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Phytochemical Analysis of *Bixa orellana* L. Seeds by GC-MS and Evaluation of Antioxidant Activity

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Abstract

Oxidative stress results from an imbalance of reactive oxygen species and antioxidant mechanisms and is associated with chronic and degenerative diseases. The use of natural and artificial antioxidants is commonly prescribed to manage it. In nature, some plants synthesize specialized metabolites with significant biological activity. This applies to *Bixa orellana* L., which contains carotenoids and terpenes. The present study purpose to per-form a phytochemical analysis of *B. orellana* seeds and determine their antioxidant activity using in vitro assays. The seed extract obtained by maceration in hexane, ethyl acetate, and methanol was analyzed by gas chromatography-mass spectrometry (GC-MS). In vitro antioxidant activity was also evaluated using the DPPH assay, with ascorbic acid as the reference control. A two-way ANOVA followed by Dunnett's multiple comparisons test was performed. A total of 141 volatile and semi-volatile compounds were identified, mainly terpenes, esters, and fatty acids, among which geranylgeraniol (27.50%), ishwarane (11.01%), and geraniol acetate (7.76%) were the most prominent. The extract showed significant dose-dependent antioxidant activity, with an IC_{50} of 0.5108 mg/mL^{-1}, compared to ascorbic acid with an IC_{50} of 0.2266 mg/mL^{-1}. The results indicate that the seeds of *B. orellana* L. possess metabolites capable of inhibiting free radicals such as DPPH.

Keywords: specialized metabolites; terpenes; free radicals

1. Introduction

Oxidative stress occurs when there is an imbalance in cells between the production of reactive oxygen species (ROS) and an organism's ability to eliminate them through endogenous or exogenous antioxidant systems [1]. Free radicals are molecules characterized by having unpaired electrons in their outer orbital, a condition that makes them highly reactive; therefore, they are also called reactive oxygen and nitrogen species (ROS/RNS) [2,3]. Their accumulation can induce oxidative damage to a variety of biomolecules, which is a precursor to neurological diseases such as Alzheimer's, Parkinson's, and multiple sclerosis [4,5]. It also contributes to aging, cancer, diabetes, high blood pressure disorders, and cardiovascular diseases [4,6].

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Antioxidants are chemical compounds capable of neutralizing reactive oxygen species through electron donation, hydrogen atom transfer, or metal ion chelation, thereby attenuating oxidative stress induced damage [3]. Among the natural sources of antioxidants, phenolic compounds, flavonoids, terpenoids, and carotenoids stand out, and their potential has been extensively studied [7–9]. These metabolites provide protection against biotic and abiotic stress in plants, and in humans, they exhibit antioxidant, anti-inflammatory, and chemoprotective properties [7,10,11].

Bixa orellana L. is a shrub belonging to the Bixaceae family, characterized by producing reddish, triangular seeds contained within a fruit capsule (Figure 1). It is cultivated in the tropics worldwide [12]. Its commercial importance lies in the dye obtained from the seed, primarily bixin and norbixin [13,14]. In addition to these carotenoids, the presence of terpenoids, flavonoids, alkaloids, and glycosides have also been reported [15–17], to which antimicrobial and antiproliferative activity is attributed [14,15,17–19].

Despite advances in phytochemical research on *B. orellana* L., comparative studies integrating specialized metabolite analysis with antioxidant activity using standardized methods are still needed. This will help to achieve a better understanding of the relationship between phytochemical composition and the ability to neutralize free radicals. Therefore, the present study purpose to perform a phytochemical analysis of *B. orellana* seeds and determine their antioxidant activity using in vitro assays.

Figure 1. *Bixa orellana* L.: (a) shrub, (b) fruit capsules and (c) seeds.

