Isolation, Characterization and Biological Activities of Food Colorants from *Bixa orellana*

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Abstract

*In the present study, the different solvent seed extracts of **Bixa orellana** were investigated for their application as food colorants. The extracts were isolated, characterized & their biological activities were determined. Extraction was performed using different solvent mixtures: $CHCl_3$/EtOH, $CHCl_3$/Acetone, Hexane/EtOAc and a base extraction (5 % KOH) to yield a reddish-orange semi-solid, with percentage yield of 9.02, 4.90, 2.98 and 26.66 % (w/w), respectively. The total carotenoids in the seed extracts were found to be 3.14 % ($CHCl_3$/EtOH), 1.42 % ($CHCl_3$/Acetone), 0.51 % (Hexane/EtOAc) and 1.76 % (Alkali extraction). Phytochemical investigation of the $CHCl_3$/EtOH seed extract over silica gel preparative thin layer chromatography led to the isolation of two compounds. The first one, compound BO-2, was identified as Bixin using spectroscopic techniques (UV, IR, MS and NMR). The second compound BO-3 was partially characterized. Bixin is one of the most important constituents of the seed, which was gravimetrically determined to be 1.62 % (w/w) from the seed. The $CHCl_3$/EtOH seed extract exhibited moderate inhibitory effect against the tested bacterial pathogens at a concentration of 50 mg/mL. The Gram-negative bacterium E. coli was found to be the most susceptible to the seed extracts, with zone inhibition of 14.0 mm (MIC = 0.25 mg/mL), while the least antibacterial activity against the Gram-positive bacteria, S. aureus, was observed, with zone of inhibition of 9.2 mm (MIC = 1.0 mg/mL). In general, the activity of the tested substances on the tested fungal pathogens were relatively weaker with the exception of seed extract against A. niger, which showed a zone inhibition of 9.2 mm (MIC = 12.5 mg/mL).*

Introduction

Many plants produce economically important organic compounds such as natural colorants, oils, resins, tannins, gums, waxes, flavors and fragrances, pharmaceuticals and pesticides. In-group of this color is a constituent and is one of the first characteristics sensed by consumers. Annatto seems to be an important natural colorant for food and drug industries owing to its potential uses as a substitute for Tartrazine, which is a synthetic colorant that is prohibited in many countries (JECFA, 1982). Simultaneous with the increasing awareness of toxicity of synthetic colors, need for colorants from natural sources has increased (Sinha *et al*., 2013). In addition to their role in coloration, natural pigments carry out a variety of important biological functions.

Bixa orellana belongs to the family Bixaceae and the genus Bixa. Under this genus, it is one of the five species known as orellana. Based on flower, fruit colour and shape, *Bixa orellana* has three varieties; one with white flowers and green capsules, second with purple flowers and brownish red capsules and third with pink flowers and red capsules (Akshatha *et al*., 2011). The chemical profile difference between these three species are not know and their similarity. Literature pertaining to the application of natural coloring is scant and the awareness of annatto as a coloring agent is limited particularly in Ethiopia. Since the demand for natural colorants is increasing, specifications that are more stringent are imposed and better understanding of their chemistry and biochemistry is required. The need for analysis of annatto is important since carotenoid content varies largely with maturity, variety, soil, light intensity among others (Dias *et al*., 2008). In addition, there are carotenoid losses during postharvest storage (Rodriguez-Amaya, 2001).

The comparison of Ethiopian introduced annatto relative to the JECFA and the largest producing country mon
ecology to be either favorable condition or not for these plant secondary metabolites in order to compare the world specifications. The study on antimicrobial activity on different strains of bacteria and fungus were also enhancing the application of natural colorant over synthetic one as an additional source of medicine and preservative usage. Based on the above assessment this work have been done in order to fulfill the profound gap by determining the overdue activity on *Bixa orellana* plant that is introduced at Wendo Genet and Tepi Agricultural Research Center.

