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# Extraction, separation and identification of carotenoids from the pulp of *Pseudospondias microcarpa* (A. Rich) Engl. red fruits using TLC and UHPLC-APCI-MS/MS

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***Abstract:***
***Background****: The fruits of Pseudospondias microcarpa (A. Rich) Engl. are highly prized by the rural population of northern Congo for their sweet and tangy flavour, as well as their sweet aroma. Studies have shown that the red colour of these fruits is due to carotenoids, but no in-depth study has been carried out in this regard. The aim of this study was therefore to characterise the red pigments in the pulp of this fruit.*
***Materials and methods:*** *Fresh fruit pulp was dried in the dark and then ground to a fine powder for pigment extraction using the conventional Soxhlet method. The resulting pigment extract was fractionated by preparative thin-layer chromatography (TLC) and analysed by analytical TLC, UV-Vis spectrophotometry and UHPLC-APCI-MS/MS on a C18 reversed-phase column.*
***Results****: A red extract was obtained with a yield of 8%, rich in pigment at a concentration of 317.11 ± 0.13 mg of carotenoids per gram of dry extract. The chemical profile revealed eleven carotenoids, comprising two carotenes and nine xanthophylls, with concentrations ranging from 0.041 to 0.213 mg/ml. Fifteen compounds were identified, including eleven carotenoids, namely phytoene, α- and β-carotene, canthaxanthin, astaxanthin, cryptoxanthin, zeaxanthin, lutein and violaxanthin.*

***Keywords****: Pseudospondias microcarpa; carotenoids, TLC; UHPLC-APCI-MS/MS; UV-Vis spectrophotometry.*

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## I. Introduction

Carotenoids are natural pigments responsible for most of the red, orange and yellow hues found in the leaves, fruits and flowers of plants, as well as for the colours of certain birds, insects, fish and crustaceans. To date, more than 850 carotenoids have been identified in nature (T. Maoka, 2020)[1], of which approximately 40 are used in the typical human diet and 20 are found in human blood and tissues. Several carotenoids act as precursors to vitamin A, which is an effective antioxidant and important for human nutrition (A. Pintea et al., 2020)[2].
In 2019, the global market for these compounds was worth US$1.44 billion and is projected to reach US$1.84 billion by 2027 (T. Brendler et al., 2019)[3]. Consequently, much of the ongoing research has focused on identifying the main sources of carotenoids for use in dietary supplementation, colouring in cosmetics, and the treatment of age-related eye diseases, cancers and skin conditions. Their absorption in the visible spectrum means they can be easily separated and quantified using cost-effective methods such as spectrophotometry and analytical and preparative thin-layer chromatography (L. Novoveskà et al., 2019, F. Nabi et al., 2020, J.L. Rowles III et al., 2020, A.J. Meléndez-Martinez et al., 2022)[4,5,6,7].

Naturally, these compounds are widely found in the red, yellow and orange fruits of the Anacardiaceae family, such as mango, cajà and mahogany apple (Maldonado-Celis et al., 2019; Schweigger et al., 2016)[8,9,]. This family

also includes *Pseudospondias*, which comprises only two species (*Pseudospondias longifolia* and *Pseudospondias microcarpa*) that produce red fruits when ripe. The fruits of *P. microcarpa* are consumed in various regions of Africa and used as fish bait. Chemical studies conducted on this fruit have shown that the essential oils derived from the epicarp, the husk, the stone and the seed contain mainly α-terpineol and borneol in the epicarp (22.9 per cent and 8.2 per cent), vaccenic acid and 2,6-dihexadecanoate of ascorbic acid in the husk (20.1 per cent and 29.8 per cent), caryophyllene oxide and α-humulene in the seed (8.4 per cent and 6.8 per cent), α-humulene and β-caryophyllene in the kernel (9.4% and 6.4%). The raw juice obtained is moderately acidic (pH 3.28), very cloudy and coloured (turbidity of 1,019 NTU).

