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PLANT SCIENCE

Cytotoxic activity in *Tagetes lucida* Cav.[◊]

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Abstract

Tagetes lucida (common name Santamaria) is a native herb widely used in the area with an important economic potential. It has several uses, such as, food, antiseptic, to control diseases infectious and spirituals. The objective of this work was to determine the agent of the cytotoxic activity from *T. lucida*. 17 straw extracts from different organs of *T. lucida* were obtained by cold maceration (methanol:chloroform), successive maceration (hexane, dichloromethane and methanol), infusion and extraction Soxhlet (ethyl acetate). The mostly of the plant extract were citotoxic to the crustacean *A. salina*. Hexanic extracts were notable for their cytotoxic effect. They were purified the 7-methoxycoumarin and 6,7-dimethoxycoumarin from leaf hexanic extract and both compounds caused citotoxicity in *A. salina*, with LC50 values of 28 and 238 µg/ml, respectively. We find that the cytotoxic effect observed in *T. lucida* is due to the coumarins.

Key words: Citotoxicity, coumarins, *T. lucida*

Introduction

It is common that any societies of any latitude have a rich tradition in medicinal plant utilization among its varied folk healing practices. A total of 3,000 species have been compiled in a database of medicinal plants employed by several Mexican ethnic groups (Lozoya and Martinez, 1994). Incredibly, of this plant group only approximately 1% of them have been studied in depth regarding their potential medicinal properties (Argueta, 1994). Into this medicinal plant core *Tagetes* spp, have been described as microbiocide and used for the treatment of gastrointestinal disorders.

Of the *Tagetes* genus there are several reports on its biocide activity. Some of them are related with their bactericide activity that including enterobactericide activity (Caceres et al., 1990, 1991). In the *Tagetes* genus, it has been research for new insecticides with specific characteristics, such as the terpenes specially limonene, caryophyllene and ocimene, which are directly responsible for this larvicide activity against *Aedes aegypti*. All of them are not harm for the environment and they have a similar or a better effect than the conventional insecticides (Rai and Acharya, 1999; Hitmi et al., 2000; Dharmagadda et al., 2004; Caballero Gallardo et al., 2011). Also, it was reported that peperitone and piperitenone obtained from *T. patula* had antifungal activity against *Botrytis cinerea* and *Penicillium digitatum*. However, in this antifugal effect it was reported a synergy of these and others components of the essential oil (Mares et al., 2004). Likewise, in the effect antifungal observed in the extracts from *T. filifolia* were involved the piperitone and piperitenone with other antifungal metabolites detected in this plant such as terpinolene, dihydrotagetone, *cis*-tagetone, limonene and *allo*-ocimene (Kishore et al., 1993; Cespedes et al., 2006).

T. lucida (synonymous: *T. florida* Sweet, *T. schiedeana* Less.) (Santamaria, one of Mexican common names) is a native herb widely used in the area and has an important economic potential. It has several traditional uses, such as, food, antiseptic, to control diseases infectious, emotional and spirituals. *T. lucida* is one of the plants most used by the Latin-American population for the treatment of gastrointestinal disorders (Giron et al., 1991; Damian Badillo et al., 2008a). Sometimes, *T. lucida* is not possible differentiate it of *T. filifolia* or *T. micrantha* or even of *Artemisia drucunculus*, therefore is necessary to confirm the specie on

