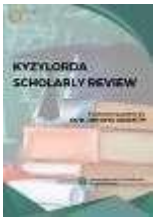
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LUTEIN AND ZEAXANTHIN IN IRAQI BARHI DATES: ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES

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Abstract: The increasing interest in environmentally friendly natural products has recently stimulated research into the phytochemical content of the date palm (Phoenix dactylifera L.). This paper presents results regarding the identification, extraction, and isolation of two main xanthophyll pigments from the Iraqi variety of dates 'Barhi' (Phoenix dactylifera L.) at the late Khalal maturity stage. Xanthophyll extraction was performed by C30 High-Performance Liquid Chromatography (HPLC) and then checked with NMR for the identification of the structure of xanthophyll pigments of β--carotene-3,3′-diol (Zeaxanthin) and its stereoisomer Lutein. The extraction yield of both xanthophyll pigments reaches 3.42 mg per 100 g of dry matter. The extract has shown promising antioxidant activity with an IC_{50} value of 42.5 µg/mL in DPPH radical scavenging assay. The mixture of xanthophyll pigments showed broad-spectrum antibacterial activity against Staphylococcus aureus, Escherichia coli, Candida albicans, and Aspergillus niger, with the low minimum inhibitory concentration of 12.5 µg/mL against S. aureus. As evident from the above findings, Barhi dates show potential for being a source of bioactive compounds, which may be utilized in pharmaceutical and nutraceutical industry.

Keywords: Phoenix dactylifera, Xanthophylls, C30-HPLC, DPPH assay, Bioactive compounds, Natural antimicrobials.

1. INTRODUCTION

Carotenoids are lipophilic tetraterpenoid pigments that has a pivot point in human health. Carotenoids such as lutein and zeaxanthin isomers are significant for being protective in eye health since lutein and zeaxanthin are known for their protective role in eyes against UV light and free radicals [1],[2]. The unique structure of their terminal ionone rings helps them to function as blue light filtering materials [3],[4]. Marigold flowers and leafy vegetables are typical lutein and zeaxanthin sources in the diet. However, it seems that date palm (Phoenix dactylifera L.) especially Iraqi 'Barhi' variety is another unknown source of carotenoids [5],[6]. In a yellow 'Khalal' stage, this variety has an increased moisture content, which is favorable for its utility in carotenoid extraction [7]. Earlier studies focused on sugar and mineral composition of dates [8],[9] but information about their xanthophyll isomers is limited.

Hence, in this paper, the researchers performed their research with the help of gas chromatography-mass spectrometry (GC-MS) technology and nuclear magnetic resonance (NMR) spectroscopy. techniques for comprehensive quantification of xanthophyll compounds present in the Barhi date fruit, and also examined antioxidant and antimicrobial properties of Barhi date against pathogenic microorganisms such as the fungus *Aspergillus niger.*

2. METHOD

2.1. Sample Preparation and Extraction

Barhi dates were brought from Basrah, Iraq. 500 g of the fresh pulp was subjected to freeze-drying and extraction process with the use of a cold solvent mixture, hexane: acetone: ethanol (2:1:1, v/v/v) [11], and to avoid oxidation, the crude extract is mixed with 0.1% butylated hydroxytoluene (BHT). Saponification is performed by using 10% methanol KOH under nitrogen to extract the chlorophylls and esterified lipids [12].

2.2. HPLC Purification

Purification was applied using an Agilent 1260 Infinity II system. Xanthophylls were purified through the YMC C30 column (250 x 4.6 mm, 5 µm) as this stationary phase provides superior shape selectivity for carotenoid isomers [13]. To facilitate this process, a mixture of methanol and methyl tert-butyl ether (MTBE) was used as the mobile phase at a velocity of 1.0 mL/min.

