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Antioxidant Compounds and Activity of the Medicinal Plants *Tagetes* sp. and *Clinopodium* sp. from Indigenous Communities in Mexico

Running title: Antioxidant compounds and activity of *Tagetes* sp. and *Clinopodium* sp.

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ABSTRACT

Knowledge about the therapeutic properties and efficacy conferred by several plants to indigenous communities is based on observations, traditional knowledge, and forms of use or application within local, cultural, and territorial contexts. However, to some extent, favorable responses to these plants depend on the form in which a plant is used and its phytochemical composition. This work aimed to evaluate the contents of total polyphenols and flavonoids and antioxidant activity and determine qualitative phytochemical profiles of *T. lucida*, *T. lunulata*, *C. mexicanum* and *C. macrostemum* from samples of plants collected in four indigenous regions of Oaxaca, Mexico. The results showed that the contents of phenolic compounds and antioxidant activity differed significantly among the evaluated species and that the effect of species was greater than the ecological-environmental effects caused by the origins of the samples. Tannins, alkaloids, steroids, terpenoids, and coumarins were qualitatively identified in all evaluated species, and emodins were detected only in *T. lucida*. Among the communities visited, it was documented that all the species are used to treat gastrointestinal disorders, and antimicrobial, calming and anti-inflammatory properties were recorded for all four species. The effectiveness conferred to these plant species in the communities to treat ailments, including endemic diseases, is partly due to the complex phytochemical composition and the beneficial properties released during infusion or decoction.

Keyword: Spectrophotometry, Traditional knowledge, Phenolic compounds, Gastrointestinal disorders

INTRODUCTION

The use of medicinal plants transcends territorial, cultural and temporal barriers in such a way that treatment with these plants is now prevalent [1]. For example, in genomic medicine, it is possible to evaluate natural extracts of fruits or plants by gene expression of antioxidant metabolic enzymes using biological models [2] to define potentialities in medicinal plants. The World Health Organization (WHO) reports that 80% or more of the countries in the world use herbal medicine as part of traditional or complementary treatments, with greater prevalence in Africa, America, some European countries and the Eastern Pacific region due to the available plants and indigenous knowledge in these areas [3]. The WHO has indicated that there is not enough information on the potential of these plants for pharmacological use and their phytochemical compositions and that research on the safety, monitoring, and efficacy of commonly used plants, such as those that are incorporated into herbal medicines or their derived products that are taken daily, is lacking [2-3]. For example, *Prosopis juliflora* (Sw) DC. (mezquite in Spanish) has potential antiproliferative effects against human prostate LNCaP cells [5], but more pharmacological studies are necessary. Zhang [6] and Lee et al. [7] pointed out that the products, practices, and practitioners of traditional and integrative medicine are part of the first medical aid provided by all societies. Among indigenous communities in Mexico, species of the genera *Tagetes* (Asteraceae) and *Clinopodium* (Labiatae) are widely used in rituals and ethnoreligious ceremonies (e.g., Day of the Dead, family festivals or social events) and traditional medicine [8-11]. Leaves, flowers, and stems of *Tagetes lucida* Cav. and *T. lunulata* Ortega, also known as 'cempoalxóchitls' (marigolds), are prepared in the form of infusions or decoctions in indigenous communities to treat gastrointestinal conditions, menstrual pain, cough, and endemic diseases locally called 'empacho', 'mal de ojo' and 'susto' (ethnodiseases) and as a relaxant [12-14]. The aerial parts of the ‘poleos’ (pennyroyal), *Clinopodium macrostemum* (Moc. & Sessé ex Benth.) Kuntze (Syn. *Satureja macrostema*) and

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Clinopodium mexicanum (Benth.) Govaerts are also used to treat stomach pain, intestinal cramps, indigestion, and gastrointestinal infections and to reduce the effects of excess alcohol consumption [10, 15, 16].

Previous studies have revealed anti-inflammatory, antibacterial and antiulcer properties and antioxidant activities in extracts of different species of *Tagetes* and *Clinopodium* [17-19]. For example, in *T. lucida*, glycosylated flavonols, phenolic acids, aromatic acids and hydroxylated coumarins have been identified, compounds that confer high antioxidant activity and produce antidepressant, anxiolytic, sedative, antifungal, and antibacterial effects, protecting against bacteria that cause intestinal infections (e.g., *E. coli*, *Salmonella* sp. and *V. cholerae*); these compounds also produce analgesic effects and help prevent diabetes and obesity [17, 20-23]. In addition, *C. macrostemum* extracts are hepatoprotective, renoprotective, and antidiarrheal and relieve intestinal pain [24-27]. Analgesic and sedative effects have been reported in *C. mexicanum* [28]. All these findings indicate that these medicinal plants have varying phytochemical compositions that need to be understood and that the ecological conditions in which the plants grow lead to variations in plant composition and compound concentration. In this context, the objective of this study was to evaluate the contents of total phenols and flavonoids and antioxidant activity and to determine a qualitative phytochemical profile of *T. lucida*, *T. lunulata*, *C. mexicanum* and *C. macrostemum* from plant samples collected in four indigenous regions of Oaxaca, Mexico.