Materials and Methods

Sample collection

A white flower, green capsule variety of *Bixa orellana* fresh fruits were collected from Wendo Genet Agricultural Research Center Gene Bank (WGARC). The bacterial test microorganisms used in this study were *Escherichia coli (*ATCC25922*), Pseudomonas aeruginosa (*ATCC25853*), Staphylococcus aureus (*ATCC25923*),* which were kindly supplied from Ethiopian Health and Nutrition Research *Institute* (EHNRI). The fungal pure culture of *Aspergilus Niger*, *Aspergilus flavus and Fusarium verticillodes* were taken from the culture grown from plant protection laboratory of Haramaya University.

Sample preparation and extraction

The seed preparation and extraction process were carried out by performing different sets of experiments and also by modifying those methods reported in the literature (JECFA, 2006**;** Smith, 2006; Jansi *et al*., 2008; Abayomi *et al*., 2014). The seeds were pulped out from the capsule and were allowed to dry in an oven at 40 °C for 24 h. Dried seed samples were initially soaked in hexane for 6 h to remove fats and waxes. The residue was subsequently and separately extracted with three different solvent mixtures (solvent mixture 1: $CHCl_3$/EtOH (1:1); solvent mixture 2: $CHCl_3$/Acetone (1:1); solvent mixture 3: Hexane/EtOAc (1:1)) by using Soxhlet apparatus for 5 h. The extracts were filtered, and Rotary evaporator removed the solvent.

The alkali extraction were performed by soaking the seeds in 0.5 % KOH solution and stirred for 30 min at 60 °C on a magnetic stirrer. The mixture was filtered, and the residue was washed with fresh 0.5 % KOH solution, stirred for 30 min, and then filtered. A fresh KOH solution was used to wash the residue while stirring for 15 min and the mixture was filtered. The filtrates were combined and 3 M HCl was used to acidify the mixture and precipitate crystals of extract. The precipitate was allowed to settle overnight and the supernatant was decanted and washed repeatedly with distilled water. The wet masses were dried in the oven at 40 °C for about 24 h. The lumps were pulverized in a mortar with pestle and the resulting powder was stored in airtight container until used for further studies (JECFA, 2006). The experiments were carried out in a completely randomized design with three replications. The yield extract was determined based on the formula

$$\text{Yield extract (\%)} = \frac{Mass\ of\ extract\ (mg).100\ \%}{Mass\ of\ seed\ (mg)}$$

Determination of total carotenoids

Seed extract (5 mg) were weighed into a small beaker. A small amount of ethanol was added to the beaker. The mixture was then stirred with a glass rod to dissolve the extract. The solutions were carefully transferred into a 100 mL volumetric flask. Ethanol was used to rinse the beaker and transferred to the flask. The volumes of solution in the flask were made up to 100 mL mark with ethanol. The flask was then covered and placed in a hot water bath at 50 ^{o}C with periodic shaking until all the extract was completely dissolved. The solution was allowed to cool at room temperature. Ethanol was used as a blank and the absorbance of the solutions were read on UV-

were assessed based on determining total percent of carotenoids through the formula

$$\text{\% of total carotenoids in terms of bixin} = \frac{A\,.\,V\,.\,100\ \%}{282.6\,.\,W}$$

Where, A is absorbance of the extract, V is the total volume (mL); W is the weight of the extract (mg) and 282.6 is the extinction coefficient of bixin.

Compound isolation & structural elucidation

Preparative TLC was prepared in the laboratory as follows: Initially the slurry was prepared by mixing 30 g silica gel and 60 mL distilled water. The slurry was then spread onto a glass plates (20 cm × 20 cm) to obtain 0.25 mm thickness. The plates were left overnight to dry and then activated by placing them in an oven with temperature 110 °C for 1 h. Isolation of compounds was conducted by dissolving 5 % crude extract in a mixture of EtOH and $CHCl_3$ (1:1) and applied it directly on preparative thin layer chromatographic plates. After dried the band subjected to isolate through a chromatographic chamber using a mixture of $CHCl_3$ and MeOH in the ratio of (47:3) as solvent systems. Chromatographic zones were visualized in daylight and then further confirmed under ultraviolet light of wavelength 254 and 366 nm. Pure compounds were isolated at R*F* value of 0.53 and 0.66, coded as BO-2 and BO-3 respectively.

