UNIVERSIDAD AUTÓNOMA DE GUERRERO

FACULTAD DE CIENCIAS QUIMICO-BIOLÓGICAS
FACULTAD DE MEDICINA
UNIDAD DE INVESTIGACIÓN ESPECIALIZADA EN MICROBIOLOGÍA

MAESTRÍA EN CIENCIAS BIOMÉDICAS

Actividad antiproliferativa y antimigratoria de extractos orgánicos de *Tagetes lucida* en líneas celulares de cáncer cervicouterino

T E S I S

QUE PARA OBTENER EL GRADO DE
MAESTRÍA EN CIENCIAS BIOMÉDICAS

P R E S E N T A:

Biol. Exp. Onelio Mora Candelario

Director de tesis: Dr. Marco Antonio Leyva Vázquez

Codirector: Dr. Macdiel Emilio Acevedo Quiroz

Chilpancingo de los Bravo, Gro., febrero, 2021

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ACTA DE APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 02 días del mes de julio de dos mil veinte, se reunieron los miembros del Comité Tutorial designado por la Academia de Posgrado de la Maestría en Ciencias Biomédicas, para examinar la tesis titulada **"Actividad antiproliferativa y antimigratoria de extractos orgánicos de *Tagetes lucida* en líneas celulares de cáncer cervicouterino"**, presentada por el alumno **Onelio Mora Candelario**, para obtener el Grado de Maestría en Ciencias Biomédicas. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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Esta investigación se realizó en el Laboratorio de Biomedicina Molecular y el Laboratorio de Biomoléculas, de la Facultad de Ciencias Químico Biológicas dependiente de la Universidad Autónoma de Guerrero (UAGro), en Chilpancingo de los Bravo, Guerrero. La caracterización fitoquímica de los extractos se realizó en el Centro de Investigaciones Químicas-Instituto de Investigación en Ciencias Básicas y Aplicadas (CIQ-IICBA) de la Universidad Autónoma del Estado de Morelos (UAEM).

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Dr. Julio Ortíz Ortíz

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Durante el periodo en que cursó la maestría en Ciencias Biomédicas, el B.E. Onelio Mora Candelario recibió beca otorgada por el Consejo Nacional de Ciencias y Tecnología (CONACyT) a los posgrados pertenecientes al Programa Nacional de Posgrado de Calidad (PNPC) con número de CVU 923201.

AGRADECIMIENTOS

Al Dr. Marco Antonio Leyva Vázquez *por darme la oportunidad de formar parte de su grupo de trabajo, por su alto compromiso conmigo, su confianza, por el apoyo, el cariño brindado y el tiempo que se tomó para la revisión del escrito.*

Al Dr. Macdiel Emilio Acevedo Quiroz *también por su alto compromiso conmigo, por sus motivaciones, por su apoyo y amistad que me permitieron aprender mucho más del proyecto, así como por sus buenas aportaciones en la revisión y redacción del escrito.*

A la Dra. Mónica Espinoza Rojo *por sus aportaciones y sugerencias en cada una de las etapas del desarrollo de este proyecto.*

Al Dr. Julio Ortíz Ortíz *por su apoyo y consejo, sus sugerencias en la revisión del escrito y por hacer un vínculo importante para la realización de este proyecto.*

Al Dr. Donaciano Flores Robles *por sus motivaciones, sus sugerencias, y por estar al pendiente durante la realización de este proyecto.*

A la Dra. Laura Patricia Álvarez Berber *por su colaboración, por permitirme realizar mi estancia en su laboratorio, aportaciones y sugerencias en la revisión de este proyecto.*

A la Dra. Mayra Yaneth Antúnez Mojica *por su colaboración, su paciencia, asesoramiento y capacitación en la caracterización fitoquímica.*

Al Dr. Miguel Ángel Mendoza Catalán *por su colaboración, por su paciencia, asesoramiento en la parte experimental, aportaciones y la revisión del proyecto.*

A la Dra. Ana Elvira Zacapala *por su paciencia, asesoramiento y capacitación en cultivo celular, y en la realización de algunos ensayos.*

Al equipo Mendoza *(Brenda, Itzel, Lore, Jonna-Ficus, Citlali, Eli, Bety, Migue, Alexis, Jonna, Erika) por hacer que los momentos dentro y fuera del laboratorio fueran únicos e inolvidables.*

A mis compañeros de generación, especialmente (Grecia, Azu, Gaby, Onniver, Clau) por su amistad, por su apoyo incondicional y por compartir conocimientos.

DEDICATORIAS

A Dios por darme la oportunidad de cumplir un legado muy importante en otra etapa de mi vida profesional.

A mi familia: *Especialmente a mi mamá Cimona Candelario Castro y mi papá Modesto Mora Aparicio, quienes siempre refrendaron su apoyo incondicional en mí, este nuevo logro es en gran parte gracias a ustedes, siempre me he sentido maravillado por la linda familia que tengo, se han preocupado de mí desde el momento en que llegué a este mundo, me han formado para saber cómo luchar y salir victorioso ante las diversas adversidades de la vida. Muchos años después sus enseñanzas no cesan, y aquí estoy, con un nuevo logro exitosamente conseguido. Les viviré eternamente agradecido, ¡los amo!.*

A mis hermanos: *Cris, Gude, Soledad, Margarita, Rosalba, Mari, Desi, Vicente, Cleo, también este trabajo se los dedico a ustedes, quienes estuvieron conmigo en los momentos más difíciles de mi vida, sus motivaciones enriquecieron mis ganas de salir adelante. Infinitas gracias, ¡los quiero!.*

