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Essential oil composition, carotenoid profile, antioxidant and antimicrobial activities of the parasitic plant *Cuscuta mitraeformis*

[Composición del aceite esencial, perfil de carotenoides, actividades antioxidantes y antimicrobianas de la planta parásita *Cuscuta mitraeformis*]

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Abstract: The chemical composition of the essential oil and carotenoid content of the parasitic plant *Cuscuta mitraeformis* are described for the first time. The essential oil was analyzed by GC-FID and GC-MS revealing nonanal (24.6%) as the main constituent followed by thymol (16.5%) and eugenol (7.5%). The total carotenoid content (130 mg 100 g⁻¹ FW) was determined by HPLC-DAD. The carotenoid fraction contained β -carotene (76.4 mg 100 g⁻¹ FW) and lutein (18.9 mg 100 g⁻¹ FW) as the most abundant compounds. A weak antioxidant activity was observed by the essential oil against DPPH radical (IC₅₀, 1.4 mg mL⁻¹), whereas a strong antioxidant activity was determined for the carotenoid fraction (IC₅₀, 60.1 μ g mL⁻¹). The essential oil inhibited the growth of *Clavibacter michiganensis*, *Pseudomonas syringae* pv. tomato and *Erwinia carotovora* with minimum inhibitory concentrations of 122.5, 184.5, 234.2 μ g mL⁻¹, respectively.

Keywords: *Cuscuta mitraeformis*, essential oil, carotenoids, antioxidant, antibacterial.

Resumen: La composición química del aceite esencial y el contenido de carotenoides de la planta parásita *Cuscuta mitraeformis* se describen por primera vez. El aceite esencial fue analizado por GC-FID y GC-MS siendo el nonanal (24.6%) el constituyente principal seguido del timol (16.5%) y el eugenol (7.5%). El contenido total de carotenoides (130 mg 100 g⁻¹ PF) fue determinado por HPLC-DAD. La fracción de carotenoides contuvo β -caroteno (76.4 mg 100 g⁻¹ PF) y luteína (18.9 mg 100 g⁻¹ PF) como compuestos mayoritarios. Fue observada una actividad antioxidante débil por parte del aceite esencial frente al radical DPPH (IC₅₀, 1.4 mg mL⁻¹), mientras que una fuerte actividad antioxidante fue determinada para la fracción de carotenoides (IC₅₀, 60.1 μ g mL⁻¹). El aceite esencial inhibió el crecimiento de *Clavibacter michiganensis*, *Pseudomonas syringae* pv. tomato y *Erwinia carotovora* con una concentración mínima inhibitoria de 122.5, 184.5, 234.2 μ g mL⁻¹, respectivamente.

Palabras clave: *Cuscuta mitraeformis*, aceite esencial, carotenoides, antioxidante, antibacterial.

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INTRODUCTION

The genus *Cuscuta* comprises around 200 members, many of them considered as medicinal sources (Ye *et al.*, 2002; Ferraz *et al.*, 2011; Raza *et al.*, 2015; Kaiser *et al.*, 2015; Yang *et al.*, 2016). These organisms are parasitic species that infest a wide range of wild and domesticated plants (Kaiser *et al.*, 2015). Conversely, the seeds of some *Cuscuta* species such as *C. chinensis* and *C. australis* are used as supplements in the pharmaceutical market (Yang *et al.*, 2016). Phytochemical studies revealed that the seeds and stalks of these plants contain cuscutine, stigmaterol, kaempferol, quercetin, dulcitol, myricetin, tannins and coumarins (Ye *et al.*, 2002; Ferraz *et al.*, 2011; Raza *et al.*, 2015). These compounds are valued as antioxidant, anti-inflammatory, antibacterial and diuretic agents (Ferraz *et al.*, 2011; Raza *et al.*, 2015). Despite the peculiar abilities of the *Cuscuta* species to infest their hosts, very few is known about the volatiles involved in the infestation process and of the pigments associated with its color. There are few reports on the essential oil composition and carotenoids from *Cuscuta reflexa* and *Cuscuta australis*, respectively (Baccarini *et al.*, 1965; Paudel *et al.*, 2014). On the other hand, recent studies propose that volatiles may have an important role in the infestation of *Cuscuta* species and in the host resistance (Kong & Zhao, 2014; Tjiurutue *et al.*, 2016). As other parasitic plants, *Cuscuta* species can adapt their metabolism to that of their hosts. Thus, many secondary metabolites could be interacting between both species (Paudel *et al.*, 2014).

