Journal of Food and Drug Analysis

Volume 32 | Issue 2 Article 4

Comparative bioactivity assessment of bixin pigment and associated phytochemicals extracted from annatto seeds using conventional and green solvents

ORIGINAL ARTICLE

Comparative bioactivity assessment of bixin pigment and associated phytochemicals extracted from annatto seeds using conventional and green solvents

Sonakshi Puri, Sumit K. Mandal, Harshit Jain, Pankaj K. Sharma, Perinkulam R. Deepa*

Department of Biological Sciences, Birla Institute of Technology and Science Pilani, Pilani Campus, Pilani, 333 031, Rajasthan, India

Abstract

Nutraceuticals, that include food ingredients and bioactives from natural products, confer physiological health benefits and protection against chronic diseases. Annatto is a tropical shrub grown in Central and South America and parts of India. Its seeds are rich in the edible carotenoid-derived apocarotenoid pigment, bixin, which is used as a natural colorant in food, textiles, and cosmetics, and is now gaining attention for its potential health-promoting attributes. Here, we compared a green solvent (ethyl lactate) based extraction of bixin and associated metabolites in annatto seeds (crushed and seed coat) with two other conventional solvents (acetone and acid-base). Bixin was characterized in the extracts using UV−visible- and FTIR-spectroscopy and thin-layer chromatography. The bixin-containing solvent extracts were then profiled for other co-existing metabolites using GC−MS analysis, which were found to be sesquiterpenes, terpenes, terpenoids, phytosterols, and tocotrienols. Their bioactivity was evaluated based on antioxidant and wound-healing efficacies and compared with pure bixin, using NIH-3T3 fibroblast cells *in-vitro*. Pure bixin, as well as the annatto solvent extracts, showed strong antioxidant and wound healing properties, wherein pure bixin and green solvent extract (ethyl lactate coat) exhibited higher levels of antioxidant activity, achieving 46.00% and 44.60% reduction in MDA levels, respectively, as well as enhanced wound-healing activity, with 54.09% and 53.60% wound closure within 24 h. The green solvent extracts of annatto seeds revealed: (a) differential bioactive profiles in annatto seeds (crushed and seed coat) in comparison with other solvents, and (b) strong antioxidant and wound healing properties. Thus, ethyl lactate extraction shows strong potential for sustainable environmental friendly production of functional foods/nutraceuticals from annatto seeds.

Keywords: Annatto, Antioxidant, Bixin, Green solvent, Wound healing

1. Introduction

Nutraceuticals are functional food ingredients and dietary supplements that contain a wide range of bioactive substances/compounds. The potential of nutraceuticals is to provide physiological health benefits and protection from chronic diseases beyond the basic nutritional value found in normal foods [1,2]. Herbal sources include anthraquinones, alkaloids, tannins, carotenoids, flavonoids, and dietary supplements such as carbohydrates, proteins, lipids, vitamins, pro-prebiotics, and mushrooms [3]. To meet the diverse health-promoting tumorigenesis and disease-preventing requirements, different types of food supplements are presently available, and many more are under intensive research and development against metabolic syndrome [4,5]. The well known food color additive bixin is sourced from annatto plant, which is emerging as a health promoting edible pigment, along with other co-metabolites [6].

Bixa orellana L. (annatto) is a shrub that belongs to the *Bixaceae* family and is native to tropical regions of Central and South America, including Peru, Kenya, Brazil, and Mexico. Interestingly, there have been reports of small-scale cultivation of *B. orellana* L. in different states of India. This plant is commonly known as annatto or achiote tree, and in

Received 29 November 2023; accepted 29 February 2024.
Available online 15 June 2024

* Corresponding author at: Dept. of Biological Sciences, Birla Institute of Technology and Science Pilani, Pilani, 333 031, Rajasthan, India.
E-mail address: deepa@pilani.bits-pilani.ac.in (P.R. Deepa).

https://doi.org/10.38212/2224-6614.3500

India, it is known as "sinduria" [7]. The seeds of the plant are highly valued for their high bixin content, which is approximately 80% in the outer layer of the seeds of the annatto seed coat [8]. A very recent study reported a quantification protocol, which can not only assess nutritionally essential minerals, but also determine possible presence of other non-essential or toxic elements, such as heavy metals in annatto seeds. The mineral content was correlated with soil treatment and agro farming methods, thereby providing a valuable insight for food regulatory assessments, and the role of soil environment [9].

Bixin (9′-cis-6,6′-diapocarotene-6,6′-dioic acid) is a carotenoid-derived apocarotenoid (C_{23}) that has a shorter molecular structure compared to usual carotenoids (C_{40}) [10]. Naturally, bixin is present in an unstable cis form. However, through the applications of heat or organic solvent extractions, it can be converted into a stable form which in turn reduces molecular instability. By converting bixin into its stable form, it can be utilized in the food and beverage industry, where it is used in various products such as cheese, butter, and baked goods [11]. It serves as a natural colorant, imparting an orange-reddish hue to these products. In addition to its industrial applications, bixin has also garnered attention for its potential health benefits [12]. For example, studies showed that bixin isolated from annatto extract was seen to have an anti-cancer effect in both *in-vitro* and *in-vivo* models by inhibiting the growth and progression of tumors [13–15]. Some studies have suggested that annatto extracts have potent antioxidant and anti-inflammatory properties [16,17], which could make them useful for preventing or treating conditions related to oxidative stress and inflammation. In an *in-silico* study, geranylgeraniol and tocotrienols purified from annatto seeds were found to have positive effects against hyperlipidemia and exhibit anti-inflammatory effects [18]. Another study showed that acetone extract of annatto and bixin have a protective effect against retinal damage cause by tunicamycin by inhibiting the early phase of apoptosis [19]. Additionally, some reports have revealed anti-microbial [20,21], and anti-mutagenic [22,23] activities of bixin. All these points strongly to health-promoting benefits of annatto.

The choice of an appropriate solvent plays a pivotal role in extracting of carotenoids and other co-metabolites from plant materials. The selection of the right solvent is essential for maximizing yield, preserving compound integrity, ensuring compatibility with analytical methods and being environmentally friendly process. There are reports of extracting bixin from annatto seeds, using organic solvents such as petroleum ether, methanol, ethanol, and more recently ethyl lactate [24–26]. Importantly, ethyl lactate is a green solvent, which has been reported for the concurrent extraction of both hydrophilic and lipophilic phytonutrients from discarded fruits and vegetables materials [27]. In another recent report, carotenoids were successfully extracted using an environmentally friendly solvent SUPRAS (supramolecular solvent system) [28]. Such green solvents based extraction are a sustainable approach for obtaining phytochemicals. However, a comparative understanding of solvent effects, based on different extraction methods, associated phytochemical profiling and their bio/pharmacological activity, is needed.

Abbreviations

AC	Acetone coat
EC	Ethyl lactate coat
ABC	Acid-base coat
ACr	Acetone crushed
ECr	Ethyl lactate crushed
ABCr	Acid-base crushed
H_2O_2	Hydrogen peroxide
MDA	Malondialdehyde
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TBA	2-thiobarbituric acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
GC–MS	Gas chromatography–mass spectrometry
FTIR	Fourier-transform infrared spectroscopy
HPLC	High-performance liquid chromatography
TLC	Thin layer chromatography
UV–Vis	UV–visible spectroscopy
PA%	Peak area percentages
Rt	Retention time in min

In our present study, our objective was to characterize bixin derived from different extracts of *B. orellana* L. seeds, namely acetone (organic solvent), HCl–KOH (acid-base), and ethyl lactate (green solvent), and to comparatively analyze the differential co-existing phytochemical constituents present in these extracts (both crushed seeds and seed coat). This was followed by evaluation of the antioxidant and anti-inflammatory/wound healing activities of bixin and annatto extracts using suitable *in vitro* models with NIH-3T3 fibroblast cells.

2. Material and methods

2.1. Chemicals and reagents

Bixin standard obtained from HPC Molecules Analytical Lab Solutions Pvt Ltd. (Hyderabad,

Telangana, India). Acetone, acetonitrile, dichloromethane (DCM), dimethyl sulfoxide (DMSO), potassium hydroxide, hydrochloric acid, monopotassium phosphate, sodium hydroxide, Tris buffer, n-butanol, methyl ethyl ketone, aqueous ammonia, trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA) were purchased from Sigma Aldrich (United States). Ethyl lactate was procured from Alfa Aesar (India). ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) MTT dye was obtained from Sisco Research Laboratories Pvt Ltd. (India). The Thin Layer Chromatography (TLC) plates (precoated with aluminum-backed silica gel 60 F_{254}) were procured from Merck (Darmstadt, Germany). For our cell culture studies we utilized mouse embryonic fibroblast cells (NIH 3T3) acquired from National Centre for Cell Science (NCCS, Pune, India). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Carlsbad, CA, USA).

2.2. Annatto seeds collection and extraction

Commercially available seeds of *B. orellana* L. (Annatto) were purchased from AJ Agri Exports (Neemuch, Madhya Pradesh, India 24.4738° N, 74.8726° E). Extracts were prepared by taking 5 g of dried annatto seeds, which were either crushed into a fine powder using waring blender or taken as whole seeds for different solvent extractions. Selection of solvents was based on published reports [17,29,30], including FAO/WHO Expert committee on Food Additives [31].

2.2.1. Organic solvent/green solvent extract

Both the crushed seeds and whole seeds were subjected to extraction three times using 20 mL of organic solvent (acetone) and green solvent (ethyl lactate) at room temperature for 60 min in an orbital shaker incubator. The supernatant was then filtered using Whatman filter paper (No.1). This step was repeated three times, and all the supernatants from both crushed seeds and seed coat were separately combined in flasks. Next, the combined supernatants were concentrated to dryness using rotary evaporator (Aditya scientific, Hyderabad, India). The concentrated extracts were subsequently stored at 4 °C for further analysis.