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which is working (Linares and Bye, 1987). *T. lucida* is an aromatic herb distributed naturally from Mexico to Honduras, at altitude between the 1000 and 2000 m. It has sessile, glabrous, oblong-lanceolate and opposite leaves (5-10 cm long) with yellow terminal flowers. It is knows that *T. lucida* is an important source of antioxidant and antifungal compounds (Aquino et al., 2002; Damian Badillo et al., 2008b). In *T. lucida* thirty compounds were identified, the methyl chavicol (95-97%) was the major constituent and, from flower oil, two bithienyls were detected as minor constituents (Ciccio, 2005; Marote et al., 2010). Recently, it was reported that stems and leaves extracts ethanolic and aqueous from *T. lucida* were toxic to T47D and HeLa cell lines, also the leaves and root hexanic extracts were toxic to crustacean *A. salina* (Vega-Avila et al., 2009; Mejia Barajas et al., 2011). These authors only reported the cytotoxic activity of the plant extracts and not identified the biologically compound actives. In a continuation of our phytochemical work and of search of biological activities in *T. lucida*, the aim of this work was to determine the causal agent of its cytotoxic activity observed *in vitro.*

Materials and Methods

Plant extracts obtaining. Plants were collected at the 3.5 km of Tiripetio–Acuitzio road in Michoacan, Mexico at October of 2008 and 2010. A voucher specimen was deposited at the Herbarium of the Institute of Ecologia, A.C., Centro Regional del Bajio, Patzcuaro, Michoacan, Mexico. The plant organs (leaves, flowers, stems and root) were dried and each one was successively macerated for 3 days with solvents of ascendant polarity at temperature room, started with hexane, then ethyl acetate and finally with methanol. Also, it was done a cold maceration for 3 days of roots and flowers with mixture of methanol:chloroform (2:1 v:v). Soxhlet extracts were obtaining from leaves and stems with ethyl acetate at 79°C for 2 h. All these extracts were filtered and concentrated with evaporator rotary. Aqueous extracts were obtaining by infusion of each plant part and then they were concentrated by lyophilization.

Extraction, isolation and identification of pure compounds

The compounds isolation form *T. lucida* was done by chromatography column technic. 0.1 g leaves hexanic extract was chromatographied in a glass column (2 cm diameter x 8 cm large) was packed with 8 g of silica gel (70X230 Merk) as stacionary phase and as movil phase was a mixture of hexan and ethyl acetate in ascendant polarity.

Two chromatographic running were done; they were collected fractions of 10 mL each; the first were 134 and the second 87 fractions. Those fractions with some residue were analyzed by ^{1}HRMN. Compounds 1 and 2 were isolated from leaf hexanic extracts.

^{1}H and ^{13}C magnetic nuclear resonance spectra were obtained in a Varian Mercury Plus 400 spectrometer. The ^{1}H and ^{13}C NMR spectra of pure compounds were obtained at 400 MHz. Deuterated chloroform ($CDCl_3$) and tetramethylsilane (TMS) were the solvents of the plant metabolites and reference, respectively.

Compound 1. (7-methoxycoumarin). Yellow crystals, (hexane-ethylacetate); mp 110-112°C, (7% yield); ^{1}H-NMR (400MHz,$CDCl_3$), δ ppm: 7.63 (d, *J* = 9.4 Hz, 1H, H-4), 7.36 (d, *J* = 8.5 Hz, 1H, H-5), 6.81 (dd, *J* = 2.4, 8.5 Hz, 1H, H-6), 6.80 (d, *J* = 2.5 Hz, 1H, H-8), 6.23 (d, *J* = 9.4 Hz, 1H, H-3), 3.85 (s,3H, OMe). **^{13}C- NMR** (400MHz $CDCl_3$), δ ppm: 162.23 (C-2), 162.05 (C-8a), 160.64 (C-7), 155.32 (C-4a), 142.84 (C-4), 128.16 (C-5), 112.51 (C-3), 112.03 (C-6), 100.24 (C-8), 55.20 (OMe).

Compound 2. (6,7-dimethoxycoumarin). Pale yellow needles (hexane-ethylacetate); mp 140-142°C, (2.5% yield); ^{1}H-NMR (400MHz,$CDCl_3$), δ ppm: 7.63 (d, *J* = 10.0 Hz, 1H, H-4), 6.86 (s, 1H, H-5), 6.84 (s, 1H, H-8), 6.30 (d, *J* = 10.0, 1H, H-3), 3.95 and 3.90 (s, 3H, OMe). **^{13}C- NMR** (400MHz $CDCl_3$), δ ppm: 161.35 (C-2), 152.80 (C-7), 149.96 (C-6), 146.29 (C-8a), 143.31 (C-4), 113.02 (C-4a), 111.38 (C-3), 107.94 (C-5), 99.95 (C-8), 56.31 (OMe), 56.31 (OMe). NMR data in agreement with those reported (Huang et al., 2006).

