another major peak at 655 nm with minor peaks at 530, 560 and 605 nm[4]. These absorptions indicated that compound 6 is pheophytin a and compound 7 is pheophytin b. Further confirmations were done by analyzing their proton NMR spectra by comparing with the data present in literature[17].

The ^{1}H-NMR spectrum of compound 6 exhibited signals for three aromatic methine groups [δ 9.76, 9.52 and 8.94], one conjugated vinyl group [δ 7.97, 6.31) and 6.16], three aromatic methyl groups [δ 3.90, 3.42 and 3.23], two methyl ester groups [δ 3.59 and 3.52], one ethyl group [1.68], two amino group protons [δ 1.10 and 1.37], one methoxy group [δ 3.91], and one downfield shifted methyl group [δ 1.59].

Compound 7, along with the common signals of compound 6, also revealed the pheophytin b type characteristics, showing signals for aldehyde proton at δ 10.25 and two aromatic methyl groups [δ 3.75, 3.27].

In the phytyl chain, signal for intermediate CH group at 1.65 ppm, intermediate CH_2 group at 1.25 ppm and side chain CH_3 group at 0.96 ppm was observed.

Among pigment molecules, two beta carotene, three xanthophylls and two pheophytin are extracted from the leaves of *P.juliflora.* These carotenoids are reported to have many physiological functions[18] and hence the leaf of this plant can serve a good source for isolation of these pigment molecules.

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