MATERIAL AND METHODS

Documentation of Communities and Collection of Plant Material

At the flowering stage, samples of *T. lucida* Cav., *T. lunulata* Ortega, *C. macrostemum* (Moc. & Sessé ex Benth.) Kuntze and *C. mexicanum* (Benth.) Govaerts (syn.: *Satureja oaxacana*; *Satureja mexicana*) were collected in two locations for each species in the Mixteca, Valles Centrales, Sierra Sur, and Sierra Norte regions from Oaxaca, Mexico. Sample collection for phytochemical analysis was performed at two locations per species from October to December 2018 in regions with different climatic conditions. In each community or locality, ecogeographic descriptors of the soil and climate were recorded based on geodetic maps provided by the National Institute of Statistics and Geography (INEGI) [29]. Furthermore, the altitude, latitude and longitude of the sites were recorded by a GPS unit. In addition, ethnobotanical information on the uses of each species was obtained through rapid surveys with key members in eight communities of the regions visited (Table 1).

Table 1 Species collected belonging to *Tagetes* and *Clinopodium* and ecological descriptions of the communities and municipalities sampled in Oaxaca, Mexico.

Species and communities at the sampling sites	Altitude (m), latitude (N), longitude (W)	Predominant climate in the communities[a]	Temperature (°C)[a] and average rainfall (mm)	Predominant soil types[b]
Tagetes lucida:				
Santa Lucía Miahuatlán	1966 16° 11' 0" 96° 38' 0"	Temperate subhumid with rainfall in summer (72.4%)	12-22 700-2000	Leptosol, luvisol and regosol
San Pedro Cajonos	2234 17° 10' 13.6" 96° 18' 10.2"	Temperate subhumid with rainfall in summer (90.3%)	16-20 1200-2000	Luvisol
Tagetes lunulata:				
San Pablo Huitzo	1813 17° 17' 4.3" 96° 57' 2.8"	Temperate subhumid (57.45%) and semiwarm subhumid (41.99%) with rainfall in summer	14-22 600-1000	Regosol, leptosol, and vertisol
Tamazulapan del Espíritu Santo	2067 17° 03' 30.1" 96° 03' 44.6"	Temperate subhumid with rainfall in summer (84.03%)	14-20 800-1500	Acrisol, luvisol, and cambisol
Clinopodium macrostemum:				
Santa Catarina Lachatao	2831 17° 07' 5.2" 96° 31' 41.8"	Temperate subhumid with rainfall in summer (95.84%)	10-20 800-1200	Acrisol and cambisol
	2261		14-24	

San Pedro y San Pablo Ayutla	17° 00’ 45” 96° 07’ 33”	Semiwarm subhumid (36.55%), temperate subhumid (28.26%), and temperate wet (24.09%) with rainfall in summer	700-1500	Acrisol, cambisol, and luvisol
Clinopodium mexicanum:				
Santo Domingo Yanhuitlán	2236 17° 33’ 1.7” 97° 22’ 17.8”	Temperate subhumid (81.04%) with rainfall in summer and other seasons	14-18 600-800	Leptosol, vertisol, and phaeozem
San Miguel Tixá	2274 17° 31’ 20.9” 97° 30’ 56.0”	Temperate subhumid (93.33%) with rainfall in summer and other seasons	14-20 700-900	Leptosol, luvisol, and vertisol

[a]INEGI [29]; [b]Soil type based on the IUSS Working Group WRB classification [30].

Preparation of Samples for Laboratory Analysis

The plant material was washed with running water and rinsed with distilled water, left to dry in the shade at room temperature, and subsequently ground in an electric mill (Krups®, model GX4100, Mexico) for 3 min (at intervals of 1.5 min) until a homogeneous powder with a particle size of 0.6 mm was obtained. The dry milled samples were stored in amber flasks at 4 °C until analysis. The determination of total polyphenols and flavonoids was made based on 0.1 g of ground sample, which was mixed with 25 mL of 80% ethanol. The evaluation of antioxidant activity (DPPH and FRAP) was performed with a 0.1 g sample mixed with 25 mL of 80% methanol. In both cases, the mixtures were homogenized (Ultra Turrax T25 Digital, IKA, Germany) for 60 s (in 30 s intervals) and centrifuged at 11500 rpm (refrigerated Eppendorf 5810R centrifuge) for 15 min at 10 °C, and the supernatant was extracted for the corresponding determinations, in each case with three replicates per extract.