2. Materials and Methods

2.1. Biological Material

Bixa orellana L. seeds collected in San Juan Comaltepec, Oaxaca, were used to obtain crude extracts from 50 g of dried and crushed seeds. An extraction train was performed in increasing order of polarity with hexane (Mc. Meyer), ethyl acetate (Mc. Meyer), and methanol (Mc. Merck), macerating for one week with solvent changes every 24 hours. The extract was filtered using Whatman No. 1 filter paper and evaporated in a rotary evaporator (Buchi B-480, Equipar, Switzerland) at 45 °C to concentrate and recover the extract for each solvent. The yield of the resulting extracts was calculated, and a pool was prepared to obtain a sample containing the mixture of metabolites extracted with the three solvents. This sample was stored in amber bottles at 4 °C until analysis.

2.2. Phytochemical Analysis

Qualitative identification of the compounds in the seed of *B. orellana* L. was performed using an Agilent 7890B gas chromatograph (GC-MS), and quantification of their relative abundances was performed using an Agilent 5977a mass selective detector (MSD).

An amount of 50 mg of the sample was dissolved in 2 mL of HPLC grade methanol (Meyer brand). The sample was passed through a PTFE microfilter and injected into the gas chromatograph in splitless mode. The chromatographic conditions were as follows: DB-WAX Ultra Inert column 60m x 250 µm x 0.25 µm, column temperature was 40 °C for 9 °C/min to 240 °C for 10 min, carrier gas was helium, flow rate was 1 mL/min, and the injection of temperature was 220 °C. The conditions of the mass selective detector were as follows: the electron ionization energy was 70 Ev, the scan range

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between 30 and 550 amu, the reading speed of 13.8 spectra/s, the temperature of the ionization chamber of 200 °C and that of the transfer line of 250 °C.

Data processing was performed using MassHunter Workstation software (Agilent Technologies, Inc. 2012). Compounds were identified based on mass spectral fragmen-tation patterns, which were compared with chemical compound information in the NIST (National Institute of Standards and Technology) database and the Flavor fragrance da-tabase [20,21].

2.3. Antioxidant Activity by DPPH

The determination of antioxidant activity was performed by DPPH (2,2-Diphenyl-1-picrylhydrazyl) according to the methodology followed by Liu et al. [22] and Ibarra Estrada et al. [23].

The DPPH radical scavenging assay provides an approximation for evaluating the antioxidant potential of an extract. The radical exhibits a violet coloration, and the reduced form is yellow; therefore, the decrease in absorbance at 517 nm determines the activity of the compound compared to the DPPH control solution [3].

A 0.1 mM methanolic solution of DPPH was prepared. The initial absorbance of DPPH in methanol was measured at 517 nm, and no changes were recorded during the assay with the samples. A standard curve was constructed from the absorbance measurements of the DPPH radical at different DPPH concentrations (2.0, 1.6, 1.2, 0.08, 0.04, 0.02, and 0 mM). The crude extract of *B. orellana* L. seed was dissolved in methanol (5mg in 1 mL) (Mc. Merk), from which three concentrations were established (0.05, 0.5 and 1 mg/mL-1).

One milliliter of each treatment was added to 3 mL of methanolic DPPH solution. The resulting mixture was incubated for 30 minutes in the dark at room temperature. Absorbance was measured at 517 nm using a spectrophotometer (Spectronic Genesis 5). Ascorbic acid was used as a reference control at the same concentrations as the seed sample. Measurements were performed in triplicate. Results were expressed as IC50 and as the percentage of DPPH inhibition compared to the control, according to the following formula:

$$\% \text{ DPPH inhibited} = (A_{\text{control}} - A_{\text{sample}}) \times 100 / A_{\text{control}}$$

where A_{control} is the absorbance of the control and A_{sample} is the absorbance of the sample

The IC_{50} was determined from the graph of the percentage of inhibition using the linear regression method with GraphPad Prism version 10.0 [24] and corresponds to the concentration at which 50% of the free radicals were neutralized.

2.4. Statistical Analysis

The evaluations were done in triplicate. To determine differences between treatments, a two-way ANOVA was performed followed by Dunnett's multiple comparisons test using GraphPad Prism version 10.0 [24].

3. Results and Discussion

3.1. Extract Yield

The pool of extracts had a total yield of 16.03% per gram of crude *B. orellana* L. seed extract.

