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Chemical screening, insecticidal and reprotoxic activities of *Tecoma stans* ethanolic leaf extract against the vector mosquito *Culex pipiens*

Nour-El Houda Hafsi[1,2] | Kaouther Hamaidia[2,3] | Noureddine Soltani[3]

[1]Laboratory of Sciences and Technical Water and Environment, Mohamed Cherif Messaadia University, Souk-Ahras, Algeria

[2]Department of Biology, Faculty of Nature and Life Sciences, Mohamed Cherif Messaadia University, Souk-Ahras, Algeria

[3]Laboratory of Applied Animal Biology, Faculty of Sciences, Department of Biology, Badji Mokhtar University, Annaba, Algeria

Correspondence
Kaouther Hamaidia, Department of Biology, Faculty of Nature and Life Sciences, Mohamed Cherif Messaadia University, Souk-Ahras, Algeria.
Email: k.hemaidia@univ-soukahras.dz

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Abstract

To select potential plant-based insecticides, *Tecoma stans* (Bignoniaceae) leaf extract was screened for its larvicidal and delayed effects against a medically important mosquito species *Culex pipiens* L. (Diptera: Culicidae). First, gas chromatography–mass spectrometry (GC–MS) was conducted on *T. stans* extract, collected in ethanol for its chemical characterization and detection of active constituents. Second, insecticidal bioassays were made with several concentrations on earlier fourth instar larvae (L4) of *Cx. pipiens* for 24 h as recommended by WHO, in order to determine the lethality parameters of the tested extract. For that, two concentrations (LC_{30} and LC_{50}) were applied on L4 for 24 h, and emerged adults were observed for their reproductive performance success like fecundity, percentage of hatching (fertility), body and gonads' volume. The biochemical composition of whole bodies of adults was investigated. Also, the specific activities of acetylcholinesterase (AChE) and glutathion S-transferase (GST), biomarkers of neurotoxicity and detoxification, respectively, were also determined in L4 at different time intervals. Qualitative and quantitative phytochemical analysis (GC–MS) of the extract unveiled nine phytoconstituents with 2-[4-cyclohexylbutanoylamino]-3-chloro-1,4-naphthoquinone (36.24%) and 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester (22.65%) as major components. The early L4 of *Cx. pipiens* were exposed to LC_{30} and LC_{50}, and after 24 h, surviving individuals have been further reared until adult emergence. All over, *T. stans* leaves ethanolic extract impairs adults reproductive traits; it adversely affected gonad's size, fecundity and fertility, which negatively affected the survival of their offspring. Lipids contents were found to increase in both male and female adults. *T. stans* ethanolic extract increased GST activity and slightly inhibited AChE activity in L4. In conclusion, the ethanolic extract of *T. stans* could be a potential candidate used as a sustainable botanical insecticide for controlling mosquito.

KEYWORDS
Culex pipiens, fecundity, fertility, GC–MS, reprotoxicity, *Tecoma stans*

INTRODUCTION

Despite decades of effective *managing* efforts using chemical insecticides as the best way to reduce adult mosquito's populations, vector-borne disease transmission persists (Ferraguti et al., 2021). To detect their occurrence and spatiotemporal distribution, updated inventories of mosquito larvae (Diptera: Culicidae) in Northeastern Algeria were carried out to perform an effective control program (Benmalek et al., 2018; Bouabida et al., 2012; Hafsi et al., 2021; Hamaidia et al., 2016; Hamaidia & Berchi, 2018; Hamaidia & Soltani, 2021). These surveys would be needed to assess the *risk* of invasive vector species *emergence* (Benallal et al., 2019; Hamaidia & Soltani, 2021; Lafri et al., 2014).

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Resistance to conventional insecticides is the most important pressure factor on mosquito control even when these compounds are used optimally (Mouhamadou et al., 2019; Richards et al., 2020). The scale of this situation emphasizes the insufficiency of the current approaches, and new further inroads must be involved to switch from chemical-based insecticides to non-conventional control methods. To achieve the expected objectives, various intervention policies and strategies are based on integrated measures such as insect growth disruptors (Hamaidia et al., 2018; Hamaidia & Soltani, 2014; Hamaidia & Soltani, 2016; Hamaidia & Soltani, 2019, 2020) or bio-pesticides (Bouguerra et al., 2018; Bouzidi et al., 2020; Draouet et al., 2020; Dris et al., 2017).

As insects could tolerate the toxicity by using several detoxification mechanisms, glutathione S-transferase (GST) is one of the main enzymes of the phase II of detoxification (Tang et al., 2020). It is usually used in the evaluation of this toxicity due to its central role in the detoxification of both endogenous and xenobiotic compounds (Turhan et al., 2020), and play key roles in the antioxidant defence system (Abdalrahman et al., 2018). Several studies show that GST could be used as a biomarker in ecological risk assessment of pesticide contaminated environment (Costa et al., 2020; Sachi et al., 2021).