In the northern highlands of Puebla, México, *Cuscuta mitraeformis* is known as “longanicilla” because of its intense yellow-orange color and its similarity with a tiny sausage. In this region, *C. mitraeformis* parasites *Sambucus mexicana* (synonym of *S. nigra*). Traditionally, the plant is boiled to obtain a yellow tincture used to dye folk wool coats. *C. mitraeformis* is also used as an ingredient for ceremonial showers because of its pleasant smell. Due to the lack of scientific data regarding the volatile profile and biological activities of *C. mitraeformis*, the main objective of this work was to contribute with the description of the principal constituents of the essential oil and the role they have in the aromatic properties and the yellow-orange color of this plant.

MATERIAL AND METHODS

Plant material

Cuscuta mitraeformis Engelm. (Convolvulaceae) was collected in Yaonáhuac, Puebla, México (19°87'16"N 97°45'16"W; 2000 masl) in February 2016. The identity of the plant was confirmed at the Herbarium of the FCME-UNAM were a reference voucher 152383 was deposited. Five different samples (n=5) were taken from their hosts (*Sambucus mexicana*) for subsequent extraction.

Essential oil extraction

Fresh stems of *C. mitraeformis* were immediately dried at 30° C for 3 days in darkness. 300 g of dried stems were extracted by hydrodistillation in a Clevenger-type apparatus for 3 h, trapping the essential oil in n-hexane. The solvent was removed under N₂ stream, the oil was dried over anhydrous sodium sulfate and stored at 4° C in amber glass vials until analyzed. The yields and density assays of the essential oil, and the preparation of stock solutions to perform antioxidant and antimicrobial tests were done according to Villa-Ruano *et al.* (2015a).

GC-FID and GC-MS analyses

The analyses were performed using a Hewlett Packard 6890 II series equipped with an HP-5 capillary column (30 m x 0.25 mm I.D., 0.25 µm of 5% phenyl-dimethylpolysiloxane). The GC-MS data were obtained in a Varian CP3800 gas chromatograph equipped with a capillary column Factor Four VF-5 ms (30 m x 0.25 mm I.D, 25 µm of 5:95 phenyl-dimethylpolysiloxane plate) coupled to a Varian quadrupole 320MS model. The mobile phase was helium at a flow rate of 1 mL min⁻¹. The GC-FID and GC-MS run conditions were as reported by Villa-Ruano *et al.* (2015a). The identity of the oil components was determined according to their mass spectra (70 eV) by comparison with the NIST 2.0 Standard Reference Database, with the literature (Adams, 2007) and by co-injection of authentic standards. The Kovats indices (RI) were obtained by running a standard mixture of n-alkanes (C₈-C₂₀) under the same analytical conditions. The quantification of the components was performed based on their GC peak areas.

Extraction and analysis of carotenoids

200 g of fresh stems were ground using a sterile mortar and pestle and immediately extracted with 600

mL of cold hexane: acetone (1:1 v/v) enriched with 0.5% (w/v) of butylated hydroxytoluene (BHT). The crude extract was incubated with vigorous shaking at 4° C for 12 h in darkness. The extract was filtered and reduced to dryness using a rotary evaporator (Buchi-R200). The extract was resuspended in diethyl ether and then, saponified with 30% methanolic KOH at room temperature in darkness. The soaps and alkalis were removed by washing this preparation several times with 50 mL of 10% NaCl solution and distilled water in accordance with Pintea *et al.* (2003). The mixture was shaken in a sedimentation funnel, the upper layer containing carotenoids was collected, reduced to dryness under N₂ stream, resuspended in 500 µL acetone and filtered using Titan® filters containing a 0.45 µm pore diameter nylon membrane. The samples were stored at -20° C in amber glass vials until analysis. Twenty microliters of the carotenoid fraction were injected by triplicate in a Hewlett Packard 1050 system coupled to an HP G1306A diode array detector and equipped with a C₁₈ Nucleosil ODS (5 µm, 250 x 4.6) column. The running conditions, the mobile phase, the temperature of the column, the flow rate and absorbance were as described by Pintea *et al.* (2003). The abundance of lutein, β-cryptoxanthin, lycopene, α-carotene and β-carotene, was estimated by calibration curves using authentic standards from Sigma-Aldrich Co.

