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Oviposition inhibitory activity of the Mexican sunflower *Tithonia diversifolia* (Asteraceae) polar extracts against the two-spotted spider mite *Tetranychus urticae* (Tetranychidae)

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ABSTRACT

The Mexican sunflower (*Tithonia diversifolia*, Asteraceae) is an invasive shrub of agricultural and non-agricultural lands in tropical countries. Besides extensive utilizations in the traditional medicine, mainly to treat malaria, the plant is believed to have a great potential in agriculture of developing countries as a green biomass to produce fertilizer, fodder and biopesticides. The plant is known to produce tagitinins, which are sesquiterpene lactones with a bitter taste endowed with toxicity against several insects such as mosquitoes, aphids, and beetles. Here, we evaluated the potential of *T. diversifolia* against the two-spotted spider mite *Tetranychus urticae* (Tetranychidae), which is one of the most economically important arthropod pests worldwide. The leaf methanolic extract and its ethyl acetate fraction were tested for acute and chronic toxicity and for oviposition inhibitory effects. The chemical composition of the extracts was analyzed by HPLC-MS^n and NMR. The main constituents were flavonoid derivatives, phenylpropanoids and sesquiterpene lactones. Among the latter, tagitinin C and tagitinin A were the major compounds. In acute toxicity assays, mortality did not exceed 50% even for the highest tested dose of 150 μg cm^{-3}. However, in chronic toxicity assays, on day 5 from application, the methanolic extract LD_{50} was 41.3 μg cm^{-3} while LD_{90} was 98.7 μg cm^{-3}. Furthermore, both *T. diversifolia* extracts inhibited oviposition in *T. urticae*. The ethyl acetate extract was the most active oviposition inhibitor, with an ED_{50} value of 44.3 μg cm^{-3} and an ED_{90} of 121.5 μg cm^{-3}. Overall, the good yield rate of the extract and the high crop yield highlighted good prospects of using the extract from this plant for the development of oviposition inhibitors against mites.

1. Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Tetranychidae), is considered as one of the most economically important arthropod pests. This mite has been reported infesting over 1200 species of plants, including cereals, legumes (with special reference to soybean), greenhouse crops, ornamental plants and fruit trees [21,49,53]. The high number of population outbreaks registered for this species are mainly due to its rapid population growth, short developmental time and long adult survival [56]. This features, coupled with male haploidy, which exposes recessive resistance genes to selection, result in a high rate of development of resistance

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to acaricides. Indeed, in more than 60 countries, it has been noted that the mites have developed resistance to more than 80 acaricides [24,43]. Therefore, the reduction of the employ of synthetic acaricides in Integrated Pest Management programs is crucial, and it is particularly recommended to alternate them with products showing different mechanism(s) of action [18,30,38]. In this framework, plant-borne products recently emerged as potential novel control tools to be used against arthropod pests, including mites [10,14,44–46].

Tithonia diversifolia (Hemsl.) A. Gray, also known as Mexican sunflower, is a shrub native to Central America and currently naturalized in tropical regions of Africa, Asia and South America. The plant is considered as an invasive species in cultivated and non-cultivated lands [6,13]. In tropical regions, given its abundance and availability, *T. diversifolia* is widely used in the traditional medicine to treat several diseases [26]. Among them, the most common therapeutic use is to cure malaria after oral administration of its decoction [27,39]. This traditional use has been validated through scientific studies [3,20,25,42] where the bioactive constituents were identified as the sesquiterpene lactones, named tagitinins [22,25]. Other traditional therapeutic uses of the plant concern the treatment of microbial infections and snakebites in Africa [34,39], diabetes in Asia [36], and skin diseases in America [26].

Given its capability to grow up very quickly, *T. diversifolia* has been experimented in agriculture to improve soil fertility [31] and as fodder for broiler chickens [19]. Recently, the *T. diversifolia* extracts attracted attention of scientists as useful tools in crop protection. This perspective of use was borne from the observation that some herbivores such as the caterpillar *Chlosyne lacinia* avoid the plant secretory structures where the bitter sesquiterpene lactones are produced [4]. These preliminary results were substantiated by other studies. For instance, the methanolic extract of leaves has been tested against the generalist phytophagous *Atta cephalotes*, showing insecticidal effects by both ingestion and contact [12]. Mikenda et al. [35] found that *T. diversifolia* extract is active against key pest species (e.g. aphids and beetles) on common bean plants (*Phaseoulus vulgaris*). Adedire and Akinneye [2] tested the leaf ethanolic extract of *T. diversifolia* on the cowpea seed beetle *Callosobruchus maculatus* that infests commonly stored legumes and found it highly effective on oviposition, adult emergence and mortality. Radhakrishnan and Prabhakaran [48] showed that the aqueous extract of the plant possesses moderate effect on the red spider mite *Oligonychus coffeae*, which is one of the major pests infesting tea plantations.