Evaluation of Phenolic Compounds and Antioxidant Activity

Total Polyphenols

The evaluation of polyphenols was performed based on the Folin-Ciocalteu method [31], which is used to measure the concentration of total phenolic compounds based on the transfer of electrons from the extract to phosphomolybdic acid/phosphatidic acid complexes in an alkaline medium, and a concentration-dependent blue color was obtained. The absorption was measured at 750 nm using a spectrophotometer (UV-1800 Shimadzu Corporation Kyoto, Japan). The concentration of each sample was obtained with reference to a calibration curve of the standard gallic acid (0.021-0.165 mg mL^{-1}), and the results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE g^{-1} dw).

Flavonoids

The estimation of flavonoids was made using two complementary colorimetric methods based on the integration of complex compounds of different chemical natures. The first method was based on the reaction of aluminum chloride ($AlCl_3$) with flavonoids [32], and it tests specifically for flavonols and luteolin (flavone). In this case, the absorbance values were recorded at 410 nm using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan), and the concentration was expressed as mg equivalents of quercetin per gram of dry weight (mg QE g^{-1} dw) with reference to a quercetin calibration curve (0.01-0.07 mg mL^{-1}). The second method was performed with sodium nitrite ($NaNO_2$) in an alkaline medium [33] and was used to test for catechins [34]. Spectrophotometric evaluations were performed at 510 nm according to the reference curve of catechin (0.01-0.12 mg mL^{-1}), and the results were expressed in mg catechin equivalents per gram of dry weight (mg CE g^{-1} dw).

Antioxidant Activity Determined by the DPPH and FRAP Methods

The DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma–Aldrich) antioxidant activity was evaluated by the method described by Brand-Williams [35]; this method involved recording absorbances at 517 nm in a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) and calculating the concentration based on the standard Trolox curve (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (0.13-1.19 µmol mL^{-1}). The antioxidant activity was also evaluated by the FRAP method (iron-reducing power) with reference to the approach proposed by Benzie and Strain [36]. Absorbance was recorded at a wavelength of 593 nm, and quantification was performed based on the Trolox calibration curve (0.05-1.0 μmol mL^{-1}). The results of both determinations were expressed as micromolar equivalents of Trolox per gram of dry weight (μmol TE g^{-1} dw).

Standard Qualitative Determination of Phytochemical Compounds

Qualitative phytochemical evaluation of the extracts was carried out by standard methods based on changes in color or the formation of precipitates, which were recorded as present (+/1) or absent (-/0). This exploratory procedure is frequently used in studies of plant extracts to determine the presence of groups of bioactive compounds in plants [37]. In this work, 7 g of each powder sample was mixed with 70 mL of 80% methanol and homogenized for 60 s (Ultra Turrax T25 Digital, IKA, Germany). The resulting extract was vacuum filtered, and liquid–liquid extraction was successively carried out with solvents of increasing polarity from hexane and chloroform to ethyl acetate, recovering the phases with each solvent. The final fractions were stored at 4 °C until evaluation.

Qualitative phytochemical analysis was performed using the methodologies reported by Savithramma [37] and Marcus [38]. Tannins: drops of 1% lead acetate were added to 1 mL of the extract, and the presence of tannins was recorded when a yellow precipitate was observed. Saponins: 1 mL of the extract was mixed with 2 mL of distilled water and stirred vigorously for 2 min; the presence of the compounds was recorded following foam formation in the solution. Terpenoids: First, 1.5 mL of the extract was mixed with 1 mL of chloroform. Then, 1 mL of concentrated H_2SO_4 was added, and the presence of terpenoids was indicated by the formation of a reddish-brown ring. Steroids: Three drops of concentrated H_2SO_4 were added to 3 mL of the extract, and a red coloration indicated the presence of steroids. Alkaloids: Three drops of 1% HCl were added to 1.5 mL of extract, and the solution was mixed; then, two drops of Wagner's reagent (2 g of I_2 and 6 g of KI dissolved in 100 mL of distilled water) were added to the solution, and the formation of a reddish-brown precipitate indicated the presence of alkaloids. Flavonoids: One or two drops of 1 M NaOH solution was added to 1 mL of extract, causing the solution to turn an intense yellow color. Then, two to three drops of 1 M HCl were added, and when the solution became colorless, it indicated the presence of flavonoids. Coumarins: 1 mL of 10% NaOH was added to 1 mL of the extract, and a yellow hue indicated the presence of coumarins.