3.2. Phytochemical Profile

Gas chromatography-mass spectrometry (GC-MS) analysis of the crude extract of *B. orellana* L. seeds identified 141 volatile and semi-volatile compounds (Figure 2) between 8.5 and 38 minutes. Few compounds were observed between 8.5 and 13 minutes. Between 14 and 20 minutes, a more complex region was observed with numerous small and medium peaks, and between 30 and 38

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minutes, high molecular weight peaks were observed. Fifteen compounds had a relative abundance greater than 1%, while the rest had lower abundances.

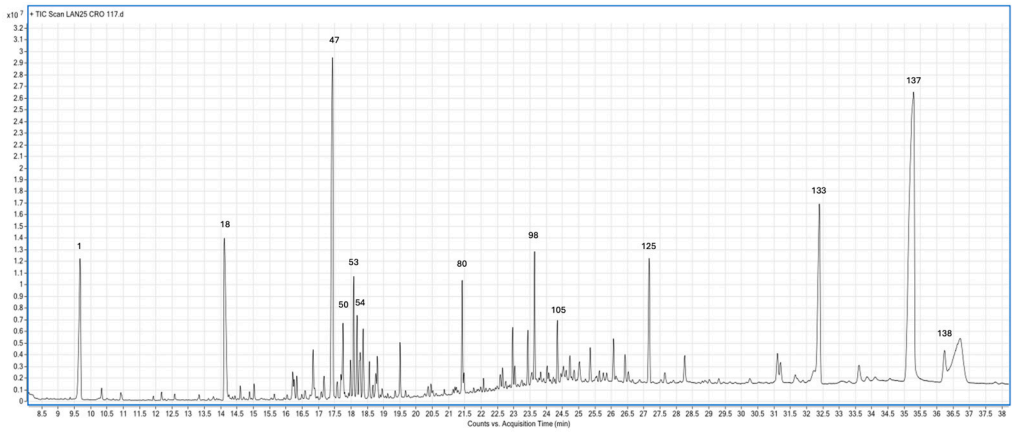

Figure 2. Chromatogram of the crude seed extract of *Bixa orellana* L., obtained by Gas Chromatography coupled to Mass Spectrometry (GC-MS). The numbers correspond to the compound number in Table 1.

Table 1 presents the 141 identified compounds. In general, the phytochemical profile is grouped into terpenes, esters, alcohols, ketones, and fatty acids. Fifty-five compounds were detected within the terpene group, with sesquiterpenes predominating at 43 compounds, representing a total relative abundance of 19.74%. Only six diterpenes were present, with a total relative abundance of 28.61%. Among the terpenes, geranylgeraniol (27.50%), ishwarane (11.016%), and geraniol acetate (7.76%) stand out with the highest relative abundance. Figure 3 shows the molecular structures of these compounds.

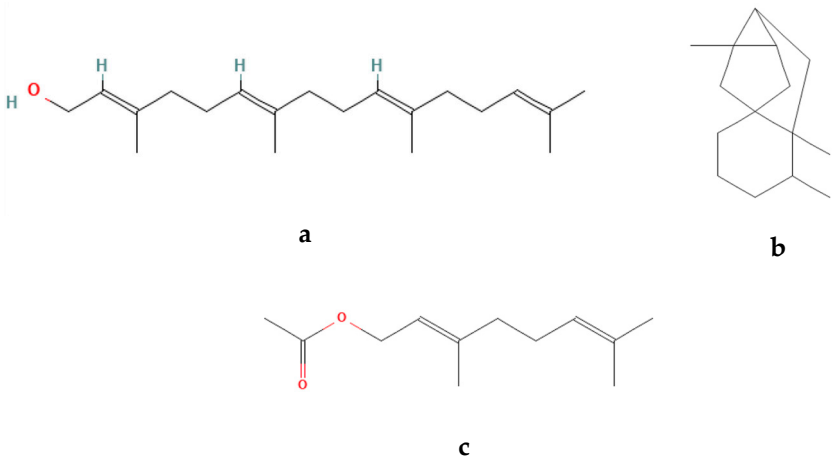

Figure 3. Molecular structure of (a) geranylgeraniol, (b) ishwarane, (c) geraniol acetate.

