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Phenolic compounds of *Tagetes lucida* Cav. with antibacterial effect due to membrane damage

[Compuestos fenólicos de Tagetes lucida Cav. con efecto antibacteriano por daños a la membrana]

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Abstract: *Tagetes lucida* Cav. (Asteracea=Compositae) is used for treating stomach infections. The study focused on evaluating the composition and antimicrobial effect of an extract of *T. lucida* Cav. The plant extracted with ethanol at 10% w/v, and the extract composition analyzed by Rp-HPLC-MS. The antibacterial effect was evaluated against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella choleraesuis* using disk diffusion, microdilution and bioautography methods. The sytox and comet assays were used to evaluate the mechanism of action. In this way, nine phenolic compounds were detected in the extract of *T. lucida*. The extract exhibited activity only on *S. aureus* (MIC of 4.000 mg/ml). The bioautography revealed that the phenolic compounds could act synergistically. The sytox and comet tests showed an antibacterial action of the extract on the bacterial membrane and DNA of this bacterial strain.

Keywords: Tagetes lucida Cav.; Chemical composition; Antibacterial effect; Action target

Resumen: *Tagetes lucida* Cav. (Asteraceae = Compositae) se usa para tratar infecciones estomacales. El estudio se centró en evaluar la composición y el efecto antimicrobiano de un extracto de *T. lucida* Cav. La planta se extrajo con etanol al 10% p/v, y la composición del extracto se analizó por Rp-HPLC-MS. El efecto antibacteriano se evaluó contra *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* y *Salmonella choleraesuis* utilizando métodos de difusión por disco, microdilución y bioautografía. Los ensayos de sytox y cometa fueron utilizados para evaluar el mecanismo de acción. De esta forma, se detectaron nueve compuestos fenólicos en el extracto de *T. lucida*. El extracto exhibió actividad solo en *S. aureus* (MIC de 4.000 mg/ml). La bioautografía reveló que los compuestos fenólicos podrían actuar sinérgicamente. Las pruebas de sytox y cometa mostraron una acción antibacteriana del extracto sobre la membrana bacteriana y el ADN de esta cepa bacteriana.

Palabras clave: Tagetes lucida Cav.; Composición química; Efecto antibacteriano; Sitio de acción

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INTRODUCTION

Compositae (Asteraceae) includes a large number of species with an antimicrobial effect such as Tagetes lucida, Calendula stellata, Calendula officinalis, Calendula arvensis, and Eclipta postrate (Lehbili et al., 2017; Lovecka et al., 2017; Abudunia et al., 2017; Chung et al., 2017). The genus Tagetes includes 56 species, and in some species flavonoids, anthocyanins, and terpenes have been detected (Shahzadi & Shah, 2015; Cicevan et al., 2016; Gakuubi et al., 2016). For years it has been shown that some Compositae species act against strains of medical importance such as Enterococcus fecalis (Lehbili et al., 2017) and Salmonella typhi (Karthikumar et al., 2007). Recently, has been detected an antibacterial effect of the essential oil of Tagetes minuta against Staphylococcus aureus, Enterobacter cloacae, Mycobacterium smegmatis, Listeria ivanovii, Streptococcus uberis, Vibrio spp and Escherichia coli (Igwaran et al., 2017), as well as antibacterial action of the extracts (butanol and ethyl acetate) from T. minuta on Micrococcus luteus, S. aureus, Bacillus subtilis and Pseudomonas pikettii. In these sense, it has been shown that flavonols are the bioactive compounds responsible for the antibacterial effect on M. luteus (Shahzadi & Shah, 2015). On the other hand, antibacterial action of Tagetes patula and Tagetes erecta against E. coli, Pastrulla multocida, B. subtilis and S. aureus has been confirmed (Ayub et al., 2017).