2.3. Spectroscopic and Structural Analysis

2.3.1. GC-MS Analysis

The purified fractions were evaluated through GC-MS using a Shimadzu GCMS-QP2020 NX System with a DB-5MS capillary column (30 m x 0.25 mm ID x 0.25 µm film thickness) following the method described by [14], [15]. Helium was used as the carrier gas (1.0 mL/min). The oven temperature program was: 150°C hold for 2 min, ramp at 10°C/min to 300°C, hold for 20 min. The ion source and interface temperature were fixed at 230°C and 280°C respectively. The mass spectra were obtained by using the Electron Ionization (EI) mode at 70eV in the range of 50 to 600m/z.

2.3.2. NMR Spectroscopy

Structural identification of xanthophyll isomers was performed using 1H-NMR and 13C-NMR spectroscopy at 400 MHz. Deuterated chloroform ($CDCl_3$) is used as a solvent, and the chemical shifts are expressed in ppm with respect to a reference internal standard [16].

2.4. Biological Activity Assays

Through a ratio of 1:1 (w/w) of the Lutein/Zeaxanthin extract containing the biological activity of isolated xanthophylls.

2.4.1. Antioxidant Capacity (DPPH Assay)

Antioxidant activity was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging technique. The DPPH solution becomes decolorized with increasing hydrogen-donating ability so that the IC_{50} values are determined in µg/mL.

2.4.2. Antimicrobial Screening (Agar Well Diffusion)

Antimicrobial activity was assessed against Microbial strains of *S. aureus, E. coli, C. albicans,* and *A. niger* were utilized for the agar well diffusion technique. Microbial suspensions having the concentration of 0.5 McFarland were inoculated into the different media namely Mueller-Hinton agar (for bacteria) and Sabouraud Dextrose agar (for fungi). Wells of 6 mm diameter were filled with 50 µl of extract at 25, 50, and 100 mg/mL. Incubation was at 37°C for 24 h (bacteria) or 28°C for 48 h (fungi). Ciprofloxacin/gentamicin and fluconazole/nystatin served as positive controls.

2.4.3. Minimum Inhibitory Concentration (MIC) Determination

Broth microdilution process was employed to determine the minimum inhibitory concentration (MIC) of xanthophylls which is defined as the least possible concentration of the xanthophyll extract (µg/mL) preventing visible microbial growth (bacterial, yeast, and filamentous fungi strains) tested.

3. RESULTS AND DISCUSSION

3.1. Structural Elucidation and Spectroscopic Characterization

An extensive number of spectroscopy methods including UV-Visible (UV-Vis), GC-MS, and NMR (1H and 13C) were performed on carotenoid samples isolated from Barhi Dates to confirm the identity and purity of the samples.

3.1.1. GC-MS Characterization and Mass Fragmentation Profiling

The purified fractions were analysed via GC-MS analysis, and their molecular structures were verified as intact by comparing their corresponding mass fragmentation patterns against the NIST library as well as against known standards.

GC-MS analysis revealed molecular ion peaks at 568.4 m/z for both lutein and zeaxanthin, corresponding to $C_{40}H_{56}O_2$. Fragmentation patterns included losses of H_2O (550 m/z), toluene (476 m/z), and xylene (462m/z). The pathways of fragmentation, as seen with carotenoid polyenes, demonstrate the many fragmentation pathways of polyene chains found in most carotenoids [16]. A distinctive fragment at 175 m/z was observed only for zeaxanthin, attributable to its symmetric β-ionone rings [15]. Lutein eluted earlier (RT = 18.42 min) than zeaxanthin (RT = 19.15 min) due to its slightly higher polarity.

The presence of these spectroscopic findings, presented in Table 1, indicates the structural purity of these isomers from Barhi date extracts.