It has a dry matter content of 3.771 per cent ± 0.013 per cent. Energy-providing nutrients (sugars, amino acids), carotenoids, coumarins, tannins, flavonoids, terpenes and sterols have been identified. The juice contains a higher concentration of polyphenols (134.8500 ± 0.0023 mg of gallic acid/100 g) than of carotenoids (5.100 ± 0.010 mg of carotene/100 g), as well as high concentrations of macrominerals (calcium: 3,570.00 ± 2.05 mg and potassium: 4,576.00 ± 9.49 mg) and trace elements (copper: 11.13 ± 0.10 mg, iron: 3.02 ± 0.03 mg and zinc: 2.57 ± 0.08 mg). The vegetable oil obtained, with a yield of 36 per cent, is green in colour and fluid. The unsaponifiable fraction accounts for only 1.2 per cent of the fat's weight. The carotenoid content is 691.92 mg/kg of oil. Eight fatty acids were identified in the vegetable oil from fruit kernels, including three major fatty acids: oleic acid (42.56 per cent); palmitic acid (33.89 per cent) and stearic acid (14.23 per cent). Among the phytosterols, β-sitosterol was found to be the predominant compound, present at a concentration of 97.3 ± 3.4 mg/g oil out of the five sterols identified.

Analysis of the tocopherols revealed the presence of three compounds: α, ϒ and d-tocopherol, at low levels of 5.0, 4.1 and 2.7 mg/g oil respectively, in this oil extract (Nkounkou Loumpangou et al., 2018; Babouongolo et al., 2021, Babouongolo et al., 2024)[10,11]. To the best of our knowledge, the nature and quantity of carotenoids in the red fruit pulp of P. microcarpa have not yet been studied. The aim of this study is to characterise the carotenoids in the fruit pulp of the species P. microcarpa from Congo-Brazzaville using chromatographic, spectrophotometric and spectrometric methods.

## II. Material And Methods

**Collection and pre-treatment of plant material**

The plant material consisted of ripe fruits of *Pseudospondias microcarpa* (A. Rich) Engl. Approximately 50 kg of fruit were harvested along the Kouyou River in Owando, a town situated in the Cuvette Centrale department, in the north of the Republic of the Congo. The botanical identity of the fruit was confirmed at the Centre for the Study of Plant Resources (CERVE) in the Republic of the Congo, where the species is registered under number 8957 dated 7 August 1961.

The pulp was separated from the fruit and dried away from light at room temperature for one week at the Plant and Life Chemistry Unit (UC2V) within the Faculty of Science and Technology at Marien Ngouabi University. These dried pulps were ground using an electric grinder and the resulting powders were stored in jars away from light.

**Extraction of total carotenoids**

A sequential extraction was carried out using absolute ethanol (in a Soxhlet apparatus), followed by liquid–liquid fractionation with hexane. Specifically, 30.06 g of dry pulp powder was placed in a Soxhlet cartridge and inserted into the Soxhlet apparatus, which was connected to a single-neck flask containing 300 mL of ethanol and fitted with a condenser. Using a flask heater, the solvent was brought to the boil to extract the pigments until the plant material was completely depleted (disappearance of the red colour) (A.O.A.C., 1990)[12]. Aluminium foil was used to minimise the influence of light on the colour of the pigments (Rodriguez-Amaya and Kimura, 2004)[13]. The total carotenoid extract was obtained following liquid–liquid extraction with hexane from the ethanolic extract. The yield of total carotenoids was calculated relative to the dry matter mass.

**Detection of total carotenoids in the extract**

10 µL of the extract, previously dissolved in acetone, was applied to a 0.25 mm Silicagel 60 F254 chromatography plate with an aluminium support (Merck). The compounds were eluted successively using two types of eluents (Sherma and Fried, 2005)[14]. The first elution on an analytical TLC plate was carried out with 100 per cent hexane and the second with a binary hexane/chloroform mixture (5:3 v/v). Rf values were determined following visual inspection of the spots on the plate to identify the carotenoids and/or their carotenoid class using data from the literature.