Tagetes lucida Cav. is an aromatic plant known as hierbanis, has been used since pre-hispanic times as a remedy to treat stomach diseases (García-Sánchez et al., 2012; Bonilla-Jaime et al., 2015). This plant grows in Guatemala, Honduras, and northern Mexico (Shahzadi & Shah, 2015; Cicevan et al., 2016). Seven coumarins, 7,8-dihydroxycoumarin, umbelliferone (7-hydroxycoumarin), scoparona (6,7dimethoxy-coumarin), esculetin (6,7-dihydroxycoumarin), 6-hydroxy-7-methoxycoumarin, herniaryne (7-methoxycoumarin) and scopoletin (6-methoxy-7-hydroxycoumarin), and three flavonoids, patuletina, quercetin and quercetagetin have been identified in the extracts of CH₂Cl₂ and MeOH from aerial parts of T. lucida (Céspedes et al., 2006). Dihydroxylated coumarins present in both extracts display antibacterial effects against B. subtilis, E. coli, Proteus mirabilis, Klebsiella pneumoniae, Salmonella typhi, Salmonella sp., Shigella boydii, Shigella sp., Enterobacter aerogenes, Enterobacter agglomerans, Sarcina lutea, **Staphylococcus** epidermidis, S. aureus, Yersinia enterolitica and Vibrio cholerae (Céspedes et al., 2006). Notwithstanding, 5,7,4'-trimethoxyflavone from ethyl acetate extracts of *T. lucida* displays activity against *S. boydii*, *S. aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, *B. subtilis*, *Sarcina lutea* and four strains of *V. cholerae* (Hernández et al., 2006).

These shreds of evidence indicate that the compounds related to the antimicrobial effect of T. lucida depend on the solvent used for their extraction. Here, it should be highlighted that extracts commonly used in traditional remedies are ethanolic extracts. However, the chemical composition, as well as their antibacterial action and mechanism of action of this extract from T. lucida are unknown (Moussaoui et al., 2010; Regalado et al., 2011). Besides, in recent years, an increase in microbial resistance has been reported worldwide (Riva et al., 2015; Day et al., 2017; Pagliano et al., 2016). For this reason, the identification of new source of antimicrobial drugs is imperative. T. lucida could represent a potential source of antibacterial bioactive compounds (Lenza et al., 2009). Therefore, the aim of the present work was to identify the main compounds of the ethanolic extract of T. lucida and evaluate the antibacterial effect and sites of action on pathogenic bacteria of clinical importance.

MATERIAL AND METHODS

Plant material and extraction

T. lucida Cav. (aerial part) was collected in Saltillo, Coahuila, Mexico, (latitude 25.421391 and longitude -101,000237) during February 2016. A specimen of the plant was registered in the Herbarium of the Autonomous University of Nuevo León with number 030054. The plant was dried at room temperature and ground in a mill (STAR Tisamatic-mill) to obtain a powder with particles of approximately 600 µm. Then, the extraction was carried out with constant agitation for 2 hours using a stirring plate (Hotplate Fisher Scientific) with 10% w/v ethanol at room temperature (25°C). The extract was filtered with a Whatman #4 paper and clarified with a Whatman G/FA glass microfibre filters. The clarified extract dried on a rotary evaporator (Büchi R-120) at 35 °C, then recovered, and the percentage yield calculated by gravimetric analysis using an analytical balance (Ohaus N1B110 Navigator) (Hernández-Ocura et al., 2017). The yield was calculated in triplicate from the weight of the plant powder. Finally, the extract was stored at -5°C until use.

Chemical characterization by Rp-HPLC-MS

The compounds contained in the ethanolic extract of T. lucida were identified by high-resolution reverse phase liquid chromatography (RP-HPLC-ESI-MS) using a method adapted for phenolic compounds. Briefly, an HPLC chromatograph (Varian) was used. The equipment includes an autosampler (Varian Pro Star 410, USA), a ternary pump (Varian Pro Star 230I, USA) and a PDA detector (Varian Pro Star 330, USA). An ion trap mass spectrometer as detector (Varian 500-MS IT Mass Spectrometer, USA) equipped with an electrospray ionization source. First, a solution of the extract at 1000 ug/ml in ethanol was prepared and filtered through a 0.45 µm nylon membrane. The compounds of T. lucida extract were separated on a Denali C18 column (150 mm x 2.1 mm, 3 µm, Grace, USA), after injection of 5 µl of extract. The oven temperature was maintained at 30°C.