The phytochemical profile obtained shows agreement with previous research on the composition of *B. orellana* L. seeds. This species has been documented to contain higher levels of sesquiterpenes, diterpenes, and fatty acid esters, as well as prenylated alcohols such as geraniol, geranylgeraniol, and their derivatives [13,25,26]. Raju et al. [26] reported the presence of ishwarane as one of the sesquiterpenes present in various parts of the plant. On the other hand, geranylgeraniol, a diterpene associated with carotenoid metabolism and the biosynthesis of bixin and norbixin [27], has been reported as one of the most abundant constituents in the volatile fraction of the seed [14,18].

A considerable proportion of fatty acids was also present, including linoleic acid at 3.06%, palmitic acid at 3.66%, and oleic acid at 0.948% relative abundance. This is consistent with the oily

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nature of the seeds and with research describing that up to 30-445% of the seed weight corresponds to unsaturated oils [13,26].

The profile obtained confirms that the seeds of *B. orellana* L. contain a mixture of volatile metabolites, highlighting the abundance of sesquiterpenes, diterpenes and lipophilic compounds.

Table 1. Compounds identified in the chromatogram obtained by gas chromatography coupled to mass spectrometry (GC-MS) of the organic crude extract of *Bixa orellana* L. seed.

Number	Name	RT Min	Percentage %	Chemical group
1	Isobutyl acetate	8.656	0.0189	Ester
2	2,2,5-trimetilhexano	8.88	0.0129	Alkano
3	2-Butenal, 2-methyl-	9.087	0.0112	Aldehyde
4	**Benzene, 1,3-dimethyl-**	9.675	**4.9286**	Aromatic hydrocarbon
5	Dodecane	10.337	0.2762	Alkane
6	2-Hexanol	10.696	0.0162	Alcohol
7	Cyclopentanol, 1-methyl-	10.927	0.1195	Alcohol
8	2-Propanone, 1-hydroxy-	12.179	0.1542	Hydroxyacetona
9	Propanoic acid, 2-hydroxy-, methyl ester, (.+/-.)-	12.281	0.0195	Fatty acid ester
10	Acetaldehyde, hydroxy-	12.389	0.08592	Hydroxyaldehyde
11	5-Hepten-2-one, 6-methyl-	12.584	0.09831	Ketone
12	Acetic acid, hydroxy-, methyl ester	13.302	0.0256	Carboxylic ester
13	Tetradecane	13.333	0.0894	Alkane
14	Ethanol, 2-butoxy-	13.621	0.0261	Alcohol
15	2,2′-Bioxirane	13.774	0.0663	Epoxide
16	Benzene, 1,3-bis(1,1-dimethylethyl)-	13.874	0.0233	Alkylbenzene
17	Dodecane, 1-iodo-	13.938	0.0215	Alkane
18	**Acetic acid**	14.107	**6.0109**	Carboxylic acid
19	2(3H)-Furanone, 5-methyl-	14.166	0.068	Lactone
20	Propanoic acid, 2-oxo-, methyl ester	14.25	0.0758	Fatty acid
21	α-Cubebene	14.435	0.0571	Sesquiterpene
22	σ-Elemene	14.599	0.2261	Sesquiterpene
23	2-Ethyl-1-hexanol	14.705	0.0575	Alcohol
24	Ylangene	14.882	0.1443	Sesquiterpene
25	α-Copaene	15.023	0.2872	Sesquiterpene
26	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	15.256	0.0613	Furano
27	cis-Muurola-4(15),5-diene	15.644	0.1055	Sesquiterpene
28	Furan, 2-ethyl-5-methyl-	15.76	0.0133	Furan
29	2-Furancarboxaldehyde, 5-methyl-	15.88	0.0092	Furan
30	1,2-Propanediol, 1-acetate	15.943	0.0351	Ester
31	Pentanoic acid, 4-oxo-, methyl ester	16.037	0.0772	Fatty acid
32	Hexadecane	16.181	0.0417	Alkane
33	(-)-Aristolene	16.208	0.5095	Sesquiterpene
34	Methyl 4-oxo-2-pentenoate	16.253	0.3422	Ester
35	β-Elemene	16.334	0.3993	Sesquiterpene
36	B-copaene	16.487	0.0888	Sesquiterpene
37	Cyclooctasiloxane, hexadecamethyl-	16.571	0.029	Xylosane
38	Caryophyllene	16.589	0.1648	Sesquiterpene
39	1,2-Ethanediol, monoacetate	16.809	0.099	Ester
40	Naphthalene	16.835	0.9478	Sesquiterpene
41	Isoledene	16.887	0.1224	Sesquiterpene
42	Benzaldehyde, 4-methyl-	16.941	0.0451	Aldehyde
43	Elixene	16.976	0.0253	Sesquiterpene
44	Isoledene	17.087	0.1501	Sesquiterpene
45	Cyclosativene	17.17	0.4759	Sesquiterpene