Statistical Analysis

Based on the data generated in the quantitative analysis of phenolic compounds and antioxidant activity, analysis of variance was performed using a random linear model, where the origins of the samples collected from each species were assumed to be nested by species. To evaluate the differences between species and sample origins, multiple comparisons of means were made with the Tukey test ($P < 0.05$). In addition, a Pearson correlation analysis ($P \leq 0.05$) was performed between average values of total polyphenols, flavonoids and antioxidant activity. For the qualitative determinations, descriptive analyses were performed. Statistical analyses were performed with the SAS statistical package [39].

RESULTS AND DISCUSSION

Community uses of Tagetes sp. and Clinopodium sp.

The indigenous communities of Oaxaca, Mexico, widely use marigolds (*Tagetes sp.*) and 'poleos' (*Clinopodium sp.*) for ornamental, medicinal, ritual and nutritional purposes, and the plants are a part of their cultures and traditions (Table 2). This finding coincides with previous reports regarding the use of these species [10, 27]. In the study region, it was observed that among Zapotec communities (in the subtropical or warm zone) of the Central Valleys and Sierra Norte of Oaxaca, Mexico, *C. macrostemum* was the main medicinal plant. In addition, in the temperate to humid temperate zones occupied by the Mixtec group, *C. mexicanum* was used exclusively. *T. lucida* (pericón) has more medicinal functions. In contrast, *T. lunulata* has more ritual and religious uses, e.g., as an altar decoration in honor of the dead in a traditional Mexican ceremony celebrated on November 1 and 2. Subsequently, the plants are taken to cemeteries, where they are used to decorate tombs. Both *T. lucida* and *T. lunulata* have a variety of medicinal uses and are distributed in temperate zones (Table 2).

Table 2 Descriptions of traditional uses of *Tagetes* and *Clinopodium* species among indigenous communities from Oaxaca, Mexico.

Species sampled	Local names in Spanish and native languages	Examples of ethnobotanical uses[1]
T. lucida	Pericón, *tiex* in Zapoteco (native language)	Food: The plant is added to boiled corn for flavor. An infusion of the sweetened aerial part of the plant is consumed. Ornamental and ceremonial: The plant is used as an adornment for the image of the Virgin Mary in the Catholic religion. Medicinal: An infusion of the aerial part of the plant relieves stomach pain, diarrhea, 'empacho', irregular heartbeat, stomach discomfort, menstrual pain and is used as an anti-inflammatory; the aerial part of the plant is soaked in 'aguardiente' (sugarcane alcohol) relieve stomach pain. The boiled root is used to treat diabetes and cancer.
T. lunulata	Cempasúchil cimarrón, flor de todos santos, flor de muerto chiquita o cempasúchil silvestre; flor de angelito, cempasúchil, cempasúchil hembra; flor de muerto; cempasuchí chiquito; flor de niño (native language).	Ritual and ceremonial: The plant is used as an adornment of the altar of the dead. Ornamental: The plant is appreciated for its aroma and the showy color of its petals. Medicinal: An infusion of the aerial part of the plant relieves stomach pain.
C. macrostemum	Poleo, hierba de borracho, rosa de borracho and *Gú guázz*, *guiezza*, *quieutzu* in Zapoteco (native language).	Food: The leaves are consumed cooked or added during the cooking of beans (*Phaseolus* sp.) and tamales and served as a condiment. An infusion of the aerial part of the plant is consumed in water, or the leaves and stems are steeped in 'aguardiente' (sugarcane alcohol) or mezcal for consumption as an alcoholic beverage. Ritual and ceremonial: It is given to guests at mayordomías, weddings and Christmas parties. Medicinal: Relieves stomach pain, diarrhea, heartburn, fever, symptoms of excess alcohol intake, and high blood pressure.
C. mexicanum	Chepito, hierba del borracho, toronjil, poleo, hierba de menta and *Gú guázz* in Zapoteco (native language)	Food: An infusion of the sweetened aerial part of the plant is consumed. Medicinal: An infusion or maceration in brandy relieves stomach pain caused by excess alcohol intake. The plant is also an analgesic and sedative and can induce sleep.

[1]A synthesis of ethnobotanical information gathered during a quick survey of species uses among indigenous communities from Oaxaca, Mexico, complemented with information provided by previous works [10, 28, 40-45].