Nowadays, there is an urgent need to develop newer and safer control tools against arthropods of agricultural and medical importance [7–9,29,47]. In this scenario, continuing our investigations on the potential application of *T. diversifolia* on the industrial level [41], here we focused on the evaluation of the acaricidal and oviposition inhibitory activity of the *T. diversifolia* polar extracts against the two-spotted spider mite *T. urticae* Koch (Tetranychidae), relying to acute and chronic toxicity tests, as well as to experiments evaluating the oviposition inhibition potential. The chemical composition of the extracts was achieved by NMR and HPLC-MS measurements (i.e. HSQC-DEPT, HPLC-ESI-MS and HPLC-DAD) and correlated with the biological activity.

2. Material and methods

2.1. Plant material

Leaves of *T. diversifolia* were gathered in Dschang, western region of Cameroon (N 05°26'18″, E 10°04'07″, 1450 m a.s.l), by one of us (P.C. Biapa Nya) in January 2016 during the dry season. Botanical authentication was performed by taxonomist Mr. Nana and a voucher specimen has been stored at the National Herbarium of Yaoundé, Cameroon, under the code 10196/HNC. Before extraction, leaves were air-dried in the dark at room temperature (≈25 °C) for 3 days and stored in wrapping papers.

2.2. Chemicals and reagents

Methanol and ethyl acetate were purchased from Sigma-Aldrich (Milan, Italy). HPLC grade acetonitrile was obtained from J. T. Baker (Phillipsburg, USA). HPLC-grade formic acid was purchased from Dikma Tech. Inc. (Beijing, China). Water (H_2O) was purified by a Milli-Q system (Millipore, Billerica, MA, USA) in our laboratory.

2.3. Preparation of extracts

Fifty grams of leaves were reduced into powder and macerated in 500 ml of methanol for 24 h. After filtration using cotton, the extract was concentrated under reduced pressure at 30 °C with a rotary evaporator and freeze-dried yielding 2.64 g crude extract (yield 5.28%). A portion of the methanolic extract (0.82 g) was macerated in 150 ml of ethyl acetate for 24 h, yielding, after filtration and evaporation, 0.38 g (46.3%) of a pasty ethyl acetate phase. The obtained extracts were stored in glass vials protected from light at −20 °C before chemical characterization and acaricidal experiments.

2.4. Experimental apparatus and chromatographic conditions

Analysis of *T. diversifolia* secondary metabolites was performed on a LC–MS system. LC–MS equipment (Varian) comprised a binary chromatographic system (Varian LC-212) coupled with a mass spectrometer Varian 500-MS (ion trap). An ion source electrospray (ESI) (Varian) was used. The MS parameters were as follows: needle potential −5.0 kV, shield 600 V, spray chamber temperature 50 °C, drying gas pressure 10 psi, drying gas temperature 350 °C, capillary voltage 80 V, RF loading 100, MS range 150–2000 Da. MS^n spectra were recorded during the chromatography run by using of the turbo-dds (tdds) utility giving the MS fragmentation pathways of ionic species whose intensity was higher than a threshold level. HPLC-DAD analysis was carried out by an Agilent 1100 series liquid chromatograph equipped with autosampler (Agilent 1100 series) and Diode Array Detector (DAD) (Agilent 1100 series). An Eclipse XDB-C8 5 μm 4.6 × 150 mm (Agilent) column was used as stationary phase. Mobile phases were: aqueous formic acid (0.1%) (A) and acetonitrile (B). The gradient elution was as follows: 0–30 min, linear gradient from 10% to 100% of B; 30–35 min, isocratic conditions at 100% of B; 35–36 min, linear gradient from 100% to 10% of B; 36–40 min, isocratic conditions at 10% of B. Flow rate: 1 ml/min. Calibration curves were obtained by standard solutions of rutin for flavonoid derivatives (at 350 nm) and chlorogenic acid for caffeoylquinic acid derivatives (at 330 nm). The concentration ranges were 11.7–117 μg/ml and 13.2–132 μg/ml for chlorogenic acid and rutin, respectively. The limits of detection (LOD) and quantification (LOQ) were 1.5 and 4.0 μg/ml, and 0.5 and 1.5 μg/ml for chlorogenic acid and rutin, respectively.