Amigos y personas cercanas a mi vida: *gracias por echarme porras, por creer en mí y créenme que cada peldaño del éxito va dedicado para todos ustedes, especialmente para Carmen, Brenda, Azu, Grecia, Fabiola, Itzel, Alma, Amarilis, Any, Rafa, Ari, Marcos, Tío Beto, Mayra, Araceli, Gude, Yosi, Bety-Alber, Ceci, Sergio, Victor, Tere, Isa, Belinda, etc., y no terminaría de mencionar.*

Onelio Mora Candelario

Índice

Dear Mr.Onelio Mora-Candelario,

You have been added as a co-author for article entitled GC-MS phytochemical profiling, antiproliferative and antimigratory effect of Tagetes lucida leaves extracts on cervical cancer cell lines submitted toPharmacognosy Magazine .

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GC-MS phytochemical profiling, the antiproliferative and antimigratory effect of *Tagetes lucida* leaf extracts on cervical cancer cell lines

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Abstract

Background: *Tagetes* species are widely used in traditional medicine and have been shown to exert diverse biological activities due to its phytochemical constituents, including those related to cancer. **Objectives:** The present study aimed to identify the bioactive compounds of the organic extract of *T. lucida* leaves with antiproliferative and antimigratory activities on human cervical cancer cell lines, SiHa and HeLa, and the non-cancer cell line, HaCaT. **Materials and Methods:** The phytochemical profile of all *Tagetes* leaf extracts was determined by GC-MC analysis and tested on SiHa, HeLa, and HaCaT cell lines using MTT and wound-healing assays. **Results:** The GC-MS analysis revealed two main constituents that were identified in all extracts were the coumarins herniarine (17.152-56.904% content) and scoparone (2.778-34.817% content), while the remainder of the identified constituents were terpenes, in which the geranyl acetate was the major constituent in the hexane extract. In terms of the antiproliferative potential of *T. lucida* extracts, our present investigation showed that all extracts decreased HeLa cell viability with a one-half Inhibitory Concentration (IC_{50}) value of <190.26 µg/mL at 24 h and of <220.41 µg/mL at 48 h in a dose-dependent manner, while SiHa and HaCaT cell viability was not affected. Also, the migration capacity of SiHa and HeLa cells decreased after 24 and 48 h with the treatments with acetonic and methanolic extracts, while in HeLa cells, a more evident effect was observed with a <80% decrease in wound closure. **Conclusions:** Our findings revealed that the coumarins present in *T. lucida* are potential candidates for inhibiting cell proliferation and migration, preferably toward cervical cancer (HeLa cell line).

Keywords: Natural products, Antiproliferative, Migration, Cervical cancer

1. Introduction

Cervical cancer (CCa) is the fourth most common cancer in women worldwide and, in Mexico, it ranks second.[1] Current chemopreventive therapies in cancer patients include surgery, radiotherapy, immunotherapy, targeted molecular therapy, and chemotherapy, among which chemotherapy, in combination with radiotherapy (chemoradiotherapy), is one of the most employed.[2] However, there is a 90% deficiency in chemotherapy treatments as a result of drug resistance developed during cancer invasion and metastasis.[3] Despite the adverse effects, currently there is a gamma of antineoplastic drugs that possess different objectives in the cell, among which we find those that cause DNA damage, such as anthracyclines, antimetabolites, antitumor antibiotics; and nutritional supplements including camptothecins, epipodophyllotoxins, platinum analogues, taxanes, and vinca alkaloids, the latter of which are derived from natural sources.[4]

Recently, the search for bioactive molecules to replace specific drugs in cancer therapy has increased significantly. Natural plant compounds play a fundamental role in defense, protection against ultraviolet radiation, parasites, and predators.[5] The growing interest in bioactive molecules is due to the presence of an infinity of secondary metabolites, such as terpenes, phenolic compounds, and alkaloids with anticancer activity.[6] Phenolic compounds are one of the most studied phytochemical families. Such compounds have various biological properties that include antioxidant, antienzymatic, antiestrogenic, and antiproliferative activities, etc. On the other hand, they stop the cell cycle and promote apoptosis as well as differentiation. This wide variety of biological functions renders them

one of the most versatile chemopreventive agents in the intervention of each of the stages of carcinogenesis.[7]

Tagetes is a genus of plants that belongs to the Asteraceae family and that comprises about 56 species.[8] This genus has been investigated as a possible source of chemical compounds with high pharmaceutical and nutritional values. The natural compounds that have been found are phenyl compounds such as coumarins,[9-13] terpenes,[9,10,13] and thiophene compounds to which various properties have been attributed, such as antibacterial, anticoagulant, anti-Alzheimer, anti-HIV and anticancer activities.[14,15,18]

Tagetes lucida Cav. is an important aromatic ritual plant that is widespread from Central to South America. It is commonly known by the name of "pericón", "yauhtli" (Náhuatl), "anís", "Santa María" or the "San Juan" herb.[12,19] In traditional medicine, *T. lucida* is one of the most widely utilized medicinal plants of western Mexico: a decoction of the entire plant or of various parts of the plant has proven useful in treating conditions of the digestive tract, the spitting up of blood, anxiety, depression, and as an anti-inflammatory and malarial remedy. In addition, pharmacological studies have reported its antibacterial, insecticidal, cytotoxic, antioxidant, antidepressant-like effects and its anxiolytic, sedative, and anti-*Candida albicans* activity.[12,20-21]

Despite the great potential of species of this genus as a source of secondary metabolites of therapeutic interest, to date there are insufficient *in-vitro* studies being conducted in the area of cancer, and at present, the background of the antiproliferative and anti-migratory activity of this genus remain poorly studied.[12,22] In addition, this species do not have sufficient phytochemical or pharmacological studies that support the properties that have been attributed to it; therefore, the objective of this work was to evaluate effects of *T. lucida* organic extracts on the proliferation and migration in two cervical cancer cell lines, that is, SiHa and HeLa, and a non-tumor HaCaT line. The results obtained from this research will generate basic information regarding the biological properties and phytochemical profiles of the species *Tagetes lucida* that have been collected; this basic information will contribute to the proper exploitation of a valuable native natural resource of Mexico.