The fresh and dry stems, leaves and flowers of *C. macrostemum*, *C. mexicanum*, *T. lucida* and *T. lunulata* are widely used in medicinal infusions and boiled solutions to counteract stomach and gastrointestinal discomfort (Table 2). According to the interviewees, *C. macrostemum* and *C. mexicanum* are preferably consumed in infusions or decoctions and served as hot drinks for the treatment of diarrhea, stomach pain caused by ingestion of food or alcoholic beverages and menstrual pain; these findings coincide with reports by UNAM [16] and Arrazola-Guendulay [10] regarding the curative uses of these plants for stomach pain, nausea, indigestion, fever, diarrhea, and intestinal cramps (including the symptoms of ethno-diseases) and to alleviate symptoms of excessive alcohol consumption. In addition, these species are used as condiments in the preparation of food; combined with ground beans (locally called 'pasta'), tamales (local dishes), or refreshing drinks; or used in the preparation of soups and vegetables similar to the so-called *quelites*, edible leaves and tender stems of the genera *Amaranthus*, *Portulaca*, *Chenopodium* and others. In addition, these species are included in the preparation of a liqueur: the leaves and stems, preferably green, are added to equal volumes of 0.25, 0.5 or 1.0 L of 'aguardiente' (alcohol of sugarcane, *Saccharum officinarum* L.) or mezcal distilled from *Agave angustifolia* Haw. That is, *Clinopodium* species have diverse uses among indigenous communities. Similar aspects were documented for *T. lucida* and *T. lunulata*, which can be prepared similarly and are used to treat stomach pain, diarrhea, menstrual pain, fever, vomiting and ethno-diseases such as 'empacho', 'mal de ojo' and 'susto' [10, 12-14].

Phenolic Compounds and Antioxidant Activity

Based on the analysis of variance, significant differences ($P \leq 0.01$) were found between the species evaluated (*T. lucida*, *T. lunulata*, *C. macrostemum* and *C. mexicanum*) and between the communities of origin of the samples of each species in terms of the contents of total polyphenols and flavonoids (quercetin and catechin) and antioxidant activity evaluated by the DPPH and FRAP methods. It is important to note that the variation attributed to the effects of the growth environment of the sampled plants was significantly lower (0.2 to 5.3%) than the variation estimated for the effect of species (94.6 to 99.3%); these findings are based on the linear model used to perform the analysis of variance (Table 3) and coincided with the results reported by Cicevan [46], who did not find a consistent difference in the contents of phenols and flavonoids between species and varieties of *Tagetes* subjected to water stress. Consequently, these results indicate that the variation in the contents of phenolic compounds and antioxidant activity between samples within the same species can be used to determine the species composition, as has been reported in other medicinal plants [47, 48].

Polyphenols and flavonoids in medicinal plants play an important communitarian role in the prevention and treatment of ethnodiseases and metabolic disorders of human health. As part of the potential application in medicinal chemistry, it is important to elucidate such a mechanism; for example, Ullah et al. [49] found that flavonoids are associated with the prevention of cancer and anti-inflammatory, antiviral, neuroprotective and cardio-preventive effects. The biological activities depend on the specific flavonoid, mode of action, bioavailability and ease of release from plant extracts. In this sense, Nuñez et al. [50] tested whether *Tagetes erecta* L. extracts have antidiabetic and anti-obesity properties using *Caenorhabditis elegans* as a biological model.

Table 3 Significance of the square means from the analysis of variance of total polyphenol and flavonoid contents and antioxidant activity in two species of *Tagetes* and two species of *Clinopodium*.

Source of variation	Total polyphenols	Flavonoid equivalents		Antioxidant activity	
		Quercetin	Catechin	DPPH	FRAP
Species	7080 ** (98.7)[1]	7236 ** (99.7)	18268 ** (94.6)	1096476 ** (99.5)	1740662 ** (99.3)
Community of origin of samples within each species	86.8 ** (1.2)	16.3 ** (0.2)	1030.3 ** (5.3)	4029.1 ** (0.4)	10445.2 ** (0.6)
Error	2.4 (0.1)	1.5 (0.1)	7.9 (0.1)	1042.1 (0.1)	273.5 (0.1)
C.V. (%)	3.6	6.1	4.1	8.9	3.7

**Significant at $P \leq 0.01$; [1]values in parenthesis indicate percentage of variance explained by the total variance estimated in the linear model; C.V. = coefficient of variation.