Commonly called yellow-bells, *Tecoma stans* (L.) Kunth (Bignoniaceae), is a popular ornamental plant and a traditional folk medicine involving various chemical classes such as polyphenols and flavonoids (Saleh et al., 2019). *It* is a promising species due to its anti-inflammatory, analgesic, anti-hyperglycemic (Saleh et al., 2019), and anti-cancer (Abisha & Raj, 2020) activities, besides its antioxidant (Abisha & Raj, 2020; Larbie et al., 2019), antibacterial (Bakr et al., 2019) and anti-fungal potentials (Abisha & Raj, 2020; Bakr et al., 2019). To assess *T. stans* extracts as safer and effective mosquitocides, several studies were undertaken on its repellent potential (Gupta & Gupta, 2020). Recently, Reis et al. (2020) demonstrated its potential against an infectious mosquito-borne flavivirus (Zika). Previously, some *T. stans* extracts were tested on mosquitoes, such as the effectiveness of petroleum ether extract on *Ae. aegypti* and *Cx. quinquefasciatus* (Hari & Mathew, 2018). *T. stans* is one of the inexpensive and easiest plants to grow (Anand & Basavaraju, 2021). Hence, the present study investigated the chemical composition and larvicidal activity of the ethanolic extract of *T. stans* leaves against the common house mosquito. To give additional information on the mode of action of this extract, we investigated the stress following exposure by measuring glutathion S-transferase (GST) and acetylcholinesterase (AChE) activities, biomarkers of detoxification and neurotoxicity, respectively. In addition, we also examined their delayed effects on adults' reproductive potential.

MATERIALS AND METHODS

Mosquito rearing

Culex pipiens L. (Diptera: Culicidae) larvae were obtained from a stock colony of the Laboratory of Applied Animal Biology and kept as described previously by Hamaidia et al. (2018). Briefly, larvae were reared under laboratory conditions of temperature (25–27°C), light cycle (10 h darkness and 14 h light), and humidity (75%–85%) in containers (150 ml of tap dechlorinated water). They were fed with 0.04 g of biscuit-dried yeast mixture (3/1 by weight) (Rehimi & Soltani, 1999). Rearing water was renewed every 4 days. Once they appear, pupae were collected and placed in a cage (30 cm × 30 cm × 30 cm) where they emerged. Adults are fed only with 10% sugar solution (Tenywa et al., 2017).

Preparation of ethanolic plant extract

The leaves were collected from Souk-Ahras (36°17′15″N 7°57′15″E) on March–May 2019. Then, they were shade-dried at room temperature and then ground to powder using an electric grinder. Extract was obtained by cold maceration of 50 g of leaf powder in 250 ml of ethanol (Llorera et al., 2014) as previously described (Draouet et al., 2020). Ethanol proved to be a better choice as an extraction solvent (Borges et al., 2020). It was used because it is, like water, versatile and relatively low-cost solvent, and also to permit comparison with previous reports made with the same solvent (Draouet et al., 2020; Silva et al., 2018; Tavares et al., 2021). After 24 h, the solution was filtered (Whatman paper No. 1), and the filtrate was stored at 4°C until future use as stock solution.

Gas chromatography–mass spectrometry characterization of *T. stans* extract

Chemical composition of ethanolic extract of *T. stans* was determined with gas chromatography–mass spectrometry (GC–MS), using Quadrupole mass spectrometer (Hewlett Packard Agilent 5973), operated at 70 eV, coupled directly to a Hewlett Packard Agilent 6890 plus gas chromatograph. This chromatograph is equipped with a splitless injector (with injection of 0.1 μl of sample, carrier gas helium N 6.0, the flow through the column 1 ml/min) and an HP-5MS capillary column (5% Phenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm, 0.25 μm film thickness). The injector temperature is 250°C and the following oven temperature profile: an isothermal held at 70°C for 5 min, followed by a ramp of 10°C/min up to 130°C, followed by an isothermal for 2 min, a second ramp to 220°C at 3°C/min, and then an isothermal was held for 4 min, followed by a third ramp of 10°C/min up to 280°C, and finally an isothermal for 7 min. Mass spectral and retention index matching to the n-alkane series for component's identification was used.

Larvicidal activity

Concentration–mortality response of *Cx. pipiens* larvae to the ethanolic extract of *T. stans* was determined (WHO, 1996). Serial concentrations (1.5, 2, 2.5, and 2.75% v/v) were tested and each concentration was added in rearing water containing 25 larvae. The test was repeated twice and four repetitions were performed for each

concentration. Equal dilutions of ethanol were applied to the vehicle control. After 24 h exposure period, dead larvae within each container were counted and mortality was corrected according to Abbott (1925). Sublethal (LC_{10} and LC_{30}) and lethal concentrations (LC_{50} and LC_{90}) and 95% confidence limits (95% FL) were estimated, and slope of the concentration-mortality lines was calculated (Swaroop et al., 1966).

Biomarker assays

The assays were performed as previously described (Dris et al., 2017). The extract was applied at its sublethal concentrations LC_{30} and LC_{50} on fourth instar larvae and its effects examined on AChE and GST activities measured at various times (24, 48, and 72 h) following treatment. In brief, determination of GST activity was assayed following the procedure of Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate in the presence of a cofactor glutathione (GSH). Larvae at different times post-treatment (24, 48, and 72 h) were pooled in three replicates each containing 15 individuals in 1 ml of phosphate-buffered saline (0.1 M; pH 6). After centrifugation at 14000 rpm for 30 min, the supernatant served as enzyme source. An aliquot of 200 μl of the supernatant was assayed with the reaction mixture consisting of 1.2 ml of the 1 mM CDNB/5 mM GSH mixture [20.26 mg CDNB, 153.65 mg GSH, 1 ml ethanol, 100 ml phosphate buffer (0, 1 M, pH 6)]. The absorbance was measured at 340 nm each min for 5 min.