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46	Glyoxal, 4-methylphenyl-	17.31	0.0259	Aldehyde
47	**Ishwarane**	17.433	**11.0164**	Sesquiterpene
48	Humulene	17.579	0.3048	Sesquiterpene
49	2-Furancarboxylic acid, 3-methyl-, methyl ester	17.639	0.0112	Ester
50	**α-Amorphene**	17.755	**1.7063**	Sesquiterpene
51	δ-Selinene	17.928	0.0724	Sesquiterpene
52	Aristolochene	17.984	0.6452	Sesquiterpene
53	**β-Cubebene**	18.082	**2.2405**	Sesquiterpene
54	**Valencene**	18.185	**1.4967**	Sesquiterpene
55	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	18.285	0.974	Sesquiterpene
56	Benzoic acid, 3-methyl-, methyl ester	18.353	0.2178	Carboxylic acid
57	**Naphthalene**	18.373	**1.1754**	Aromatic hydrocarbon
58	δ-Cadinene	18.563	0.6178	Sesquiterpene
59	γ-Cadinene	18.675	0.1652	Sesquiterpene
60	1,2-Cyclopentanedione	18.754	0.3006	Ketone
61	(-)-.α-Panasinsen	18.808	0.775	Sesquiterpene
62	Valencen	18.895	0.0481	Sesquiterpene
63	Ethanone, 1-(4-methylphenyl)-	18.956	0.1712	Ketone
64	2-Hexanone, 6-(acetyloxy)-	19.127	0.0699	Ketone/ester
65	4-Methylphenyl acetone	19.355	0.1071	Ketone
66	Acetic acid	19.464	0.0477	Aromatic hydrocarbon
67	1H-Cyclopropa[a]naphthalene	19.508	0.9378	Sesquiterpene
68	Calamenene	19.577	0.0111	Sesquiterpene
69	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	19.676	0.1084	Monoterpene
70	2,5-Dihydroxyheptane	19.763	0.0511	Alcohol
71	Epicubebol	20.245	0.0349	Alcohol
72	2,6-Bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol	20.322	0.0108	Phenol
73	2,5-Dihydroxyheptane	20.368	0.2035	Alcohol
74	Furan, tetrahydro-2,5-dimethyl-	20.458	0.1935	Ester
75	Naphthalene	20.625	0.0329	Aromatic hydrocarbon
76	Carotol	20.864	0.0966	Sesquiterpene
77	β-Calacorene	21.124	0.0448	Aromatic hydrocarbon
78	Cadina-1(10),6,8-triene	21.191	0.1192	Sesquiterpene
79	1,5-Hexanediol	21.234	0.1271	Alcohol
80	**Benzaldehyde**	21.415	**2.0419**	Aldehyde
81	γ-Gurjunene	21.468	0.3336	Sesquiterpene
82	1,1,4,7-Tetramethyldecahydro-1H-cyclopropa[e]azulene-4,7-diol	21.771	0.0685	Sesquiterpene/alcohol
83	Geranil hexanoato	21.873	0.0463	Ester
84	Cyclododecasiloxane	21.901	0.0158	Xylosane
85	Cyclohexylideneacetone	21.981	0.0852	Ketone
86	1,3,6,10-Cyclotetradecatetraene	22.071	0.2154	Diterpene
87	1,3,6,10-Cyclotetradecatetraene	22.487	0.0918	Diterpene
88	Dihydroxyacetone	22.584	0.3637	Ketone
89	1,2,4-Trimethoxybenzene	22.655	0.3557	Benzene
90	5-Cyclodecen-1-ol	22.759	0.0946	Sesquiterpene
91	Piperazine, 1,4-dimethyl-	22.826	0.053	Ester
92	**Espatulenol**	22.965	**1.0143**	Sesquiterpene
93	γ-Costol	23.027	0.3133	Alcohol
94	1-Nonanol	23.125	0.0319	Alcohol
95	Cembrene	23.252	0.1186	Diterpene