The elution was carried out with a gradient of formic acid (0.2%, v/v, solvent A) and acetonitrile (solvent B). As an initial gradient, 97% A and 3% B added for 0-5 min, then a linear gradient of 91% A and 9% B for 5-15 min, followed by a linear gradient of 84% A and 16% of B for 15-45 min and ending with a linear gradient of 50% A and B. The column was then washed and reconditioned. The flow rate was maintained at 0.2 ml/min. The compounds were monitored at 245, 280, 320, and 550 nm during the elution and detected at 280 nm. The fractions eluted injected into the source of the mass spectrometer without splitting automatically. The mass spectra were acquired in the negative ion mode $[M-H]^{-1}$ to obtain the m/z of each compound. Nitrogen was used as nebulizing gas and helium as damping gas. The ion source parameters were: spray voltage 5.0 kV, and capillary voltage and temperature were 90.0 V and 350°C, respectively. Data were collected and processed using MS Workstation software (V 6.9). The compounds were identified according to the retention times and on the interpretation of the m/z stored in the chromatographic database. Additionally, the identification was supported by information previously reported in the literature (Guajardo-Rios et al., 2017)

Antibacterial activity evaluation Bacterial strains

Antibacterial effect of the ethanolic extract from *T. lucida* was tested on *S. aureus* (ATCC 6538), *E. coli* (ATCC 11229), *P. aeruginosa* (ATCC 15442) and

Salmonella choleraesuis (ATCC 1070) strains provided by the Faculty of Chemistry of the Autonomous University of Coahuila. The above strains were kept at 4° C on Mueller Hinton agar (Bioxon®). From each strain, a bacterial suspension of $1x10^{6}$ colony forming units (CFU/ml) as prepared using the Mc Farland scale of 0.5 using Mueller-Hinton broth. Bacterial suppression of each strain was used in disc diffusion test, minimum inhibitory concentration (MIC), bioautography, sytox green, and comet assays.

Disk diffusion test

This test was carried out on Petri dishes prepared with Mueller-Hinton agar under aseptic conditions. In each plate, 100 μ l of the microbial suspension of each strain was inoculated and distributed using a Drigalski handle. On the inoculated Petri dishes were placed 7 mm diameter filter paper discs previously impregnated with 10 μ l of the extract at 2.000 mg/ml (Bauer *et al.*, 1966, Silva-Belmares *et al.*, 2014). 10 ml of ceftriaxone (4.000 mg/ml) and ethanol (10%) were used as positive and negative controls, respectively. Petri dishes incubated for 24 h at 37°C and the inhibition zone was measured using a Vernier. The most sensitive strains were used to evaluate the MIC, bioautography, the sytox method and comet assay.

Minimal inhibitory concentration (MIC)

The minimum inhibitory concentration was measured by the broth microdilution method in a 96-well plate (Klancnik *et al.*, 2010). Both the extract and the positive control (ceftriaxone) were tested at 0.003, 0.008, 0.015, 0.031, 0.062, 0.125, 0.250, 0.500, 1.000, 2.000 and 4.000 mg/ml. Therefore, from both samples, serial dilutions (100 ml) were made from a stock solution of 8,000 mg/ml, and Mueller Hinton broth was tested as a negative control. Just then, 100 µl of a bacterial suspension of 1×10^6 CFU/ml was added. The microplates incubated at 37°C for 24 h, and the absorbance measured at 625 nm in a microplate reader (BioTek Synergy HTX Multi-Mode Reader).

Bioautography

The antibacterial effect of the *T. lucida* extract fractions was evaluated by bioautography, as they contained a mixture of compounds. For this, a thin layer chromatography (TLC) of the extract of *T. lucida* was used. Briefly, the extract at 2.000 mg/ml

was applied on a 25x75 mm silica gel plate and eluted with petroleum ether:ethyl acetate (2:1) in an elution chamber. The retention factor (Rf) was measured to identify each fraction. Then, the TLC was placed in a Petri dish under aseptic conditions, covered with a layer of Mueller-Hinton agar and inoculated with 100 μ l of a 1x10⁶ CFU/ml bacterial suspension. The petri dish incubated during 24 h at 37°C and the antibacterial fractions identified by a zone of inhibition of bacterial growth. The test was carried out in triplicate.