Materials and methods

2.1 Plant material.

The *T. lucida* leaves were collected in Petaquillas (17°28'26' N, 99°27'43' O), Guerrero state, Mexico, in December 2018. Identification of the plant was authenticated by Arturo Hernández-Abarca, the Biologist at the Herbarium of the Institute of Scientific Research, Natural Sciences Area (Autonomous University of Guerrero). A voucher specimen (11555) was deposited at this same Institute for future reference.

2.2 Extracts preparation

The *T. lucida* plant material (1.4 kg), dried at room temperature (24°C) and ground, was successively macerated with hexane, dichloromethane, acetone, and methanol (reactive-grade, 500 mL for 72 h, three times each). The macerated volume was filtered and the organic phase was concentrated at 60°C under reduced pressure in a rotating evaporator. The Hexane (H), dichloromethane (D), acetone (A), and methanol (M) extracts were stored in amber-colored vials at -20°C until their use.

2.3 Gas Chromatography-Mass Spectrometry analysis (GC-MS)

The phytochemical profile of *T. lucida* extracts revealed the presence of bioactive components. GC-MS analysis was carried out using an Agilent Technology 6890 gas chromatograph interfaced to a 5973N mass spectrometer equipped with a HP-5MS capillary nonpolar column (30 m; ID: 0.25 mm; film thickness: 0.25 μm), connected to an ion trap detector operating in the electron impact mode at 70 eV; the carrier gas was He, with a flow rate of 1 mL/min and an injection volume of 20 μL (in HPLC-grade Hexane). The oven temperature was programmed from 50 to 230°C with an increase of 2°C min^{-1}. The results were compared by using NIST/EPA/NIH Mass Spectral Library version 1.7a/ChemStation.

2.4 Cell culture and exposure to extracts

Human SiHa, HeLa, and HaCaT cells were cultured in Dulbecco's Medium Modified Eagle Medium nutrient mixture F-12 (DMEM/F12) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS), 1% (v/v) antibiotic (Ampicillin/Streptomycin), and incubated at 37°C in a 5% CO_2 atmosphere and at 100% humidity.

2.5 Cell proliferation assay

The proliferation effect of the extracts on SiHa, HeLa, and HaCaT cells were assessed using an MTT (3-(4,5-diMethylThiazol-2-yl)-2,5-diphenylTetrazolium bromide) assay, according to Mossman (1983); a total of 1 x 10^4 cells per well were seeded in a 96-well flat-bottom culture plate in 100 μL of medium and incubated at 37°C overnight. The *T. lucida* extracts in 100 μL of fresh medium were added to reconstitute the final concentrations (5-320 μg/mL) during 24 and 48 h. Medium containing vehicle solvent DiMethylSulfOxide [DMSO <1% (v/v)] was added as untreated controls (V, Vehicle). After treatment, the medium was replaced with fresh medium and the MTT reagent (100 μL) in each well and was incubated for 4 h at 37°C. The formazan crystals were diluted with isopropanol (100 μL), and the supernatant's Optical Density (OD) was measured at 545 nm using a Statfax 2100 microplate reader (Awareness Technology, Palm City, FL, USA). A Paclitaxel (5 μL) compound was used as a positive control.[23] Each concentration was tested with three replications in each experiment, and the experiment was performed independently at least three times. The concentration of the extract required for a one-half Inhibitory Concentration (IC_{50}) was calculated by means of the linear equation ($Y = mX + b$) using GraphPad Prism ver. 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

2.6 Migration: Wound-Healing Assays

Cells were grown in 12-well plates until they reached ~90% confluence, and then, in a linear wound, the cell monolayer was scratched with a pipette tip (2 mm). After this, the cells were preincubated with Cytosine β-D-Arabinofuranoside (AraC) (5 μg/mL) for 2 h to eliminate the migration induced by the cell proliferation; these were then washed twice with Phosphate-Buffered Saline (PBS) solution. The cells were treated with *T. lucida* extracts (5-20 μg/mL) with FBS (1%) and incubated for 24 h and 48 h at 37°C. Cell migration above the denuded area was visualized and photographed under a phase-contrast inverted microscope (NIKON, Ts2FL, USA) at 0, 24, and 48 h. The width of the wound was measured with ImageJ ver. 1.44p software and with the MRI wound healing tool, compared to zero time. The experiment was repeated at least three times.[24,25]

2.7 Statistical analysis

Data analysis was performed using the GraphPad Prism ver. 6.0 statistical software program. One-way Analysis Of VAriance (ANOVA) was employed with the Dunnett test. The data were shown as the mean ± Standard Deviation (SD). A statistically significant difference was considered when $p < 0.05$. Analysis and adjustment of the images were carried out in ImageJ software. All experiments were carried out in triplicate.