Citotoxicity assay

Eggs of *A. salina* [Salt Creek Co™] and synthetic sea water Red Sea Salt™ [Red Sea Fish Pharm] were obtained in the local market. It was performed in accordance with the Meyer method (1982). Phototrophic nauplii (instar I and II) were collected from sea water with a light source (1000-4000 lux) at 25 ºC for 24 h. 10 – 15 nauplii (100 µL) were placed in wells of an ELISA plate and 3 µL of the extracts dissolved in dimethyl sulfoxyde (DMSO) were added (1, 10, 100 and 1000 µg). Berberine sulfate was used as a positive control and DMSO as negative control. The nauplii were incubated at room temperature for 24 h and the dead and live nauplii were counted. 100 µL of methanol were added to annihilate the surviving nauplii. Survival percentage was determined by the ratio of live nauplii between total nauplii (Geran et al., 1972).

Statistical analysis

In order to determinate the cytotoxic activity of the extracts plant and pure compounds, at least ten nauplii were tested. Concentration-response data were analyzed with the PROBIT program and a factorial analysis with $P < 0.01$ was used for determinate the statistical significance, and they are showed as mean ± standard error.

Results

It was obtained 17 extracts from *T. lucida* using several extraction methods. It was observed that the flower, leaf and root hexanic extracts, leaf and root dichloromethanic extracts and root metanolic extract, all of them had a lethal effect on the survival of *A. salina*, (Table 1). Ethyl acetate and aqueous extracts were innocuous for the crustacean. However, to determining the LC_{50} values on the crustacean, it was highlighted the hexanic extracts, see Table 2.

Table 1. *T. lucida* extracts tested against the survival of the crustacean *A. salina*.

Conc.µg/ mL	Survival (%)															
	[1]Hexanic				[1]Dichloromethanic				[1]Methanolic				[2]Methanol/ Chloroform		[3]Ethyl acetate	
	F	L	R	S	F	L	R	S	F	L	R	S	F	R	L	S
1000	3	0	0	0	0	0	0	0	44	92	0	0	3	0	0	3
100	3	10	0	89	30	5	0	92	100	95	52	19	38	73	57	97
10	63	59	76	100	82	80	97	100	100	100	85	70	100	100	93	100
1	93	88	100	100	92	100	100	98	100	100	92	90	100	100	92	100
0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

R= roots, S= stems, L = leaves, F = flowers. 1 = Maceration with successive extraction, 2 = Cold maceration, 3 = Soxhlet extraction

Table 2. Median lethal concentration for *A. salina* of the *T. lucida* extracts.

Plant organ	LC_{50} (µg/mL)				
	[1]Hexane	[1]Dichloromethane	[1]Methanol	[2]Methanol/Chloroform	[3]Ethyl acetate
Flowers	26	88	877	88	>1000
Leaves	16	52	>1000	>1000	223
Roots	40	64	96	386	>1000
Stems	509	672	46	>1000	590

1,2,3 = same annotation that in Table 1

By its lethality the leaves hexanic extract were selected for the purification of the major compounds with cytotoxic activity. They were isolated two major compounds; one was obtained of the chromatographic fraction 45 to 48 with polarity 80:20 and another was obtained of chromatographic fraction 19 to 22 with polarity 20:30, both with regard to the mixture of solvents hexane:ethyl acetate. They were identified by ^{1}H and ^{13}C NMR spectra as 7-methoxycoumarin and 6, 7-dimetoxycoumarin (Figure 1).