2.5. Qualitative and quantitative NMR analysis

NMR analysis was obtained on a Bruker AVANCE III spectrometer operating at 400.13 MHz for ^1H NMR and 100 MHz for ^{13}C. 2D spectra, HSQC-DEPT, HMBC, COSY and TOCSY were used for compound identification in mixture. Samples were dissolved in deuterated methanol and used for analysis. For quantitative purposes, previously published conditions were used [16]. Briefly, extract was dissolved in deuterated chloroform at a final

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concentration of 30 mg/ml in a 5-mm NMR tube, and the solvent signal ^{1}H at 7.26 ppm was used for pulse calibration. ^{1}H spectra were run with a standard pulse sequence with acquisition time 4.90 s, 16 scans, 10 s D1. The same spectral calibration as that used for ^{1}H NMR experiments were then used for 2D measurements. Signals ascribable to tagitinin derivatives were deduced based on literature. The quantitative ^{1}H NMR measurements were performed using caffeine as internal standard, because its ^{1}H NMR signals (methoxy groups and aromatic proton) are clearly separated from those of extract components. A stock solution of caffeine in deuterated chloroform was prepared and 500 μl of this solution (3.1×10^{-5} mol) were added to an NMR tube with 30 mg of the extract. Each measurement was performed in triplicate [17].

2.6. Mites

Two-spotted spider mites, *Tetranychus urticae* Koch, were obtained from the cultures maintained at the Crop Research Institute (Czech Republic). The two-spotted spider mite used in the experiments were reared on bean plants (*Phaseolus vulgaris* L. var. Carmen) in a growth chamber (22–25 °C; 16 h photoperiod) [43].

2.7. Acute and chronic toxicity

The toxicity, measured as mortality after 24 h (acute toxicity) or 120 h (chronic toxicity) of exposure, was determined by tarsal application to adults of *T. urticae* [43]. The experiment was done in blackberry leaf discs (*Rubus fruticosus* L.) sized 1 cm^{-2}. The *T. diversifolia* methanolic and ethyl acetate extracts were dissolved in acetone. Subsequently, an automatic pipette was used to uniformly apply to the cut pieces always 10 μl of acetone containing a defined dissolved amount of the extracts in order to obtain a concentration series equivalent to the doses of 150, 100, 80, 50, 25 and 12.5 μg cm^{-2}. After application, the discs were placed in Petri dishes (5 cm in diameter) with an agar layer 0.3 cm thick on the bottom (to maintain the freshness of the discs and standard ambient humidity). Only acetone was applied to the control discs.

After evaporation of the solvent (approximately 10 min from application), a fine brush was used to transfer 10 females of *T. urticae* (2–3 days old) on each of the treated sides of the leaf discs. The Petri discs were placed in a growth chamber (L16:D8, 25 °C). The cut leaf discs were checked after 24 h from application, determining the number of dead adults. Death was recorded when the larvae did not respond to prodding with forceps. Each experiment was repeated 5 times.

2.8. Oviposition inhibitory potential

In order to determine the effect of the *T. diversifolia* extracts on the oviposition capacity of *T. urticae*, an experiment was carried out using a methodological procedure identical to that described above for acute and chronic toxicity, with some modifications. Five females (3–4 days old) were transferred using a fine brush onto each of the cut bean leaf discs sized 1 cm^{-2}. The leaf discs were obtained from those bean leaves that had been treated identically as described for acute and chronic toxicity and after drying of the spray, using a cork borer. The cut discs with the females were placed in Petri dishes with an agar bottom. The females were removed after 48 h and the laid eggs were counted. Subsequently, the number of eggs was determined for individual concentrations, and the lethal concentration causing oviposition inhibition by 50% or 90% compared with the control was estimated using Probit analysis. The Petri dishes were placed in a growth chamber (L16:D8, 25 °C). The experiment was repeated five times.

2.9. Data analysis

Experimental tests demonstrated that more than 20% of the controlled mortality was discharged and repeated. When the controlled mortality reached 1–20%, the observed mortality was corrected by Abbott's formula [1]. Probit analysis of dose-mortality data was conducted to estimate the LD_{50} and LD_{90} values and associated 95% confidence limits for each treatment [23]. Probit analysis of dose-oviposition inhibition data was conducted to estimate the ED_{50} and ED_{90} values and associated 95% confidence limits for each treatment.

3. Results and discussion

3.1. Characterization of the extracts by NMR and HPLC-MS

The *T. diversifolia* methanolic extract was analyzed using different techniques. As first, HPLC-MS^n analysis was performed both in negative and positive ion mode using ESI source. Exemplificative chromatograms are reported in Fig. 1A and B. Fragmentation pathways of phenolics and of the two most abundant sesquiterpenes were obtained allowing the tentative identification of 13 constituents that are reported in Table 1. Comparison with available reference compounds allowed the identification of several constituents. From this first analysis, the phytocomplex of *T. diversifolia* appears to be composed of several flavonoid derivatives, phenylpropanoids and sesquiterpene lactones. The amounts of flavonoids and phenylpropanoids were measured by HPLC-DAD using rutin and chlorogenic acid as reference compounds. An exemplificative chromatogram showing the main peaks and their UV spectra is reported in Fig. 2, whereas the amount of the main phenolic constituents is reported in Table 2.