Characterizations of the isolated compounds were governed by spectroscopic techniques through the overdue conditions. NMR spectra were recorded on a Bruker Avance DMX 400 FT-NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C at room temperature using deuterated $CHCl_3$. A region from 0 to 12 ppm for ^{1}H and 0 to 205 ppm for ^{13}C was employed for scanning. Signals were referred to an

coupling constants (*J*) in Hz. Multiplicities of ^{1}H NMR signals are indicated as *s* (singlet), *d* (doublet), *dd* (doublet of doublets), *t* (triplet), and *m* (multiplate). IR spectra were recorded among 400-4000 cm^{-1} in KBr pellets. UV-Visible spectra were scanned between 370-800 nm at room temperature/in chloroform solvent. ESI-MS were recorded on an Ultimate 3000 LC-MS. The measurement was carried out by an electrospray ionization method with positive mode. The source voltage and temperature were fixed at 3kV and 250ºC.

Free radicals scavenging activity on 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

The DPPH radical scavenging activities of the $CHCl_3$/EtOH (1:1) extract was determined by the method described by Cuendet *et al*. (1997). 50 of seven concentrations (4000, 3000, 2000, 1000, 500, 250, 125, 62.5 and 31.25 µg/mL) of the test samples were mixed with 5 mL of 0.004 % methanol solution of DPPH. The mixture was incubated for 30 minutes at 37 °C. After incubation, the absorbance of the mixture was read at 517 nm using UV-Vis spectrophotometer. Similarly, Ascorbic Acid (standard) was prepared at different concentrations (125 - 1000 µg/mL). Tests were carried out in triplicate and average values were taken. Inhibition of DPPH radical was calculated using the equation

$$I\ (\%) = 100 \times (Ao$$

Where Ao is the absorbance of DPPH solution, and as is the absorbance of the tested sample. The IC_{50} value that represented the concentration of the samples that caused 50 % inhibition was determined for all test samples.

Examination of anti-microbial activity

The selected bacteria were grown on MHA media, through a Petri dish within 24 h incubation at 37 °C. The bacteria inoculums suspension were prepared by transferring a loop full of cells from the stock cultures to distilled water until it reached the turbidity equal to that of the standard 0.5 McFarland solution monitored by spectrophotometer at a wavelength of 600 nm which is (0.08-0.13 absorbance) equivalent to 10^6-10^8 CFU/mL. The isolated fungal were grown on a Petridish through incubation for 7 days at 26 °C. The inoculums were prepared by dissolving a loop of fungus in distilled water until the concentration of the zoospore suspension was adjusted to approximately 10^6 zoospores, which were confirmed by a Haemocytometer. The inoculums were used immediately.

Antibacterial activities of the samples were evaluated by using disc diffusion (Kirby Bauer) method against the test strains as described by Tamil *et al*. (2011). *In vitro*

antimicrobial activity was screened using MHA. The MHA plates were prepared by pouring 18 mL of molten media into sterile petriplates. The plates were allowed to solidify for 5 min and 0.1 % inoculums suspension of tested organisms were swabbed uniformly and the inoculums were allowed to dry for 5 min.
the seed extract and 1mg/mL of the isolated compound (BO-2) were loaded on 6 mm sterile individual paper discs (HiMedia) and thoroughly dried in air draft to remove traces of the solvent. Negative control was prepared using respective solvent, which is

The fortified discs were placed on the surface of medium using a disc template and incubated at 37 °C for 24 h. Inhibition zones formed around the discs were measured with transparent ruler (in millimeters) (Kumaraswamy *et al*., 2002).

Determination of anti-fungal activity

Antifungal assay was performed through the standard procedure that is described by (Hari Babu *et al.*
isolated compound (BO-2) were loaded to a sterile paper disc independently. 18 mL of Potato Dextrose Agar medium were poured into sterile Petri dish. After solidification, a loop full of culture was swamp uniformly on the surface of the plate. Negative Controls was maintained with a solution of Potato Dextrose Agar and chloroform. Propiconazole maintained a positive control. The fortified discs were placed on the surface of medium using a disc template and incubated at 26 ºC. Growth was monitored for 24, 48 and 72 h, depending on the period required for the visible growth. The growths of treated samples were compared with their respective control plates.