Table 1. GC-MS profiling of the compounds

Compound	RT (min)	Molecular Formula	MW (g/mol)	Major Fragment Ions (m/z)
Lutein	18.42	C40H56O2	568.87	568, 550, 476, 462, 444, 119, 105
Zeaxanthin	19.15	C40H56O2	568.88	568, 550, 476, 462, 175, 121, 91

3.1.2. Nuclear Magnetic Resonance (1H and 13C-NMR) Characterization

The NMR spectra provided definitive structural verification for the isolated xanthophylls. In the 1H-NMR spectrum, the olefinic protons appeared as characteristic overlapping peaks in the region of δ 6.10–6.65 ppm, a signature of the conjugated polyene system found in carotenoids.

1H-NMR spectra displayed characteristic olefinic proton signals at δ 6.10–6.65 ppm, indicative of the conjugated polyene system. For lutein, diagnostic signals at δ 1.62 ppm (H-18′) and δ 0.85 ppm (H-16′) distinguished its ε-ring from the symmetric β-rings of zeaxanthin, which showed gem-dimethyl singlets at δ 1.07 ppm.

As indicated by the 13C-NMR results summarized in Table 2, the carbon skeletons of the two

isomers were well-resolved. The Lutein spectrum exhibited a larger number of distinct carbon signals (40 unique signals) because of its asymmetric structure relative to the Zeaxanthin spectrum (fewer unique signals; multiple signals representing two equivalent carbons, i.e., 2C).

Table 2. Nuclear magnetic resonance characterization

Position	Lutein (δ)	Zeaxanthin (δ)	Assignment
C-3, C-3'	65.1, 65.9	65.1 (2C)	Hydroxyl-bearing carbons
C-6'	124.5	126.2 (2C)	Ring olefinic carbon
C-8, C-8'	137.8, 137.6	137.6 (2C)	Polyene chain
C-12, C-12'	137.6, 128.7	137.6 (2C)	Polyene chain
C-30, C-30'	28.7, 24.3	28.7 (2C)	Methyl groups

According to Table 2, Lutein has a C-4' to C-6' non-equal structure, which causes a downfield signal shift of approximately 124.5 ppm. In contrast, the downfield signal shift of Zeaxanthin's symmetric β-rings is at 126.2 ppm. It also shows that in the case of both Lutein and Zeaxanthin, the carbons associated with their respective hydroxyl groups (C-3 and C-3') are both visible in the same region of the spectrum (65.1 and 65.9 ppm for Lutein, respectively). Additionally, these values are closely matched to previous measurements reported in scientific literature [17].

3.2. Antioxidant Evaluation

DPPH assay results demonstrated dose-dependent radical scavenging, with IC_{50} = 42.5 µg/mL (Table 3). The inhibition percentages from the purified extract ranged from 18.4% at a concentration of 10 µg/mL to 89.1% at a concentration of 100 µg/mL. Based on calculations, the IC50 was 42.5 µg/mL, suggesting that xanthophyll extracts possess a high capacity to donate hydrogen atoms and stabilize free radicals. Although the xanthophylls exhibited lower activity than the ascorbic acid control (IC50 = 12.4 µg/mL), they were very comparable in their activity to other naturally occurring carotene-rich extracts, indicating their potential usefulness in protection against oxidative stress and associated cellular damage [18], [19].

Table 3. DPPH assay at different concentrations

Concentration (µg/mL)	Radical Scavenging Activity (% Inhibition)	IC50 (µg/mL)
10	18.4 ± 0.9	42.5
25	34.2 ± 1.2	
50	58.7 ± 1.5	
75	76.3 ± 1.1	
100	89.1 ± 0.8	
Ascorbic Acid	--	12.4

3.3. Biological Activity of Barhi Date Xanthophyll Extract

Combining both lutein and zeaxanthin at a 1:1 ratio showed strong, broad-spectrum, and potent antimicrobial activity towards bacterial and fungal pathogens. The synergistic effects between lutein and zeaxanthin increased the level of dose-dependent inhibition of these pathogens when using combinations of these xanthophylls (Table 4).