Further informations were obtained by ^{1}H NMR and 2D experiments namely HSQC-DEPT, HMBC and COSY. The ^{1}H NMR spectrum showed signals ascribable to sesquiterpene sp^2 protons namely, the doublets at δ 6.92 (d, $J = 16.5$), 6.36 (d, $J = 1.3$), and the signals in overlapped zone at δ 6.27, 5.86 and 5.82. The directly linked carbons observed in the HSQC-DEPT were 161.3 (CH), 124.0 (CH_2), 129.7 (CH), 137.4 (CH) and 124.0 (CH_2). A representative portion of HSQC-DEPT spectrum is reported in Fig. 3 showing the considered diagnostic signals. COSY and HMBC correlations allowed to assigned those signals to positions 9, 13, 10 and 3 of tagitinin C. Linkage with the isobutyryl moiety is observed from the signal at δ 5.38 (δc 73.8) assigned to position 6. Other signals in the mixture that can be assigned to tagitinin A, are the protons at δ 5.56 and 6.29 (H-13), the methyne supporting the ester linkage at δ 5.62 (δc 70.22) the methyl groups 14 and 15 at δ 1.45 and 1.10, respectively. Fig. 3 represents an enlargement of HSQC spectrum with indicated assignments related to diagnostic sp^2 proton signals of the two sesquiterpenes.

From a quantitative point of view, due to the non-availability of tagitinins as reference compounds, the quantification of tagitinin C and A was performed by ^{1}H NMR using caffeine as internal standard using a previously published method [16]. The amount of tagitinin A and C and phenolic compounds in the methanolic and ethyl acetate extracts is reported in Table 2. Due to the higher lipophilicity of the ethyl acetate extract compared with the methanolic one, the most hydrophilic compounds (i.e. quinic acid derivatives and glycosylated flavonoids) were not detected. On the other hand, lipophilic flavonoids (hispidulin and isorhamnetin) were found as well as sesquiterpene lactones.

3.2. Toxicity and inhibition of oviposition on Tetranychus urticae

Although both tested extracts caused mortality in *T. urticae*

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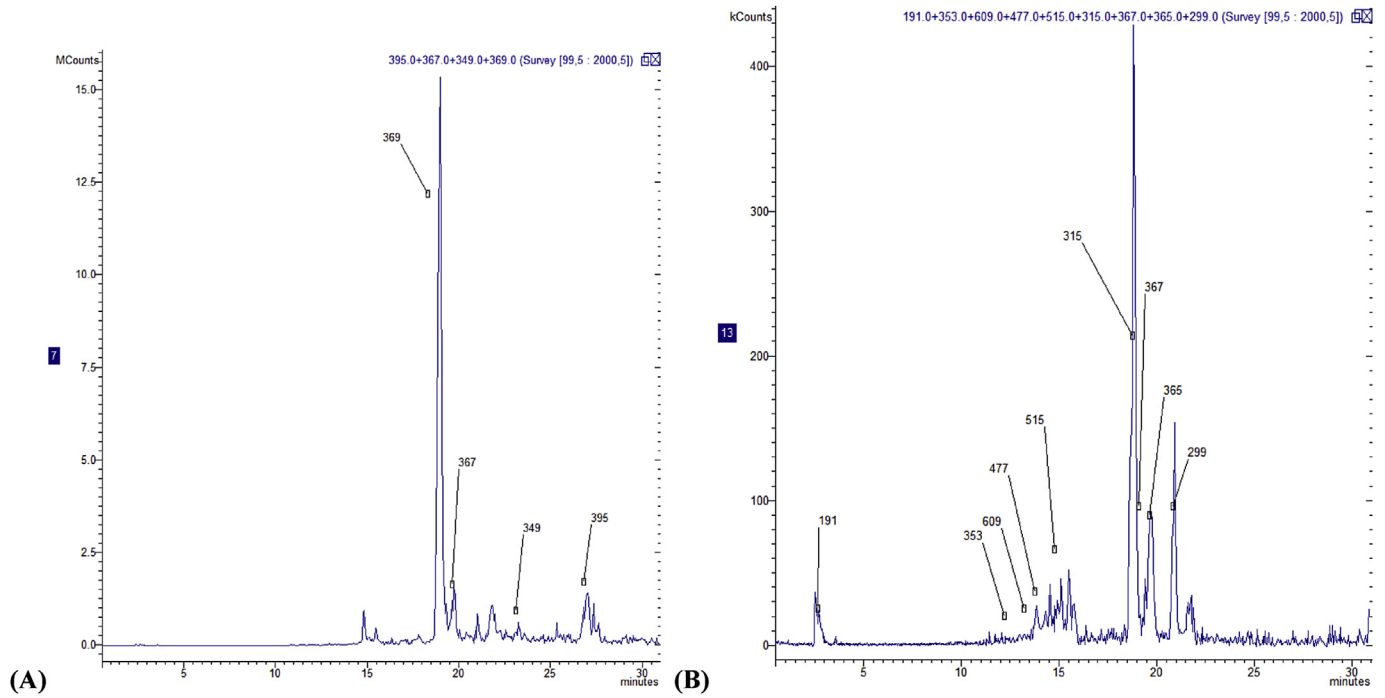