**Fractionation of the extract**

100 mg of the extract, previously dissolved in acetone, was applied to a 20 × 20 cm chromatography plate consisting of 2 mm thick Silica Gel 60 F254 on a glass support (Merck). The compounds were eluted successively using the same eluents as in the analytical thin-layer chromatography (TLC). The coloured spots obtained were scraped off and the fractions (carotenoids) were recovered after filtration with acetone for the non-polar fractions and ethanol for the polar fractions (Sherma and Fried, 2005)[14].

**Determination of total carotenoids in the extract and fractions**

The total carotenoids were determined using the method described by Rodriguez-Amaya (D.B. Rodriguez-Amaya and M. Kimura, 2004). The absorbances of the solutions obtained after appropriate dilution of the extract or fraction in 10 ml of absolute ethanol were measured directly at 450 nm (the wavelength at which β-carotene exhibits maximum absorption). The total carotenoid content is determined on the basis of the linear calibration curve (y = ax + b) obtained using the β-carotene standard at different concentrations (0.50; 0.25; 0.125; 0.0625 mg/mL) under the same conditions as the sample. The carotenoid contents in the extract and its fractions are expressed, respectively, as β-carotene equivalent per gram of extract (EBC/g) and as a percentage (%) (Rodriguez-Amaya and Kimura, 2004)[13].

**Identification of the carotenoids in the extract**

Solutions of 100 µM were prepared under dim lighting by dissolving 1 mg of the total carotenoid extract. Each stock solution was diluted to a concentration of 2 µM using methanol. These solutions were analysed by UHPLC-APCI-MS/MS (Thermo Ultimate 3000 ultra-high-pressure liquid chromatography coupled with a Bruker amaZon speed MS mass spectrometer), on a reverse-phase C18 column (Phenomenex Kinetex Phenyl Hexyl 2.6 µm C18 100 A; 150 × 3 mm) over an analysis time of 25 minutes, with an m/z scan ranging from 150 to 600 in both positive and negative ion modes. The gradient mobile phase consisted of water, formic acid and acetonitrile. The initial elution solvent was WATER/ACN (95/5), changing to WATER/ACN (5/95) at 20 minutes and to WATER/ACN (0/100) at 25 minutes. The column temperature was maintained at 30 °C, the flow rate at 0.6 mL/min and the detection wavelength at 450 nm. The injection volume was 1.0 µL. The APCI operated in positive and negative ionisation mode under standard scanning conditions (scan time of 10 ms for MS and 100 ms for MS/MS). The following source parameters were used: corona discharge 4–6 µA; nebuliser temperature 400 °C; capillary temperature 300 °C; capillary voltage 45 V; tubular lens voltage 85 V. Compounds were identified by comparing the experimental mass spectra with data from the literature.

**Processing of the results**

The results were processed using Excel 2016.

## III. RESULTS

**Extraction yield**

The carotenoid extract obtained from the pulp of *P. microcarpa* fruit was a red-coloured paste (figure no 1) with a characteristic fruit odour, yielding 8.82 per cent.



**Figure no 1: Carotenoid pigment extract**

**Chemical profile of carotenoids by analytical CCM**

The various Rf values, together with the colours of the spots observed on the chromatogram in Figure no 2, are presented in Table no 1. Visual inspection of the chromatogram revealed eleven spots of varying colours (yellow, orange-yellow and red) characteristic of carotenoids. This profile would suggest a diversity or a high degree of isomerisation amongst the compounds.