2.2.2. Acid-base extract

Both crushed seeds and whole seeds extracts were prepared using method by Abayomi et al. [17]. A total of 5 g of seeds were subjected to extraction three times with 20 mL of 1 M KOH at a temperature of 70 °C for 30 min on a magnetic stirrer. The resulting supernatant was then filtered using Whatman filter paper (No.1). This filtration step was repeated three times, and all the supernatants from both the crushed seeds and whole seeds were combined separately in flasks. To acidify extracts, 3 M HCl was added, leading to the formation of crystals. The filtrate was allowed to settle overnight at room temperature. Afterwards, supernatants were decanted, and the filtrate was washed with distilled water 3–4 times. The extract was then placed in an oven at 45 °C for 48 h to dry. The resulting lumps were subsequently ground into a fine powder using mortar and pestle. The powdered extract was stored at 4 °C for further analysis.

The overall yield of the extract was calculated using the formula: Extract yield % = (Extract mass/dry mass) *100.

The quantification of bixin was done using a bixin standard curve and the following formula: Bixin yield (%) = (concentration of Bixin/weight of seeds) *100.

2.3. UV–visible spectrophotometry analysis

UV–visible spectroscopy was conducted using UV-VIS-NIR spectrophotometer (Shimadzu 3600). The UV–vis spectra of bixin (standard) were compared with annatto extracts was obtained using quartz cuvettes with a path length of 1 cm. A concentration of 0.1 mg/mL for both bixin and the other extracts was prepared by dissolving them in a mixture of 9 parts acetonitrile and 1 part dichloromethane (DCM). Absorbance spectra were recorded within the range of 350–550 nm range. To ensure accuracy, background measurements were taken using pure solvents in which sample is dissolved prior to each sample measurement.

2.4. Fourier-transform infrared spectroscopy (FTIR) analysis

For the comparative analysis of the FTIR spectra of bixin (standard) and annatto extracts, a concentration of 0.1 mg/mL for both bixin and the other extracts was achieved by dissolving them in a solution consisting of 9 parts acetonitrile and 1 part dichloromethane (DCM). The FTIR spectra were then obtained using FTIR spectrophotometer (Alpha Bruker, Germany) with scans conducted over the wavenumber range of 400–4000 cm^{-1}. This technique aimed to discern the characteristic functional groups and molecular structure of bixin present in the annatto extracts.

2.5. Thin layer chromatography

To identify bixin in the annatto extracts, analytical TLC was performed. Prior to TLC, 1 mg/mL extracts were dissolved in a mixture of 9 parts of acetonitrile and 1 part of dichloromethane (DCM). A volume of 3 μL of the sample was then applied to the TLC plate. After the sample application on the adsorbent, the TLC plate was placed in a solvent system consisting of n-butanol, methyl ethyl ketone and 10% aqueous ammonia in a volume ratio of (3:2:2) as recommended by the FAO/WHO Expert committee on Food Additives [31]. The plate was kept in solvent system until the solvent front ascended about 7 cm. Subsequently, the R_f value was calculated using the formula: R_f = (distance travelled by the solute/distance travelled by the solvent) × 100. The R_f value of extracted bixin was then compared with the R_f value of the standard bixin for analysis.

2.6. High-performance liquid chromatography (HPLC)

The annatto extracts and standard bixin were analyzed using HPLC (Agilent 1260 Infinity II) equipped with a Poroshell 120 EC-C_{18} column (4.6 × 150 mm 4-Micron). A 0.1 mg/mL sample was dissolved in a mixture of 9 parts of acetonitrile and 1 part of dichloromethane (DCM). Prior to injection, the sample was filtered through a 0.22um filter. The injection volume was 2 μL, and the mobile phase consisted of eluent A (acetonitrile + 0.1% formic acid) and eluent B (water + 0.1% formic acid). The analysis was performed based on modified protocol [32] using a gradient sequence: 0 min A-B (0:100), 15 min A-B (95:5), 20 min A-B (95:5), with a total run time of 25 min. The flow rate was set at 0.7 mL/min. Detection of the samples was carried out at 460 nm using a variable wavelength detector.

2.7. Gas chromatography–mass spectrometry GC/MS analysis

GC–MS analysis was performed on all the extracts using the GCMS-TQ8040 NCI (Shimadzu) equipment in pulsed spitless mode. A concentration of 0.1 mg/mL for each extract was prepared by dissolving them in 100% acetone. The instrument was equipped with an Rxi-5Sil MS column (Restek, U.S.A) with dimensions of 30 mm length, 0.25 mm inner diameter (ID), and a 0.25 μm film thickness (df). The following parameters were used in this analysis: an injector temperature of 250 °C, a detector temperature of 300 °C, an interface temperature of 240 °C, an ionization voltage of 70 eV, and a mass range of m/z 33–500 measured at a rate of 2.76 scans/s. The column flow rate was set at 1.3 mL/min, and helium was used as the carrier gas. For each extract, a sample injection volume of 1 μL was introduced into the injector port. The run method followed the temperature program described as follows: initially held at 40 °C for 2 min, then gradually increased at a rate of 5 °C/min until reaching 180 °C where it was held for 2 min. Subsequently, the temperature was further raised at a rate of 5 °C/min until reaching 300 °C, and it was maintained for 10 min [23,33].The fragmentation patterns of the mass-spectra were compared to the NIST library for identification.

2.8. Cell culture

Murine fibroblast NIH/3T3 cells (obtained from NCCS, Pune, India) were cultured in DMEM (Dulbecco's modified eagle medium) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin G and streptomycin). The cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2.

2.9. Cell viability assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was done to assess cytotoxicity in the presence of treatment groups, namely bixin and different annatto extracts. NIH/3T3 cells were initially seeded in a T-25 flask with a surface area of 25 cm^2 at a density of 6×10^4 cells/mL of medium. After 24 h, when the desired cell confluency was reached, the cells were trypsinized using 0.25% trypsin and transferred to a 96-well plate at a density of 8×10^3 cells per well. Following an additional 24 h incubation, the test samples were dissolved in dimethyl sulfoxide (DMSO) and diluted in the culture media to obtain final concentrations ranging from 5 ug/mL to 20 ug/mL, ensuring that concentration of DMSO remained below 1%. After 24 h of incubation with the test compounds, the cell was treated with a mixture of 9 parts growth media and 1-part MTT dye (5 mg/mL) and further incubated for 3 h. Following the incubation period, the media containing the MTT dye was removed, and the purple-colored formazan crystals were dissolved by adding DMSO. The absorbance of the resulting solution was measured at 570 nm using (Multiskan FC, Thermo Scientific, DE) [34].

The following formula was used to calculate percentage cell viability: (mean absorbance of viable cells)/(means absorbance of control cells) × 100.

2.10. Lipid peroxidation assay

To assess the antioxidant activity of bixin and annatto extracts, the thiobarbituric acid reactive substances (TBARS) assay was performed in cultured cells *in vitro* as described previously [34]. This assay measures the levels of malondialdehyde (MDA), as an indicator of lipid peroxidation and oxidative damage in the test samples. NIH/3T3 cells were used for the assay. The control group consisted of cells without any treatment with H_2O_2 or test samples bixin and annatto extracts. To create an oxidative stress-induced model, NIH/3T3 cells were exposed to 100 μM H_2O_2 for 3 h and 6 h' time points. In the experimental groups, NIH/3T3 cells were pre-treated with pure bixin and different solvent extracts for 3 h, followed by exposure to 100 μM H_2O_2 for another 3 h and 6 h (Fig. 1). After the treatment period, cells were washed with phosphate-buffered saline (PBS) and subsequently lysed using RIPA buffer, The resulting lysates were sonicated and centrifuged at 13,000×*g* at 4 °C for 15 min, and the supernatant was collected in fresh microcentrifuge tubes to remove cell debris. Protein content was then normalized for each treatment group. To measure lipid peroxidation, a total of 350 μL of Tris buffer (0.15 M), 50 μL of monopotassium phosphate (10 mM), and 50 μL of cell lysate were mixed and shaken for 20 min. Subsequently, 250 μL of 10% trichloroacetic acid (TCA) and 375 μL of 1% thiobarbituric acid (TBA) under acidic conditions were added to the reaction mixture. The resulting mixture was heated in a boiling water bath for 15 min to generate a pink-colored adduct. The absorbance of the adduct was measured spectrophotometrically at 530 nm (Multiskan FC, Thermo Scientific, DE). The obtained values were then compared to a standard MDA curve to quantify the level of MDA in the samples, in μM/mL.

2.11. Wound healing/scratch assay

For the scratch/wounding assay, NIH/3T3 cells were seeded into a 24-well plate at a density of 5×10^4 cells per well. Once reached approximately 80–85% confluency, a wound was created using a 200 μL pipette tip, using the same tip to create wound in other wells. After the wound was made, the media was removed from each well and washed twice with PBS. Subsequently, the wells were treated with test samples (bixin, and annatto extracts) all at a concentration of 10 μg/mL. Photographs were taken at the wound site at 0, 24 and 48 h using an inverted microscope, and the migration of cells at the wound site was compared with the control group. The wounding area was then quantified using ImageJ software.

2.12. Statistical analysis

The *in vitro* experiments were performed at least thrice, in triplicate sets. Statistical analysis was performed using one-way or two-way ANOVA to compare the experimental groups, and their statistically significant differences in values were expressed as: $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). GraphPad Prism v9.5.1, 2023 was used for analysis.

3. Results and discussion

Annatto seeds, as a source of natural edible pigment bixin, along with the other co-metabolites, not only adds vibrant hues to our foods but also has several nutraceutical and industrial applications. In the present study, we used three distinct solvents for extraction of bixin and co-metabolites namely, acid -base, acetone, and a green solvent, ethyl lactate. The experimentation included both crushed seeds as well as seed coat. The highlight of the study is

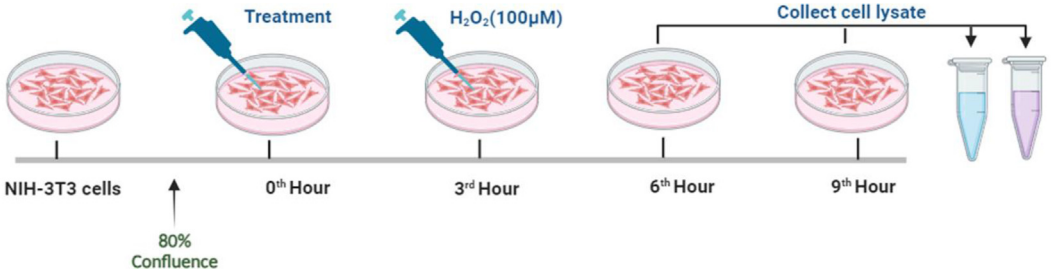

Fig. 1. ***Experimental induction of oxidative stress in normal fibroblast cells and treatment with bixin and annatto extracts.*** *This schematic represents the timelines in our experiment work flow. After confluence, NIH-3T3 cells were treated with bixin/annatto extracts. The oxidative stress inducer hydrogen peroxide was added 3 h later. The biochemical assays were performed in samples obtained in the next 3 and 6 h. The figure was created using Bio Render.*

that a comparative profiling under similar experimental conditions was performed between the three solvents, by performing GC–MS analysis of bioactives in the different extracts, and evaluation of their bioactivity in *in vitro* cell culture models of oxidative stress and wound healing.