Fig. 1. HPLC-ESI-MS of the *Tithonia diversifolia* methanolic extract, in positive **(a)** and negative **(b)** ion mode. Identified compounds are indicated with their *m/z* values (see Table 1 for additional details).

Table 1
Constituents identified in the *Tithonia diversifolia* methanolic extract by HPLC-MS.

Retention time	Compound	Polarity	[M−H] or [M+H]	Fragments
2.53	Quinic acid[a]	Negative	191	85
12.0	Chlorogenic acid[a]	Negative	353	191−179
13.9	Rutin[a]	Negative	609	301-271-255-179-151
14.5	Quercetin-glucuronide	Negative	477	301-179-151
14.6	1,5-Dicaffeoylquinic acid[a]	Negative	515	353-191-173-127
14.8	3,4-Dicaffeoylquinic acid	Negative	515	353-191-179-173
15.7	3,5-Dicaffeoylquinic acid[a]	Negative	515	353-191-173-127-111-85
18.8	Isorhamnetin	Negative	315	300-272-228
19.5	Tagitinin A	Negative	367	279-261-235
		Positive	369	281-263-245
19.7	Tirotundin 3-*O*-methylether	Positive	367	279-261-243-233-215
		Negative	365	277-233-215-191-176
20.8	Hispidulin	Negative	299	284
24.6	Tagitinin C	Positive	349	261-243-215-173
27.4	3-*O*-Methyl titonin	Positive	395	293-275-261-199-181

[a] Confirmed by injection of reference compound.

adults, significant differences were found between them. The methanolic and ethyl acetate extracts showed only low acute toxicity (Table 3), and observed mortality did not exceed 50% even for the highest tested dose of 150 μg cm^{-3}; lethal doses thus could not be objectively estimated. However, mortality of *T. urticae* adults increased in time and on day 5 from application and significant differences were found in biological efficacy of individual extracts, which can be considered as a manifestation of chronic toxicity. The methanolic extract was the most toxic against mites, with a LD_{50} value of 41.3 μg cm^{-3} and a LD_{90} of 98.7 μg cm^{-3} (Table 3). In addition, both *T. diversifolia* extracts were able to inhibit oviposition in *T. urticae*. At variance with chronic toxicity assays, here the ethyl acetate extract was the most active oviposition inhibitor against *T. urticae*, with an ED_{50} value of 44.3 μg cm^{-3} and an ED_{90} of 121.5 μg cm^{-3} (Table 3).

Mortality that starts to be observed only several days from application and is usually associated with a repellent and anti-feedant effect or inhibition of oviposition of the insects on the treated plants is usually a manifestation of efficacy of substances contained in the extracts. These substances play an important role in natural defensive capacity of plants against pathogens and pests [51]. Substances synthesized by plants in the scope of antixenosis also include compounds that were contained in our tested *T. diversifolia* extracts. Generally, secondary metabolites showing chronic toxicity due to their ability to inhibit food intake or growth of juvenile stages or causing various disorders of the insects' behavior usually exert an effect in higher doses or concentrations [18,45], which is in accordance with our results.

Comparing our estimated lethal doses of *T. diversifolia* extracts to other plant extracts, we see that our samples showed comparable or possibly a little worse efficacy. For example, Chen and Dai [15] tested the *Cinnamomum camphora* (L.) J.Presl extract against