**Table no 2:** Rf values and colours of carotenoids detected by CCM

| Rf | Colours | Carotenoids |
|---|---|---|
| 0,97 | Yellow | Carotene |
| 0,91 | **Yellow-orange** | |
| 0,76 | **Red-orange** | Xanthophylls |
| 0,71 | **Red-orange** | |
| 0,61 | **Red-orange** | |
| 0,53 | **Red-orange** | |
| 0,45 | **Red** | |
| 0,25 | **Yellow-orange** | |
| 0,10 | **Yellow** | |
| 0,07 | **Red** | |
| 0,04 | **Yellow-orange** | |
| *1st elution: 100% hexane*<br>*2nd elution: Hexane/$CHCl_3$ (5/3)*<br>*Observation in visible light* | | |

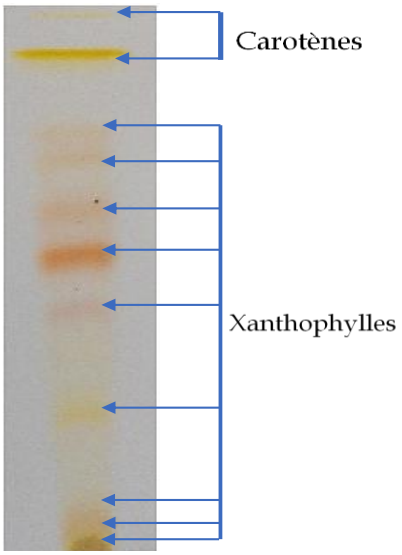



**Figure no 2: Chromatographic profile of carotenoids**

**Chemical profile of carotenoids by preparative CCM**

Analysis of these spots by preparative chromatography using the elution systems described reveals colour bands with different Rf values for the carotenes and very similar Rf values for the xanthophylls in this plant (Figure no3). Following separation and recovery using acetone or ethanol, ten fractions of yellow, red and orange colours were obtained (Figure no 4).

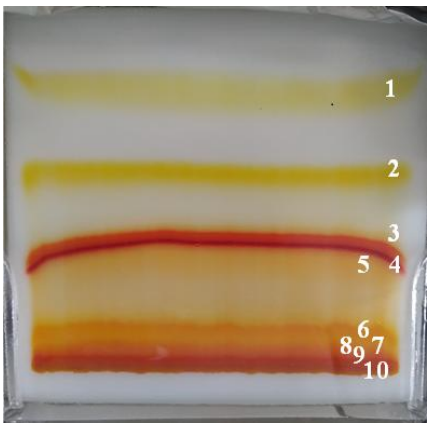



**Figure 3: Chromatographic profile of carotenoids on preparative CCM**

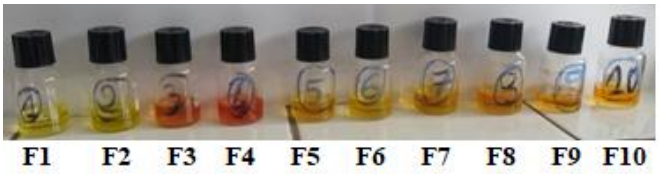



Figure 4: Colours of the ethanolic solutions of the fractions obtained

**Carotenoid content in the total extract and in the fractions**

The total carotenoid content in the extract obtained from the pulp of this fruit is 317.11 ± 0.13 mg β-carotene equivalent/g dry extract, representing 31 per cent carotenoids in one gram of dry extract. By contrast, the carotenoid contents of the individual fractions range from 6 to 17 per cent (Figure no 5). It can be seen that the fraction richest in carotenoids is F4 and the poorest is F1.