Antioxidant and antimicrobial assays

The essential oil and the carotenoid fractions were assayed for these activities basically in accordance with Villa-Ruano *et al.* (2015a). A solution of 0.1M DPPH (Sigma-Aldrich Co) was used for antioxidant assays without BHT with the fractions resuspended in acetone. Dose-response curves were performed with each component (0.02 to 2 mg mL⁻¹) in a final volume of 0.3 mL DPPH using a 96-well plate in quintuplicate. Same dose-response curves were performed with ascorbic acid as a standard of reference. The plates were incubated at 25° C in darkness for 1 hour. The absorbance was recorded at 517 nm and then used to calculate the percentage of inhibition as previously described by Villa-Ruano *et al.* (2015a). The IC₅₀ values were obtained by linear regression using the software GraphPad 6.0. The IC₅₀ values were compared and validated by ANOVA-Tukey Test (p < 0.01) using the same statistical software.

The antibacterial assays were carried out using *Ewinia carotovora* ssp *carotovora*, *Clavibacter michiganensis* AB299158, and *Pseudomonas syringae* pv. *tomato* DC3000 by the broth microdilution method using resazurin as an indicator of cell viability and dose-response curves of 0.02 to 2 mg mL⁻¹ (Sarker *et al.*, 2007; Villa-Ruano *et al.*, 2015b). The incubation was performed with 5 x 10⁵ UFC in a 96-well plate in quintuplicate at a final volume of 0.3 mL. Dose-response curves (0.001-1 mg mL⁻¹) were also performed with rifampicin (for *E. carotovora* and *C. michiganensis*) and Agrigen Plus® (for *P. syringae*) as standards of reference. Absorbance was measured at 545 nm. The MIC value was considered as the initial concentration able to avoid any absorbance change in the dose response curves.

RESULTS AND DISCUSSION

The GC-MS profile of the essential oil from *C. mitraeformis* revealed 23 known volatile compounds (Table 1) and 94.7% of the constituents were identified. The monoterpenes were more abundant than sesquiterpenes and oxygenated hydrocarbons (Table 1). Nonanal was the main component (24.6%) followed by thymol (16.5%) and eugenol (7.5%). Hexanal, heptanal, nonanal and linalool have been previously detected in the essential oils from the aerial parts of its host *Sambucus nigra* (synonym of *S. mexicana*) grown in France (Toulemonde & Richard, 1983). However, the variation in type and content of volatiles usually depends on the specific geographical location, on biotic and abiotic factors and on the phenological stage of the host plants (Tjiurutue *et al.*, 2016). As an example, the chemical profile of the essential oil from *S. nigra* grown in Turkey showed similar levels of hexanal, limonene, and linalool but contrarily, very low levels of nonanal (1.2%) than the reported here for *C. mitraeformis* (Ağalar *et al.*, 2014). Other *Sambucus* species such as *S. ebulus* from Iran, exhibited a similar abundance of p-menthane monoterpenes, linalool, and eugenol than that of the parasitic *C. mitraeformis* (Feizbakhsh *et al.*, 2014). In the same way, the chemical profile of the essential oil from *C. mitraeformis* was quite different to that of *Cuscuta reflexa* collected in Nepal (Paudel *et al.*, 2014). Remarkably, some volatiles such as limonene, linalool, vanillin and eugenol were coincidentally found in both oils. Nonanal, the principal volatile of the essential oil from *C.*

Table 1
Volatile constituents of the essential oil from *Cuscuta mitraeformis*.