Minimum Inhibitory Concentration (MIC) of the crude extract and isolated compound were determined by agar dilution method, where serial dilutions (100 mg/mL - 0.1 mg/mL) were prepar
sterile paper disc. 18 mL of medium were poured into sterile Petri dish. After

plate and incubated. The minimum concentrations that have inhibitory effect against the microorganisms (no growth) were recorded as the MIC value of the extract and isolated compound (BO-2).

Statistical analysis

Significance difference between extract yield and total carotenoid yield via different solvent combination and extraction methods were analyzed by SAS, version 9. Statistical significance was defined as $p < 0.05$.

Results and Discussion

The alkali and three extracting solvent methods, Hexane/EtOAc (1:1), $CHCl_3$/Acetone (1:1) and $CHCl_3$/EtOH (1:1) were evaluated for their effectiveness to extract carotenoids from *B. orellana* seeds. The polarity of solvent significantly affects the extract yield and the total carotenoid from the seed at $P < 0.01$ and total carotenoids from the seed extract at $P < 0.05$. Similarly the method of extraction also affect significantly for the crude extract yield and total carotenoid from the extract. But the total carotenoids from the seeds were not significantly ($P < 0.05$) affected by the method of extraction at.

There was a significant difference in the extracting ability of $CHCl_3$/EtOH (9.02 %; w/w from the seed) compared with the other two solvent systems (Table 1). Swati *et al.* (2013) reported similar results, where 9.5% yield was found for MeOH extract. It is interesting to note that the alkali extraction method gave the highest extract yield (26.66 %; w/w from the seed) **(**Table 2) which is about three times more than that offered by $CHCl_3$/EtOH solvent extraction. However, it is important to point out that the highest extraction yield was not translated to higher carotenoid yield (1.76 %; w/w); the alkali based method may just extract a high concentration of organic acids. This finding may suggest that the seeds are rich with organic acids, some of which may have little or no coloring values.

The $CHCl_3$/EtOH solvent system was superior in its ability to extract carotenoids (3.14 %; w/w from the seed) and it was significantly more efficient than the alkali extraction method and the other solvent system towards extracting the carotenoids. These findings could be supported by other studies reported in the literature, where chloroform and ethanol mixture have been found to be superior in extracting carotenoids, within the range of 2.98-5.91 % yield . As of similar to 3-4% of total pigment content of seeds of *B. orellana* originated from Peru and in Ethiopia it has a value of 3.14 %. This result is complies with the international benchmark for annatto pigment export more than 2.5 % of pigment content (Heywood, 1993). Based on the trends of our present study, it could be suggested that $CHCl_3$/EtOH solvents are superior to recovering a higher extraction yield of carotenoid components from annatto. The total carotenoid yield was significantly affected by var. type and environmental condition (Table 3). Pink flower with pink capsule var. has yielded higher carotenoid than the white flower with green capsule var.

Table 1. Effect of solvent polarity on extract and carotenoids yields.

Solvent Combination	Polarity Index (εo)	Yield Extract (%)	Total Carotenoids Yield (%)	Carotenoids yield from Seed Only (%)
Hexane/EtOAc	0.28	2.98^{b}	17.95^{b}	0.51^{b}
CHCl3/Acetone	0.35	4.91^{b}	28.69^{ab}	1.42^{b}
CHCl3/EtOH	0.41	9.02^{a}	34.76^{a}	3.14^{a}
LSD (0.05)	_	2.08	12.91	1.21

Means followed by the same letter within a column are statistically non-significant at P<0.05.

Table 2. Effect of extraction methods on extract and total carotenoids yields.

Extraction Method	Aril Extract Yield (%)	Total Carotenoids Yield (%)	Carotenoids Yield from Seed Only (%)
Solvent Extraction	9.02[b]	34.76[a]	3.14[a]
Alkali Extraction	26.66[a]	7.09[b]	1.76[a]
LSD (0.05)	10.51	16.85	2.84

Means followed by the same letter within a column are statistically non significant at P<0.05.