The AChE activity analysis was carried out following the method of Ellman et al. (1961) using acetylthiocholine as previously described (Habes et al., 2006). Pooled fourth instar larvae (each containing 15 individuals per repetition, 3 repetitions per series) were homogenized in this solution containing: 38.03 mg ethylene glycol tetra-acetic acid (EGTA), 1 ml Triton X-100, 5.845 g NaCl, and 80 ml Tris buffer (10 mM, pH 7). After centrifugation (5000 rpm for 5 min), the AChE activity was measured in aliquot (100 μl) of resulting supernatant added to 100 μl of 5–5′-dithiobis-(2-nitrobenzoïc acid) (DNTB) in Tris buffer (0.01 M, pH 8) and 1 ml Tris (0.1 M, pH 8). After 5 min, 100 μl acetylthiocholine was added. Absorbance was measured at 412 nm for 20 min. Each assay was performed in three replicates, and the results are expressed as mM/min/mg of proteins. The protein content was evaluated according to Bradford (1976) using bovine serum albumin as standard (BSA, Sigma).

Morphometric measurements

Newly exuviated fourth instar larvae were treated as described above with LC_{30} and LC_{50} of the ethanolic extract of *T. stans* for 24 h. Experiments related to the evaluation of toxicity of insecticides are traditionally accessed by the estimative of lethal doses like the median lethal concentration (LC_{50}) (Desneux et al., 2007). Due to the limitations of the traditional methods, recent studies in the past three decades are assessing the sublethal effects of insecticides upon several important biological traits (De França et al., 2017; Hamaidia & Soltani, 2019). In addition, this sublethal concentration (LC_{30}) was chosen to assess the delayed effects on a sufficient number of survivors following treatment of larvae (Draouet et al., 2020; Kissoum et al., 2020). Dead larvae were removed and the surviving ones were rinsed with tap water and then reared in clean water to reach adulthood. Newly emerged adults (males and females, <8 h old), from both treated and control series, were collected and briefly immobilized on ice to determine the biometrical measurements. Subsequently, they were weighed, and their wing lengths were measured to estimate their body size (Briegel, 1990). Then, gonads (ovaries and testis) of each adult were dissected out and their linear measurements were taken and their volumes calculated (Lass & Brinsden, 1999). The assays were repeated three times. Microscopic observations were carried out, to detect any morphological aberrations following treatments.

Fecundity and fertility estimation

The fecundity and fertility were determined in quadruplicate. In short, treated larvae with LC_{30} of *T. stans* ethanolic extract were reared to emergence, and then 15 females and 15 males were transferred into cubic cage (30 cm × 30 cm × 30 cm) for mating and oviposition without any blood feeding to evaluate the autogeny capacity which is estimated from the first eggs' batch laid by *Culex pipiens* females (Beji et al., 2017; Draouet et al., 2020; Tewfick et al., 2019). Many factors could interfere with "the number of laid eggs", and the main one is the quantity of ingested blood. A container of dechlorinated water (200 ml) and a 120 ml cup with 10% sugar solution were placed within each cage (Tenywa et al., 2017). The number of egg rafts in each cage was counted to estimate the autogeny capacity. The fecundity was calculated by counting the number of eggs laid per female, and the fertility was determined as the number of hatched eggs per female.

Metabolic profile determination

After treatment of newly eclosed fourth instar larvae for 24 h, newly emerged adults were collected from treated series for biochemical analyses as well from control ones. Lipids, carbohydrates, and proteins were extracted (Shibko et al., 1966) and quantified as previously described (Hamaidia et al., 2018). Briefly, samples ($n = 10$; adults <3 h) were weighed and extracted in 1 ml of trichloroacetic acid (20%). Protein content was determined colorimetrically (Bradford, 1976) using coomassie brilliant blue (G 250; Merck, Germany) with bovine serum albumin (Sigma, St Louis, Missouri) as a standard. Carbohydrates quantification was measured according to Duchateau and Florkin (1959) by reaction with anthrone reagent and glucose as standard. Lipids were quantified using the vanillin method (Goldsworthy et al., 1972) and the table oil Afia as a standard. Three replicates were performed.

Data analysis

Statistics were carried out with MINITAB software (version 16, PA State College, USA). Larvicidal analysis was performed using probit

TABLE 1 Compounds identified in leaves ethanolic extract of *T. stans* in GC–MS analysis

Pk#	RT	Area%	Library/ID	Ref#	CAS#
1	9.8215	2.9515	2-Isopropylbenzaldehyde	21673	006502-22-3
2	12.9780	12.3101	Benzene, 3-ethyl-1,2,4,5-tetramethyl-	30701	031365-98-7
3	13.4608	7.4044	Benzonitrile, 2,4,6-trimethyl-, N-oxide	30010	002904-57-6
4	18.9229	5.1813	Tridecanoic acid	65565	000638-53-9
5	20.6611	7.9287	7,10,13-Hexadecatrienoic acid, methyl ester	96893	056554-30-4
6	20.8483	5.1082	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	120892	001191-41-9
7	23.9505	**22.6595**	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	105069	004376-20-9
8	28.3503	**36.2439**	2-[4-Cyclohexylbutanoylamino]-3-chloro-1,4-naphthoquinone	144518	025304-04-5
9	28.4650	0.2126	Silicic acid, diethyl bis(trimethylsilyl) ester	114916	003555-45-1
Total		100			

Note: Search Libraries: C:\Database\NIST02.L/Major compounds in bold.