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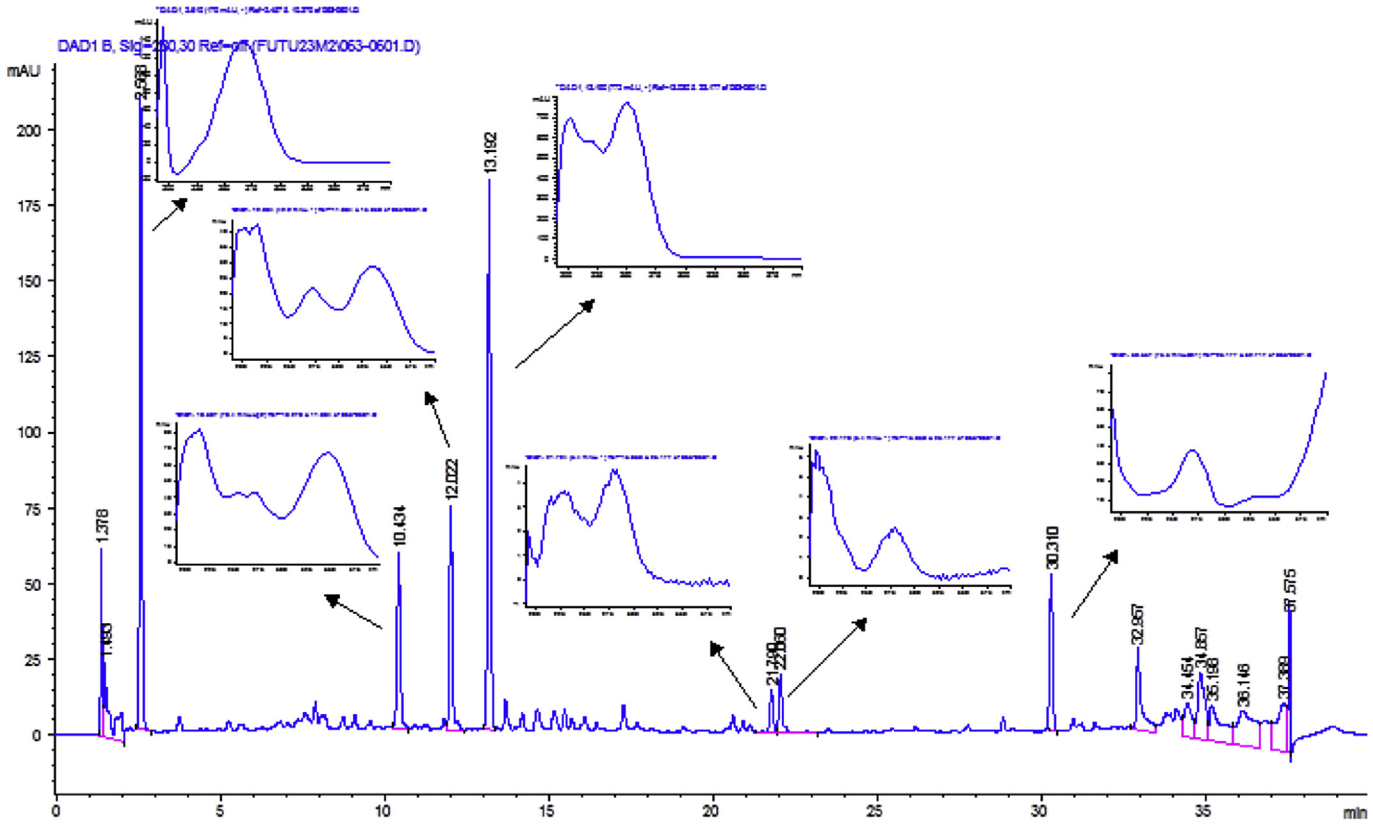

Fig. 2. HPLC-DAD of the *Tithonia diversifolia* methanolic extract.

Table 2
HPLC-DAD quantitative determination of the main constituents of the *Tithonia diversifolia* extracts.

Compound	Methanolic extract metabolite contents mg g^{-1}	Ethyl acetate extract metabolite contents mg g^{-1}
Phenolic acids		
Chlorogenic acid	0.05 ± 0.01	n.d.[a]
1,5-Dicaffeoylquinic acid	0.33 ± 0.01	n.d.
3,4-Dicaffeoylquinic acid	0.40 ± 0.01	n.d.
3,5-Dicaffeoylquinic acid	1.42 ± 0.01	n.d.
Chlorogenic acid derivatives content	2.2	n.d.
Flavonoids		
Rutin	0.52 ± 0.01	n.d.
Quercetin glucuronide	0.08 ± 0.01	n.d.
Isorhamnetin	7.92 ± 0.01	3.96 ± 0.01
Hispidulin	3.08 ± 0.01	3.11 ± 0.01
Flavonoid total content	11.6	11.6
Tagitinin A	0.83 ± 0.01	1.16 ± 0.01
Tagitinin C	1.10 ± 0.01	1.69 ± 0.01

[a] n.d. = not detected.