3.1. Physiochemical characterization of annatto seed extracts

3.1.1. Differential solvent extracts from annatto seeds

The yield of crude extracts and bixin yield obtained from the three different extraction methods, namely acetone (an organic solvent), HCl–KOH (acid-base), and ethyl lactate (a green solvent) are shown in Fig. 2. The results indicate that the acetone extracts show the highest crude extract yield, with 15.36% for the seed coat and 19.56% for the crushed seeds, compared to the ethyl lactate extracts, which yielded 14.62% for the seed coat and 16.5% for the crushed seeds, as well as the acid-base extracts, which yielded 12.82% for the seed coat and 15.28% for the crushed seeds. The contribution of acetone to the extraction of bixin and bioactives has been comparatively reported [19,29]. Interestingly, while the extract yield in ethyl lactate seed coat extract lower than that of acetone seed coat extract, it is still comparable. This finding indicates that ethyl lactate can still extract a considerable amount of bioactives, but slightly at lower efficiency compared to acetone. Thus, ethyl lactate can serve as a viable green alternative to organic solvent extraction process, albeit with a slightly reduced extract yield.

As a green solvent, ethyl lactate has several advantages over traditional solvents such as acetone and petroleum ether. It is biodegradable, non-toxic, has low volatility, and can be produced sustainably from non-petroleum sources such as fermentation of agro biomass [35]. Consequently, it has lower environmental impact and is less harmful to human health. Ethyl lactate was also reported as a green solvent to remove the total petroleum hydrocarbons from oil contaminated soil, for environmentally friendly remediation of soil [36].

Ethyl lactate is considered comparable in polarity with acetonitrile, and forms different types of chemical interactions (hydrogen bonds, van der Waals forces) thereby enabling miscibility with polar/hydrophilic and nonpolar/hydrophobic carotenoids [37]. However, ethyl lactate based extraction of bioactives from annatto seeds is under-researched. Further, ethyl lactate extracts of annatto also need to be evaluated for their bioactivity/pharmacological activity, which has been presented in this study.

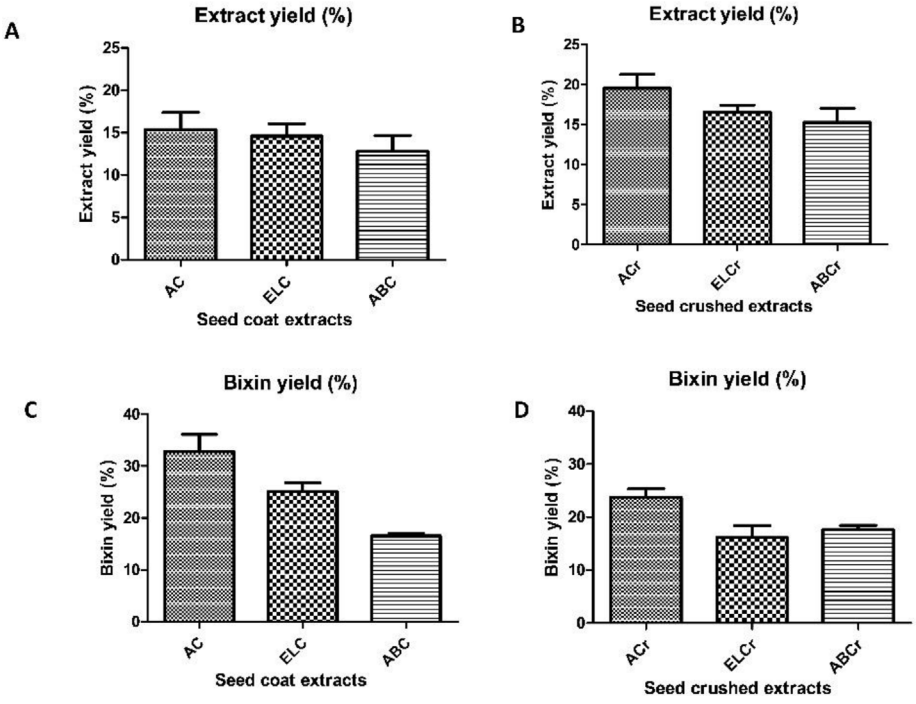

Fig. 2. ***Yield of bixin following solvent extraction.*** *Yield (%) of extracts obtained through different solvent extractions in both annatto seed coat and seed crushed (A–B) and bixin yields (%) from different annatto extracts (C–D). AC-acetone coat, ELC-ethyl lactate coat, ABC-Acid-base coat, ACr-Acetone crushed, ELCr-ethyl lactate crushed, ABCr-Acid-base crushed. Values are expressed as mean ± SD of three independent extractions.*

The UV–Visible spectrophotometry analysis reveals a significant increase in bixin extraction yield in the seed coat extracts than the seed crushed extracts, as illustrated in Fig. 2(C) and (D). Bixin has been reported to be found abundant in the seed coat and constitutes about 80% of the carotenoid pigments found in annatto seeds [38]. Our results indicate an increased bixin yield using acetone solvent for extraction, with 32.85% in seed coat extracts and 23.71% in crushed seed extracts. In contrast, ethyl lactate solvent extracts showed a slightly lower bixin yield of 25.08% in seed coat extracts and 16.15% in crushed seed extracts. The acid-base extracts exhibited a lower bixin yield, with 16.56% in seed coat extracts and 17.66% in crushed seed extracts, when compared to the yield obtained from the other solvent extracts.

In Fig. 3(a), the UV–Visible absorbance spectra show the bixin (standard) and annatto extracts at a concentration of 0.1 mg/mL in acetonitrile/DCM (9:1). Following the methodology outlined by FAO/WHO, the absorption maxima for bixin in 100% acetone are anticipated to occur at 425 nm, 457 nm and 487 nm [31]. Our UV–Vis analysis results show the peak signatures of the standard bixin exhibit absorption maxima at 460 nm and 490 nm, which are similar to the peaks observed in the annatto extracts. The acetone seed coat extract showed bixin with highest absorption peak, which has been comparatively studied [39]. Additionally, the ethyl lactate seed coat shows a lower absorption peak compared to the acetone coat extract but higher than the other extracts.

Bixin exhibits higher affinity for polar organic solvents due to the presence of a carboxylic acid group and a methyl ester group in its structure Fig. 3(b). These functional groups contribute to its relatively polar nature, distinguishing it from other carotenoids. It is worth noting that the yield of bixin extracted from the annatto seeds is influenced by the polarity of the solvent used. Interestingly, it has been observed that both the least polar and the most polar solvents result in the lowest yield of bixin [40]. This suggests that acetone and ethyl lactate are good extraction solvents for extracting apocarotenoid bixin from annatto seeds. These solvents can efficiently penetrate plant cells, disrupting the structural integrity of the cell walls and facilitating the release of desired compounds [41,42]. Moreover, considering the principles of acid-base reactions, stronger acids tend to react with the conjugate bases of weaker acids. So, acetone which has a pKa of 19.20, is considered a weaker acid compared to bixin (pKa 4.9010) [29]. In recent reports, green extraction solvents has been successfully utilized in the extraction of carotenoids from tomato and other plants as reported in studies by Silva et al. and Murador et al.

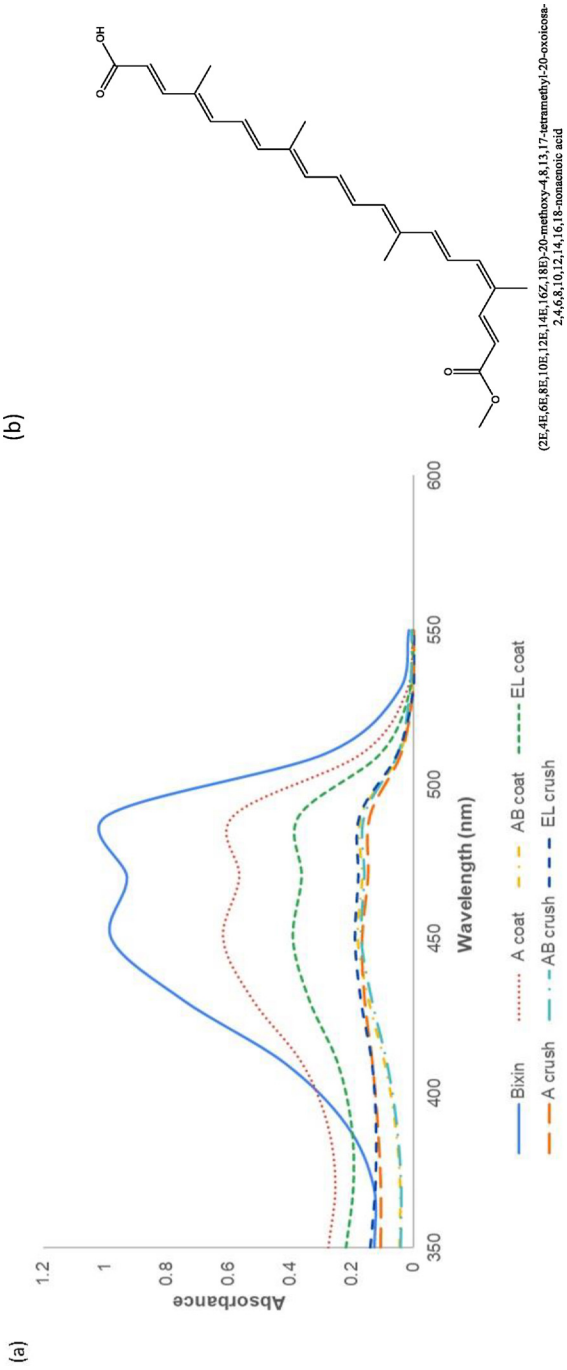

Fig. 3. Spectrophotometric characterization of bixin in different solvent extracts of annatto. (a) UV–visible spectra of the various annatto extracts compared with UV–visible spectra of pure bixin (reference). (b) Chemical structure of bixin.