TABLE 2 Lethality parameters of ethanolic extracts of *T. stans* applied to fourth instar larvae of *cx. Pipiens* after 24 h: Lethal concentrations (LC %) together with their corresponding 95% fiducial limits (FL [95%]), coefficient of determination (R^2), hill slope and the regression equation (n = 4 repeats each containing 25 individuals)

Concentrations	Values	FL (95%)	R^2	Hill slope	Regression equation
LC_{10}	1.68	[1.17–2.41]	0.996	3.04	$y = 5.466x + 2.488$
LC_{30}	2.31	[1.61–3.32]			
LC_{50}	2.88	[2.04–4.05]			
LC_{90}	4.94	[3.26–7.47]			

analysis (Finney, 1971) to determine LC_{30} value and its 95% FL. Comparison between the different series, presented as mean ± SD, was made using one-way analysis of variance (ANOVA) followed by a post-hoc honestly significant difference (HSD) Tukey's test, and the level of significance considered is $p \leq 0.05$.

RESULTS

GC–MS analysis

Phytochemical screening of the ethanolic extract of *T. stans* was performed by GC–MS Data showed the presence of nine phytoconstituents (Table 1). Two dominant compounds were found: 2-[4-cyclohexylbutanoylamino]-3-chloro-1,4-naphthoquinone (36.24%), 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester (22.65%).

Larvicidal bioassay

Larvicidal efficacy of ethanolic extract of *T. stans* was explored following the WHO standard protocol. It was conducted on newly eclosed fourth instar larvae of *Cx. pipiens*. The LC_{10}, LC_{30}, LC_{50}, and LC_{90} values together with corresponding 95% fiducial limits (95% FL) and the slope of the concentration-mortality lines were summarized in Table 2. Data of regression analysis revealed that the mortality rate was positively correlated with the extract concentration ($R^2 = 0.996$).

TABLE 3 Effect of *T. stans* ethanolic extract (LC_{30} and LC_{50}) on the specific activity of GST (μM/min/mg of proteins) in the whole body of *Cx. pipiens* fourth instar larvae (mean ± SD, 3 repetitions each containing 15 individuals)

Exposure time (h)	Control	LC_{30}	LC_{50}
24	49.65 ± 13.57^a A	73.29 ± 0.58^b A	215.47 ± 14.13^c A
48	61.16 ± 16.36^a A	69.37 ± 27.65^a A	218.95 ± 41.50^b A
72	52.38 ± 14.19^a A	70.01 ± 0.82^a A	143.94 ± 6.24^b B

Note: For each line, values followed by different minuscule letters are significantly different at $p < 0.05$, while for each series, values followed by different majuscule letters are significantly different at $p < 0.05$.

Determination of GST and AChE activities

To give additional information on the mode of action of *T. stans* extract, two selected biomarkers were measured. As reported in Table 3 and in each treatment, there were no significant differences in the specific activities of GST with time ($p > 0.05$).

However, a significant increase in GST activity only was noted with both extract' concentrations with concentration-effect relationship after 24 h ($F_{2,8} = 7.51$, $p = 0.031$, control vs LC_{30}: $q_{cal} = 4.467$, control vs LC_{30}: $q_{cal} = 4.888$) according to ANOVA and Tukey tests. After 48 h ($F_{2,8} = 32.78$, $p = 0.001$, $q_{cal} = 9.141$) and 72 h ($F_{2,8} = 7.86$, $p = 0.021$) of exposure, only the highest concentration (LC_{50}) increase GST activity. For AChE activity (Table 4), an inhibitory effect was revealed after application of the extract ($F_{2,8} = 6.30$, $p = 0.033$) without concentration-effect relationship (LC_{30} vs LC_{50}: $p > 0.05$). According to ANOVA, both tested concentrations inhibited significantly the specific activity of AChE after 72 h (LC_{30}: $F_{2,8} = 5.64$; $p = 0.046$, $q_{cal} = 4.837$; LC_{50}: $F_{2,8} = 9.67$, $p = 0.013$).

TABLE 4 Effect of *T. stans* ethanolic extract (LC_{30} and LC_{50}) on the specific activity of AChE (μM/min/mg of proteins) in the whole body of *Cx. pipiens* fourth instar larvae (mean ± SD, 3 repetitions each containing 15 individuals)

Exposure time (h)	Control	LC_{30}	LC_{50}
24	24.18 ± 2.93^a A	24.90 ± 2.60^a A	19.42 ± 1.97^b A
48	22.49 ± 2.55^a A	23.22 ± 3.91^a A	19.90 ± 1.52^b A
72	22.15 ± 2.04^a A	19.97 ± 3.58^b B	15.14 ± 2.07^c B

Note: For each line, values followed by different minuscule letters are significantly different at $p < 0.05$, while for each series, values followed by different majuscule letters are significantly different at $p < 0.05$.

Morphometric measurements

A strong insecticidal effect of the ethanolic extract of *T. stans* on all tested parameters was observed (Table 5). The weight of females emerged from treated fourth instar larvae was slightly increased as compared to control series ($F_{2,6} = 28.77$, $p = 0.004$) without concentration effect (LC_{30} vs. LC_{50}: $q_{cal} = 0.65$). But their body volume was reduced significantly ($F_{2,49} = 39.78$; $p < 0.001$, LC_{30} vs. LC_{50}: $q_{cal} = 2.21$). Moreover, ovaries were smaller in treated groups than those from control females ($F_{2,91} = 31.65$; $p < 0.001$) (Figure 1).