Tetranychus cinnabarinus. The authors identified five compounds from the *C. camphora* extract, all endowed with acaricidal activity. At 7 days after treatment in a potted seedling experiment, the LC_{50} values of 2,4-di-*tert*-butylphenol and ethyl oleate were found to be 1850 and 2481 mg kg^{-1}, respectively. The acaricidal and ovicidal efficacy of the methanolic extract obtained from *Ammi visnaga* seeds against *T. urticae* was also tested. By analyzing the extract, two major substances belonging to the group of furanochromenes were determined as khellin and visnagin. Adult mortality was studied both for the extract and for visnagin and khellin. The efficacy increased with time; LD_{50} levels after 72 h from application were estimated as 17, 10 and 98 μg cm^{-2}, for the extract, visnagin and khellin, respectively. The extract as well as both furanochromenes inhibited the development of eggs and caused their mortality, while LD_{50} was estimated as 13.3, 0.5 and 1.8 μg cm^{-2}, for the extract, visnagin and khellin, respectively [43].

However, is important to note that, besides the low toxicity of the tested extracts, their oviposition inhibition potential was very good, with special reference to the ethyl acetate extract (ED_{50} = 44.3 μg cm^{-3}). Chemical analysis of this extract highlighted a different qualitative profile compared with the methanolic one, with absence of glycosylated flavonoids and caffeoylquinic acid derivatives, and enrichment in tagitinin C and A. Therefore, the latter compounds appear to play the major role as repellent and antifeedant agents against the two-spotted spider mite. Therefore, the relatively good yield rate of the extract (about 5%) and the high crop yield (5 t of dry matter ha^{-1}) [31] highlighted promising prospects of using low doses of the *T. diversifolia* polar extracts for

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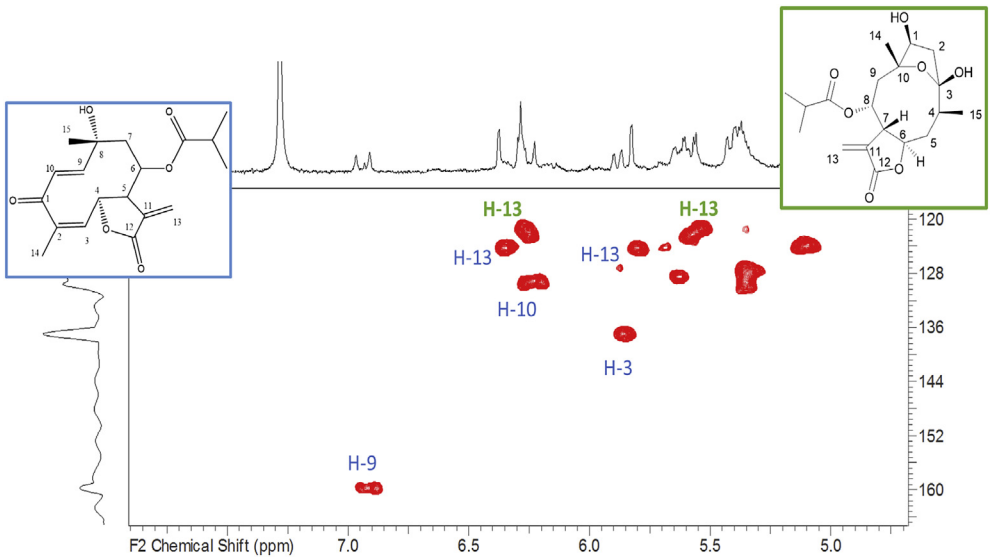

Fig. 3. HSQC-DEPT (MeOD) of the *Tithonia diversifolia* methanolic extract. The green letters indicate signals due to tagitinin A while blue letters are referred to tagitinin C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Acute toxicity, chronic toxicity and inhibition of oviposition evoked by the *Tithonia diversifolia* methanolic and ethyl acetate extracts on the two-spotted spider mite *Tetranychus urticae* Koch.

Solvent	Dose (μg cm^{-2})	Acute toxicity		Chronic toxicity				Inhibition oviposition			
		Mortality[a] (±SE)	LD_{50}[b]	Mortality[a] (±SE)	LD_{50}[b] (CI_{95})	LD_{90}[b] (CI_{95})	Chi	Inhibition[a] (±SE)	ED_{50}[c] (CI_{95})	ED_{90}[c] (CI_{95})	Chi
Methanol	150	38.6 ± 3.8	>150	100.0 ± 0.0	41.3 (39.8–45.6)	98.7 (93.3–101.5)	0.895	62.8 ± 3.3	108.3 (98.6–121.7)	>150	2.325
	100	18.9 ± 2.5		95.7 ± 5.2				48.2 ± 3.2			
	80	5.8 ± 1.6		75.6 ± 3.9				38.7 ± 5.2			
	50	2.7 ± 0.9		58.3 ± 2.8				21.5 ± 5.3			
	25	2.5 ± 0.6		39.8 ± 5.6				9.8 ± 2.2			
Ethyl acetate	150	27.6 ± 2.8	>150	80.7 ± 6.8	85.3 (81.4–89.8)	153.5 (141.1–178.3)	0.965	100.0 ± 0.0	44.3 (41.8–51.9)	121.5 (111.6–138.9)	3.253
	100	21.5 ± 3.5		68.3 ± 8.2				87.3 ± 5.2			
	80	10.3 ± 5.2		46.6 ± 6.3				66.3 ± 4.7			
	50	0.0 ± 0.0		32.5 ± 6.2				46.2 ± 5.5			
	25	0.0 ± 0.0		16.5 ± 2.9				26.9 ± 3.3			