[43,44]. Therefore, both acetone and ethyl lactate can be utilized as an effective solvent for extracting bixin.

3.1.2. Characterization by FTIR spectroscopy

Fig. 4 displays the overlapping FTIR spectra of annatto extracts. The spectrum assignments made based on the reported literature [25,39,45]: at 3547 cm^{-1} the —O—H vibration is observed, at 3002 cm^{-1} = C—H stretching, at 2252 cm^{-1} the H—C—H bending vibration, at 1633 cm^{-1} the carboxylic C = O group, at 1439 cm^{-1} C = C stretching is observed, at 1039 cm^{-1} and 919 cm^{-1}, at 1378 cm^{-1} C—H bending of the methyl groups, at 1039 cm^{-1} C—O—C ester group, at 919 cm^{-1} the out of plane vibration of the = C—H group. The FTIR spectra indicate a distinction between the crude extracts and the standard bixin spectrum, suggesting the presence of other bioactives in the extracts. This suggests that the annatto extracts contain other derivatives of bixin, as well as minor carotenoids and related bioactives, as previously reported [46,47].

3.1.3. Thin layer chromatography analysis of annatto seed extracts

In order to identify the presence of bixin in crude annatto extracts, analytical TLC was performed. The chromatographic profile of the samples was observed under visible light, revealing two distinct yellow spots: a dark yellow band and a light-yellow spot (detected below the dark yellow spot) is shown in Fig. 5. Bixin, being the dominant carotenoid of the annatto extract, appeared at R_f 0.57 and norbixin appeared at R_f 0.4, which is consistent with standard bixin and previously reported literature [31,48]. Since organic solvents are aimed at extracting lipophilic compounds, both acetone and ethyl lactate extracts showed prominent presence of bixin spot, while the relatively polar norbixin may not be efficiently extracted using organic solvents and hence the mild colored spot. In acid-base extractions, both bixin and norbixin spots are distinctly visible, which is consistent with the literature [17].

3.1.4. HPLC analysis

The HPLC analysis of the annatto extracts allowed the separation of bixin, with a distinct peak observed at retention time of 18.23 min, which matched the retention time of the standard bixin. In the seed coat extracts, the bixin peak accounted for 90.76% of total area separated in the acetone extract, 81.21% in the ethyl lactate extract, and 55.03% in the acid-base seed coat extract. In the crushed seed extracts, the bixin peak comprised 53.99% in the

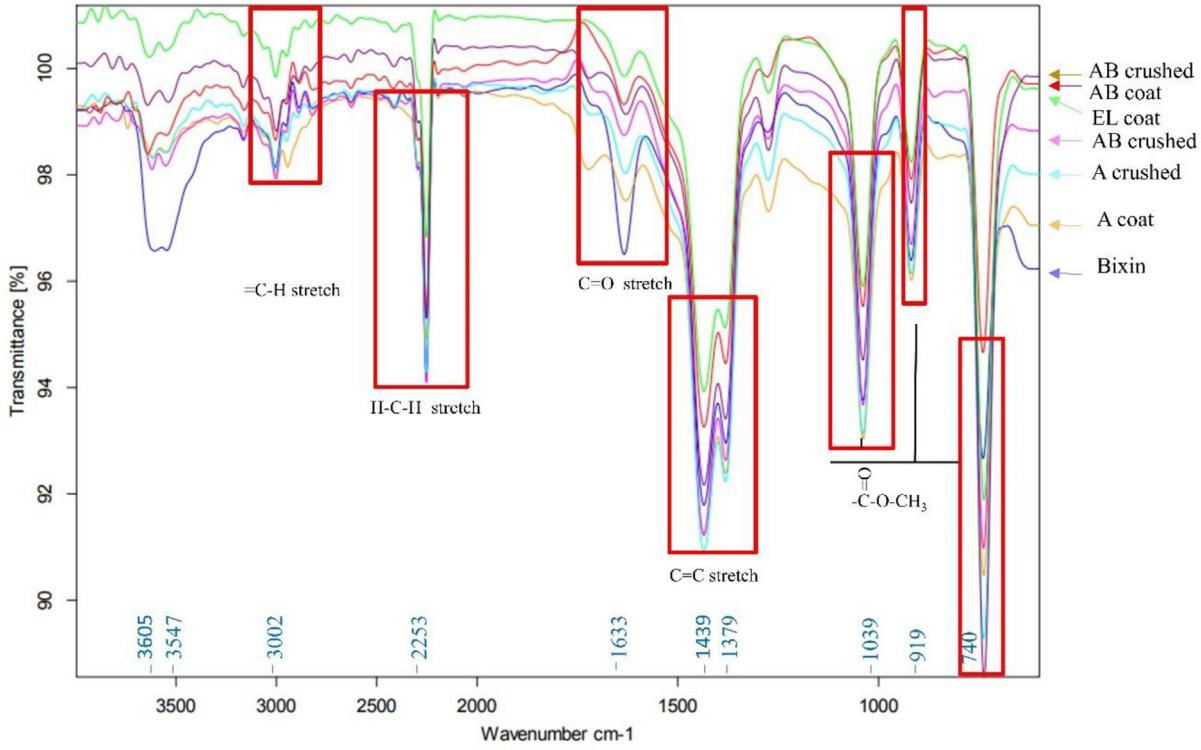

*Fig. 4. **Comparative FTIR spectra of pure bixin with different annatto extracts.** The red boxes indicate characteristic peaks of the functional groups related to bixin.*

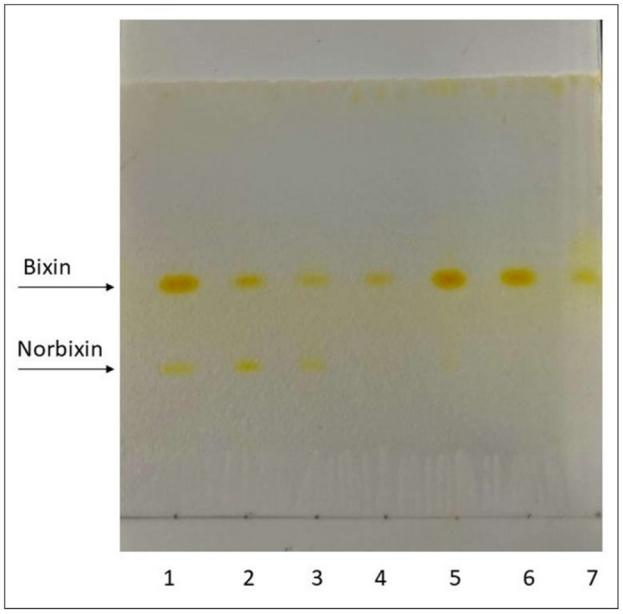

Fig. 5. ***Bixin characterization in different solvent extracts of annatto by thin layer chromatography (TLC).*** *TLC plate with different annatto extracts developed in n-butanol, methyl ethyl ketone and 10% aqueous ammonia (3:2:2). 1-Standard bixin, 2-Acid-base coat, 3-Acid-base crushed, 4-Ethyl lactate crushed, 5-Ethyl lactate coat, 6-Acetone coat, 7-Acetone crushed.*

acetone extract, 52.97% in the acid base extract and 49.46% in the ethyl lactate extract. The bixin peak area was significantly higher in the seed coat extracts, particularly in the acetone seed coat extract, compared to the levels observed in the crushed seed extracts, as shown in Fig. 6.

3.1.5. Bioactives profiling by GC–MS analysis

Overall, a total of 272 different compounds were identified in the extracts by GC–MS analysis (please refer to Fig. 7 for the chromatograms while a detailed compilation of phytochemicals, as well as the list of the top 50 abundant compounds, can be found in the **supplementary file** [https://doi.org/10.38212/2224-6614.3500]). The majority of volatile compounds found in these extracts were terpenes/terpenoids, sesquiterpenes, tocotrienols, steroids, with small concentrations of fatty acid esters, alcohols, ketones, alkanes, alkenes and heterocyclic or aromatic compounds. These co-pigments and compounds coexisting with bixin may be the breakdown products of higher carotenoids [49,50]. Out of the top 50 abundant compounds, sesquiterpenes constituted approximately 38% of the total volatiles in the acetone seed coat and acetone seed crushed extract. In contrast, the acid-base seed coat and crushed extract contained about 12% of sesquiterpenes. The ethyl lactate coat and ethyl lactate crushed extract had approximately 18% and 12% sesquiterpene respectively. Major sesquiterpene includes geranylgeranyl acetate, caryophyllene oxide, spathulenol, isospathulenol, beta-springene, alpha-springene, beta-santalol, ishwarane, globulol. Sesquiterpenes have been studied for their potential in anti-inflammatory, antioxidant, antiproliferative, weight-modulating, and antimicrobial activities [51–53].

Among these, the phytocannabinoid, caryophyllene oxide has strong affinity for cannabinoid receptor 2, thereby demonstrating anti-cancer, anti-inflammatory effects [51]. Additionally, studies indicate that extracts enriched with spathulenol from plants *Psidium guineense Sw* (80.71%), *Salvia mirzayanii* (62%) possess antioxidant, antiproliferative, and antimicrobial effects [52,54].