Analysis of mechanism of action Effect on the bacterial membrane

Damage to the bacterial membrane was detected qualitatively with a specific dye known as Sytox green. According to the results of CMI, we evaluated the effect of the extract at 1,000, 2,000, and 4,000 mg/ml in a 96-well microplate. Therefore, 100 µL of each concentration and 100 µL of a microbial suspension at 1x10⁶ CFU/ml were added per well. Mueller-Hinton culture broth tested as a negative control and Triton X-100 as a positive control. After 24 h of incubation at 37°C, 5 ml of Sytox® green dye (1 mM) was added to each treatment (nucleic acid staining, Cat. No. S7020) (Diaz et al., 2018) and incubated in the dark for 30 min. Both the bacteria treated with the extract of T. lucida and those treated with the positive and negative controls were observed by optical microscopy in a bright field and fluorescent at 100x using a microscope (AxioLab A1 Carl Zeiss, China).

Effect on the bacterial DNA

The effect on DNA was evaluated by the comet assay. The plates for the comet test (2.5×7) were covered with the Comet Oxiselect Kit agarose gel (free of additives) and kept their cavities free. Then, in each cavity, 75 µl of the gel contained in each deposited sample was added. The plates were stored at 4°C for 30 min to maintain solidification. The plate covered with a lysis buffer (pH 10) was incubated at 4°C for 10 h in a dark room. The lysis buffer was replaced by an enzyme buffer. Every plate was incubated at 37°C during 2 h (in a dark room), and then buffer removed. The plate was placed in a chamber for electrophoresis and covered with a running buffer. During electrophoresis, 12 volts were applied for 60 min. The electrophoresis gel was washed three times with distilled water for 2 min and then washed with 70% ethanol for 10 min. Then, the Vista-Green DNA dye was stained with 0.5 µl and observed at 100 x under a fluorescence microscope (AxioLab A1 Carl Zeiss, China) (Solanky & Haydel, 2012; Min-Jeong *et al.*, 2016).

Statistical analysis

All results were expressed as the mean and the standard deviation (n=3). Statistical comparison of disc diffusion and MIC data were performed with an analysis of variance (ANOVA) followed by post hoc test of Tukey. Statistical significance was accepted at p < 0.05.

RESULTS AND DISCUSSION *Plant material and extraction*

The ethanolic extract of *T. lucida* showed a yield of $7.3 \pm 0.01\%$ that resembles the extracts of other Compositae (García-Pérez *et al.*, 2016). The ethanolic extract of *T. lucida* showed a yield percent similar to the methanolic extract since they have chemical compounds with comparable polarities (Téllez-López *et al.*, 2013).

Chemical characterization by Rp-HPLC-MS

The Rp-HPLC study detected medioresinol (1); 3,4-DHPEA-EA (2); gardenina B (3); tangeretin (4); 6 "-O-acetylglycine (5), petunidine 3,5-O-diglucoside (6), 4-O-glycoside of gallic acid (7), tartaric caffeic acid (8) and sculin (9), in the ethanolic extract (Figure No. 1) which have not been previously identified in T. lucida However, the extract could contain compounds not detected by this method and/or chromatographic column. For instance, phenolic compounds with antimicrobial effect have been detected in some organic extracts of T. lucida (CH₂Cl₂, MeOH, and ethyl acetate) (Céspedes et al., 2006: Hernández et al., 2006). In the present study, essential oils were not detected in the ethanolic extract since they could have been removed during drying of the extract by rotary evaporation. Still, other extracts from T. lucida contain essential oils such as estragole, methylenenolol, anethole (Bicchi et al., 1997), chavicol (Cicció, 2004), eugenol and myrcene (De la Luz et al., 2011).

