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

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Abstract

The usage of vast amount of synthetic dye causes pollution that disturbs the ecological balance and health hazards for human beings. Indeed there is a growing trend towards the usage of natural colorants of plant origin in various food industries. One of such plant that falls in this category is *Bixa orellana* (Annatto) whose seed aril extracts are used as a natural food colorants. Aril of seeds of *B. orellana* was subjected to extraction using three 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% (w/w; $CHCl_3$/EtOH), 4.90% (w/w; $CHCl_3$/Acetone), 2.98% (w/w; Hexane/EtOAc) and 26.66% (w/w; alkali extraction). The total carotenoids were found to be 3.14% ($CHCl_3$/EtOH), 1.42% ($CHCl_3$/Acetone), 0.51% (Hexane/EtOAc) and 1.76% (Alkali extraction) in the seed extracts. Phytochemical investigation of the $CHCl_3$/EtOH seed extract over silica gel preparative thin layer chromatography led to the isolation of two compounds, of which one compound BO-2 was identified as Bixin by using spectroscopic techniques (UV, IR, MS and NMR). 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. It is important to note that the antioxidant activity of the seed extract was showed a weak free radical scavenging with an IC50 value of 3124.31 µg/mL, which is about 50 times less than that offered by the standard ascorbic acid (IC50=577.04 µg/mL). 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 *Escherichia coli* was found to be the most susceptible to the seed extract, with zone of 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). In conclusion, the present findings support the huge potential of B. orellana as a natural food colorant.

Keywords: Antimicrobial; Antioxidant; *Bixa orellana*; Carotenoids; Colorant; DPPH assay; Disk diffusion

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 these 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 [1-5]. Simultaneous with the increasing awareness of toxicity of synthetic colors, need for colorants from natural sources has increased. In addition to their role in coloration, natural pigments carry out a variety of important biological functions.

Literature pertaining to the application of natural coloring is scant and the awareness of annatto as a coloring agent is also limited particularly in Ethiopia. Since the demand for natural colorants is increasing, more stringent specifications for these products are imposed and also better understanding [6-10] 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. Also, there are carotenoid losses during postharvest storage.

The comparison of Ethiopian introduced annatto relative to the JECFA and the largest producing country monograph is used to give information's of the Ethiopian agro ecology to be either favorable condition or not for these plant secondary metabolites in order to [11,12] compete 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 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

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(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 [12-15]. 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 the solvent were removed by Rotary evaporator.

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 and 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 [16-18] 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. The experiments were carried out in a completely randomized design with three replications. The extract yield was determined based on the below formula.

Extract yield (%)=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°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-Vis spectrophotometer at λ max of 460 nm. The color values were assessed based on determining total percent of carotenoids through the formula;

% of total carotenoids in terms of bixin=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.

Isolation of compound

Home-made PTLC 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 Rf value of 0.53 and 0.66, coded as BO-2 and BO-3 respectively.

Structural elucidation

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 ^1H and 100 MHz for 13°C at room temperature using deuterated $CHCl_3$. A region from 0 to 12 ppm for ^1H and 0 to 205 ppm for 13°C was employed for scanning. Signals were referred to an internal standard tetra methyl silane (TMS). Chemical shifts are reported in δ units and coupling constants (J) in Hz. Multiplicities of ^1H NMR signals are indicated as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), and m (multiplate). IR spectra were recorded between 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 3 kV and 250°C.

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 described method. 50 µL 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 × (Ao –As)/Ao

Where Ao is the absorbance of DPPH solution (containing all reagents except the test sample), and as is the absorbance of the tested sample. The IC50 value which represented the concentration of the samples that caused 50% inhibition was determined for all test samples.

Examination of Antimicrobial Activity

Inoculums preparation

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 106 zoospores which were confirmed by a Haemocytometer. The inoculums were used immediately.

Determination of antibacterial activity

Antibacterial activities of the samples were evaluated by using disc diffusion (Kirby Bauer) method against the test strains. *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. 20 µL of 50 mg/mL the seed extract and 1 mg/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 chloroform. 25 mg/mL solution of Penicillin (20 µL/disc) was used as positive control. 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).