In males, according to ANOVA, there was a significant reduction in whole body volume in treated series as compared to control ones ($F_{2,61} = 72.19$; $p < 0.001$) without concentration-effect relationship (LC_{30} vs LC_{50}: $q_{cal} = 4.04$). The whole-body weight was slightly

TABLE 5 Effect of *T. stans* ethanolic extract (LC_{30} and LC_{50}) on morphometric parameters in *Cx. pipiens* adults (mean ± SD)

	Female			Male		
Parameters	Control	LC_{30}	LC_{50}	Control	LC_{30}	LC_{50}
Weight (mg)	2.16 ± 0.32^a	2.71 ± 0.68^b	3.15 ± 0.09^b	1.45 ± 0.17^a	1.80 ± 0.06^b	1.76 ± 0.14^b
Body volume (mm^3)	79.72 ± 11.92^a	50.22 ± 9.96^b	58.39 ± 4.12^b	54.32 ± 10.65^a	24.73 ± 6.87^b	37.74 ± 8.81^b
Gonad volume (μm^3)	40.00 ± 13.96^a	16.65 ± 9.32^b	15.78 ± 2.64^b	6.88 ± 2.23^a	3.63 ± 1.92^b	3.45 ± 1.25^b

Note: For each line and sex, values followed by different letter are significantly different at $p < 0.05$.