Table 3. Effect of location and var. on extract yield and total carotenoid yield from aril of seed of Bixa orallana

Location	Altitude (m.a.s.l.)	Var.	Yield Extract (%)	Total Carotenoids Yield (%)	Carotenoids Yield from Seed Only (%)
Bebeqa	1190	Pink flower	11.44[a]	21.00[c]	2.40[c]
Tepi	1205	Pink flower	10.78[a]	29.99[b]	3.23[a]
Wendo genet	1800	White flower	8.84[b]	34.47[a]	3.05[b]
LSD (0.05)	-	-	1.31	0.25	0.03

Means followed by the same letter within a column are statistically non significant at P<0.05.

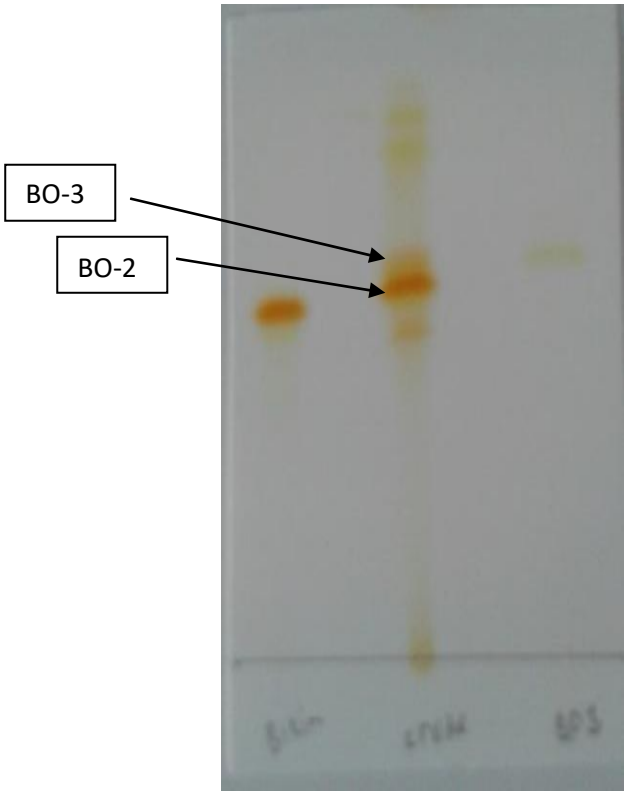

Figure 1. TLC profile of the crude extract (A) $CHCl_3/CH_3OH$ (47:3)

Compound **BO-2** was isolated as a purple reddish amorphous solid with an R*f* value of 0.53 ($CHCl_3/CH_3OH$; 47:3). A molecular formula of $\mathbf{C_{25}H_{30}O_4}$ was deduced for compound **BO-2** by positive-mode high resolution electrospray ionization mass spectrometry (HRESI-MS) (obtained mass *m/z* 395.2577 $[M+H]^+$, exact calculated

mass *m/z* 395.222235 $[M+H]^+$), as indicated in appendix figure 1. Compound **BO-2**

spectrum (Appendix Figure 2) that are a characteristic absorption of a carotenoid moiety (Soumaya *et al.*, 2010). The IR spectrum of compound **BO-2** (Appendix Figure 3) revealed a broad absorption band at 3436 cm^{-1} due to the presence of a stretching vibration of O-H group associated to a carboxylic functional group, a sharp peak at 2924 cm^{-1} shows the presence of C-H group stretching vibration. Furthermore, a band at 1716 cm^{-1} absorbed to a stretching vibration of the C=O group, a band at 1608 cm^{-1} to a stretching vibration of C=C group and a band at 1159 cm^{-1} to a starching vibration of C-O group.