3.3.1. Antibacterial and Antifungal Efficacy

The antimicrobial activity of the extract was determined to be dependent on concentration, with the maximum activity achieved at 100 mg/mL of extract The combined extract showed concentration-dependent growth inhibition (Table 4). Highest activity was observed against *S. aureus* (22.4 ± 0.6 mm inhibition zone at 100 mg/mL). MIC values were 12.5 µg/mL (*S. aureus*), 100 µg/mL (*E. coli*), 50 µg/mL (*C. albicans*), and 75 µg/mL (*A. niger*) (Table 5), while the positive control Ciprofloxacin had a zone of inhibition of 25.0 ± 0.5 mm.

Table 4. Dose-dependent inhibition of different microorganisms

Microorganism	25 mg/mL	50 mg/mL	100 mg/mL	Control (Cip.)	Control (Fluc.)
S. aureus	12 ± 1.0	16 ± 0.5	22.4 ± 0.6	25 ± 0.5	-
E. coli	08 ± 0.5	12 ± 1.1	11.2 ± 0.4	28 ± 0.4	-
C. albicans	10 ± 0.8	14 ± 0.6	18.2 ± 0.5	-	20 ± 0.5
A. niger	09 ± 0.5	11 ± 0.7	16.5 ± 0.7	-	22 ± 0.8

Cip: Ciprofloxacin (5 µg); Fluc: Fluconazole (25 µg).

3.3.2. Minimum Inhibitory Concentration (MIC)

MIC tests determined the minimal amount needed to inhibit microbial growth (Table 5). The highest level of susceptibility was determined for *S. aureus* at 12.5 µg/mL, which corresponds to the results obtained from the diffusion assays. The hydrophobic nature of these carotenoids facilitates their integration into microbial lipid bilayers, disrupting membrane integrity [9], [20].

Table 5. Minimum inhibitory concentration of different microorganisms

Microorganism	MIC (µg/mL)	Standard Control (µg/mL)
S. aureus	12.5	1.0 (Vancomycin)
E. coli	100.0	2.5 (Gentamicin)
C. albicans	50.0	5.0 (Nystatin)
A. niger	75.0	5.0 (Nystatin)

3.4. Mechanism of Action

The antimicrobial effects are attributed to the lipophilic nature of xanthophylls, which allows membrane integration and disruption, as supported by prior studies [9], [2]. With respect to A. In this case, the process would significantly affect the structural instability of the ergosterol-rich area of the cell membrane which further increases membrane permeability. The loss of key cytoplasmic materials (for example, intracellular ions and proteins) causes with its cell death [2].

3.5. Discussion

3.5.1. Extraction Efficiency and Carotenoid Yield

The biomass was added to 50 ml conical flasks and then 20 ml of the solvent system was added to each flask. The use of non-polar hexane and moderate polarity acetone and ethanol can recover lipophilic carotenoids in a single step while minimizing the co-extraction of more polar carbohydrates and proteins [11]. Ultrasonication and other assisted extraction techniques have been proposed to enhance carotenoid recovery from plant tissues [21]. The incorporation of 0.1% butylated hydroxytoluene (BHT) is crucial to protect carotenoid compounds which degrade easily under light, heat and oxygen [22], [8]. Treatment of the carotenoid fraction with 10% methanolic KOH for saponification successfully removed the esterified fatty acids and chlorophylls and produced a clean carotenoid fraction which is suitable for analysis by HPLC [12]. Using a C30 stationary phase column in semi-preparative HPLC, lutein and zeaxanthin were achieved as well-defined, baseline-separated peaks. Columns in the C30 phase allow superior selectivity for shape when compared to standard C18 columns, which is required for isomers with similar structure [4], [13]. Although the Barhi dates have quantifiable amounts of xanthophylls (3.42 mg/100g DW), it has a lower yield than commercial sources such as marigold petals or spinach [23], [24]. Nonetheless, the Barhi date's wealth of nutraceutical constituents and other bioactive materials makes it a powerful functional food [25].