The findings indicate the rich diversity of terpenes and terpenoids present in annatto extract, with some compounds being more predominant in specific extract. One of the major components was 1,6,10,14,18,22-tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E) -(+/–.)-, which constituted approximately 37% of the peak area in the acetone crushed extract and 33.9% in acetone coat extract. This compound was also present in other extracts but in lesser amounts. A study identified the diterpenoid trans-geranylgeraniol (19.69%) in the petroleum ether extract of ground annatto seeds [23]. Another study revealed the presence of trans-geranylgeraniol (0.75%) in the methanolic fraction of annatto seed extract [49]. Our results showed a higher percentage of trans-geranylgeraniol in the ethyl lactate coat extract (34% of the peak area), followed by 13.8% in the acid-base crushed extract. This compound has been reported to exhibit anticancer, anti-inflammatory activities as well as benefits against hyperlipidemia [18,55]. Additionally, smaller amounts of other terpenes/terpenoids, such as squalene, and farnesyl palmitate, were also present in the annatto extracts that are worth mentioning owing to their wide range of potential health benefits [56] and industrial applications such as aroma [8,57].

Tocotrienols, specifically the delta and gamma forms, which are members of the vitamin E family, are found in extracts derived from annatto plant. Studies have highlighted their potential health benefits. For instance, research suggests that tocotrienols extracted from annatto seeds improve glucose uptake in diabetic mice by mitigating inflammatory responses [58]. Moreover, these tocotrienols have been shown to decrease triacylglycerol accumulation in both adipoctyes and hepatocytes and also offer antioxidant activity [59]. In our findings, delta-tocotrienol exhibits higher area percentage compared to gamma-tocotrienol. The area percentage of delta-

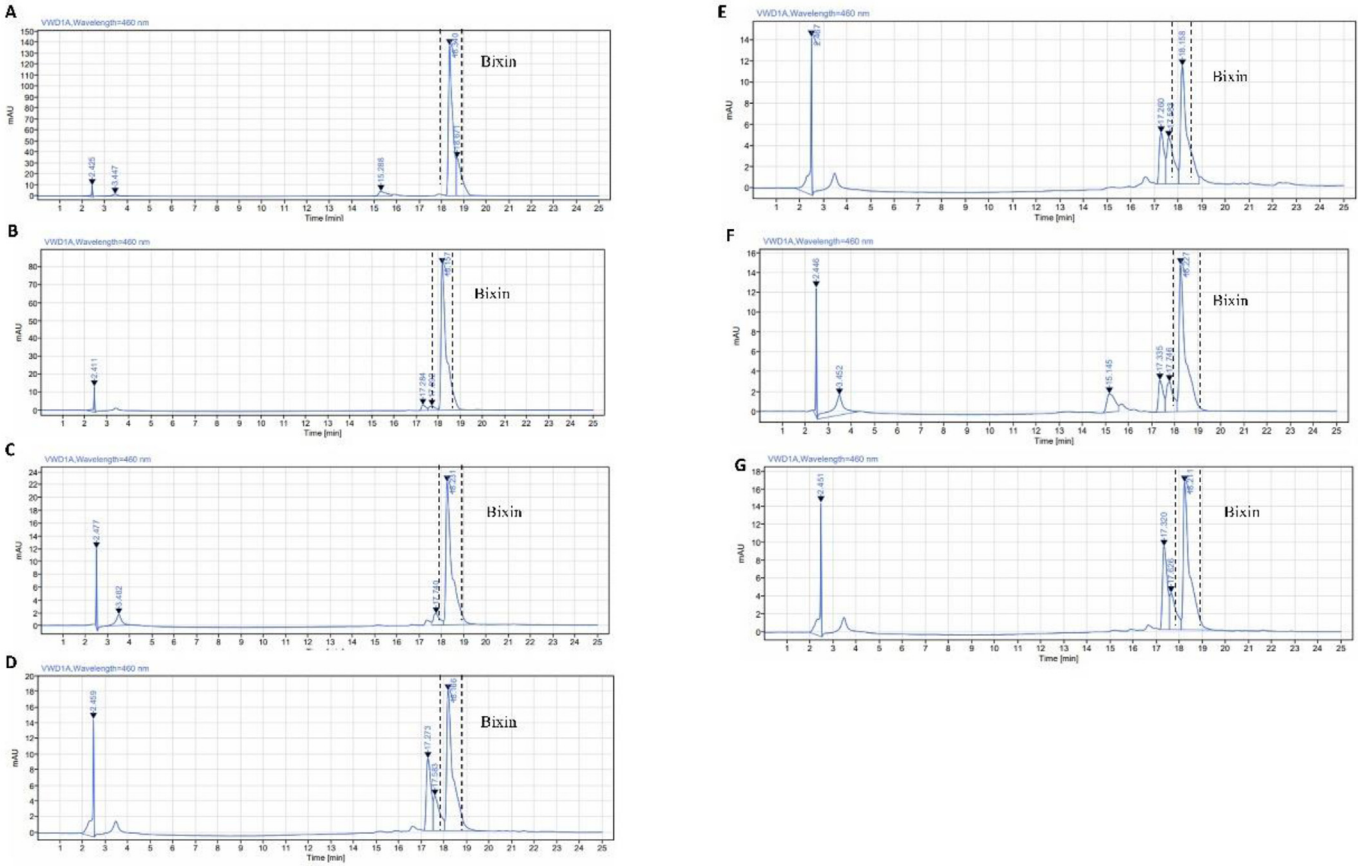

Fig. 6. ***Bixin characterization in different solvent extracts of annatto by high performance liquid chromatography (HPLC).*** *HPLC chromatogram of the different annatto extracts. A-pure bixin, B-acetone/coat, C-ethyl lactate/coat, D-acid-base/coat, E-ethyl lactate/crushed, F-acid-base/crushed, G-acetone/crushed.*

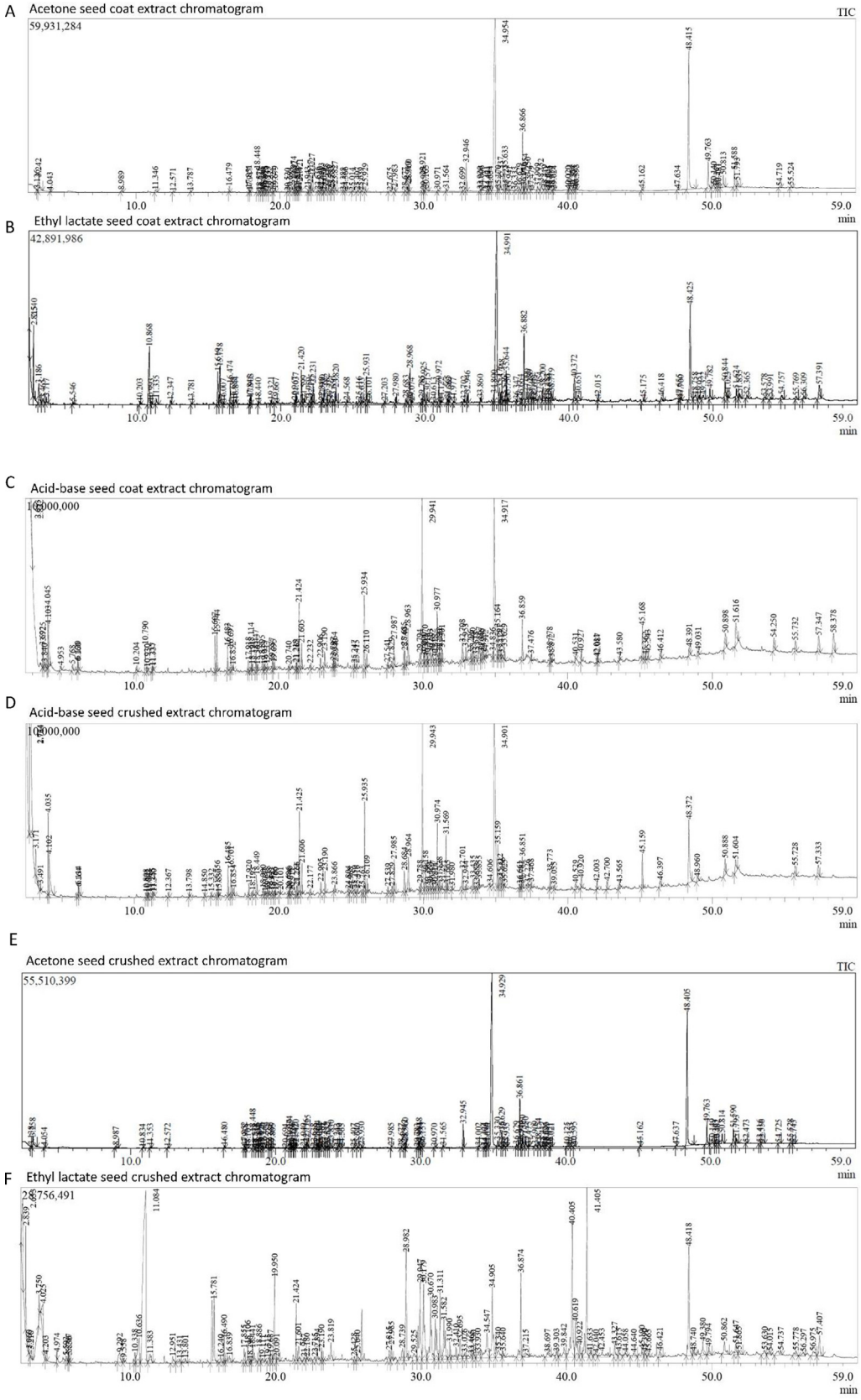

Fig. 7. ***GC–MS chromatograms of different annatto seed/crushed extracts****. A-acetone/coat extract, B-ethyl lactate/coat extract, C-acid-base/coat extract, D-acid-base/crushed extract, E-acetone/crushed extract, F-ethyl lactate/crushed extract.*

tocotrienol in both acetone crushed extract (20.6%) and acetone coat extract (18.2%), is higher than that of other two extracts. Similarly, gamma-tocotrienol demonstrates higher area percentages in acetone crushed (2.72%) and acetone coat (2.7%) extracts. While this compound is also present in ethyl lactate crushed and coat extracts, it is present in lesser levels. Gamma-tocotrienol is not detected in acid-base crushed and coat extracts in the top 50 compounds. A recent report [24] demonstrated that a pre-processing step of probe sonication, followed by solvent extraction (particularly isopropyl acetate and ethyl lactate) in annatto seeds greatly improved the yield of delta tocotrienol.