Compound	IR1 ^a	IR2 ^a	LRI	Area (%)	Identification ^b
Hexanal	802	804		2.4	RI, MS
Heptanal	898	900		3.2	RI, MS
Limonene	1030	1032		5.6	RI, MS, AS
Eucalyptol	1039	1041		3.8	RI, MS, AS
Linalool	1098	1099		1.6	RI, MS, AS
Nonanal	1102	1103		24.6	RI, MS
α -Terpineol	1189	1192		2.7	RI, MS
Estragole	1195	1196		0.7	RI, MS
Geraniol	1249	1251		2.9	RI, MS, AS
Piperitone oxide	1253	1255		3.5	RI, MS
Thymol	1292	1290		16.5	RI, MS
Eugenol	1356	1357		7.5	RI, MS
α -Copaene	1376	1377		2.7	RI, MS
Decanoic acid	1386	1387		0.9	RI, MS
Dodecanal	1406	1405		1.6	RI, MS
Vanillin	1412	1414		2.6	RI, MS, AS
Aromadendrene	1434	1435		0.7	RI, MS
α -Humulene	1454	1455		2.6	RI, MS
(Z)-Nerolidol	1533	1533		1.2	RI, MS
α -Cadinene	1538	1539		0.9	RI, MS
Ledol	1561	1562		1.3	RI, MS
Spathulenol	1576	1577		4.6	RI, MS
Caryophyllene oxide	1581	1582		0.6	RI, MS
Monoterpenes				36.6	
Sesquiterpenes				14.6	
Oxygenated hydrocarbons				32.7	
Phenylpropanoids				10.80	
Total				94.7	

^a IR1 and IR2 retention indices for HP-5 and Factor Four VF-5ms columns, respectively.

^b Identification by retention index (RI) and mass spectrometry (MS, 70eV) or by co-injection of authentic standards (AS).

mitraeformis has previously been involved in the growth inhibition of some opportunistic species of *Penicillium* (French *et al.*, 1978). Also, the oxygenated hydrocarbon has shown antidiarrheal activity on induced diarrhea CD1 mice (Zavala-Sánchez *et al.*, 2002). Nonanal is known to be an aromatic compound used in the cosmetic industry due to its nice smell (Omonov *et al.*, 2014). Considering the volatility of nonanal and the traditional use of *C. mitraeformis* in medicinal showers, it is probably that this compound may have a role in the pleasant aroma of the plant. The same compound has recently been

linked to the modulation of the abscisic acid (ABA) response in some plants such as *Oryza sativa* (Kong & Zhao, 2014). These authors propose that ABA avoids the genetic expression of the rice lipoxygenase 3 (OsLOX3) and rice hydroperoxide lyase 1 (OsHPL1), which are involved in the biosynthesis of nonanal (Kong & Zhao, 2014). This evidence suggest the involvement of nonanal and related volatiles in the chemical defense and probably in the abilities of *Cuscuta* species to colonize their hosts (Tjiurutue *et al.*, 2016). Further studies are required to elucidate

the specific participation of nonanal in the parasitic activity of *C. mitraeformis*.

The carotenoid profile (Figure 1) revealed β -carotene as the main pigment of the voluble stalks from *C. mitraeformis* (Table 2). The total carotenoid content was around 130 mg 100 g⁻¹ FW, these amounts were similar to those reported for carrot (Fikselová et al., 2008). The abundance of β -carotene and lutein was comparable of those previously reported for *Cuscuta australis* (Baccarini et al., 1965). On the other hand, the presence of β -carotene, β -cryptoxanthin and lycopene was coincident with the carotenoids reported for *Cuscuta reflexa* (Murkherjee et al., 2008). The levels of these carotenoids were comparable with those found in the edible GAC fruit (*Momordica cochinchinensis*) (Aoki et al., 2002). *C. mitraeformis* is not used as an edible plant in the northern highlands of Puebla, however, the use as an alternative source of vitamin A precursors should be considered. Interestingly, the