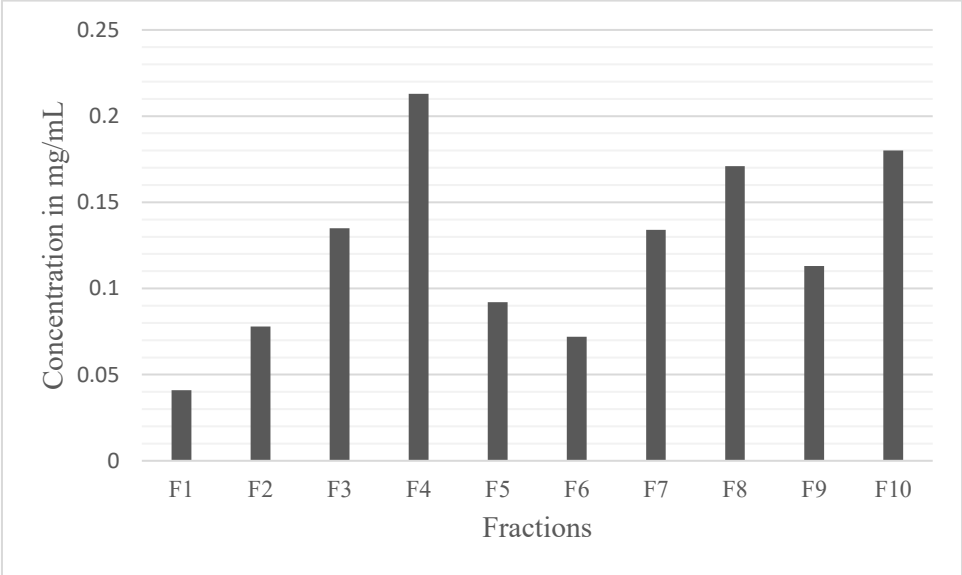



Figure no 5: Carotenoid contents of the fractions obtained from the extract

**Identification of the carotenoids detected in the extract**

The chromatographic and spectrometric data for the total carotenoid extract, obtained by UHPLC-APCI-MS/MS on a C18 reversed-phase column, are shown in Figures 5 and 6. Analysis of the chromatogram shows that fifteen (15) compounds were detected, of which thirteen (13) were identified as carotenoids, with m/z values ranging from 150 to 600 (Table no 3). According to the literature, in reverse-phase chromatography, the order of elution depends on hydrophobicity, which explains why xanthophylls elute before carotenes. Based on their polarity within the xanthophyll group, the compounds are obtained in the following order: those with polyhydroxy, hydroxy-epoxy and monohydroxy groups, followed by cetocarotenoids and esters. Whereas within the carotenoid group, cyclic carotenoids elute faster than linear carotenoids and those with fewer conjugations (phytoenes and phytofluenes) (Khachik et al., 1991; Bohoyo-Gil et al., 2012; Neto et al., 2016)[15,16,17]. By combining the order of elution, retention time, MS fragments and the analytical CCM chromatogram, peak 15 (tR = 23.3 min) corresponds to phytoene with a molecular weight of m/z = 544, corresponding to the molecular peak $[M + H]^+$ at 545 on the mass spectrum (Pintea, 2020; Lara-Abia et al., 2021; Bationo et al., 2022)[18, 19,20]. This compound is colourless in the visible spectrum, highly hydrophobic and cannot be identified on the chromatogram shown in Figure 2. Peaks 13 (tR = 19.8 min) and 14 (tR = 20.0 min) exhibit two MS spectra that are almost superimposable in negative mode. These peaks, with a molecular weight of 536, correspond respectively to those of β-carotene (peak 14) and α-carotene (peak 13), with a mass peak at m/z = 523.24 corresponding to the loss of a methyl group $[M – 15 – H]^-$. These isomers appear yellow on analytical TLC at Rf values of 0.97 and 0.91, respectively, as shown in Figure no 2 (Minyuk, 2018; Mezadri, 2004; Daood et al., 2013; Lara-Abia, 2021; Bationo et al., 2022)[20, 21,22]. Peaks 10, 11 and 12 at tR values of 19.1, 19.3 and 19.5 min, respectively, exhibit MS fragments at m/z = 578 in negative ion mode, corresponding to the loss of a hydroxyl group $[M – 18]^-$. These mass spectra do not show a molecular peak but may be attributable to the three esterified isomers of astaxanthin with a molecular weight of 596. On TLC, these compounds appear as orange spots (Figure no 2) (Pérez-Gálvez and María Roca, 2018; Van