2. Results and discussion

The hexane, dichloromethane, acetone, and methanol *T. lucida* extracts produced yields of 5.5%, 16.26%, 3.45%, and 5.44%, respectively. Gas Chromatography-Mass Spectrometry (GC-MS) analyses are depicted in Table 1. The phytochemical study by GC-MS analysis revealed 23 identified constituents in which different fatty acids, heterocyclic compounds, and esters, among others, were present. Interestingly, the highest percentage (~70%) of the coumarins herniarine (**15**) and scoparone (**17**) (Figure 1) were predominant in the dichloromethane, acetone, and methanol extracts compared to these compounds, while the geranyl acetate (**4**) was the major constituent identified in the hexane extract. These compounds were previously reported in the *Tagetes* genus.[26,27]

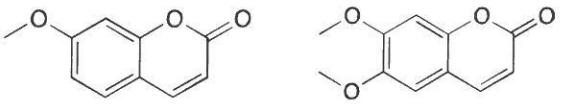
Phenolic compounds identified from studied species of the *Tagetes* genus are mainly thiophenes, flavonoids, and coumarins, which normally are described as antifungal and antibacterial compounds.[28,29] However, this is, to our knowledge, the first work in which a high content of these coumarins is reported both in *T. lucida* and other *Tagetes* species.[30-33]

Table 1. Compounds detected by GC-MS in the *Tagetes lucida* extract

No	Component	Content (%) RT (min)				
		H	D	A	M	
1	Linaool	0.127 8.463				
2	2,6-Dimethyl-3,5,7-octatriene-2-ol, E	0.810 10.112				
3	Geraniol	1.358 10.762				
4	Geranyl acetate	21.230 12.542	5.918 12.509	9.300 12.503	7.206 12.246	
5	Nerolidol acetate	0.076 12.614				
6	β-Elemene	2.493 12.719				
7	Isohomogenol	0.557 12.818				
8	Caryophyllene	9.887 13.146	3.744 13.127	3.515 13.120	1.340 13.114	
9	α-Humulene	1.074 13.560				
10	β-Cubebene	7.931 13.915	2.173 13.903	1.494 13.120	1.035 13.895	
11	α-Cadinene	1.735 14.381			0.541 14.105	
12	E-Nerolidol	6.410 14.821	2.916 14.808	2.673 14.808	2.777 14.802	
13	(-)-Spathulenol	1.692 15.117	0.469 15.110	0.729 15.104	0.370 15.097	
14	Caryophyllene oxide	0.943 15.189	0.844 15.183	0.843 15.176	0.447 15.176	
15	Herniarin	17.152 16.897	38.815 16.956	56.904 16.891	53.639 16.838	
16	Phytol	7.000	3.396	3.575	4.190	0.708

		17.830	17.817	17.817	17.810	20.516
17	Scoparone	2.778 19.367	34.817 19.498	12.638 19.360	21.855 19.347	
18	Geranyl linallol	5.811 19.781	3.833 19.774	4.332 19.761	3.444 19.754	
19	Squalene	3.736 29.259				
20	α-Tocopherol	7.199 33.122	3.075 33.389	3.603 33.082		
21	Tricyclo[5.2.2.0(1,6)]undecano-3-ol,2-methylene-6,8,8,-trimethyl-			0.394 16.352		
22	α-Muurolene				1.156 14.375	
23	Ethyl α-linolenate				1.290 20.398	

H: Hexane; D: Dichloromethane; A: Acetone, and M: Methanol extracts.



15: Herniarin **17: Scoparone**

Figure 1. Chemical structure of coumarins with a high content in *Tagetes lucida* extracts.

3.1 Antiproliferative activity

MTT assays were performed to evaluate the antiproliferative activity of *Tagetes* extracts on SiHa, HeLa, and HaCaT cell lines for 24 and 48 h. However, the extracts after 24 h of treatment did not show an effect on antiproliferative activity (data not shown). In HeLa cells treated during 48 h with 160 and 320 μg/mL of all of the extracts, cell viability of less than 50% and 40%, respectively, was observed. Similarly, in SiHa cells treated with 160 and 320 μg/mL, cell viability of less than 60% and 50%, respectively, was observed, while in HaCaT cells treated with 160 μg/mL of the hexane, dichloromethane, and acetone extracts, cell viability remained above 60% (Figure 2).

Moreover, all extracts induced morphologic changes on cancer cells lines, such as a decrease in cell size, a rounded shape, and the formation of intracellular vacuoles suggestive of apoptosis (data not shown). It was reported that regulation of growth in SiHa and HeLa is due to variation in p53 activation due to the low levels of p53, which induces cell-cycle arrest, whereas high levels of p53 induce apoptosis.[34-35] Hence, the antiproliferative effect of *T. lucida* could be due to that p53 is activated more in HeLa than in SiHa in response to treatments.