seeds of *Cuscuta chinensis* have been consumed for centuries as a traditional herbal medicine to treat diverse health problems. Currently, alimentary supplements based on the seeds of *C. chinensis* are available in the pharmaceutical market (Yang et al., 2016). These seeds contain potent antioxidants such as quercetin, kaempferol, sesamin and its glycosides (Ye et al., 2002). Due to the high endogenous levels of phytate, oxalate and toxic alkaloids in other parasitic plants, the anti-nutrient properties of the *C. mitraeformis* stalks should be determined (Ferraz et al., 2011; Ohikhena et al., 2017). Also, possible fluctuations in the metabolism of carotenoids should be explored, since different biotic (hosts) and abiotic factors such as light intensity and the geographical location could produce changes in the color of the stalks (Snyder et al., 2005; Tjiurutue et al., 2016). The identification of carotenoids in the stalks of *C. mitraeformis* partially explain the color of the yellow tincture used to dye wool coats.

Table 2
Carotenoids from the stems of *Cuscuta mitraeformis*.
Results are expressed in mg 100 g⁻¹ fresh weight (FW).

Compound	mg 100 g ⁻¹ FW
Lutein	19.9 ± 1.3
β -Cryptoxanthin	4.8 ± 0.01
Lycopene	3.8 ± 0.2
α -Carotene	3.3 ± 2.1
β -Carotene	77.4 ± 5.7

^a Means plus standard deviation of five different plant samples.

The essential oil showed a weak antioxidant activity against DPPH (IC₅₀ = 1.4 ± 0.1 mg mL⁻¹; Figure 2), but the activity of the carotenoid fraction was better (IC₅₀ = 60.1 ± 0.5 μ g mL⁻¹; Figure 2). However, ascorbic acid had the best antioxidant activity (p < 0.01) than the two latter agents (IC₅₀ = 12.6 ± 0.5 μ g mL⁻¹; Figure 2). Despite these differences the chemical preparations from *C. mitraeformis* could be alternatively used as antioxidants. Up to our knowledge, there is no evidence of the antioxidant activity of nonanal, which was the most abundant compound in the essential oil. Nevertheless, other essential oils containing equivalent amounts of p-menthane monoterpenes, eugenol, limonene, and linalool have shown significant antioxidant activities (Wei & Shibamoto, 2007). The *in vitro* antioxidant capacity of the

carotenoid fraction from *C. mitraeformis* was reasonably similar to that of the algae *Gracilaria birdiae* under controlled conditions (Guaratini et al., 2012). Previous reports suggested than the antioxidant activity of the ethanolic extract from *C. reflexa* and *C. racemosa*, was due to their phenolic content (Ferraz et al., 2011; Raza et al., 2015; Yang et al., 2016). Nevertheless, our work presents an unexpected antioxidant activity of the essential oil and of the carotenoid fraction, containing β -carotene as the main component.

In order to determine the antibacterial activity of these extracts, the carotene fraction and the essential oil were tested. The first preparation had no effect on the growth of the assayed bacterial strains at the assayed concentrations (0.02-2 mg mL⁻¹), whereas the essential oil showed an evident inhibition

effect in all the phytopathogenic bacteria (Table 3). However, a more efficient MIC was observed for the assayed standards of reference (Table 3). Previous antibacterial reports using the crude ethanolic extracts from *Cuscuta racemosa* showed a weak antibacterial capacity on *Staphylococcus aureus* (Ferraz et al., 2011). Contrary to those results, the essential oil from *C. mitraeformis* showed a moderated antibacterial activity on *Clavibacter michiganensis*, *Pseudomonas syringae* pv. tomato and *Erwinia carotovora*. Other

studies on the antimicrobial activity of carotenoid fractions have shown inhibitory effects against ampicillin resistant strains of *Bacillus cereus* ATCC 13061 and *Staphylococcus aureus* ATCC 6538 (Djifaby et al., 2012). However, the effect of carotenoids in the assayed phytopathogens has been poorly studied. Contrarily, the effect of essential oils on these and other phytopathogenic microorganisms has been relatively more explored (Villa-Ruano et al., 2015a; Villa-Ruano et al., 2015b).