Some plant sterols, including gamma-sitosterol, stigmasterol, fucosterol and campesterol, have been identified in annatto extracts. Among the top 50 compounds, the proportion of phytosterols is marginally higher in acetone-crushed extract (8%) and ethyl lactate-crushed extract (6%) when compared to seed coat extracts, where it accounts for 6% for acetone-coat and 4% for ethyl lactate coat extracts. The content of steroids in acid-base extracts, both from seed coat and crushed samples, is lower, approximately around 2%. The identification of phytosterols in annatto extracts holds significance due to their potential involvement in a range of biological activities. Notably, these compounds can exert cholesterol-lowering effects, which is primarily attributed to sitosterol and campesterol [60]. Additionally, phytosterols possess anti-inflammatory properties, thereby contributing to the mitigation of inflammatory conditions [61]. Moreover, their antioxidant activity serves to counteract detrimental free radicals in the body [62]. These phytosterols have also demonstrated promise in terms of anti-cancer properties [63].

p-Xylene were also identified among the top 50 compounds in acetone-crushed and seed coat extracts, as well as in ethyl lactate coat extracts. This compound is reported to be the result of degradation of higher molecular weight carotenoids [64].

To summarize, annatto seeds can serve as a valuable source of above-mentioned bioactives that can be tapped suitably using various extractions methods.

3.2. Experimental validation of antioxidant and anti-inflammatory activity of bixin and different annatto extracts

3.2.1. Non-cytotoxic (safe) dosage of bixin and annatto extracts determined in normal fibroblast (NIH/3T3) cells

Before proceeding to evaluate the antioxidant and anti-inflammatory effects of bixin and different annatto extracts on NIH/3T3 cells, we conducted an MTT colorimetric assay to determine the non-cytotoxic dosage. The safe dosage was defined as the concentration at which at least 80% or more cells remain viable. After incubating the test samples for 24 h, we observed a dose-dependent decrease in cell viability. Hence, for further *in vitro* studies, we opted for a non-cytotoxic dosage of 10 μg/mL. This concentration ensured that cells are viable more than 80% for both bixin and the other annatto extracts (Fig. 8).

3.2.2. Lipid peroxidation - TBARS assay

The levels of MDA, a commonly used biomarker for assessing lipid peroxidation were evaluated in our study. Our H_2O_2-induced oxidative stress model showed a significant increase in MDA concentration ($p \leq 0.001$), confirming the effectiveness of our cell culture model in inducing oxidative stress. However, upon treating the models with the apocarotenoid bixin and different solvent extracts of annatto seeds (crushed or seed coat), we observed a remarkable suppression of oxidative stress-mediated lipid peroxidation (Fig. 9). For the 3-h post H_2O_2 exposure time point, the reduction levels of MDA ranged from 20.90% to 44.20%. Likewise, at the 6-h post H_2O_2 exposure time point, the reduction levels ranged from 30% to 46%. Relative to all the extracts, pure bixin exhibited the highest antioxidant activity with reductions of 44.20% and 46% at the 3 h and 6 h post H_2O_2 exposure, respectively. The ethyl lactate- and acetone-coat extracts demonstrated significant reduction in MDA levels [24], with decrease of 44.60% at 6 h post H_2O_2 exposure and 43.15% at 3 h post H_2O_2 exposure, which may be due to the abundance of bixin pigment in the annatto seed coat, along with the presence of other bioactives [21].

Our results showed that bixin, an apocarotenoid, exhibits potent antioxidant properties that may be

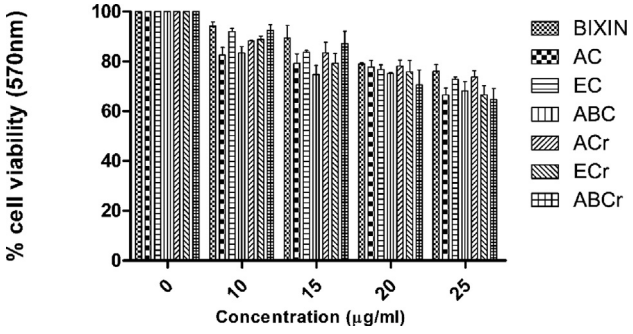

*Fig. 8. **Concentration dependent effects of the different annatto extracts on cell viability.** Values are expressed as mean ± SD of three independent experiments performed in triplicates. AC-acetone coat, ELC-ethyl lactate coat, ABC-Acid-base coat, ACr-Acetone crushed, ELCr-ethyl lactate crushed, ABCr-Acid-base crushed.*

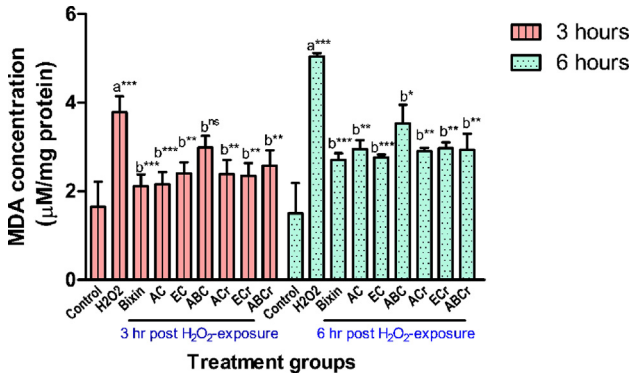

*Fig. 9. **Evaluation of antioxidant potential of pure bixin and solvent extracts of annatto seeds.** MDA levels in oxidative stress induced group and its reduction in the treated groups indicates the antioxidant activity of bixin and annatto extracts. Values are expressed as mean ± SD of minimum of two to three independent experiments, each done in triplicate sets. Statistical comparisons are as follows: a-with respect to control; b-with respect to H_2O_2-induced oxidative stress group (symbols *, **, and *** represent statistical significance at $p \leq 0.05$, 0.01, and 0.001 respectively).*

attributed to its conjugated double bonds. In the presence of free radicals, bixin functions as an electron donor, neutralizing these harmful molecules and prevents oxidative damage. Its stability and lipophilic nature further enable it to protect cell membrane from lipid peroxidation and consequent oxidative damage. This antioxidant capacity positions bixin as a valuable candidate for potential health benefits and protection against oxidative-stress related diseases [16]. The association of oxidative stress with metabolic syndrome is well known [65], though the cause and effect roles of reactive oxygen species (ROS) need investigation in each metabolic condition [66]. Thus, the antioxidant efficacy shown by pure bixin and the solvent extracts point to their potential applications in preventing lipid peroxidation and protection against oxidative stress, implicating their role as antioxidant in nutraceutical/functional food formulations, antioxidant food additives that require antioxidant component.

3.2.3. Wound healing assay

In this study, we conducted an *in-vitro* wound healing assay using a monolayer of NIH-3T3 fibroblasts cells with a "scratch" assay to evaluate the wound healing potential of both pure bixin and various annatto extracts. We compared the percentage of area closure with untreated control cells as shown in (Fig. 10).

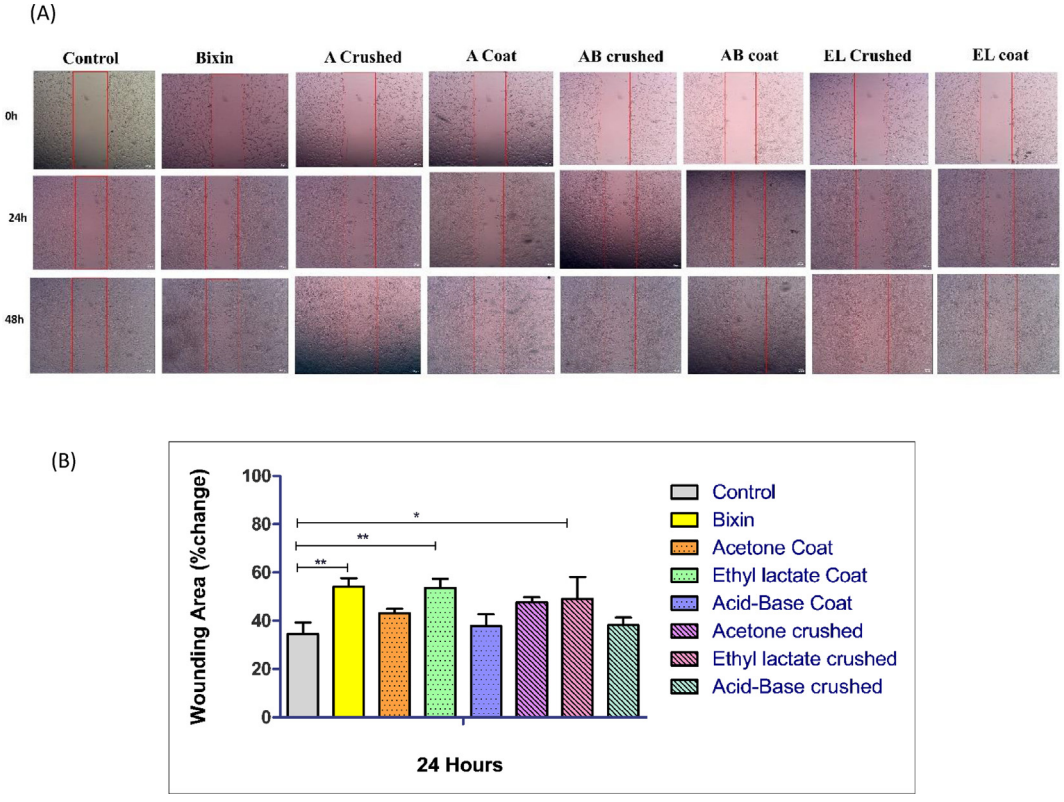

*Fig. 10. **Evaluation of wound healing efficacy of bixin and annatto extracts** (A) Representative images of wound healing assay of NIH-3T3 mouse fibroblast cells treated with bixin and different annatto extracts (scale bar: 100 μm; magnification: 10×). (B) Percentage area closure after 24 h was quantified using ImageJ software. Values are expressed as mean ± SD of three independent experiments performed in triplicates. Statistical difference between values were expressed as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).*

Our experimental results showed that scratch closed significantly faster when treated with pure bixin, showing a 54.09% closure at concentration of 10 μg/mL, as opposed to control cells, which exhibited a 36.4% closure rate. Among the different annatto extracts tested, the ethyl lactate seed coat extract showed an increased wound closure of 53.6%, while the slowest closure rate was observed in the acid-base extracts, with a 38% closure at the same concentration of 10 μg/mL. Previous literature suggests that carotenoids possess antioxidant properties [67]. In our study, both bixin, an apocarotenoid, and annatto extracts exhibited antioxidant activity. Wound formation triggers the production of free radicals associated with the inflammatory process. If the anti-inflammatory and antioxidant functions are not active, it can lead to oxidative stress and persistent inflammation, slowing down the healing process and causing additional damage to the tissues [68]. Antioxidants play a crucial role in mitigating the effects of free radicals, thereby aiding the wound healing process. It is interesting to note a formulation where nanofiber based scaffold with bixin as an antioxidant has been reported for wound healing applications, that can have treatment implications to heal diabetic wounds [69].