It was observed that the coumarins had a lethal effect dependent of the concentration on the crustacean *A. salina* (Figure 2). 7-methoxycoumarin presented a LC_{50} value of 28 µg/mL on *A. salina*, while the 6,7-dimethoxycoumarin had a LC_{50} value of 238 µg/mL.

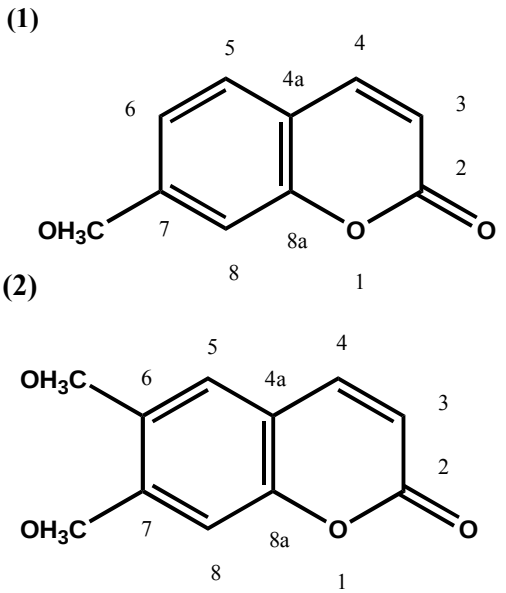

Figure 1. Coumarins from *T. lucida*. Major compounds isolated from *T. lucida* were 7-methoxycoumarin **(1)** and 6,7-dimethoxycoumarin **(2)**.

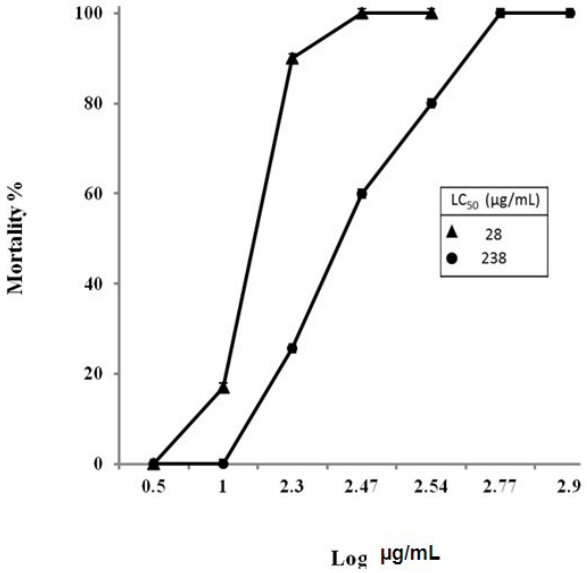

Figure 2. Response of *A. salina* to the coumarins purified from *T. lucida*. Concentration-response curves of 7-methoxy coumarin **(▲)** and 6,7-dimethoxy coumarin **(●).** Experiments were done for triplicate with 10 nauplii per replicate. Data are expressed as means of the percent of mortality ± s.e; $P < 0.01$ versus control.

Discussion

Forever, the plants are renewable resources and had been a valuable natural resource to human being, both for its feed, to cure its ills and several human uses. A lot native plants are used for medicinal properties, however, it is unknown their bioactive components. This fact is an opportunity area to search bioactive plant metabolites in the medicinal plants of ethnical pharmacopeias, specify bioactive metabolites with cytotoxic, antitumoral and anticancer activities.