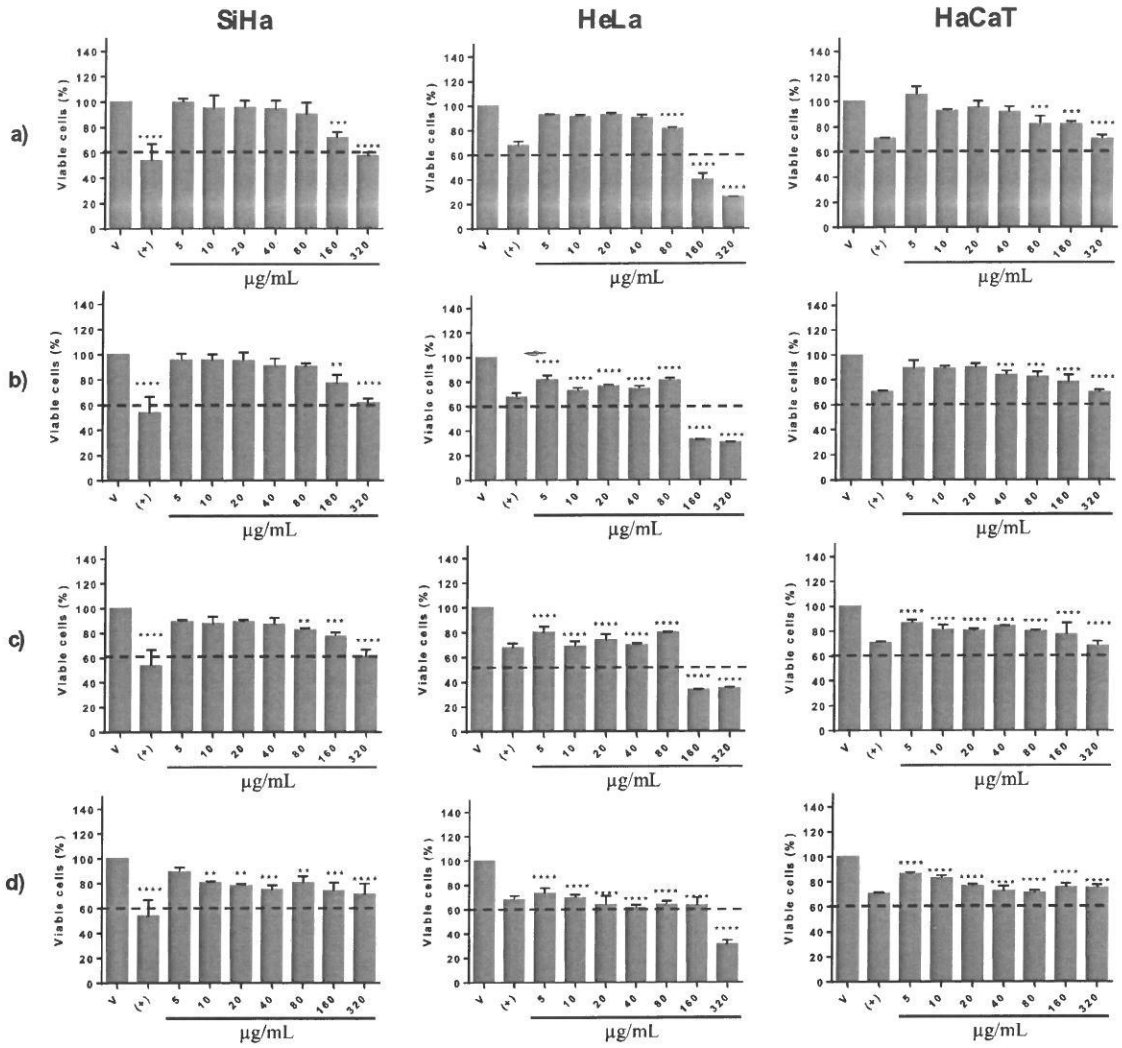


Figure 2. Antiproliferative effect of *Tagetes lucida* extracts on HeLa, SiHa, and HaCaT cells. MTT assay; cells treated with *T. lucida* extracts during 48 h. Hexane a), Dichloromethane b), Acetone c), and Methanol d) extracts. V: Vehicle, (DMSO), +: positive control, Paclitaxel (5 µg/mL). One-way ANOVA, Dunnett test: *p <0.05; **p <0.01, and ***p <0.001, and ****p <0.0001 vs. V.

The best antiproliferative effect in a dose-dependent manner compared to untreated control cells was induced by all *T.lucida* extracts for HeLa at 24 h (IC_{50} <190.26 µg/mL) and at 48 h (IC_{50} <220.41 µg/mL), while for SiHa, there was no effect (Table 2).

Table 2. Antiproliferative effect of organic extracts using the MTT assay

Extracts	IC_{50} ± SD (µg/mL)					
	24h			48h		
	SiHa	HeLa	HaCaT	SiHa	HeLa	HaCaT
H	na	190.26±2.35	na	na	220.41±3.21	na
D		174.21±3.02			217.41±2.95	
A		178.28±3.12			176.55±3.31	
M		189.70±1.84			203.116±2.26	

Positive control: Paclitaxel (5 µg/mL). na: Non-active (IC_{50} >250 µg/mL).

Due to that CCa is the second major cause of deaths due to cancer in women worldwide and to the VPH-16 (SiHa) and VPH-18 (HeLa) genotype is attributed the major risk factors, which account for nearly 70% of cancers, and SiHa contains around 1-2 integrated copies of the HPV 16 genome whereas HeLa has 10-50 integrated copies of HPV 18, the result is that the rate of replication is higher in HeLa cells because it becomes a more aggressive cancer.[34,35] Our results are interesting because the evaluated extracts were only active against the HeLa cell line (Table 2).