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96	Selin-6-en-4.alpha.-ol	23.431	0.9214	Sesquiterpene
97	Germacreno-δ-4-ol	23.558	0.2542	Alcohol
98	**Palmitic acid**	23.637	**2.3622**	Fatty acid
99	Glycerol α-monoacetate	23.826	0.1558	Alcohol
100	α-Springene	23.917	0.0507	Diterpene
101	Ethyl palmitate	24.02	0.2777	Fatty acid ester
102	4(15),5,10(14)-Germacratrien-1-ol	24.075	0.1772	Sesquiterpene
103	Isospathulenol	24.222	0.1035	Sesquiterpene
104	1-Naphthalenol	24.271	0.0543	Sesquiterpene
105	**Isospathulenol**	24.35	**1.131**	Sesquiterpene
106	1-Naphthalenol	24.459	0.1421	Sesquiterpene
107	Glycerol 1-monoacetate	24.539	0.3332	Ester
108	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	24.621	0.1979	Ketone
109	Humulane-1,6-dien-3-ol	24.736	0.5011	Sesquiterpene
110	α-Muurolene-14-ol	24.865	0.2078	Alcohol
111	Glycerin	25.004	0.2592	Alcohol
112	Sobrerol	25.024	0.216	Alcohol
113	β-Selinene	25.035	0.2466	Sesquiterpene
114	Viridiflorol	25.358	0.7184	Sesquiterpene
115	Farnesyl acetone	25.559	0.12	Ketone
116	7R,8R-8-Hydroxy-4-isopropylidene-7-methylbicyclo [5.3.1]undec-1-ene	25.641	0.1988	Alkene
117	2-Decenoic acid	25.763	0.1744	Fatty acid
118	2-Naphthalenol,	25.865	0.2292	Sesquiterpene
119	Methyl stearate	26.074	0.8862	Fatty acid ester
120	Isobutyl palmitate	26.195	0.0468	Ester
121	Elaidic acid	26.427	0.5943	Fatty acid
122	Elaidic acid	26.527	0.1887	Fatty acid
123	Dehydrofukinone	26.537	0.1071	Ketone/sesquiterpene
124	Cyclododecasiloxane	26.953	0.014	Xylosane
125	**Linoleic acid**	27.169	**3.0674**	Fatty acid
126	5-Hydroxymethylfurfural	27.527	0.0946	Aldehyde/alcohol
127	Linoleic acid	27.649	0.2297	Fatty acid
128	Linolenic acid	28.261	0.7189	Fatty acid
129	trans-2-Dodecenoic acid	29.014	0.0979	Fatty acid
130	Eicosanoic acid, methyl ester	29.306	0.1185	Fatty acid
131	Geranyl-.α.-terpinene	31.203	0.6396	Diterpene
132	Geranylgeranyl formate	31.656	0.2687	Terpenoid ester
133	Gernylgeraniol acetate	32.397	**7.7694**	Acetate ester
134	Acetic acid, 2-(1-buten-3-yl)-2-nitro-, ethyl ester	33.614	0.7082	Ester
135	Farnesyl propionate	33.856	0.17078	Sesquiterpene
136	Adipic acid	34.102	0.1543	Carboxylic acid
137	**Geranylgeraniol**	35.293	**27.5022**	Diterpene
138	**Palmitic acid**	36.239	**1.3027**	Fatty acid
139	Tyrosol, acetate	37.8	0.0942	Ester
140	Tyrosol, acetate	37.8	0.0941	Ester
141	Phthalic acid, butyl 2-pentyl ester	37.998	0.0365	Ester

3.3. Antioxidant Activity

Table 2 presents the inhibitory concentrations 50 (IC_{50}) of the crude extract of *B. orellana* L. seed and ascorbic acid, both at three concentrations (1.0, 0.5 and 0.05 mg/mL^{-1}).