T. lucida is one of the 28 species of *Tagetes* that habit in Mexico (Turner, 1996). In this country, it is used as medicinal, insecticide and ornamental plant. The economic importance of this plant is given for its potential as spice, nutraceutical and flowering herb. Today, this plant is used as a spice in the Southern states of USA, Costa Rica and Southern states of Mexico. It is cultivated commercially in Costa Rica as a spice herb and sold in the supermarket as a substitute of tarragon (*A. dracunculus*). In Guatemala, the extracts of this plant are sold as infusion, tincture and elixir. These products are used for stomach pains, gastritis, menstrual pains, to treat infections and dysmenorrhea (Caceres, 1996). This species has shown an important chemical variability in according to geographic and ecologic distribution. This fact is an advantage chemical and agronomic to select varieties plant and it is possible found novel secondary metabolites. Major secondary metabolites obtained of *T. lucida* collected from different sites belong to chemical families, such as; flavonoids, terpenes, phenolic compounds and coumarins (Abdala, 1999; Aquino et al., 2002; Ciccio, 2005; Cespedes et al., 2006).

Initially, it was observed that the plant extract obtaining by maceration successive process allowed detecting the cytotoxic activity form *T. lucida*. In this work we screened in the core plant extracts from *T. lucida* taken in count the Meyer criterion, which considered those plant extracts biologically actives that have an effect on the survival of *A. salina* at 1000 µg/mL o less (Meyer et al., 1982). With this criterion all plant extracts had a cytotoxic effect but the exception was the leaves methanolic extract. However, all of them the most lethal were the flower, leaves and root hexanic extracts, leaves and root dichloromethanic extracts and finally the stem methanolic extract. A criterion second of scrutiny was taken in count. This is accorded for the United States National Cancer Institute plant screening program, it advice that a plant extract is generally considered to have a cytotoxic effect at IC_{50} value, following incubation between 48 to 72 h, of 20 µg/mL or less (Geran et al., 1972). *T. lucida* leaf hexanic extract was selected to be fractioned for to search its compounds with cytotoxic activity. They were isolated from it, two coumarins; 7-methoxycoumarin and 6,7-dimethoxycoumarin. Coumarin such as 7-hydroxy coumarin and 7-methoxycoumarin had been reported in *T. lucida* and others *Tagetes* spp. The coumarins have different pharmacologic effects. It has been reported properties like antiinflammatory, antioxydants, antiallergics, hepatoprotectors, antivirals, cytotoxic, antitumoral, anticancer and sensibilizers to ultraviolet light (Mohler et al., 1992; Thornes et al., 1994; Maucher and Von, 1994; Kofinas et al., 1998; Kasahara et al., 2002; Kostova, 2005; Uchiyama et al., 2002).

Pure 7-methoxycoumarin and 6,7-dimethoxycoumarin from *T. lucida* had a cytotoxic effect that depended of the concentration**.** The cytotoxic effect was different between the pure coumarins, because that 28 µg/mL 7-methoxycoumarin cause the mortality of the 50% of the nauplii, while the 238 µg/mL 6,7-dimethoxycoumarin cause the same effect. This result is agreed with the report of Watanabe et al. (2005). They mentioned that the oxygen-containing functional group at the 7-position of the coumarin core was advantageous for the cytotoxic activity.

The US NCI plant screening program has established that, a pure compound is generally considered as candidate molecule to continue investigating it as potential anticancer agent if it has a cytotoxic effect with a LC_{50} value, following between 48 to 72 h of 4 µg/mL o less (DTP NCI/NIH). In accord to this criterion broadly accepted and based on our results, it is conclude that the 6,7-dimethoxycoumarin and 7-methoxycoumarin from *T. lucida* had of lower to moderate cytotoxic effect on *A. salina*, respectively.

However, the coumarins from *T. lucida* are susceptible of chemical modification for increase their cytotoxic activity on *A. salina* and tumor human cell lines, but innocuous to normal human cell lines. Currently, we are working on this aspect of the semi synthesis chemical of coumarins from *T. lucida* with these biological activity characteristics.

Nutritional aspect of *T. lucida* is a pharmacologically and nutriaceutiacally important if it is used as spice; because this plant could contributes to maintenance of health human being. For all this, *T. lucida* is proposal as an alternative plant culture new with advantages with regard to other regional and conventional crops. This work contributes to basic information for to promote it as crop and its use as herbal remedy, nutraceutical and food reinforcement in accord to official standard.

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