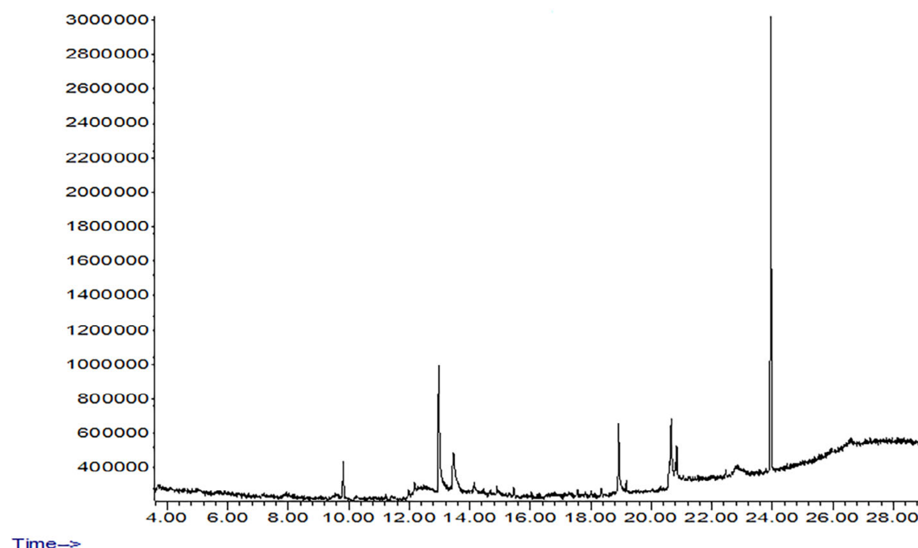

FIGURE 1 Chromatogram of *T. stans* leaf' ethanolic extract

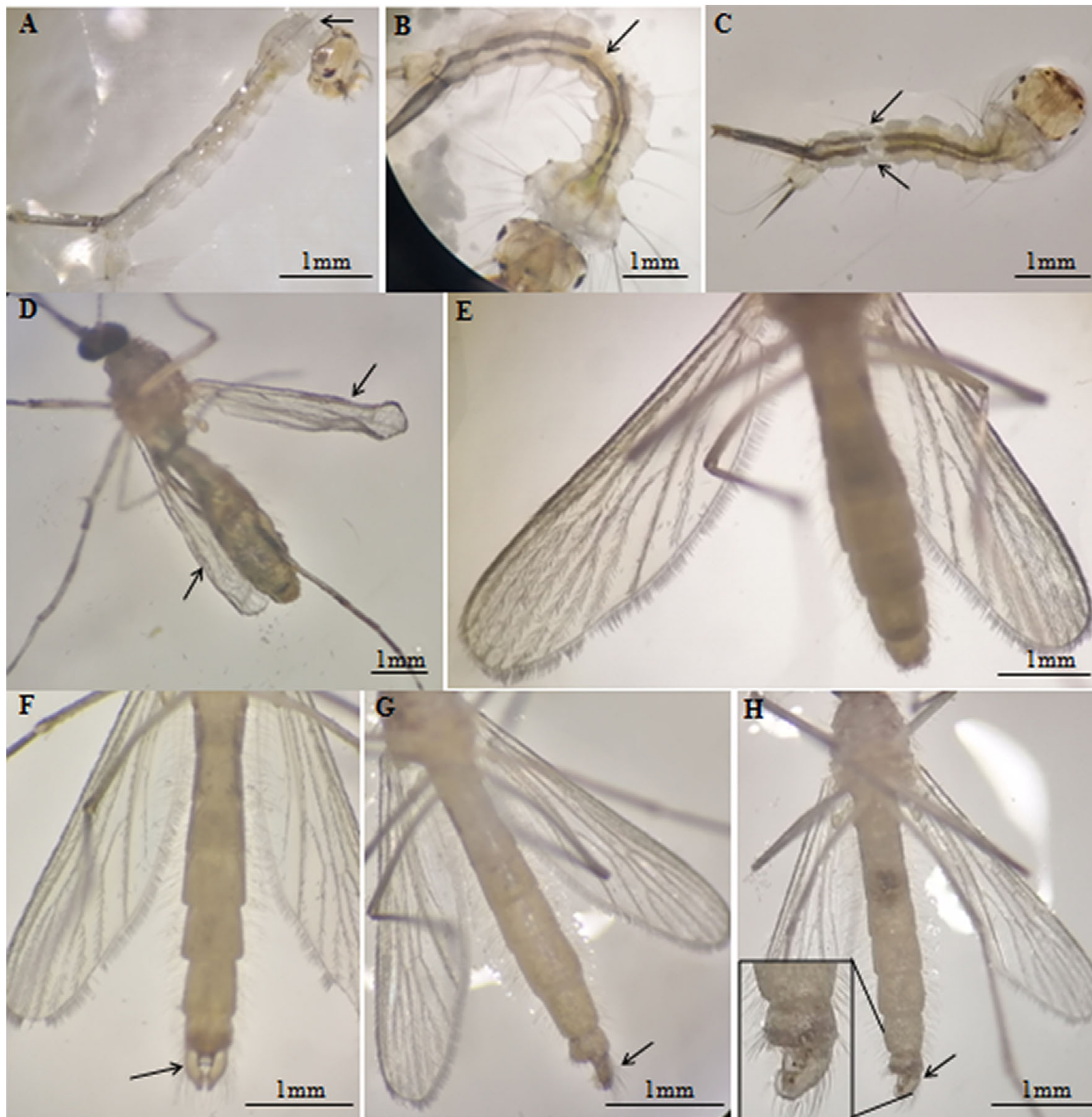

FIGURE 2 Morphological abnormalities in *Cx. pipiens* following treatment with T. stans ethanolic leaf extract. (A) Curved larval body, (B and C) Lesion of larvae respiratory tube, (D) Curly wings, (E) Normal wings, (F) Normal male genitalia, (G and H) Deformed male genitalia

increased after *T. stans* extract application ($F_{2,8} = 5.88$; $p = 0.038$; LC_{30} vs LC_{50}: $q_{cal} = 3.89$). Also, the tested extract reduced significantly the whole body ($F_{2,61} = 72.19$; $p < 0.001$) and testes' volumes ($F_{2,98} = 54.83$; $p < 0.001$).

Furthermore, It was recorded some abnormalities in treated groups (Figure 2). Larvae with curved body (A) or with lesion of respiratory tube (B and C), adults with curly wings (D), and males with deformed genitalia (G and H) were detected.

Estimation of fecundity and fertility

As mentioned in Table 6, the ethanolic extract from leaves of *T. stans* significantly decreased the fecundity ($F_{2,82} = 4.64$; $p < 0.001$) and the hatchability of the laid eggs ($F_{2,59} = 16.12$; $p < 0.001$) over the control with concentration-effect relationship according to Tukey's test (LC_{30} vs. LC_{50}: $q_{cal} = 7.78$). In addition, the treatment significantly reduced females' autogeny capacity ($F_{2,94} = 18.76$; $p = 0.005$).

Determination of metabolic profile

The ethanolic extract of *T. stans* did not affect ($p > 0.05$) the contents of the carbohydrates assayed in adults' whole body as compared to the controls (Table 7). For the protein content, a significant increase was recorded in females only after application of LC_{50} ($F_{1,4} = 10.48$; $p = 0.011$; $q_{cal} = 4.80$). While it was noted a significant increase for lipids contents in females ($F_{1,4} = 31.05$; $p = 0.0006$; $q_{cal} = 10.96$) as well for males ($F_{1,4} = 8.83$; $p = 0.016$; $q_{cal} = 5.90$).

TABLE 6 Effect of *T. stans* ethanolic extract (LC_{30} and LC_{50}) on reproductive parameters in *Cx. pipiens* females (mean ± SD)

Reproductive parameters	Control	LC_{30}	LC_{50}
Fecundity (Number of eggs/female)	53.47 ± 17.59[a]	45.57 ± 7.45[b]	46.16 ± 7.56[b]
Fertility (%)	97.69 ± 2.96[a]	86.10 ± 10.56[b]	54.12 ± 8.52[c]
Autogeny (%)	83.33 ± 5.77[a]	68.45 ± 5.15[b]	71.43 ± 12.62[b]

Note: For each line, values followed by different letter are significantly different at $p < 0.05$.

TABLE 7 Effect of *T. stans* ethanolic extract (LC_{30} and LC_{50}) on the main constituent contents in whole body of *Cx. pipiens* adults (mean ± SD, μg/mg, 3 repetitions each containing 10 individuals)

	Female			Male		
Contents	Control	LC_{30}	LC_{50}	Control	LC_{30}	LC_{50}
Carbohydrates	20.01 ± 5.75[a]	19.75 ± 4.44[a]	23.02 ± 2.94[a]	24.09 ± 10.22[a]	25.55 ± 15.09[a]	28.69 ± 9.89[a]
Lipids	45.23 ± 5.75[a]	67.58 ± 4.44[b]	89.31 ± 4.36[c]	52.29 ± 10.22[a]	78.81 ± 15.09[b]	96.64 ± 7.24[c]
Proteins	46.02 ± 7.71[a]	41.00 ± 13.57[a]	63.78 ± 7.53[b]	35.09 ± 13.64[a]	33.71 ± 5.04[a]	30.35 ± 2.69[a]

Note: For each line and the same sex, values followed by same letter in each line are not significantly different ($p > 0.05$).