Determination of antifungal activity

Antifungal assay was performed through the standard procedure. 20 µL of 50 mg/mL of the seed extract and 1 mg/mL of 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. A positive control was maintained by Propiconazole. The fortified discs were placed on the surface of medium using a disc template and incubated at 26 °C. Growth was monitored for 24, 48 and 72 h, depending on the period of incubation time required for the visible growth. The growths of treated samples were compared with their respective control plates.

Determination of minimum inhibitory concentration (MIC)

Minimum Inhibitory Concentration (MIC) of the crude extract and isolated compound were determined by agar dilution method, where serial dilutions (100 mg/mL to 0.1 mg/mL) were prepared. 20 µL each concentration were loaded independently on sterile paper disc. 18 mL of medium were poured into sterile Petri dish. After solidification, 20 µL loop full of culture was swamp uniformly on the surface of the 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).

Design of the study and 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 methods of extraction also affect [19] significantly for the crude extract yield and total carotenoid from the extract. But the total carotenoids from the seeds were not significantly affected by the method of extraction at $P<0.05$ (Appendix Table 1).

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). Similar results were reported 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 bench mark for annatto pigment export more than 2.5% of pigment content. 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.

Compound BO-2 was isolated as a purple reddish amorphous solid with an Rf value of 0.53 ($CHCl_3$/CH_3OH; 47:3). A molecular formula of $C_{25}H_{30}O_4$ 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 exhibited three absorption bands at λ max of 445, 471 and 503 nm in its UV/Vis spectrum (Appendix Figure 2) that are a characteristic absorption of a carotenoid moiety. 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, as well as 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.

Solvent Combination	Polarity Index (εo)	Extract Yield (%)	Total Carotenoids yield from Extract (%)	Total Carotenoids yield from Seed (%)
Hexane/EtOAc	0.28	2.98[b]	17.95[b]	0.51[b]
$CHCl_3$/Acetone	0.35	4.91[b]	28.69[ab]	1.42[b]
$CHCl_3$/EtOH	0.41	9.02[a]	34.76[a]	3.14[a]
LSD (0.05)	-	2.08	12.91	1.21

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

Extraction Method	Aril Extract Yield (%)	Total Carotenoids yield from Extract (%)	Total Carotenoids yield from Seed (%)
Solvent Extraction	9.02b	34.76a	3.14a
Alkali Extraction	26.66a	7.09b	1.76a
LSD (0.05)	10.51	16.85	2.84
Means followed by the same letter under the same column are statistically non-significant at P<0.05 according to least significant difference (LSD) test			

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

^{1}H NMR spectrum of compound BO-2 (Appendix Figure 4) showed a singlet spectra which resonates at δ 3.81, corresponding to a methoxyl group. The presence of two sets of trans-vinyl protons adjacent to two carbonyl were evident from ^{1}H NMR spectrum (H-2: δ 5.90, d, J=16 Hz; H-3: δ 7.48, d; J=16 Hz and H-18: δ 7.99, d, J=16 Hz; H-19: δ 5.94, 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: δ 123.39, C-3: δ 151.09 and C-18: δ 142.39; C-19: δ 124.21). Complete ^{13}C NMR chemical shift assignments of compound BO-2 was done as shown in Table 3. From ^{1}H NMR, 13C NMR, IR and UV spectra data of compound BO-2, its structure was established as bixin (Methyl hydrogen-6, 6'-diapo-Ψ, Ψ-carotenedioate) as indicated (Figures 1-3). This was further confirmed by comparing the NMR data with those reported in the literature for the same compound.

Note: nr=not well resolved; J-coupling constant in parenthesis.