3.5.2. Structural Confirmation via Spectroscopic Analysis

GC–MS and NMR data aligned with reference spectra [14], [17], [15]. The unique 175 m/z fragment in zeaxanthin and distinct 13C shifts (C-6′: 124.5 ppm in lutein, 126.2 ppm in zeaxanthin) confirmed isomer identities [26]. Recent studies emphasized the value of HPLC-DAD-MS for accurate identification of carotenoids in complex herbal matrices [27].

The cleavage of carotenoids along the conjugated polyene backbone is indicated by the fragmentation patterns which includes the losses of water (m/z 550), toluene (m/z 476) and xylene (m/z 462) [16], [28]). The 175 m/z unique fragment which is only found in zeaxanthin spectrum results from cleavage of both β-ionone rings symmetry, and acts as a distinguishing feature which differentiates zeaxanthin from asymmetric lutein [15]. The finding that Lutein (RT 18.42 min) eluted first before zeaxanthin (RT19.15 min) on the DB-5MS non-polar column further corroborated this due to the slightly higher polarity created by the asymmetric ε-ring [13]. Advanced purification approaches such as high-speed counter-current chromatography have also been reported as efficient techniques for zeaxanthin isolation from natural matrices [29].

The structure was further confirmed using 1H-NMR analysis. The δ 6.0–6.7 ppm region signals demonstrated characteristics typical of the long-conjugated polyene chain responsible for the antioxidant properties and yellow-orange pigmentation [17]. The photoprotective role of the xanthophyll cycle in plants further explains the stability and biological relevance of lutein and zeaxanthin [30]. The signal at δ 1.62 ppm (H-18′) and the signal at δ 0.85 ppm (H-16′) are diagnostic for the ε-ring of lutein, differentiating lutein from zeaxanthin which has a symmetric β,β-configuration. In addition, the asymmetric lutein showed 40 different signals in the 13C-NMR. Fewer signals were observed in zeaxanthin due to its symmetry. The C-6′ signal (lutein; 124.5 ppm, zeaxanthin; 126.2 ppm) chemical shift indicates a very different electronic environment between the ε- and β-rings, as also recorded in other studies [26].

3.5.3. Antimicrobial Activity and Mechanism of Action

The findings indicate broad-spectrum antimicrobial activity, which is quite interesting. The bioactivity is primarily due to the lipophilic characteristic of xanthophylls. Because of the long, hydrophobic polyene chain, these molecules insert into the lipid bilayer in microbial membranes, disturbing membrane fluidity and integrity [9], [20].

The discovery denotes its ability to interfere with critical processes connected with membranes, including the uptake of nutrients and energy production, leading to cell death. It is effectual against the Gram-positive bacteria S. aureus rather than *E. coli* (MIC 12.5 μg/mL). The mechanism is in line with this microorganism E. coli. There is an extra barrier presented by the outer membrane of Gram-negative bacteria containing high levels of lipopolysaccharides which prevents hydrophobic compounds from penetrating [12].

Antifungal effects against *Candida albicans* and *Aspergillus niger* were significant too. The suggested mechanism entails the infringement of the fungal cell membrane that contains ergosterol [2]. The incorporation of carotenoids disrupts the packing of ergosterols and leads to much higher permeability of the membrane, as well as to leaching of vital cellular components [31].

The activity against fungi was lower than *S. aureus* has likely got this resistance due to the presence of rigid cell wall made of chitin and β-glucans that provides structural protection and limits access of cell membrane [32].

3.5.4. Comparison with Conventional Antimicrobials

The inhibition zones of the carotenoid extract at 100 mg/mL are not less than those reported for other natural extracts such as essential oils and polyphenols. However, they are smaller than those of the standard antibiotics Ciprofloxacin (25–28 mm) and Fluconazole (20–22 mm) under the same conditions. Nevertheless, natural antimicrobials have a greater value than direct potency. Carotenoids that are natural in origin are non-toxic to human cells, do not cause microbial resistance and have many bioactive effects like antioxidant and anti-inflammatory effects [18], [19].