Breemen et al., 2012; Minyuk, 2018; Lara-Abia, 2021)[19,21,24,25]. Similarly, for peaks 7, 8 and 9 at tR values of 18.4; 18.6 and 18.8 min, respectively; canthaxanthin isomers with a molecular weight of 564 are identified, with a molecular peak at 561 corresponding, in negative ion mode, to the loss of two protons $[M - 2H]^-$ and a fragment mass at m/z = 471. These compounds also appear as orange spots on CCM (Figure no 2) (Minyuk, 2018; Lara-Abia, 2021)[19,21]. Peaks 5 and 6 at retention times of 17.6 and 17.8 minutes, respectively, correspond to lutein and zeaxanthin. These isomers with a molecular weight of 568 can be identified by their molecular peaks in negative ion mode $[M]^-$ and their order of elution. They appear as yellow-orange spots (Rf = 0.25) on CCM (Mendes et al., 2007; Bationo et al., 2022)[20, 26]. Peak 4 at tR 17.0 min is characteristic of cryptosxanthin, identifiable by its mass fragment m/z = 535 in positive ion mode $[M + H - 18]^-$, corresponding to the loss of the alcohol function (Pérez-Gálvez and María Roca, 2018; Mezadri, 2004; Pintea, 2020; Bationo et al., 2022)[18, 20, 22,24]. Peak 3 at tR 16.2 min corresponds to violaxanthin, with a molecular weight of 600, which was identified on the basis of its mass fragment of 565.72, indicating the loss of two hydroxyl groups $[M - H - 36]^-$ (Mezadri, 2004; Lara-Abia, 2021; Bationo et al., 2022)[19, 20, 22]. In contrast, peaks 1 and 2 correspond to two xanthophylls exhibiting fragmentation patterns different from those of the other 13 carotenoids; no identifying features were found to elucidate their structure based on the data consulted in the literature.

Table no 3: LC-MS characteristics of the carotenoids detected in the fruit pulp extract

| N° | $t_{R\ (min)}$ | Carotenoids | Formula | MM | Importants Fragments m/z |
|---|---|---|---|---|---|
| 1 | 13,7 | Undetermined | - | - | 332.35 ; 509.28 ; 531.25; 540.16 |
| 2 | 14,6 | Undetermined | - | - | 473.27; 534.67 ; 541.23; 570.76 |
| 3 | 16,2 | Violaxanthin | $C_{40}H_{56}O_4$ | 600,870 | 249.90 ; 384.80 ; 510.77 ; 565.72 |
| 4 | 17,0 | Cryptoxanthin | $C_{40}H_{56}O$ | 552,850 | 282.22 ; 457.32 ; 509.28 ; 535.35 |
| 5 | 17,6 | Lutein | $C_{40}H_{56}O_2$ | 568,871 | 457,21 ; 468,75 ; 490,80 |
| 6 | 17,8 | Zeaxanthin | $C_{40}H_{56}O_2$ | 568,871 | 457,21 ; 510,77 ; 547,22 ; 568,77 |
| 7 | 18,4 | Canthaxanthin | $C_{40}H_{52}O_2$ | 564,839 | 455,23 ; 471,23 ; 539,23 ; 561,22 |
| 8 | 18,6 | Canthaxanthin | $C_{40}H_{52}O_2$ | 564,839 | 471,23 ; 539,22 ; 561,23 |
| 9 | 18,8 | Canthaxanthin | $C_{40}H_{52}O_2$ | 564,839 | 471,23 ; 539,22 ; 561,21 |
| 10 | 19,1 | Astasanthin | $C_{40}H_{52}O_4$ | 596,838 | 457,27 ; 536,76 ; 578,79 |
| 11 | 19,3 | Astasanthin | $C_{40}H_{52}O_4$ | 596,838 | 457,26 ; 537,76 ; 578,77 |
| 12 | 19,5 | Astasanthin | $C_{40}H_{52}O_4$ | 596,838 | 457,25 ; 536,73 ; 578,75 |
| 13 | 19,8 | α-carotene | $C_{40}H_{56}$ | 536,872 | 455,24 ; 510,77 ; 523,24 |
| 14 | 20,0 | β-carotene | $C_{40}H_{56}$ | 536,872 | 455,24 ; 523,24 |
| 15 | 23,3 | Phytoene | $C_{40}H_{64}$ | 544,936 | 457,31 ; 506,57 ; 545,46 |