Based on the high coumarin content in the GC-MS analysis and in previous investigations results, we can infer that coumarins are directly related to the antiproliferative effect. Coumarins, depending on their structure, can act on various tumour cells through different mechanisms.[36-38] Hence, a selective e difference of the cytotoxic effect on the tumor cells (SiHa and HeLa) could be more noticeable by isolated coumarins from T. lucida. Thereby, these results were consistent with those of other studies, where coumarins are reported to exhibit negligible or mild adverse effects in humans employing doses of up to 7 g daily, after 2 weeks of continued treatment; even though they were excellent agents for treating the adverse effects caused by radiotherapy.[35-39]

Although coumarins are considered one of the most important phytochemicals deriving from the *Tagetes* genus, cancer studies are focused on flavonoids and thiophenes of mainly *Tagetes minuta* and *Tagetes erecta* species,[9,10,13,40,41] among the studies included, in which activity is attributed to these compounds against HeLa cells;[11,14,41] this could be due to the low coumarin quantities found in the studied species. Coumarin antitumor activity is extensively explored by many researchers at present due to that they are considered promising anticancer compounds; however, the compounds utilized in the research of these authors were not isolated from natural species.[42] This is the first study, to our knowledge, which recognizes antitumor activity in identified coumarins from *T. lucida* extracts, and the first, to our knowledge, in which two cervical-cancer and one non-cancer cell line were employed. Although these results propose *T. lucida* extracts as an alternative or complementary therapy against cancer, cytotoxicity and molecular mechanisms must be analyzed in more study models.

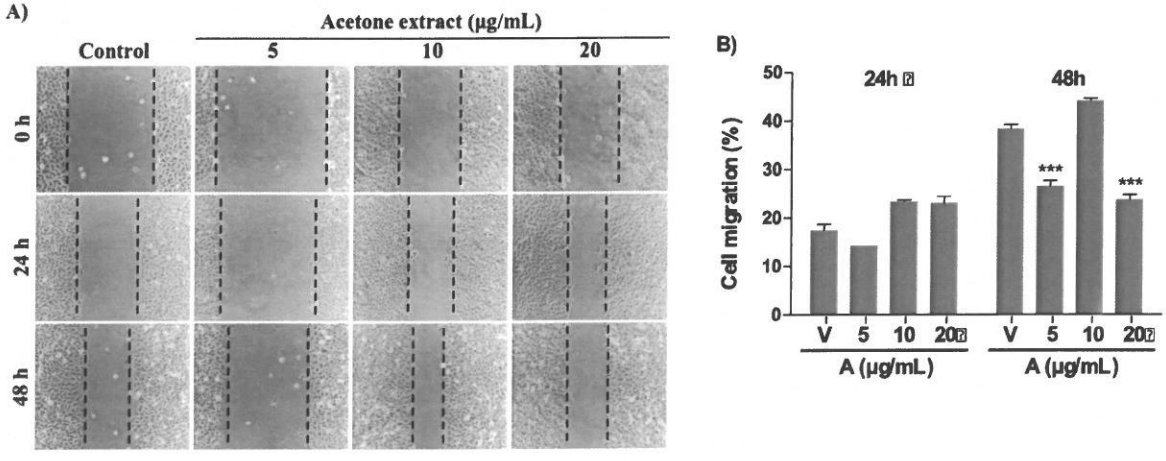
Additionally, the GC-MS analysis revealed that several terpenes presented in *Tagetes* extracts have been reported to possess attractive anticancer properties. Considering monoterpenes, linalool exhibited an antiproliferative effect on human melanoma cells (RPMI 7932) at IC_{50} = 5.60 μM and on prostate cancer cells (DU145) at IC_{50} = 28.3 and 10.5 μM at 12 and 24 h, respectively;[43,44] and geraniol and geranyl acetate were reported due to their ability to trigger apoptosis, DNA damage and cell-cycle arrest in colon-cancer cells (Colo-205) at IC_{50} values of 20 and 30 μM, respectively.[45] Likewise, sesquiterpenes such as spathulenol were disclosed as effective against the ovarian-cancer cell line (OVCAR-3) at IC_{50} = 49.30 μg/mL,[46] while nerolidol was noted to have anticancer properties against skin melanoma cells (B16-F10, IC_{50} >25 μM), hepatocellular carcinoma (HepG2, IC_{50} >25 μM), human promyelocytic leukemia cells (HL-60, IC_{50} = 21.99 μM), and human erythroleukemic cells (K562, IC_{50} = 17.58 μM),[47] and, in combination with their isomers cis- and trans-nerolidol, exhibited an anticancer effect against HeLa cells (IC_{50} = 1.5 μM) and breast-carcinoma cells (BT-20, IC_{50} = 1.5 μM).[48] Further, the *in-vitro* studies revealed a reduction of the incidence of intestinal neoplasia (50%) and a reduction of several tumors/rat (about 53%) in rats fed nerolidol.[49]

Moreover, although the anticancer properties of β-caryophyllene and the β-caryophyllene oxide are poorly recognized, a great deal of evidence has demonstrated that both sesquiterpenes possessed