[a] Average mortality (corrected by Abbott's formula) or inhibition oviposition (all in % ± the Standard Error).
[b] Lethal doses LD_{50} (LD_{90}) in μg cm^{-2} causing 50% (90%) mortality of *T. urticae* adults 24 h (for acute toxicity) and 5 days (for chronic toxicity) after application.
[c] Effective doses ED_{50} (ED_{90}) in μg cm^{-2} causing 50% (90%) inhibition oviposition of *T. urticae* in compared by control. CI_{95} = 95% confidence intervals, extract activity is considered significantly different when the 95% CI fail to overlap. Chi = square value, not significant (P > 0.05).

the development of oviposition inhibitors effective in mite control programs.

To our mind, the acaricidal and oviposition inhibitory effects of *T. diversifolia* extracts on *T. urticae* may be mainly due to the sesquiterpene lactones tagitinins, which are reported as biologically active molecules characterizing the genus *Tithonia* [13] and, more generally, the Asteraceae family. Notably, this group of secondary metabolites plays an important role in the plant defense. Their bitter taste makes the plant less palatable to herbivores. This might explain the antixenosis observed on *T. urticae.* Indeed, sesquiterpene lactones showed antifeedant activities on several arthropod pests [52]. Actually, tagitinin C, the parent molecule of this class of secondary metabolites occurring in the genus *Tithonia*, exhibited pronounced antifeedant activity on the caterpillar *Chlosyne lacinia* (Lepidoptera). Interestingly, when tagitinin C decreases in the plant, infestation by caterpillar increases [4]. According to Schmidt [50], we hypothesized that the biological activity of the sesquiterpene lactones may be mediated by general chemical mechanisms like alkylation of biological macromolecules (e.g. Michael type additions) or by receptor-meditated interactions. Thus, further studies are scheduled on tagitinins in order to clarify their mechanism of action on the two-spotted spider mite *T. urticae* as well as on non-target organisms.

Regarding the possible role played by the other constituents detected in the extracts, little is known on their specific effect against *T. urticae*. In general, phenolic acids and flavonoids are believed to be responsible for reduction in the arthropod's growth and reproduction [5,54]. As concerns the oviposition inhibitor activity, it has been found a negative correlation between the phenolic content in some cultivated plants and mite oviposition [33].

Caffeoylquinic acids that are marker compounds in the Asteraceae family and are found in the *T. diversifolia* methanolic extract, are capable of reducing the growth of the cabbage looper, *Trichoplusia ni*, when incorporated into artificial diet [11]. One of the most common representative of this group, i.e. chlorogenic acid, was reported to be involved in resistance of horticultural crops towards predators. In damaged plant tissue, chlorogenic acid is converted into orthoquinones that are able to alkylate $-NH_2$ and $-SH$ groups of proteins and amino acids leading to alteration of solubility and

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digestibility by arthropods [32]. Chlorogenic acid and rutin were reported as the most abundant constituents in strawberry and tomato plants resistant against infestation and oviposition by *T. urticae* [28,55]. Hispidulin derivatives were found as abundant components in plant extracts (e.g. *Cnidoscolus aconitifolius* (Mill.) I.M. Johnst) highly toxic and with oviposition inhibitor activity against the two-spotted spider mite [40]. Isorhamnetin and quercetin are flavonoids involved in the defense mechanisms of conifers and their synthesis is considered as a specific defense response of many plants against insect herbivores [37].

Overall, our results highlighted the promising potential of *T. diversifolia* as an effective and cheap bioresource for the development of oviposition inhibitors against mites. On the other hand, further studies focusing on the mechanisms of action of plant secondary metabolites, with special reference to tagitinin C and tagitinin A, against the mite pest *T. urticae* are urgently needed, as well as the validation of the efficacy of *T. diversifolia* extracts and related compounds in the field.

Conflict of interest

The authors declare no competing interests.

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