MM: Molecular mass

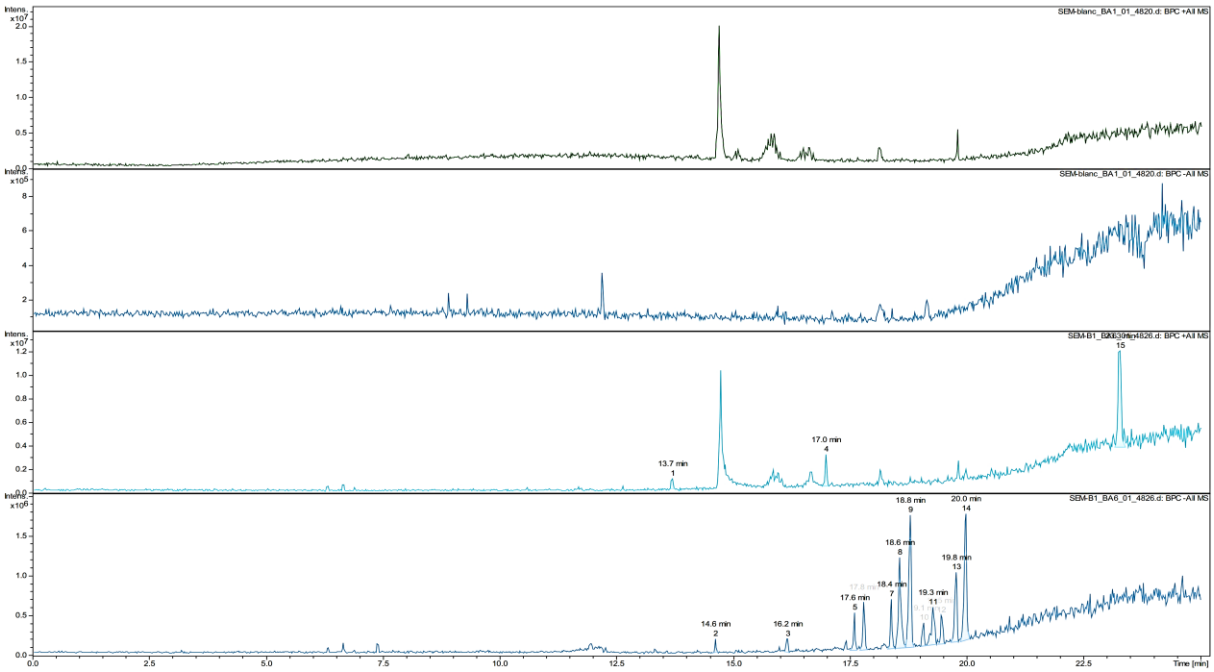



Figure no 6: LC chromatogram of the compounds detected in the fruit pulp extract.

## IV. Discussion

The odour emitted by this extract is very similar to that of the fruit itself; however, it is not identical to that obtained by Babouongolo et al. from the fruit pulp of the same plant (Babouongolo et al., 2021)[11]. This demonstrates that the aroma of this plant's fruit stems from these non-volatile compounds.

The spots with Rf values of 0.97 and 0.91 are attributable to carotenes, specifically β-carotene and α-carotene, respectively. The latter is qualitatively more abundant than β-carotene in the sample (Rajput et al., 2022; Sachindra et al., 2004)[27, 28]. The other nine red-orange, yellow and red spots are attributable to xanthophylls. These compounds are more abundant and more diverse than the carotenes in this extract. These compounds, with Rf values ranging from 0.76 to 0.07, are thought to be, respectively, astaxanthin di-ester, lutein or its isomer zeaxanthin, violaxanthin ester or its isomers, and neoxanthin (Sachindra et al., 2004, Azevedo-Meleiro et al., 2004, Rajput et al., 2022) [27, 28, 29]. This finding confirms that the Anacardiaceae family contains a significant amount of carotenoids.