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The seed extract shows clear, dose dependent antioxidant activity, with inhibition reaching 59.70% at 1 mg/mL^{-1}, decreasing to 47.50% at 0.5 mg/mL^{-1}. At 0.05 mg/mL^{-1}, activity barely reaches 37.45%. Ascorbic acid, on the other hand, exhibits greater antioxidant activity, which is expected given its high reducing capacity as a pure compound.

Regarding the IC_{50}, the value obtained for ascorbic acid (0.2266 mg/mL^{-1}) indicates that it has a high antioxidant potential, as very little is required to achieve 50% inhibition. The IC_{50} of the crude seed extract (0.5108 mg/mL^{-1}) shows that more than twice the concentration of ascorbic acid is needed to inhibit 50% of DPPH. The larger standard deviation indicates greater variability in the extract's behavior, which is typical of complex mixtures.

Table 2. Percentage of inhibition and IC_{50} of the *Bixa orellana* L. seed treatments on DPPH.

Extract or compound	Concentration (mg/mL^{-1})	DPPH radical inhibition (%)	IC50 ± DE (mg mL^{-1})
Ascorbic acid	1.0	99.50	0.2266 ± 0.0303
	0.5	97.00	
	0.05	89.00	
Seed	1.0	59.70	0.5108 ± 0.1891
	0.5	47.50	
	0.05	31.45	

SD = Standard deviation. Percentage inhibition values are presented as means. IC_{50} values are presented as means ± standard deviation.

Figure 4 graphically represents the percentage of DPPH radical inhibition and confirms the trend of the values in Table 2. Therefore, the seed extract of *B. orellana* L. exhibits significant, concentration dependent antioxidant activity, suggesting that some secondary metabolites can donate hydrogens or electrons to neutralize free radicals. According to the molecular structure of geranylgeraniol (Figure 3), which contains -OH groups, it has the capacity to donate H to neutralize free radicals; furthermore, the presence of double bonds may enhance its radical scavenging capacity, thus promoting antioxidant activity [3].

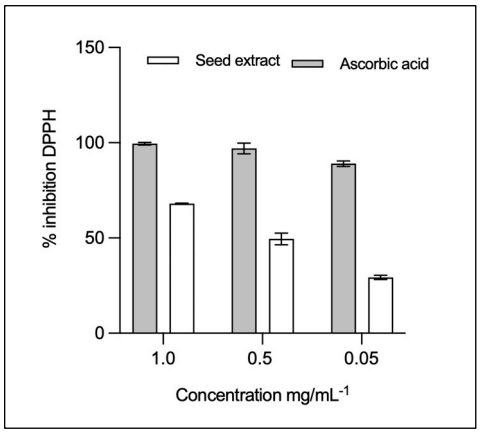

Figure 4. Percentage of DPPH inhibition of three concentrations (1, 0.5 and 0.05 mg/mL^{-1}) of the organic crude extract of *Bixa orellana* L. seed.

4. Conclusions

Gas chromatography-mass spectrometry (GC-MS) analysis of *B. orellana* L. seeds reveals a diverse phytochemical composition of volatile and semi-volatile compounds, predominantly terpenes and fatty acids. The abundance of specialized metabolites such as geranylgeraniol and ishwarane suggests their contribution to the antioxidant activity observed in the DPPH assay.

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Although the crude extract of B. *orellana* L. seed showed a lower antioxidant capacity than ascorbic acid, its action demonstrates that the compounds present in the volatile fraction can participate in free radical neutralization mechanisms.

The results obtained indicate that the seed of *B. orellana* L. could be a potential source of cosmetic or pharmaceutical applications, although further studies are needed to explore its mechanism of action and its stability in biological systems.

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