Species of *Tagetes* and *Clinopodium* are used by indigenous communities in Oaxaca, Mexico, for the treatment of gastrointestinal disorders (Table 2), and the healing effects of these plants are directly based on their phytochemical composition. For example, the phenol and flavonoid contents of these species are associated with antimicrobial and anti-inflammatory properties and relaxing effects [27-28, 51-52]. The results show that the contents of polyphenols and flavonoids in *Tagetes* ranged from 29.6 to 71.7 mg GAE g^{-1} and from 17.8 to 95.6 mg QE/CE g^{-1}, respectively; in *Clinopodium*, the variation ranged from 30.1 to 39.5 mg GAE g^{-1} and from 6.3 to 96.1 mg QE/CE g^{-1}. *T. lucida* had higher contents of total polyphenols and flavonoids than *T. lunulata*, a pattern that is reflected in greater antioxidant activity; similar patterns were also recorded in *C. mexicanum* with respect to *C. macrostemum* (Table 4). The determination of general or specific compounds in medicinal plants guides future studies or specific pharmacological tests. For example, in traditional Chinese medicine, *Clinopodium chinense* is reported to be a therapeutic option for cardiac fibrosis due to first reports of an antifibrosis mechanism in rat models with myocardial injury [Li et al. [53]. However, more evaluations are necessary because not always all species of the same genera confer similar effects; for example, Morales-Torres et al. [54] determined the absence of nephroprotective effects in the evaluation of aqueous extracts of *Clinopodium vimineum* (L.) Kuntzie using a renal disorder model with Wistar rats.

Table 4 Differences in the contents of phenolic compounds and antioxidant activity among *Tagetes* and *Clinopodium* species collected in Oaxaca, Mexico.

Species evaluated	Total polyphenol content (mg GAE g^{-1})	Flavonoid equivalent		Antioxidant activity	
		Quercetin (mg QE g^{-1})	Catechin (mg CE g^{-1})	DPPH (µmol TE g^{-1})	FRAP (µmol TE g^{-1})
Tagetes lucida	71.7 a[1]	48.9 a	95.6 a	725.8 a	901.7 a
Tagetes lunulata	29.6 c	17.8 b	32.9 c	257.2 b	264.5 c
Clinopodium macrostemum	30.1 c	6.3 c	51.7 b	179.9 c	233.4 d
Clinopodium mexicanum	39.5 b	6.7 c	96.1 a	282.9 b	380.6 b

[1]In columns, means with the same letter are not significantly different (Tukey's test, P ≤ 0.05).

The variation in phenolic compounds and antioxidant activity between samples collected in different communities reflected different patterns between species. For example, the contents of total polyphenols and quercetin-equivalent flavonoids and antioxidant activity differed between *T. lucida* samples according to community origin; the values of these measures were also significantly higher in *T lucida* than in *T. lunulata, C. macrostemum* and *C. mexicanum*. Therefore, to a great extent, the differences occur at the species level and are not due to the different ecological origins of the samples, although it was necessary to obtain different samples to estimate the variation in composition within each sample. Within each species, there were significant differences in the total polyphenol content according to the sample origin, but there were no differences in the contents of quercetin-equivalent flavonoids within the species *T. lunulata*, *C. macrostemun* and *C. mexicanum*. The antioxidant activity in the samples of *T. lucida*. and *C. mexicanum* differed significantly according to one or both evaluation methods (DPPH or FRAP), but this result was not observed for *T. lunulata* or *C. macrostemum* (Table 5). This indicates that the growth environment of the plant has an influence on its contents of phenolic compounds and antioxidant activity but that the pattern changes from species to species. These patterns have some similarities to those reported by Dunkić [55], who differentiated *Clinopodium dalmaticum, C. pulegium, C. serpyllifolium* and *C. thymifolium* by the contents of tannins, phenolic acids, flavonoids, and total polyphenols, and to those reported by Cicevan [46] and Li et al. [56], who established differences between *Tagetes erecta, T. patula* and *T. tenuifolia* according to total phenol and flavonoid contents.

Table 5 Variation in the contents of phenolic compounds and antioxidant activity within species of *Tagetes* and *Clinopodium* collected in Oaxaca, Mexico, according to the community of origin of the samples.

Species and community of origin of the samples	Total polyphenols (mg GAE g^{-1})	Flavonoid equivalent		Antioxidant activity	
		Quercetin (mg QE g^{-1})	Catechin (mg CE g^{-1})	DPPH (µmol TE g^{-1})	FRAP (µmol TE g^{-1})
Tagetes lucida					
Santa Lucia Miahuatlán	68.4 b[1]	50.5 a	92.9 c	739.7 a	878.6 b
San Pedro Cajonos	74.9 a	47.4 b	98.2 b	711.9 a	924.9 a
Tagetes lunulata					
San Pablo Huitzo	31.0 e	18.6 c	31.5 g	264.7 bc	276.2 e
Tamazulapám del Espíritu Santo	28.3 f	16.9 c	34.2 g	249.7 c	252.8 ef
Clinopodium macrostemum					
Santa Catarina Lachatao	28.7 f	5.6 d	48.7 f	171.6 d	229.7 f

San Pedro y San Pablo Ayutla	31.4 e	7.0 d	54.6 e	188.1 d	237.2 f
Clinopodium mexicanum					
Santo Domingo Yanhuitlán	41.7 c	6.9 d	110.6 a	307.0 b	421.0 c
San Miguel Tixa	37.2 d	6.5 d	81.5 d	258.9 c	340.1 d

[1]In columns, means with the same letter are not significantly different (Tukey's test, $P \leq 0.05$).