Antibacterial activity evaluation Disk diffusion test

The ethanolic extract of *T. lucida* inhibited the growth of *S. aureus* with a zone of inhibition of 11.50 \pm 0.20 mm. However, it did not affect the growth of *E. coli*, *S. choleraesius* and *P. aeruginosa*. As we expected, ceftriaxone inhibited the growth of all microorganisms tested with a zone of inhibition \geq 30



Figure No. 1 Chemical structures detected in the ethanolic extract from *Tagetes lucida* Cav. by Rp-HPLC-MS Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas / 584

while negative control did not mm, show inhibitionzones. Our results are similar to those of the methanolic extract of T. lucida, since it gives rise to zones of 10 mm inhibition (Vega-Menchaca, 2013). On the other hand, zones of inhibition $\ge 9 \text{ mm}$ of the ethanolic extract of T. lucida on S. typhi, Salmonella enteritidis, E. coli, Shigella dysenteriae and Shigella flexneri were detected (Capunzo et al., 2003), while the extract in MeOH/CH₂Cl₂ (1:4) at 0.4 µg/disc inhibited the growth of E. coli and Proteus mirabilis by 40%, K. pneumoniae by 31.1%, Salmonella sp. in 35.5% at 72 h of cultivation (Céspedes et al., 2006). The essential oil of *T. lucida* reduced the growth of *S.* aureus with zones of inhibition of 17 mm, as well as of E. coli and B. subtilis with zones of inhibition of 14 mm (Elsayed et al., 2015).

Minimal inhibitory concentration (MIC)

Table No. 1 shows the percentages of inhibition of the extracts from *T. lucida* on *S. aureus*, which was the only strain sensitive to this treatment. Here, it should be highlighted that the antibacterial action was

dependent on the concentration used since lower doses to 1.000 mg/ml did not inhibit bacterial growth (data not shown). However, inhibited bacterial growth in a 61 \pm 1.73, 93.00 \pm 1.00, and 100.00 \pm 0.00% at 1.000, 2.000, and 4.000 mg/ml, respectively. In this way, the MIC of the ethanolic extract of T. lucida on S. aureus was 4.000 mg/ml (MIC_{100}) . Accordingly, with the above, other Compositae species have a MIC of 1 mg/ml against S. aureus (Tereshuk et al., 1997; Capunzo et al., 2003; Hernández et al., 2006). Interestingly, the MIC values were 100 and 200 mg/ml on Campylobacter jejuni (UVG 62-1773-6), and 450 mg/ml on E. coli (ATCC 25922), Aeromonas hominis (ATCC 7966), and P. aeruginosa (27853) for the methanolic and chloroform extract from T. lucida suggesting that the depend on both the chemical MIC values composition of each extract and the bacterial susceptibility. Furthermore, ceftriaxone (MIC of \leq 0.003 ± 0.000 mg/ml) exhibited a higher potency than the ethanolic extract from *T. lucida*.

Table No. 1Percentages of bacterial inhibition of the ethanolic extract from Tagetes lucida Cav.

Concentration mg/ml	Bacterial inhibition (%)
1.000	61.00 ± 1.73
2.000	93.00 ± 1.00
4.000	100.00 ± 0.00
4.000	100.00 ± 0.00

The data are expressed as the mean \pm standard deviation, n = 3

Bioautography

The eluent of petroleum ether-ethyl acetate (2:1) separated the fractions of the extract of *T. lucida* better other eluents since the retention factor between the bands was (Rf) ≥ 0.1 . Therefore, it was more efficient, as previously reported by Wagner & Bladt (2001). In these conditions, five fractions of ethanolic extract from *T. lucida* with Rf values of 0.50, 0.67, 0.75, 0.80, and 0.95 were separated by TLC. Juárez-Sánchez (2015) separated three fractions after degreasing the plant with hexane with similar Rf values (Rf of 0.50, 0.68, and 0.77), indicating that the extraction procedure influences the fractions obtained from an extract determined due to its chemical composition (Dewanjee *et al.*, 2015).

The fractions evaluated by bioautography did not have an antibacterial effect. Here, can be argued that the antibacterial effect on *S. aureus* of the extract from *T. lucida* could be due to a synergism between the five fractions based in that: 1) the antibacterial effect of the extract was observed in diffusion assay, and 2) it has been observed that other fractions or compounds display synergistic antibacterial effects (Silva-Belmares *et al.*, 2014; Jesionek *et al.*, 2015; Zater *et al.*, 2016). On the other hand, another explanation for this observation is that the stationary phase retained the compounds containing in the five fractions, so they did not migrate to the culture medium to exert their antibacterial effect. In any case, future experiments using isolated fractions are warranted.