cytotoxic activities against several types of cancer cells.[50] For example, β-caryophyllene was reported to lead to strong growth inhibition in two colon-cancer cell lines, including HCT-116 (IC_{50} = 19 μM) and HT-29 (IC_{50} = 63 μM), as well as in pancreatic-cancer cells, PANC-1 (IC_{50} = 27 μM) and, in combination with their isomers α-humulene and iso-caryophyllene, were more effective in the reduction of MCF-7 human breast-cancer cell line proliferation (90% at 10 μg/mL) than when employed separately.[51,52] In addition, β-caryophyllene oxide was reported as exerting a cytotoxic effect on various cancer cell lines, such as HeLa (IC_{50} = 13.55 μM), HepG2 (human liver-cancer cells, IC_{50} = 3.95 μM), AGS (human gastric-cancer cells, IC_{50} = 12.6 μM), SNU-1 (human gastric cancer cells, IC_{50} = 16.79 μM), SNU-16 (human stomach-cancer cells, IC_{50} = 27.39 μM), and A-2780 (human ovarian-cancer cells, IC_{50} = 40.6 μM).[53,54] In human brain-cancer studies, β-elemene displayed an antiproliferative effect against human brain-tumor cells, that is, A172 (80.8 μg/mL), CCF-STTG1 (82.8 μg/mL), and U-87MG (88.6 μg/mL),[55] while in human hepatocellular carcinoma studies, α-humulene demonstrated an antiproliferative effect on Huh7 (IC_{50} = 15.09 μg/mL), SMMC-7721 (IC_{50} = 17.31 μg/mL), HepG2 (IC_{50} = 11.22 μg/mL), and Hep3B (IC_{50} = 13.78 μg/mL).[56] Finally, the diterpene phytol and triterpene squalene exhibited potent antiproliferative activity against human lung carcinoma cells (A549, IC_{50} = 70.81 and 60.7 μM at 24 and 48 h, respectively)[57] and against breast-cancer cells (MDA-MB-231 and MCF-7, IC_{50} = 4.35 and 6.05 mg/mL, respectively).[58]

3.2 Antimigratory activity

The effect of all of the extracts of *T. lucida* on the migration capacity of SiHa and HeLa cells was tested at concentrations of 5, 10, and 20 μg/mL for 24-48 h. However, the hexane and dicloromethane extracts did not demonstrate an effect on the cell-migration capacity (data not shown). No significant decreases were observed in the migration capacity of SiHa cells treated with the acetone extract at 24 h; nonetheless, compared to the control (39% decrease in wound closure), the treatment decreased migration capacity at 48 h, observing a 26% and 23% decrease in wound closure at concentrations of 5 and 20 μg/mL, respectively (Figures 3A and 3B). Additionally, treatment with the methanolic extract (20 μg/mL) decreased the migration capacity of SiHa cells from 24 h (14% less wound closure compared to the control), and this effect was maintained at 48 h, although at a lower proportion (around 9% less wound closure compared to the control) (Figures 3C and 3D).



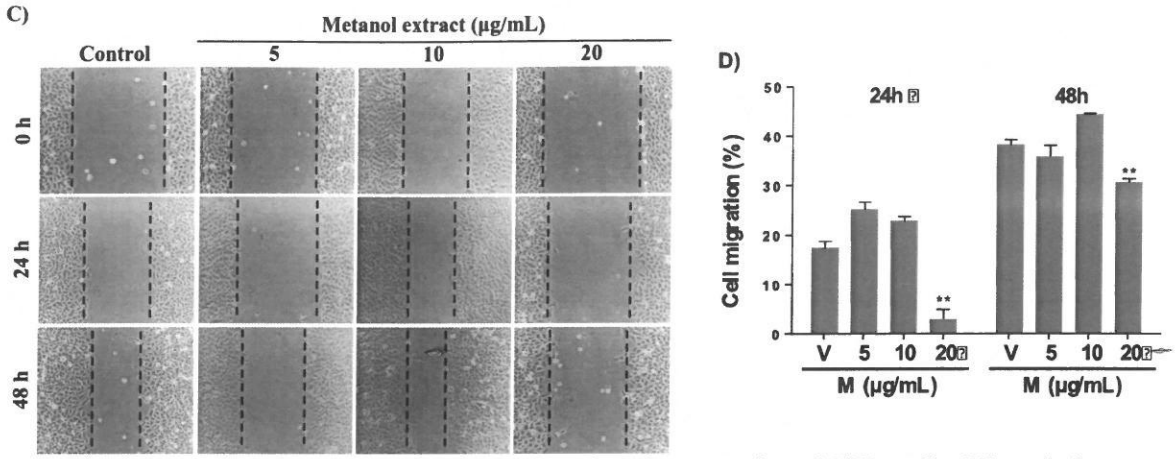
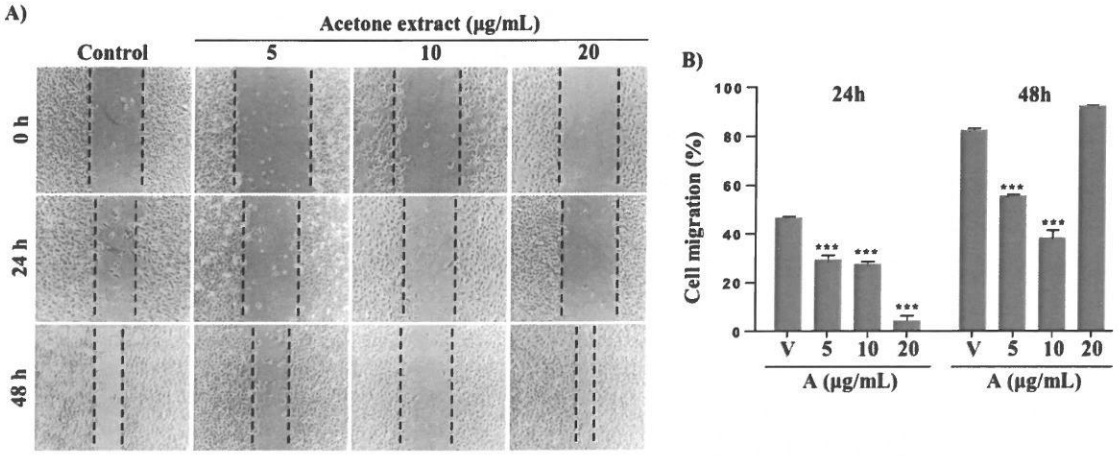


Figure 3. Effect of *Tagetes lucida* extracts on the migration capacity of SiHa cells. Wound closure test. Negative control (FBS 1%). Microscopic images of the effect of acetonic (A) and methanolic (C) extracts at 10X magnification. Graphic representation of the migration percentage of cells treated with the acetonic (B) and methanolic (D) extracts. Values were expressed as the mean ± Standard Deviation (SD) of three independent experiments. One-way ANOVA, Dunnett multiple comparison test; $^{**}p < 0.01$ and $^{***}p < 0.001$. *Differences with respect to the control treatment.