4. Conclusions

The present study brought out the differential bioactive profiles in the crushed and seed coat extracts of annatto, using different solvents such as acid-base, acetone, and ethyl lactate (green solvent). Pure bixin and ethyl lactate extracts of annatto seeds showed relatively better antioxidant and wound healing properties when compared with the other solvent extracts. The green solvent extraction used here for annatto seeds is a sustainable approach that can be extended to other natural pigments. The edible pigment bixin and annatto seed extracts with bixin and co-metabolites, may be tapped further for nutraceutical and food additive applications, as well as innovative formulations including nanoformulations that need synergy of antioxidant and wound healing efficacy.

Statements & declarations

Data availability statement

The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Conflicts of 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.

CRediT authorship contribution statement

P R Deepa, Sonakshi Puri: Conceptualization; **Sonakshi Puri, Sumit Kumar Mandal, Harshit Jain**: Methodology & Experimentation; **Sonakshi Puri, Sumit Kumar Mandal, Harshit Jain**: Data curation Software; **Sonakshi Puri, Sumit Kumar Mandal, P R Deepa**: Writing and original draft preparation; **P R Deepa, Pankaj Kumar Sharma**: Visualization, Investigation Supervision; **Sonakshi Puri, Sumit Kumar Mandal, P R Deepa, Pankaj Kumar Sharma**: Writing- Reviewing and Editing.

Funding

No funding was obtained for this study.

Conflicts of 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.

Acknowledgements

SP and SKM acknowledge the Institute Fellowship from BITS Pilani. The authors thank Birla Institute of Technology and Science (BITS), Pilani – Pilani Campus for providing research support and instrumentation facility.

References

[1] Espín JC, García-Conesa MT, Tomás-Barberán FA. Nutraceuticals: facts and fiction. Phytochemistry 2007;68:2986–3008. https://doi.org/10.1016/j.phytochem.2007.09.014.
[2] Souyoul SA, Saussy KP, Lupo MP. Nutraceuticals: a review. Dermatol Ther 2018;8:5–16. https://doi.org/10.1007/s13555-018-0221-x.
[3] Sachdeva V, Roy A, Bharadvaja N. Current prospects of nutraceuticals: a review. Curr Pharmaceut Biotechnol 2020;21:884–96. https://doi.org/10.2174/1389201021666200130113441.
[4] Cheng J, Liu D, Zhao J, Li X, Yan Y, Wu Z, et al. Lutein attenuates oxidative stress and inhibits lipid accumulation in free fatty acids-induced HepG2 cells by activating the AMPK pathway. J Funct Foods 2019;60:103445. https://doi.org/10.1016/j.jff.2019.103445.
[5] Srinivasan K. Cumin (Cuminum cyminum) and black cumin (Nigella sativa) seeds: traditional uses, chemical constituents, and nutraceutical effects. Food Qual Saf 2018;2:1–16. https://doi.org/10.1093/fqsafe/fyx031.
[6] Vilar DDA, Vilar MSDA, Moura Tfadle, Raffin FN, De Oliveira MR, Franco CFDO, et al. Traditional Uses, chemical

constituents, and biological activities of Bixa Orellana L.: a review. Sci World J 2014;2014. https://doi.org/10.1155/2014/857292.
[7] Kumar U, Singh RS, Mandal J, Nayak AK, Jha AK. Removal of As(III) and Cr(VI) from aqueous solutions by Bixa orellana leaf biosorbent and As(III) removal using bacterial isolates from heavy metal contaminated site. J Indian Chem Soc 2022;99:100334. https://doi.org/10.1016/j.jics.2021.100334.
[8] Shahid-ul-Islam, Rather LJ, Mohammad F. Phytochemistry, biological activities and potential of annatto in natural colorant production for industrial applications - a review. J Adv Res 2016;7:499−514. https://doi.org/10.1016/j.jare.2015.11.002.
[9] Baptistão M, Lorençatto R, Eberlin MN, Simionato AVC. Multielemental characterization of bixa orellana L.(urucum) seeds by inductively coupled plasma mass spectrometry. Food Bioprocess Technol 2023:1−10.
[10] Scotter M. The chemistry and analysis of annatto food colouring: a review. Food Addit Contam 2009;26:1123−45.
[11] Marcolino VA, Zanin GM, Durrant LR, Benassi MDT, Matioli G. Interaction of curcumin and bixin with β-cyclodextrin: complexation methods, stability, and applications in food. J Agric Food Chem 2011;59:3348−57.
[12] Saini RK, Prasad P, Lokesh V, Shang X, Shin J, Keum Y-S, et al. Carotenoids: dietary sources, extraction, encapsulation, bioavailability, and health benefits—a review of recent advancements. Antioxidants 2022;11:795.
[13] Qiu Y, Li C, Zhang B, Gu Y. Bixin prevents colorectal cancer development through AMPK-activated endoplasmic reticulum stress. BioMed Res Int 2022:2022. https://doi.org/10.1155/2022/9329151.
[14] Kumar Y, Phaniendra A, Periyasamy L. Bixin triggers apoptosis of human Hep3B hepatocellular carcinoma cells: an insight to molecular and in silico approach. Nutr Cancer 2018;70:971−83. https://doi.org/10.1080/01635581.2018.1490445.
[15] Kusmita L, Franyoto YD, Mutmainah M, Puspitaningrum I, Nurcahyanti ADR. Bixa orellana L. carotenoids: antiproliferative activity on human lung cancer, breast cancer, and cervical cancer cells in vitro. Nat Prod Res 2022;36:6421−7. https://doi.org/10.1080/14786419.2022.2036144.
[16] Chisté RC, Mercadante AZ, Gomes A, Fernandes E, da Costa Lima JLF, Bragagnolo N. In vitro scavenging capacity of annatto seed extracts against reactive oxygen and nitrogen species. Food Chem 2011;127:419−26.
[17] Abayomi M, Adebayo AS, Bennett D, Porter R, Shelly-Campbell J. In vitro antioxidant activity of Bixa orellana (Annatto) seed extract. J Appl Pharmaceut Sci 2014;4:101−6.
[18] Batista MA, Dos Santos Avt de LT, Nascimento Al Do, Moreira LF, Souza IRS, da Silva HR, et al. Potential of the compounds from bixa orellana purified annatto oil and its granules (Chronic®) against dyslipidemia and inflammatory diseases: in silico studies with geranylgeraniol and tocotrienols. Molecules 2022;27. https://doi.org/10.3390/molecules27051584.
[19] Tsuruma K, Shimazaki H, Nakashima K, Yamauchi M. Annatto prevents retinal degeneration induced by endoplasmic reticulum stress in vitro and in vivo. 2012. p. 713−24. https://doi.org/10.1002/mnfr.201100607.
[20] Galindo-Cuspinera V, Rankin SA. Bioautography and chemical characterization of antimicrobial compound(s) in commercial water-soluble annatto extracts. J Agric Food Chem 2005;53:2524−9. https://doi.org/10.1021/jf048056q.
[21] Quintero Quiroz J, Naranjo Duran AM, Silva Garcia M, Ciro Gomez GL, Rojas Camargo JJ. Ultrasound-assisted extraction of bioactive compounds from annatto seeds, evaluation of their antimicrobial and antioxidant activity, and identification of main compounds by LC/ESI-MS analysis. Int J Food Sci 2019;2019:5−7. https://doi.org/10.1155/2019/3721828.
[22] Azzarà A, Chiaramonte A, Filomeni E, Pinto B, Mazzoni S, Piaggi S, et al. Increased level of DNA damage in some organs of obese Zucker rats by γ-H2AX analysis. Environ Mol Mutagen 2017;58:477−84.
[23] Kumar Y, Periyasamy L. GC-MS analysis and in-vitro cytotoxic studies of bixa orellana seed extract against cancer cell line. Int J Pharm Pharmaceut Sci 2016;8:408−13.
[24] Gómez-Linton DR, Navarro-Ocana A, Román-Guerrero A, Alavez S, Pinzón-López L, Mendoza-Espinoza JA, et al. Environmentally friendly achiote seed extracts with higher δ-tocotrienol content have higher in vitro and in vivo antioxidant activity than the conventional extract. J Food Sci Technol 2021;58:2579−88.
[25] Husa NN, Hamzah F, Said HM. Characterization and storage stability study of bixin extracted from bixa orellana using organic solvent. IOP Conf Ser Mater Sci Eng 2018;358. https://doi.org/10.1088/1757-899X/358/1/012035.
[26] Alcázar-Alay SC, Osorio-Tobón JF, Forster-Carneiro T, Meireles MAA. Obtaining bixin from semi-defatted annatto seeds by a mechanical method and solvent extraction: process integration and economic evaluation. Food Res Int 2017;99:393−402.
[27] Kua YL, Gan S, Morris A, Ng HK. Ethyl lactate as a potential green solvent to extract hydrophilic (polar) and lipophilic (non-polar) phytonutrients simultaneously from fruit and vegetable by-products. Sustain Chem Pharm 2016;4:21−31.
[28] Leite PIP, Barreto SMAG, Freitas PR, de Araujo ACJ, Paulo CLR, de Almeida RS, et al. Extraction of bioactive compounds from buriti (Mauritia flexuosa L.) fruit by eco-friendly solvents: chemical and functional characterization. Sustain Chem Pharm 2021;22:100489.
[29] Chuyen HV, Eun JB. Effects of different extraction methods on the extraction yield, degradation of bixin and formation of harmful volatile compounds in the extracts from annatto seeds. Food Res 2021;5:42−8. https://doi.org/10.26656/FR.2017.5(6).017.
[30] Ishida BK, Chapman MH. Carotenoid extraction from plants using a novel, environmentally friendly solvent. J Agric Food Chem 2009;57:1051−9. https://doi.org/10.1021/jf8026292.
[31] FAO/WHO. General specifications and considerations for enzyme preparations used in food processing in Compendium of food additive specifications. 67th meeting, vol. 3; 2006.
[32] Varzakas T, Kiokias S. HPLC analysis and determination of carotenoid pigments in commercially available plant extracts. Curr Res Nutr Food Sci J 2016;4:1−14.
[33] Giorgi A, De Marinis P, Granelli G, Chiesa LM, Panseri S. Secondary metabolite profile, antioxidant capacity, and mosquito repellent activity of Bixa orellana from Brazilian Amazon region. J Chem 2013;2013. https://doi.org/10.1155/2013/409826.
[34] Mandal SK, Puri S, Kumar BK, Muzaffar-Ur-Rehman M, Sharma PK, Sankaranarayanan M, et al. Targeting lipid-sensing nuclear receptors PPAR (α, γ, β/δ): HTVS and molecular docking/dynamics analysis of pharmacological ligands as potential pan-PPAR agonists. Mol Divers 2023. https://doi.org/10.1007/s11030-023-10666-y.
[35] Villanueva-Bermejo D, Reglero G, Fornari T. Recent advances in the processing of green tea biomolecules using ethyl lactate. A review. Trends Food Sci Technol 2017;62:1−12.
[36] Jalilian Ahmadkalaei SP, Gan S, Ng HK, Abdul Talib S. Investigation of ethyl lactate as a green solvent for desorption of total petroleum hydrocarbons (TPH) from contaminated soil. Environ Sci Pollut Res 2016;23:22008−18.
[37] Kua YL, Gan S, Ng HK, Morris A. The potential of ethyl lactate as a green solvent to extract carotenoids and vitamin e from crude palm oil. Semenyih, Malaysia: Proc ISPFVF, Univ Nottingham Malaysia Campus; 2014. p. 11−3.
[38] Satyanarayana A, Prabhakara Rao PG, Rao DG. Chemistry, processing and toxicology of annatto (Bixa orellana L.). J Food Sci Technol 2003;40:131−41.
[39] Rahmalia W, Fabre J-F, Mouloungui Z. Effects of cyclohexane/acetone ratio on bixin extraction yield by accelerated solvent extraction method. Procedia Chem 2015;14:455−64. https://doi.org/10.1016/j.proche.2015.03.061.
[40] Cardarelli CR, de Toledo Benassi M, Mercadante AZ. Characterization of different annatto extracts based on