^{1}H NMR spectrum of compound **BO-2** (Appendix Figure 4) showed a singlet spectra
s
of *trans*-vinyl protons adjacent to two carbonyl were evident from ^{1}H NMR spectrum (H-2: 5.90, *d*, *J* = 16 Hz; H-3: *d*; *J* = 16 Hz and H-18: *d*, *J* = 16 Hz; H-
d; *J* = 16 Hz). Other important ^{1}H NMR signals are assigned as shown in table 3. ^{13}C NMR spectrum of compound **BO-2** (Appendix Figure 5) showed the presence of 25 carbon atoms, including two carbonyl carbons resonates at 168.00 ppm (C-20) and 181.11 ppm (C-1). In addition to a methoxyl carbon signal (51.64 ppm), two sets of carbon-carbon double bonds adjacent to two carbonyl were noted in the ^{13}C NMR spectrum (C-2: 123.39, C-3: 151.09 and C-18: 142.39; C-19: 124.21). Complete ^{13}C NMR chemical shift assignments of compound **BO-2** was done as shown in table 3. From ^{1}H NMR, ^{13}C NMR, IR and UV spectra data of compound **BO-2**, its structure was established as bixin (Methyl hydrogen-6, 6'-diapo- -carotenedioate) as indicated in figure 2. This was further confirmed by comparing the NMR data with those reported in the literature for the same compound (Jondiko and Pattenden, 1989).

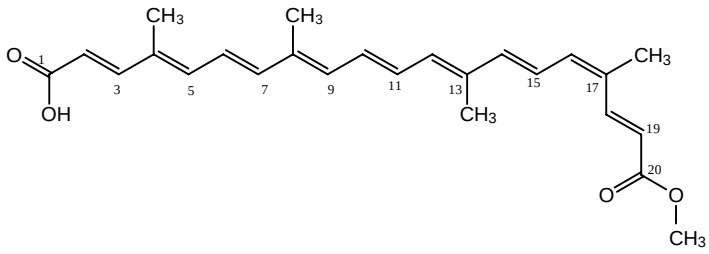

Figure 2. Chemical structure of compound BO-2.

Table 4. ^{1}H and ^{13}C NMR spectral data of compound BO-2 in CDCl3.

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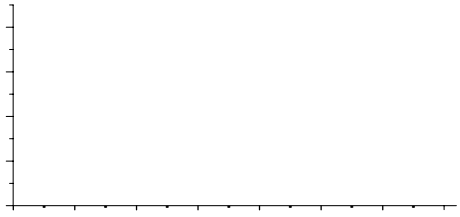
C Atom	^{1}H NMR (δ, ppm)		^{13}C NMR (δ, ppm)
	BO-2	Bixin (Literature)	BO-2
1	-	-	181.12
2	5.90 d ,(J= 16 Hz)	5.68 d (J = 15.8 Hz)	123.39
3	7.48 d (J = 16 Hz)	7.22 d (J = 15.8 Hz)	151.09
4	_	_	137.95
4-CH3	1.98 s	1.8-2.0 m	22.71
5	6.62 nr	6.30-7.00 m	130.73
6	6.70 nr	6.30-7.00 m	131.46
7	6.58 nr	6.30-7.00 m	136.57



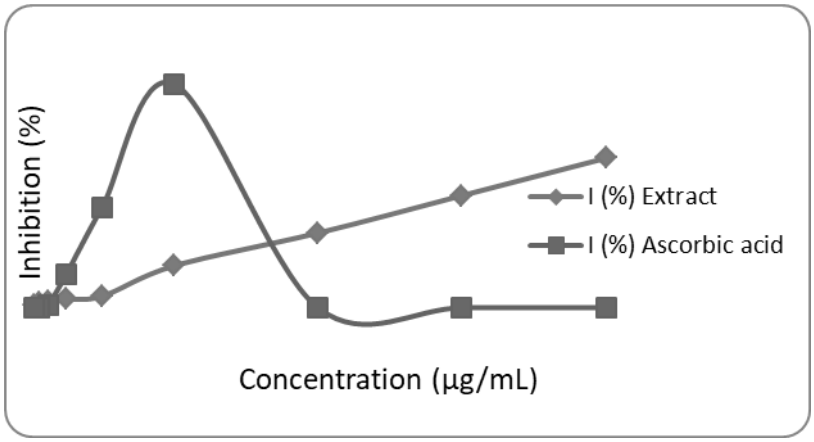


Figure 3. Plot of inhibition of DPPH scavenging activities of seed extracts of *Bixa orellana and* Ascorbic acid