In twelve varieties of *Tagetes patula, T. tenuifolia and T. erecta*, Cicevan [46] estimated that the total phenol content ranged from 2.52 to 8.38 mg GAE g^{-1} and that the total flavonoid content ranged from 2.72 to 7.88 mg CE g^{-1}; in this study, the observed variations were slightly higher, with the ranges for *T. lucida* and *T. lunulata* ranging from 28.3 to 68.4 mg GAE g^{-1} and from 31.5 to 98.2 mg CE g^{-1}, respectively. This finding indicates that despite the methodological differences between laboratories, there are higher concentrations of these compounds in native Mexican species than in the species evaluated by Cicevan [46], and this finding may also partially explain the beneficial effects conferred by these species in traditional medicine.

The correlation analysis showed that the contents of total polyphenols were positively and significantly correlated with the antioxidant activity estimated by FRAP ($r = 0.99$ **, $P \leq 0.01$) and DPPH ($r = 0.96$ **, $P \leq 0.01$.) methods, and similar patterns were observed for the contents of flavonoids evaluated according to the quercetin standard (QE) with the DPPH ($r = 0.95$ **, $P \leq 0.01$) and FRAP ($r = 0.92$ **, $P \leq 0.01$) methods and the catechin standard (CE) with the FRAP ($r = 0.68$ **, $P \leq 0.01$) and DPPH ($r = 0.59$ **, $P \leq 0.01$) methods. The results show a close association of polyphenols and flavonoids with antioxidant activity, but antioxidant effects of other compounds are not ruled out, as indicated by the findings of Cicevan [46] in species of *Tagetes* and Dunkić [55] in species of *Clinopodium*.

The differential polarity of each solvent used (hexane, chloroform and ethyl acetate) helped to qualitatively determine the presence of different phytochemical compounds. Hexane allowed us to determine the presence of saponins, steroidal terpenoids and alkaloids in *T. lucida* and *T. lunulata,* but the same solvent only helped to determine the presence of alkaloids in *C. mexicanum* and *C. macrostemum*. Specifically, alkaloids were present in all the species evaluated when hexane and ethyl acetate were used but not when chloroform was used, and this result may guide future studies to evaluate the specific concentrations of this group of compounds. Similarly, the results show that the use of chloroform and ethyl acetate as extraction solvents is recommended for the evaluation of the contents of tannins, terpenoids, coumarins and flavonoids in both *Tagetes* and *Clinopodium*. However, the presence of saponins was detected in *C. mexicanum, C. macrostemum* and *T. lucida* only with chloroform. Emodins were detected only in *T. lucida* with ethyl acetate (Table 6). Therefore, between and within the species evaluated, the same group of phytochemical compounds was not always observed in samples from two geographical origins. For example, saponins and steroids in *T. lunulata* were detected with hexane, while saponins and emodins in *T. lucida* were detected with chloroform and ethyl acetate; these compounds were not observed with this method in *T. lunulata*, and in this species, steroids were observed using ethyl acetate. These findings indicate that the samples collected probably did not have a sufficient concentration of compounds to be detected by the qualitative techniques used. However, the use of ethyl acetate and chloroform as extraction solvents shows reactive potential for quantitative determinations of tannins, saponins, terpenoids, steroids, alkaloids, coumarins and flavonoids.

Table 6 Screening of phytochemical compounds based on methanolic extracts of hexane, chloroform, and ethyl acetate solvents in *Tagetes* and *Clinopodium* species from Oaxaca, Mexico.

Phytochemical compounds	*C. mexicanum*		*C. macrostemum*		*T. lucida*		*T. lunulata*	
	Santo Domingo Yanhuitlán	San Miguel Tixá	Santa Catarina Lachatao	San P. y San P. Ayutla	Santa Lucía Miahuatlán	San Pedro Cajonos	San Pablo Huitzo	Tamazulapam del Espíritu Santo
	Hexane							
Tannins	0	0	0	0	0	0	0	0
Saponins	0	0	0	0	0	1	0	1
Terpenoids	0	0	0	0	1	1	1	1