DISCUSSION

Secondary natural products extracted from plants possess great potential as insect pest-control agents as they could be safer compared to the current conventional ones, and to overcome several health and risk concerns (Duarte et al., 2020; Silvério et al., 2020). Data from GC–MS allowed the identification of nine phytochemical components in *T. stans* leaves ethanolic extract with 2-[4-cyclohexylbutanoylamino]-3-chloro-1,4-naphthoquinone and 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester (DMEHE) known also as mono(2-ethylhexyl)phthalate as major components. Both compounds were revealed in several plant species known by their insecticidal/acaricidal activity. 2-[4-Cyclohexylbutanoylamino]-3-chloro-1,4-naphthoquinone was detected in extracted essential oil with ethanol from *Calotropis procera* (Alhazmi et al., 2018), *Azadirachta indica* (Babatunde et al., 2019), and *Hypericum perforatum* leaves (Ebadollahi et al., 2019) that showed potency against *Wohlfahrtia magnifica* larvae (Carnevali et al., 2019). The main constituent in the essential oils of *Hertia cheirifolia* leaves, with a potent acaricidal activity against *Tetranychus urticae* (Rincón et al., 2019), was found to be DMEHE (33.71%) (Segueni et al., 2017). This constituent was detected recently in petroleum ether extract obtained from *Acacia* spp. bark (Prayitno et al., 2021). Also, DMEHE was identified in ethyl acetate leaf extract of *Amaranthus viridis* (58.52%) which exhibited an antifungal activity (Akbar et al., 2020), and a strong larvicidal activity against *Spodoptera exigua* (Lepidoptera) (Rachokarn et al., 2008). Pure DMEHE caused human endocrine disruption (Beg & Sheikh, 2020), cytotoxic (Krishnan et al., 2014), antibacterial (Verma et al., 2014), and antifungal (Akpuaka et al., 2012) activities. Also, DMEHE as the main di(2-ethylhexyl) phthalate metabolite, is a known reproductive toxicant (Kalo et al., 2019). Also, polyphenols and flavonoids were predominant in *T. stans* leaf crude ethanolic extract and fractions (Mukul & Basavaraju, 2021). It was reported that solvent type, plant variety, location, and climatic conditions influence the quality of extracts (Jedidi et al., 2020). Moreover, the effectiveness of each extract of the same plant on the same insect species should be different depending on the solvent used (Karthi et al., 2020; Kumar et al., 2011) and correlated to its phytoconstituents (Bakr et al., 2019).

T. stans has gained attention as an insecticide. Its crude (Abere & Enoghama, 2015) and aqueous (Navaneethan et al., 2016) leaf extracts have been evaluated against several species. The tested extract showed an interesting and relevant larvicidal activity. Likewise, the petroleum ether extract of *T. stans* was found to present maximum activity on *Ae. aegypti* and *Cx. quinquefasciatus* (Hari & Mathew, 2018). In another axis, ethanolic extract, ethyl acetate, and aqueous fractions from *Tecoma* species leaves, exhibited potent in vitro antiviral activity against the Zika virus (Reis et al., 2020). Ovicidal activity and oviposition deterrence efficiency of aqueous leaf extracts of *T. stans* were reported on *Ae. aegypti* (Navaneethan et al., 2016). Its crude extract produced antifeedant, repellent, and insecticidal activities against *Sitophilus zeamais* and *Acanthscelides obtectus* (Abere & Enoghama, 2015).

GSTs are ubiquitous multifunctional detoxification enzymes that their relationship with the resistance of insects to conventional insecticides was largely investigated (Adeyi et al., 2015). They have been recognized with their important role in xenobiotic detoxification (Tang et al., 2020). Results from the *T. stans* leaf extract treatment showed an induction of GST activities in *Cx. pipiens* larvae. Similar results were observed in *Micromelalopha troglodyta* in response to tannic acid stress (Tang et al., 2020). Previously, it was reported an induction in GST activity in *Cx. pipiens*, *Cs. Longiareolata*, and *Ae. caspius* treated with *Mentha rotundifolia* (Kharoubi et al., 2020). Moreover, Liu et al. (2020) showed that ar-turmerone significantly increased the level of GST enzyme in *Culex pipiens pallens* larvae. It was shown, also, an induction of this detoxifying enzyme activity in *Spodoptera litura* after application of purified compounds of *Alpinia galangal* (Datta et al., 2020).

Detoxification enzymes in *Aedes aegypti*, *Anopheles dirus*, and *Culex quinquefasciatus* were significantly induced by hexane crude extract of *Alpinia galangal* (Poonsri et al., 2019).

Data from the present study revealed that *T. stans* extract had a slight effect on the AChE activity of fourth instar larvae of *Cx. pipiens*. It inhibited the specific activity of AChE during the first 72 h after exposition. *However, this effect was low, suggesting that it is not likely the main mode of action of this material. Veratrum nigrum* alkaloidal extract showed an excellent inhibitory activity on AChE in male adults and 4th nymphs cockroaches (Cai et al., 2018). Powdered roots of *Stemona collinsiae* extracted with hexane, dichloromethane, and methanol indicated an active AChE inhibitory activity (Kongkiatpaiboon et al., 2013). It was reported that the dichloromethane crude extract of *Tithonia diversifolia* presented the best AChE inhibition against *Atta cephalotes* compared to its fractions (Pantoja-Pulido et al., 2020).