According to the work of Schweiggert et al. on cashew apples from Costa Rica, the carotenoid content depends on the colour of the fruit: yellow cashews (2.8 mg/100 g dry weight), red (3.2 mg/100 g dry weight) and orange (5.3 mg/100 g dry weight). These authors identified seven carotenoids in these fruits, of which β-carotene, α-carotene, cryptoxanthin, lutein, antheraxanthin, zeaxanthin and violaxanthin are the most commonly found carotenoids in the Anacardiaceae family (Schweiggert et al., 2016)[30]. Da Costa et al. report a content of 28 µg/g DM in the fruits of Spondias mombin and additionally identify zeinoxanthin (Da Costa and Mercadante, 2018)[31]. In the recent review by Maldonado-Celis et al. on bioactive compounds, ripe mangoes were found to have the highest content, as determined by Manthey and Perkins-Veazie in 2009 for the Ataulfo variety (26.1 mg/100 g FM), with the most common carotenoids being α-carotene, β-carotene, γ-carotene, auroxanthin, antheraxanthin, neoxanthin, lutein, violaxanthin and zeaxanthin (Maldonado-Celis et al., 2019)[32].

To the best of our knowledge, no study has reported on the identification of carotenoids in P. microcarpa. These original findings show that the pulp of *Pseudospondias microcarpa* may be a source of natural carotenoids for commercial use in food supplementation, clinical trials or as chemical standards.

Studies have shown that the compounds identified in this extract have multiple health and nutritional benefits. Consuming fruit and vegetables rich in α-carotene is a way of preventing premature death (Pintea et al., 2020)[2]. β-cryptoxanthin is used as a colouring agent, antioxidant, anti-inflammatory and anti-cancer agent. These compounds are also found in oranges, mandarins, persimmons, papayas, red peppers, apples and peaches. It is structurally similar to β-carotene, lutein and zeaxanthin and has high bioavailability. Furthermore, the carotenoids (astaxanthin, β-carotene-15,15′-epoxide and 7,7′,8,8′-tetrahydro-β,β-carotene) isolated from the leaves of *Spondias mombim* are pro-apoptotic; which lends credence to the chemopreventive capabilities of carotenoids (Schultz, 2018)[33].

The high bioactivity of the identified carotenoids and their widespread application are driving the development of production methods, suggesting that the pulp of *Pseudospondias microcarpa* (A. Rich) Engl. could henceforth serve as a reliable source. The method used for the extraction and isolation of carotenoids from this source could find applications in the scientific community for the production of standards in analytical chemistry; in the food industry for enhancing the nutritional and rheological properties of foods; and finally in medicine for the formulation of medicines and for clinical trials, as carotenoids are expensive due to the high cost of their production.

## V. Conclusion

Carotenoids are among the compounds responsible for the yellow, orange and red colours of fruit. Many fruits in the Anacardiaceae family contain high levels of these pigments, thereby increasing their nutritional value. The dietary, pharmaceutical and medicinal value of these compounds means that a great deal of research is being undertaken to identify new sources of carotenoids. Chemical and biological characterisation shows that the epicarp of the fruit of the species *Pseudospondias microcarpa* is a source of carotenoids possessing good free-radical scavenging activity, with a diverse profile of carotenes and xanthophylls. This study identified fifteen compounds, eleven of which were carotenoids, namely phytoene, α- and β-carotene, canthaxanthin, astaxanthin, cryptoxanthin, zeaxanthin, lutein and violaxanthin. The chemical protocol employed in this study classifies the species P. microcarpa as a significant source of natural carotenoid pigments that can be used in food for dietary supplementation and colouring, in cosmetics, and in the treatment of age-related eye diseases, cancers and skin conditions.

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