Steroids	0	0	0	0	1	1	0	1
Alkaloids	1	1	1	1	1	1	1	1
Coumarins	0	0	0	0	0	0	0	0
Flavonoids	1	0	0	0	0	0	0	0
Emodins	0	0	0	0	0	0	0	0
	Chloroform							
Tannins	1	1	1	1	1	1	1	1
Saponins	1	1	1	1	1	1	0	0
Terpenoids	1	1	1	1	1	1	1	1
Steroids	0	0	0	0	0	0	1	0
Alkaloids	0	0	0	0	0	0	0	0
Coumarins	1	1	1	1	1	1	1	1
Flavonoids	1	1	1	1	1	1	1	1
Emodins	0	0	0	0	0	0	0	0
	Ethyl acetate							
Tannins	1	1	1	1	1	1	1	1
Saponins	0	0	0	0	0	0	0	0
Terpenoids	1	1	1	1	1	1	1	1
Steroids	0	1	1	1	0	0	1	1
Alkaloids	1	0	0	1	1	1	1	1
Coumarins	1	1	1	1	1	1	1	1
Flavonoids	1	1	1	1	1	1	1	1
Emodins	0	0	0	0	1	1	0	0
Sum of compound presences	12	11	11	12	14	15	13	14

1 = presence; 0 = absence.

The quantification of phenolic compounds and flavonoids, qualitative determination of compounds (tannins, saponins, terpenoids, steroids, alkaloids, coumarins and emodins), and high antioxidant activity indicate that the evaluated extracts are complex matrices of compounds; this finding partially explains the use of *T. lucida*, *T. lunulata*, *C. macrostemum*, and *C. mexicanum* in the traditional medicine of the indigenous communities of Oaxaca, Mexico. For example, flavonoids in *T. lucida*, including quercetagetin 7-O-β-D-glucoside, quercetagenin-7-O-β-D glucopyranoside, 6-hydroxykaempferol-7-O-β-D-glucopyranoside and 5,7,4'-trimethoxyflavone, which have high antioxidant capacities, are analgesic and inhibit the growth of enterobacteria and pathogenic fungi [20, 21, 23, 51, 57]. Coumarins (7,8-dihydroxycoumarin, 7,8-dihydroxy-6-methoxycoumarin, and 7-isoprenyloxycoumarin) have also been detected in *T. lucida*. 6,7-Dimethoxycoumarin, herniarin and 6,7,8-trimethoxy-coumarin have anti-inflammatory, analgesic, vasorelaxant, anxiolytic and sedative properties [11, 20, 48, 51, 58]. All these findings support the possible medicinal effects conferred on these plants by the indigenous communities of Mexico.

Terpenes confer antimicrobial properties to the plants that possess them. In this study, terpenes were present in the chloroform and ethyl acetate fractions in all the species evaluated. Villa-Ruano [59] identified the terpenes menthone and piperitone oxide in *C. macrostemum*, and the results of the study partially explained the medicinal use of this plant in Oaxaca, Mexico, for stomach pain. The same effect has been described for *C. mexicanum*; the steroid saponins *β-sitosterol* and *daucosterol*, which have antimicrobial potential, have been detected in this species [27].

Additionally, it was determined that *T. patula* and *T. lunulata* contain alkaloids (Table 6), compounds associated with anesthetic, stimulant, antimicrobial and anti-inflammatory properties. In the communities visited, infusions and preparations of these plants were used to treat stomach disorders, which could be related to the presence of these compounds. Similarly, Faizi and Naz [60] identified japhrin alkaloids with antimicrobial potential in *T. patula*.

CONCLUSION

Based on the exploration, documentation, collection and phytochemical evaluation of two species of *Tagetes* and two species of *Clinopodium*, it is concluded that *T. lucida*, *T. lunulata*, *C. macrostemum and C. mexicanum* are used for the treatment of disorders and gastrointestinal diseases, including ethno-diseases (e.g., 'empacho'), by indigenous communities of Oaxaca, Mexico. Among species, significant differences were found in total polyphenol and flavonoid contents and antioxidant activity. The effect of species on the phenolic content and antioxidant activity was significantly higher than the effect of sample location or ecological-environment effect. *T. lucida* had the highest phenolic compound and antioxidant activity values, while *C. macrostemum* had the lowest. Tannins, alkaloids, steroids, terpenoids, and coumarins were detected in all the evaluated species, and emodins were detected only in *T. lucida*. One or more of these compounds have antioxidant and antimicrobial properties, thus validating the traditional use of these native species for treating disorders or stomach pain as indicated, in local terms, by the indigenous communities visited.

Conflicts of Interests

The authors have not declared any conflicts of interest.

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The authors confirm that all the communities mentioned in this study gave us their consent to perform the work and allowed us to conduct interviews and share parts of their knowledge.

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