Compound BO-3 was obtained as an orange reddish amorphous solid with an Rf value of 0.66 in the solvent system ($CHCl_3$/CH_3OH; 47:3). ESI-mass spectrum (Appendix Figure 6) of compound BO-3 gave a pseudo molecular ion [M+H]+ at m/z=338.3632. The UV/Vis spectrum (Appendix Figure 7) of compound BO-3 revealed absorption bands at 444 and 427 nm, indicating the presence of a carotenoid moiety. The IR spectrum (Appendix Figure 8) of compound BO-3 showed the presence of a hydroxyl group (3435 cm^{-1}), C-H stretching of alkane (2923 cm^{-1}) and a C=C stretching of unsaturated hydrocarbon (1632 cm^{-1}). ^{1}H NMR spectrum (Appendix Figure 9) of compound BO-3 showed the presence of aliphatic protons from δ 0.8-2 ppm and δ 4.8-7.5 due to the presence of highly conjugated olefinic protons, in addition the compound has two alcoholic groups protons (δ 3.43 and 3.69 ppm). 13C NMR spectrum of compound BO-3 showed two olefinic carbons, which resonates at δ 114.11 and 138.33 ppm. Other important proton and carbon signals are shown in Appendix Figures 9 and 10. The structural elucidation of compound BO-3 is partially characterizes.

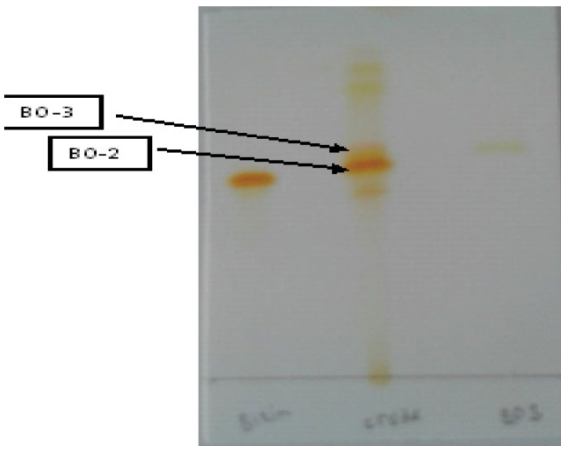

Figure 1: TLC profile of the crude extract (A) $CHCl_3$/CH_3OH (47:3).

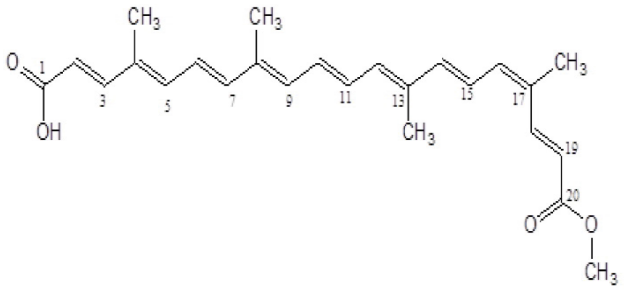

Figure 2: Chemical structure of compound BO-2.

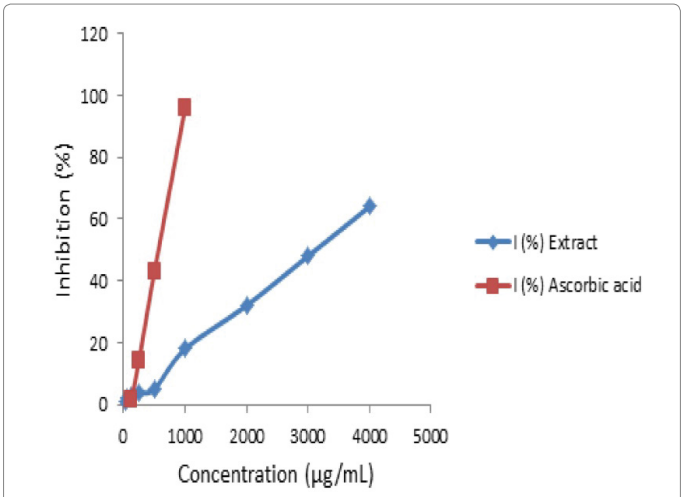

Figure 3: Plot of inhibition of DPPH scavenging activities of seed extracts of *Bixa orellana* and Ascorbic acid.