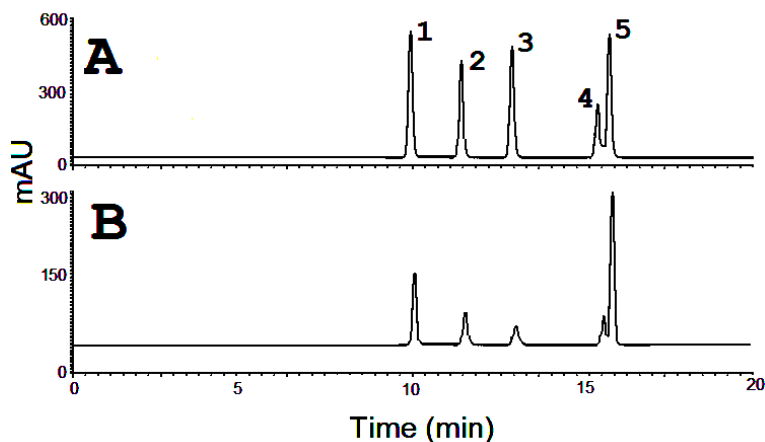


Figure 1

HPLC profile of carotenoids from *C. mitraeformis*. A, pure standards of lutein (1), β-cryptoxanthin (2), lycopene (3), α-carotene (4) and β-carotene (5). B, carotenoid fraction from *C. mitraeformis*.

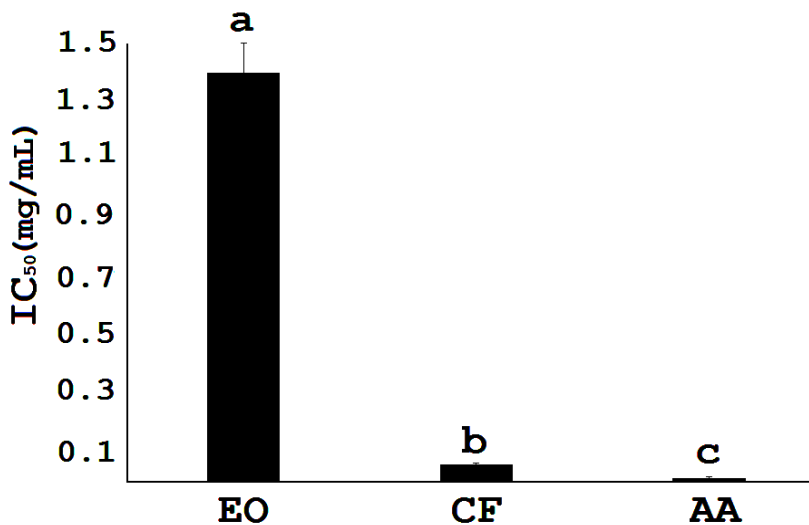


Figure 2

IC₅₀ values for the antioxidant activity of the essential oil (EO) and carotenoid fraction (CF) from *C. mitraeformis*. These values are compared with the standard activity of ascorbic acid (AA). Bars indicate the standard deviation of five repetitions (n = 5) and diverse letters indicate significant differences between treatments by ANOVA-Tukey test (p < 0.01).

Table 3
Antibacterial activity of the essential oil from *Cuscuta mitraeformis*.

Compound	MIC ($\mu\text{g mL}^{-1}$)	Standard ($\mu\text{g mL}^{-1}$)
<i>Erwinia carotovora</i> spp <i>carotovora</i>	234.2 \pm 1.3 ^a	9.1 \pm 0.5 ^R
<i>Clavibacter michiganensis</i> AB299158	122.5 \pm 0.6 ^a	5.6 \pm 0.3 ^R
<i>Pseudomonas syringae</i> pv. tomato DC3000	184.5 \pm 0.9 ^a	17.5 \pm 0.4 ^{Ag}

^aMeans of experiments performed in quintuplicate plus standard deviation. ^{Ag}Agrigen Plus. ^RRifampicin.

CONCLUSION

This work reports on the volatile and the carotenoid contents of the parasitic plant *Cuscuta mitraeformis*, a medicinal plant used by the native people from the northern highlands of Puebla, México. The antioxidant activity of the plant is strong and related to its carotenoids, whereas the antibacterial activity is linked to the cytotoxicity of the essential oil components. Herein we report for the first time the chemical profiles and biological activities of this specie.

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