Combining therapies is a very promising area for future use. According to studies, it was found that carotenoids like β-lactams or quinolones can increase the permeability of membranes. It is said that carotenoids can also inhibit the action of efflux pumps [33]. Combining antibiotics with

probiotics will reduce the need for high-antibiotic doses and postpone the emergence of antibiotic-resistant strains which is a global threat.

3.5.5. Implications for Date Palm as a Bioactive Source

Barhi dates represent a sustainable, value-added source of nutraceuticals, particularly in date-producing regions like Iraq [13], [34]. Whole-fruit extracts may offer enhanced bioactivity due to phytochemical synergy [7]. According to studies, dates are high in lutein and zeaxanthin, but they have a more complex phytochemical composition and contain several polyphenols, flavonoids and dietary fibers [35], [9], [18]. Processing conditions such as roasting were reported to influence the phenolic and antioxidant profile of date by-products [36].

The combination of these substances implies that whole fruit extracts or obtained fractions may be more effective therapeutically compared to pure substances [7]. A systematic screening of date varieties and their habitats should be performed because the carotenoid content varies considerably depending on such environmental conditions as soil composition, weather and farming practices [37], [10].

3.5.6. Limitations and Future Directions

Although this study gives strong evidence for bioactive properties of xanthophylls of Barhi date, limitations remain. The antimicrobial tests performed in vitro can never mimic what happens in living organisms perfectly. Further research would need to subject the active fractions to in vivo infection models to evaluate their therapeutic potential and safety [19]. Additionally, despite the fact that the proposed microbicide mechanism is based on known physicochemical principles owing to the need of direct experiments, it still has to be proved through in vitro and in vivo experiments [19]. Mechanistic investigations involving microscopy and metabolomics are encouraged. Improving bioavailability of the drug by means of nanoencapsulation or lipid-based delivery medications should also be included in plans for research [38] along with extensive toxicological testing.

Carotenoids' bioavailability and pharmacokinetics from date matrix have not been studied. Lutein and zeaxanthin, due to their lipophilic nature, are generally poorly absorbed. Their absorption varies significantly based on the food matrix, co-ingestion of lipids and inter-individual factors [39], [24]. In order to resolve these issues and improve therapeutic effectiveness, research on advanced formulation strategies (such as nano-encapsulation, liposomal delivery or SEDDS) is warranted [38]. Ultimately, it is necessary conduct detailed toxicological studies to ensure concentrated extracts are safe. While dietary carotenoids usually have low toxicity, high doses can cause other effects. Before a potential application in human clinical trials can take place, it is necessary that these long-term safety studies are done in animal model and subsequently phase I clinical trials.

4. CONCLUSION

This research successfully demonstrated the complete quantification and high-resolution structural elucidation of Lutein and Zeaxanthin from Iraqi Barhi dates. The results highlight the "Khalal" stage as an optimal harvesting time. The structural identities of the isomers were confirmed using C30-HPLC, GC-MS, and NMR spectroscopy.

The isolated xanthophylls showed notable antioxidant and wide range of antimicrobial actions against pathogenic bacteria and fungi, like *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, and *Aspergillus niger*.

These findings indicate that Lutein and Zeaxanthin from Barhi dates can serve as efficient eco-friendly alternatives to synthetic preservatives and as lead compounds for nutraceutical and pharmaceutical applications. Further studies should be conducted to test their effectiveness in vivo, to improve their bioavailability, and to check for their safety.

AUTHOR CONTRIBUTIONS

Wafaa H Khassaf: Investigation, formal analysis, writing – original draft. Ammar B. Altemimi: conceptualization, supervision. H. N. K. Al-Salman: Investigation, writing – review and editing.

Entessar Mohammad Al Jbawi: Visualization. Hassan Raza: writing – review and editing.

CONFLICT OF INTEREST

There are no conflicts of interest to declare

DATA AVAILABILITY STATEMENT

The data findings of this study are available from the corresponding author upon request.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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