Target action evaluation

Because the ethanolic extract from *T. lucida* only inhibited the growth of *S. aureus* in the test of disc diffusion plate and microdilution, the strain was used to determine the site of action through the Sytox Green test (plasmatic membrane) and comet assay

(bacterial DNA). The Sytox green test showed that the ethanolic extract from *T. lucida* permeabilizes the membrane (observed as fluorescence) of *Staphylococcus aureus* in a concentration-dependent manner (1.000 and 2.000 mg/ml). The group treated with the negative control showed no fluorescence because its membrane remains intact (Figure No. 2).

The microscopic observations indicate that the ethanolic extract from *T. lucida* causes the formation of pores in the bacterial membrane because the fluorogenic dye (Sytox Green) only penetrates the compromised membranes and fluoresces when it binds to DNA (Kim *et al.*, 2015). The effect on the plasma membrane resembles the reported by (Lee *et al.*, 2015). The effect of the extract in *S. aureus* could be due to some phenolic compounds detected by RpHPLC-MS since phenolic groups affect the stability of membrane proteins through the formation of pores resulting in bacterial death (Ye *et al.*, 2011; Casero *et al.*, 2013; Diaz *et al.*, 2018).

On the other hand, the ethanolic extract from *T. lucida* breaks the bacterial deoxyribonucleic acid (DNA) because the bacteria embedded in the agarose and subjected to electrophoresis gave rise to the formation of a comet-like structure observed by fluorescence microscopy. The comet assay is specific to assess the decomposition of the DNA chain (Solanky & Haydel, 2012) because the fluorescence intensity of the tail relative to the comet's head is a reflection of DNA breakage since comets are formed by breaking the DNA loops (see Figure No. 3).



Figure No. 2

Effect of the ethanolic extract from *T. lucida* on the *S. aureus* bacterial membrane detected by SYTOX Green absorption. In the upper panels, the images observed correspond to optical microscopy (100x). In the lower panels, the images observed correspond to fluorescent field microscopy (100x). Panel A shows the bacteria treated with the negative control. Panel B shows the strain treated with the ethanolic extract from *T. lucida* at 1 mg/ml. Panel C shows the strain treated with the ethanolic extract from *T. lucida* at 2 mg/mL



Figure No. 3

Effect of the ethanolic extract from *T. lucida* on the bacterial DNA detected by the comet assay. Panel 1 shows the control with an ethanolic extract from *Tagetes lucida* Cav. without *Staphylococcus aureus*. Panel 2 shows control with *S. aureus* without extract. Panel 3 shows the ethanolic extract from *T. lucida* against *S. aureus*

It has been shown that 3,4-DHPEA-EA inhibits the growth of S. aureus with MIC values between 125 and 250 µg/ml (Bisignano et al., 2014), whereas medioresinol does at 5.0 µg/mL (Hwang et al., 2013). Tangeretin has a biopreservative potential so that it could also be involved in the antibacterial effect of the ethanolic extract from T. lucida (Jayaprakasha et al., 2000). Additionally, 3,4-DHPEA-EA and tyrosol show antioxidant activity (Masella et al., 1999), so they have an anti-aging property (Nianhan et al., 2014), while, tangeretine acts as a suppressant for breast cancer (Lakshmi & Subramanian, 2014). As well, coumarins have been detected in some extracts from flowers, leaves, and stems of T. lucida (Mejia-Barajas et al., 2012; Téllez-López et al., 2013). All the compounds detected in

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the extract of *T. lucida* have a polyphenolic chemical structure. Therefore, they could destabilize plasma membranes to cause microbial death, as some flavonoids are known to cause cell membrane damage (Lee *et al.*, 2015).

CONCLUSION

The present study shows that the ethanolic extract from *T. lucida* has an antibacterial effect against *S. aureus*. This antibacterial action of *T. lucida* is related to membrane disruption and DNA damage, probably induced by phenolic compounds contained in this plant. On this basis, *T. lucida* represents a potential source of antibacterial compounds that could be used for the development of pharmaceutical products against *S. aureus*.

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