With regard to the HeLa cell line compared to the control (46% decrease in wound closure), the acetonic-extract treatment decreased the cell-migration capacity at 24 h, from 5-10 µg/mL (29 and 27% of wound closure, respectively) to 20 µg/mL (4% of wound closure), where a better effect was observed (42% less wound closure, compared to that of the control). The effect of 5 and 10 µg/mL (55 and 38% of wound closure, respectively) was maintained at 48 h; however, no significant decrease was observed at 20 µg/mL compared to the control (82% decrease in wound closure) (Figures 4A and 4B). Likewise, the methanolic-extract treatment decreased wound closure by 28% and 8% at 24 h compared to the control (46% decrease in wound closure) at concentrations of 10 and 20 µg/mL, respectively. This effect was maintained at 48 h, but with a better significant decrease at a concentration of 20 µg/mL (2% of wound closure), compared to the control (82% decrease in wound closure). At this time, a decrease in cell-migration capacity was observed at a concentration of 5 µg/mL (49% less wound closure) compared to that of the control (Figures 4C and 4D).



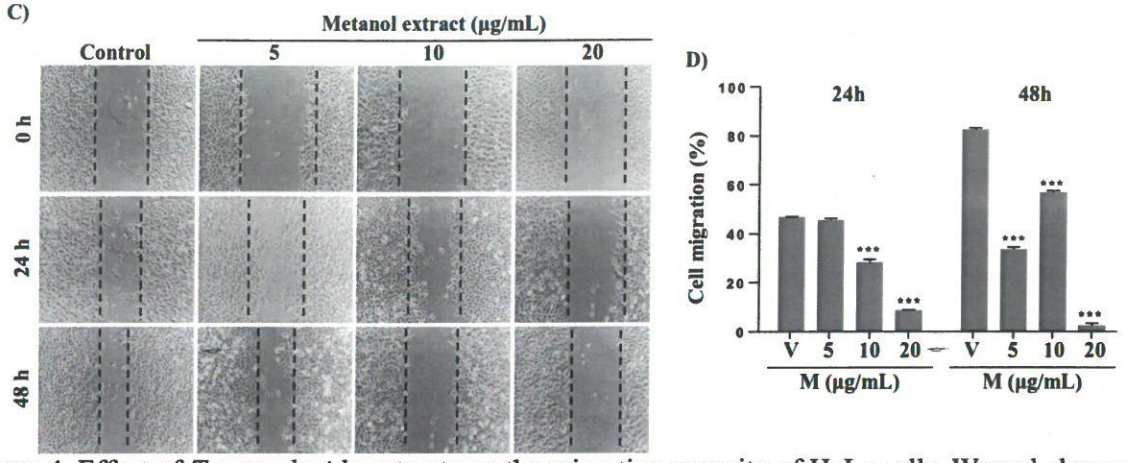


Figure 4. Effect of *Tagetes lucida* extracts on the migration capacity of HeLa cells. Wound closure test. Negative control (FBS 1%). Microscopic images of the effect of acetonic (A) and methanolic (C) extracts at 10X magnification. Graphic representation of the migration percentage of cells treated with acetonic (B) and methanolic (D) extracts. Values were expressed as the mean ± Standard Deviation (SD) of three independent experiments. One-way ANOVA, Dunnett multiple comparison test; ***p <0.001. *Differences with respect to the control treatment.

The results suggest that the metabolites present in the acetonic and methanolic extracts decrease the migration capacity of SiHa and HeLa cells, demonstrating a more evident effect on HeLa cells after 24 h and 48 h of treatment (<80% decrease in wound closure) even at low concentrations, while in SiHa cells, they decreased the cell- migration capacity only after 24 and 48 h of treatment (<16% decrease in wound closure) and only at the highest concentration tested. The possible antimigratory effect of the acetonic and methanolic extracts could be due to the presence of the coumarins herniarin and scoparone, the most abundant compounds identified in the acetonic and methanolic extracts, since it has been reported that treatment with these compounds in laryngeal-cancer cells (RK33) decreased cell migration in a dose-dependent manner.[59]

3. Conclusions

The phytochemical study revealed the presence of high quantities of compounds **15** and **17** in all of the bioactive extracts of *T. lucida* leaves, the principal coumarins suspected of inhibiting cell proliferation and migration preferably toward cervical cancer (HeLa cell line). This study contributes to the phytochemical and biological knowledge of *T. lucida* leaves and to the research of native species of Mexico. Thus, our findings provide a strong basis for further exploration of *T. lucida*, justifying its potential use as an alternative or complementary therapy against cervical cancer. Nevertheless, more studies are required to determine the components responsible for this biological activity, as well as the molecular and cellular mechanisms involved.

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