B. orellana seed extracts obtained from solvent extraction exhibited scavenging activity in dose-dependent manner with a ranging of 1- 64% for the tested concentrations of 31.5-4000 µg/mL. It is important to note that the seed extract showed a free radical scavenging with an IC50 value of 3124.31 µg/mL, which is about 6 times less than that offered by ascorbic acid (IC50=577.04 µg/mL), which is consistent with standard values. The DPPH assay is based on the reduction of DPPH in methanol solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form (DPPH-H) in the reaction. Since phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups and may contribute directly to antioxidative action. Therefore, weak free radical scavenging activity of the extract may be due to the absence or low concentration of phenolic compounds in the seed extract of B. orellana and the interference of Apo and Diapo carotenoids at 517 nm.

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 (Tables 3-5). 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 standard work reported with a MIC of 256 µg/mL. The seed extract also 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. But the secondary metabolites have better efficiencies on an activity against microbes due to their synthesis associated to defense mechanism of the plant.

C Atoms	^{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-CH_3	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
8	-	-	137.15
8-CH_3	1.28 s	1.8-2.0 m	12.66
9	6.38 nr	6.30-7.00 m	130.46
10	6.88 nr	6.30-7.00 m	131.46
11	6.88 nr	6.30-7.00 m	131.46
12	6.38 nr	6.30-7.00 m	130.73
13	-	-	137.15
13-CH_3	1.28 s	1.8-2.0 m	12.63
14	6.58 nr	6.30-7.00 m	136.57
15	6.70 nr	6.30-7.00 m	131.46
16	6.62 nr	6.30-7.00 m	130.73
17	-	-	140.51
17-CH_3	1.28 s	1.8-2.0 m	29.68
18	7.99 d (J=16 Hz)	7.80 d (J=15.8 Hz)	142.39
19	5.94 d (J=16 Hz)	5.80 d (J=15.8 Hz)	124.21
20	-	-	168.01
20-OCH_3	3.81 s	3.66 s	51.64

Table 3: ^{1}H and ^{13}C NMR spectral data of compound BO-2 in $CDCl^{3}$.

Conclusion

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 *Bixa 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, *B. orellana* grown in Ethiopia has a huge potential as a natural food colorant.

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Test organisms	Diameter of zone of inhibition (mm)		
Bacterial strains	**Seed extract (50 mg/mL)**	**Isolated compound (BO2) (1 mg/mL)**	**Penicillin (25 mg/mL)**
Escherichia coli	14	2	26.5
Pseudomonas aeurginosa	11.7	4	36.5
Staphylococcus aureus	9.2	3	24
Fungal strains	**Seed extract (50 mg/mL)**	**Isolated compound (1 mg/mL)**	**Propiconazole (0.02%; v/v)**
Aspergilus flavus	-	-	34
Aspergilus niger	9.2	-	22
Fusarium verticollodes	4.3	-	44

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

	MIC (mg/mL)							
Test organisms	**Seed extract**				**Isolated compound (B.O-2)**			
Bacterial strains	0.125	0.25	0.5	1	0.125	0.25	0.5	1
Escherichia coli	+	-	-	-	+	+	+	-
Pseudomonas aeurginosa	+	+	-	-	+	+	-	-
Staphylococcus aureus	+	+	+	-	+	+	+	-
Fungal strains	6.25	12.5	25.5	50	0.125	0.25	0.5	1
Aspergilus flavus	+	+	+	+	+	+	+	+
Aspergilus niger	+	-	-	-	+	+	+	+
Fusarium verticollodes	+	+	-	-	+	+	+	+
+=Growth (2-5 mm); -=No growth of inhibition								