The *in vitro* antibacterial activity of the seed extracts against three bacterial strains was assessed by using the disk diffusion method. The seed extract exhibited moderate inhibitory effect against the tested bacterial pathogens at a concentration of 50 mg/mL (Table 4). Among the tested bacterial strains, *E. coli*, which is the Gram-negative bacteria, was found to be the most susceptible to the seed extract, with zone of inhibition of 14.0 mm (MIC = 0.25 mg/mL). This result is in agreement with the work reported by Gelmy *et al*
showed the least antibacterial activity against the Gram-positive bacteria, *S. aureus*, with zone of inhibition of 9.2 mm (MIC = 1.0 mg/mL), which was consistent with the data reported by Rajendra (2014). Owing to the antibacterial effects of the seed extract, further phytochemical investigation was carried out, which led to the isolation of two carotenoids, of which the major compound is identified as bixin. As shown in Table 4, both tested Gram-positive and Gram-negative bacteria were less sensitive to the isolated compound (BO-2), ranging 2 - 4 mm zone inhibition at concentration of 1 mg/mL.

The seed extract of *B. orellana* also showed variation in the level of activity against the three fungal strains tested (Table 4). The seed extract exhibited weak activity against *A. Niger* with zone of inhibition of 9.2 mm (MIC = 12.5 mg/mL), however no activity was observed against *A. flavus*. All the tested fungi were resistant to the isolated compound (BO-2). In general, the carotenoids originated from plant are classified either primary or secondary metabolites. From these came up with an agreement that the extract favoring primary metabolite constituent have less activity against the microbes due to their synthesis as a requirement of the plant itself. Nevertheless, the secondary metabolites have better efficiencies on an activity against microbes due to their synthesis associated to defense mechanism of the plant.

Table 4. Diameters of zone of inhibition of seed extract of *B. orellana* and compound isolated from it against some microbes.

Test organism	Diameter of zone of inhibition (mm)		
	Seed extract (50 mg/ml)	Isolated compound (BO2) (1 mg/ml)	Penicillin (25 mg/ml)
Bacterial strains			
Escherichia coli	14.0	2.0	26.5
Pseudomonas aeurginosa	11.7	4.0	36.5
Staphylococcus aureus	9.2	3.0	24.0
Fungal strains	Seed extract (50 mg/mL)	Isolated compound (1 mg/mL)	Propiconazole (0.02%; v/v)
Aspergilus flavus	-	-	34.0
Aspergilus niger	9.2	-	22.0
Fusarium verticollodes	4.3	-	44.0

Table 5. Minimum inhibitory concentrations (MICs) of the seed extract of *B. orellana* and compound isolated from it against some microbes.

Test organism	MIC (mg/mL)							
	Seed extract				Isolated compound (B.O-2)			
Bacterial strains	0.125	0.25	0.50	1.00	0.125	0.25	0.50	1.00
Escherichia coli	+	-	-	-	+	+	+	-
Pseudomonas aeurginosa	+	+	-	-	+	+	-	-
Staphylococcus aureus	+	+	+	-	+	+	+	-
Fungal strains	6.25	12.50	25.50	50.00	0.125	0.25	0.50	1.00
Aspergilus flavus	+	+	+	+	+	+	+	+
Aspergilus niger	+	-	-	-	+	+	+	+
Fusarium verticollodes	+	+	-	-	+	+	+	+

+ = Growth (2-5 mm); - = No growth of inhibition

Conclusions

Extraction solvent has a significant influence on the extraction of carotenoids from *B. orelana*. Previous studies have mostly focused on single solvent system; however, this study clearly indicates that solvent systems involving $CHCl_3$/EtOH are more effective towards extracting optimal amount of carotenoids from *B. orellana*. Coloring strength was expressed in terms of total carotenoids, the result of which was comparable and meets the required standard set by the international community. However, the weak DPPH activity of the $CHCl_3$/EtOH seed extract might be due to the absence or low concentration of phenolic compounds in the seed extract of *B. orellana* and the interference of carotenoids at 517 nm. It was also interesting to note that almost all tested microbes are less sensitive to either of the crude extract and the isolated compound. In conclusion, *Bixa orellana* grown in Ethiopia has a huge potential as a natural food colorant.

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