antioxidant and colour properties. LWT–Food Sci Technol 2008;41:1689–93.
[41] Dunn JL, Turnbull JD, Robinson SA. Comparison of solvent regimes for the extraction of photosynthetic pigments from leaves of higher plants. Funct Plant Biol 2004;31:195–202.
[42] Saini RK, Keum Y-S. Carotenoid extraction methods: a review of recent developments. Food Chem 2018;240:90–103.
[43] Silva YPA, Ferreira TAPC, Celli GB, Brooks MS. Optimization of lycopene extraction from tomato processing waste using an eco-friendly ethyl lactate–ethyl acetate solvent: a green valorization approach. Waste Biomass Valoriz 2019;10:2851–61. https://doi.org/10.1007/s12649-018-0317-7.
[44] Murador DC, Salafia F, Zoccali M, Martins PLG, Ferreira AG, Dugo P, et al. Green extraction approaches for carotenoids and esters: characterization of native composition from orange peel. Antioxidants 2019;8:613.
[45] Gómez-Ortíz NM, Vázquez-Maldonado IA, Pérez-Espadas AR, Mena-Rejón GJ, Azamar-Barrios JA, Oskam G. Dye-sensitized solar cells with natural dyes extracted from achiote seeds. Sol Energy Mater Sol Cells 2010;94:40–4. https://doi.org/10.1016/j.solmat.2009.05.013.
[46] Kiokias S, Proestos C, Varzakas T. A review of the structure, biosynthesis, absorption of carotenoids-analysis and properties of their common natural extracts. Curr Res Nutr Food Sci J 2016;4:25–37.
[47] Rivera-Madrid R, Aguilar-Espinosa M, Cárdenas-Conejo Y, Garza-Caligaris LE. Carotenoid derivates in achiote (Bixa orellana) seeds: synthesis and health promoting properties. Front Plant Sci 2016;7:1406.
[48] Pattanaik L, Pradhan S, Shaoo NK, Naik SN. Extraction and rapid quantification of pure bixin from bixa orellena L. Indian J Nat Prod Resour 2018;9:290–5.
[49] Nathan VK, Rani ME, Rathinasamy G, Narayanan Dhiraviam K. Antioxidant and antimicrobial potential of natural colouring pigment derived from Bixa orellana L. Seed Aril. Proc Natl Acad Sci India Sect B Biol Sci 2019;89:137–43.
[50] Galindo-Cuspinera V, Lubran MB, Rankin SA. Comparison of volatile compounds in water-and oil-soluble annatto (Bixa orellana L.) extracts. J Agric Food Chem 2002;50:2010. –5.
[51] Fidyt K, Fiedorowicz A, Strządała L, Szumny A. β-caryophyllene and β-caryophyllene oxide—natural compounds of anticancer and analgesic properties. Cancer Med 2016;5:3007–17.
[52] do Nascimento KF, Moreira FMF, Santos JA, Kassuya CAL, Croda JHR, Cardoso CAL, et al. Antioxidant, anti-inflammatory, antiproliferative and antimycobacterial activities of the essential oil of Psidium guineense Sw. and spathulenol. J Ethnopharmacol 2018;210:351–8.
[53] Franco-Arroyo NN, Viveros-Paredes JM, Zepeda-Morales ASM, Roldán E, Márquez-Aguirre AL, Zepeda-Nuño JS, et al. β-Caryophyllene, a dietary cannabinoid, protects against metabolic and immune dysregulation in a diet-induced obesity mouse model. J Med Food 2022;25:993–1002.
[54] Ziaei A, Ramezani M, Wright L, Paetz C, Schneider B, Amirghofran Z. Identification of spathulenol in Salvia mirzayanii and the immunomodulatory effects. Phyther Res 2011;25:557–62.
[55] Giriwono PE, Shirakawa H, Ohsaki Y, Sato S, Aoyama Y, Ho HJ, et al. Geranylgeraniol suppresses the expression of IRAK1 and TRAF6 to inhibit NFκB activation in lipopolysaccharide-induced inflammatory responses in human macrophage-like cells. Int J Mol Sci 2019;20:1–10. https://doi.org/10.3390/ijms20092320.
[56] Lozano-Grande MA, Gorinstein S, Espitia-Rangel E, Dávila-Ortiz G, Martínez-Ayala AL. Plant sources, extraction methods, and uses of squalene. Int J Agron 2018;2018:1–13. https://doi.org/10.1155/2018/1829160.
[57] Bitencourt APR, Duarte JL, Oliveira AEMFM, Cruz RAS, Carvalho JCT, Gomes ATA, et al. Preparation of aqueous nanodispersions with annatto (Bixa orellana L.) extract using an organic solvent-free and low energy method. Food Chem 2018;257:196–205. https://doi.org/10.1016/j.foodchem.2018.02.067.
[58] Shen CL, Kaur G, Wanders D, Sharma S, Tomison MD, Ramalingam L, et al. By decreasing theAnnatto-extracted tocotrienols improve glucose homeostasis and bone properties in high-fat diet-induced type 2 diabetic mice inflammatory response. Sci Rep 2018;8:1–10. https://doi.org/10.1038/s41598-018-29063-9.
[59] Pang K-L, Chin K-Y. The role of tocotrienol in protecting against metabolic diseases. Molecules 2019;24:923.
[60] Zhang R, Han Y, McClements DJ, Xu D, Chen S. Production, characterization, delivery, and cholesterol-lowering mechanism of phytosterols: a review. J Agric Food Chem 2022;70:2483–94.
[61] Othman RA, Moghadasian MH. Beyond cholesterol-lowering effects of plant sterols: clinical and experimental evidence of anti-inflammatory properties. Nutr Rev 2011;69:371–82.
[62] Yoshida Y, Niki E. Antioxidant effects of phytosterol and its components. J Nutr Sci Vitaminol 2003;49:277–80.
[63] Shahzad N, Khan W, Shadab MD, Ali A, Saluja SS, Sharma S, et al. Phytosterols as a natural anticancer agent: current status and future perspective. Biomed Pharmacother 2017;88:786–94.
[64] Scotter MJ, Wilson LA, Appleton GP, Castle L. Analysis of annatto (Bixa orellana) food coloring formulations. 2. Determination of aromatic hydrocarbon thermal degradation products by gas chromatography. J Agric Food Chem 2000;48:484–8.
[65] Ando K, Fujita T. Metabolic syndrome and oxidative stress. Free Radic Biol Med 2009;47:213–8.
[66] Carrier A. Metabolic syndrome and oxidative stress: a complex relationship. Antioxidants Redox Signal 2017;26:429–31.
[67] Paiva SAR, Russell RM. β-carotene and other carotenoids as antioxidants. J Am Coll Nutr 1999;18:426–33.
[68] Ellis S, Lin EJ, Tartar D. Immunology of wound healing. Curr Dermatol Rep 2018;7:350–8.
[69] Pinzón-García AD, Cassini-Vieira P, Ribeiro CC, de Matos Jensen CE, Barcelos LS, Cortes ME, et al. Efficient cutaneous wound healing using bixin-loaded PCL nanofibers in diabetic mice. J Biomed Mater Res Part B Appl Biomater 2017;105:1938–49.