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

References

1. Abayomi M, Adebayo AS, Bennett D, Porter R, Shelly-Campbell J (2014) *In vitro* antioxidant activity of *bixa orellana* (annatto) seed extract. JAPS 4: 101-106.
2. Cuendet M, Hostettmann K, Potterat O (1997) Iridoid glucosides with free radical scavenging properties from *Fagvaea blumei*. Helv Chim Acta 80: 1144-1152.
3. Dias MG, Filomena M, Camoes GFC, Oliveira L (2008) Uncertainty estimation and in-house method validation of HPLC analysis of carotenoids for food composition data production. Food Chem 109: 815-824.
4. Gelmy LC, Jose EZ, Jessica L (2014) *In vitro* evaluation of *Bixa orellana* L (Annatto) seeds as potential natural food preservative. Journal of Medicinal Plant Research 8: 772-779.
5. Hari babu B, Vasu babu A, Sobhan babu A, Rambabu A (2011) Phytochemical and antifungal screening of leaves of *Givotia Rottleriformis Griff*. Journal of Pharmacy Research 4: 2146-2148.
6. Heywood VH (1993) In: flowering plants of the world. New York Oxford University press (New York), 426.
7. Sigurdson GT, Tang P, Giusti MM (2017) Natural colorants: Food colorants from natural sources. Annu Rev Food Sci Technol 8: 261-280.
8. Shahid-ul I, Rather LJ, Mohammad F (2016) Phytochemistry, biological activities and potential of annatto in natural colorant production for industrial applications-A review. J Adv Res 7: 499-514.
9. Rivera-Madrid R, Aguilar-Espinosa M, Cardenas-Conejo Y, Garza-Caligaris LE (2016) Carotenoid derivates in achiote (Bixa orellana) Seeds: Synthesis and Health Promoting Properties. Front Plant Sci 7: 1406.
10. Jondiko IJO, Pattenden G (1989) Terpenoids and an apocarotenoid from seeds of *Bixa orellana*. Phytochemistry 28: 3159-3162.
11. Kumaraswamy Y, Cox PJ, Jaspars M, Nahar L, Sarker SD (2002) Screening seeds of scottish plants for antibacterial activity. Journal of Ethnopharmacol 83: 73-77.
12. Morales M, Galdos MDC, Lopez I, Pina JC, Vazquez R (2016) In Vitro Antimicrobial Action of Bixa Orellana L. IJPNI 3: 3.
13. Pasaporte MS, Rabaya FJR, Toleco MM, Flores DM (2014) Xanthophyll content of selected vegetables commonly consumed in the Philippines and the effect of boiling. Food Chem 158: 35-40.
14. Sinha K, Chowdhury S, Saha PD, Datta S (2013) Modeling of Microwave-Assisted Extraction of Natural Dye From Seeds of *Bixa orellana* (Annatto) Using Response Surface Methodology (RSM), and Artificial Neural Network (ANN). Industrial Crop Production 41: 165-171.
15. Pandey RP, Parajuli P, Shin JY, Lee J, Lee S, et al. (2014) Enzymatic biosynthesis of novel resveratrol glucoside and glycoside derivatives. Appl Environ Microbiol 80: 7235-7243.
16. Soumya V, Venkatesh P, Hari PK, Shrishailappa B (2010) Microwave facilitated extraction of bixin from *Bixa Orellana* and its *in-vitro* antioxidant activity. Der Pharmacia Lettre 2: 479-485.
17. Swati DN, Shrivastava B, Pankaj S, Hemant KJ, Ganesh N (2013) Pharmacognostical and phytochemical investigation of leaves of *Bixa orellana* Linn. Int J Pharm Sci Rev Res 22: 247-252.
18. Tamil SA, Dinesh MG, Satyan RS, Chandrasekaran B, Rose C (2011) Leaf and seed extracts of *Bixa Orellana* L. Exert antimicrobial activity against bacterial pathogens. JAPS 1: 116-120.
19. Wagh SS, Vadnere GP, Patil AV, Jain SK (2012) *In vitro* free radical Scavenging and Antioxidant activity of *Cicer arietinum* L. (Fabaceae). International Journal of PharmTech Research 4(1): 343-350.