Body volume plays a significant role in the reproductive capacity of insects, and female size is the most important constraint on insect potential fecundity (Honěk, 1993). Furthermore, wing length is a reliable predictor of mosquito size and nutritional status (Hernández-Martínez et al., 2019). In the present investigation, the adults of both sexes of *Cx. pipiens* of treated series showed reduction in body and gonad volumes with the consequent impairment of their fecundity, fertility, and autogeny capacity. *Culex pipiens* autogenously lay its first batch of eggs without blood feeding (Gao et al., 2019). Decrease of fertility in the present study may be due to a diminution of ecdysteroid titre necessary for reproduction, which can basically be explained by the impairment of prothoracic glands (PGs) or decrease in synthesis of vitellogenic precursors or their incorporation into oocytes. It was demonstrated that DMEHE (a major compound in *T. stans* ethanolic extract) disrupts antioxidant enzymes, inducing an oxidative stress which could inhibit the follicle growth (Luderer, 2014; Wang et al., 2012). Also, the lipids contents in their whole bodies were reduced. In an interspecific scaling study, egg number, egg size and ovary volume were correlated with body size in Diptera (Berrigan, 1991). Likewise, potential fecundity and number of ovarioles have been reported to increase along with female body weight (Honěk, 1993). It was demonstrated that *I. cairica* crude extract affected the whole life history of both dengue vectors, *Ae. albopictus* and *Ae. aegypti* including reduction in their reproductive capacity (Zuharah et al., 2016). The effectiveness of each extract of the same plant on the same insect species should be different depending on the solvent used (Karthi et al., 2020; Kumar et al., 2011) and correlated to its phytoconstituents (Bakr et al., 2019).

Further, several abnormalities to the whole body of treated larvae, pupae, and adults were noted (Figure 2). These results were probably due to the damage to the air tube organization and impairment of the remodelling process during metamorphosis. All results from the present study could be associated with disturbed hormonal balance. Ecdysone (20E) and juvenile hormone (JH), two key insect hormones, are involved in developmental hormone balance and interplay orchestrating reproductive maturation (Areiza et al., 2015). The PGs, which produce ecdysteroids, and the *corpus allatum*, which releases juvenile hormones (JH) are the main important insects' endocrine glands (Klowden, 2008). In many insect species, ecdysteroids are required for female reproduction (Hentze et al., 2013). During insect development, periodic pulses of 20E are released from the PGs under the episodic activation by prothoracicotropic hormone PTTH (Marchal et al., 2010; Ou et al., 2016). In connection with noticed lesions of the larval PGs, *T. stans* ethanolic extract may display reproduction inhibitory activity limiting insect reproduction and survival. Same effects were reported after application of *Agerantum conyzoides* extract on *Anopheles* species (Muema et al., 2016). Some works suggested that the insects' endocrine system (especially PGs) is synchronized with their physiological state and the environmental signals (Di Cara & King-Jones, 2016; Klowden, 2008). Thus, the reproduction is triggered only in suitable conditions (Klowden, 2008).

Energetic profile in insects reflects their fitness towards habitat manipulation programs including insecticides application. The amount of the main metabolites (proteins, carbohydrates, and lipids) control several vital functions, such overwintering, mating behaviour, and flight (Lee, 2019). In the present study, lipid levels in *Cx. pipiens* adults were increased following treatment with *T. stans* ethanolic extract. The increase in total lipids rates might be due to mobilization of reserves for detoxifying process' energy. Similar results were shown after application of methanolic extract of *Azadirachta indica* on *Anopheles stephensi* and *Culex quinquefasciatus* larvae (Sharma et al., 2011). But, a depletion of both, total carbohydrate and lipid contents, in *Cx. pipiens* were reported after treatment with *Lantana camara* ethanolic extract (Draouet et al., 2020). Also, *Citrus limonum* essential oil caused in *Sitophilus granarius* a decline of lipid and carbohydrate contents (Guettal et al., 2020). Based on obtained results of Shahat et al. (2020), methanol, ethyl acetate, chlorobenzene, and hexane extracts from leaves of *Origanum syriacum*, *Pergularia tomentosa*, *Senna italica*, and *Otostegia fruticose* decreased the total carbohydrate, protein, and lipid contents against *Cx. pipiens* (Shahat et al., 2020). Likewise, feeding *Agrotis ipsilon* larvae on *Conyza aegyptiaca*, *Melia azedarach*, and *Vinca rosae* induced a significant reduction in the total protein, lipids, and carbohydrates (Ramadan, 2020).

CONCLUSION

Our study showed that *T. stans* ethanolic extract disturbed biological parameters and reduced reproductive capacity of *Cx. pipiens*. Biomarkers tests revealed a slight neurotoxic action and detoxification enzyme activation. The morphological and physiological abnormalities present evidence that this extract could be effective in controlling mosquitoes particularly by its obviously larvicidal and delayed effects and it offers promise as a potential biocontrol agent against mosquitoes. Further experiments like the ecdsyteroid measurements, are needed to give additional information on its mode of action. These ecdysteroid measurements will be made as soon as experimental conditions allow it.

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CONFLICT OF INTEREST

The authors declare that there have no conflicts of interest regarding the publication of this article.

ETHICS STATEMENT

All authors have seen and approved the final version of the submitted paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, K.H., upon reasonable request.

ORCID

Kaouther Hamaidia https://orcid.org/0000-0002-2994-5126

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