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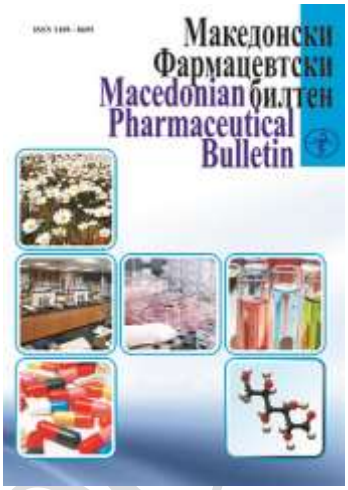

Title: **Inhibition of seed germination, toxicity on *Artemia salina* and phytochemical prospecting with from Cuban plants as indicator of antitumor activity**

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Inhibition of seed germination, toxicity on *Artemia salina* and phytochemical prospecting with from Cuban plants as indicator of antitumor activity

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Abstract

Cuba has a high biodiversity and many plants are widely known and used in folk medicine and for commercial manufacturing of phytomedicines. However, many plants have not been studied for their pharmacological properties, particularly endemic plants. Two simple, inexpensive and rapid biological assays, inhibition of germination of tomato and lettuce seeds and toxicity on the brine shrimp *A. salina*, have been evaluated as prescreens for possible antitumor activity. Extracts of six plant species collected in Havana, Cuba were subjected to the brine shrimp lethality test and inhibition of seed germination in order to detect potential sources of novel cytotoxic and cytostatic antitumor compounds, respectively. The larvicidal activity, based on the percentage of larval mortality, was evaluated after 24 h exposure to the treatments. In the case of inhibition of seed germination the readings were made 48 h after exposure. Semi-quantitative phytochemical prospecting was done by color and precipitation reactions for chemical functional groups. All species tested showed some cytotoxic and cytostatic effects. Two extracts showed high cytotoxicity in the *Artemia salina* test, the methanol extract from flowers of *Tithonia diversifolia* (TD) with IC_{50} (Inhibitory concentration 50) or LD_{50} (Lethal doses 50) of 1.14 µg/mL and methanol extract of stems from *Castela lucida* (CL) with LD_{50} of 0.052 µg/mL. However, the more promising species was the *Tabebuia hypoleuca* (TH) with cytostatic effect superior of 65% and good cytotoxic effect with the leaf extract, with the prominent compound classes' triterpenes, tannins, phenols, and alkaloids.

Keywords: Cuban plants; cytotoxic; *Artemia salina*; cytostatic; seed germination; chemical compounds

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Introduction

The search for new drugs which are plant-derived has been receiving renewed interest among researchers throughout the world in view of the need for discovering potent new drugs to combat the menace of drug resistant pathogenic microorganisms, as well as antitumor and anticancer agents (Hamidi et al., 2014).

Despite the advance of modern drug discovery technologies, such as rational drug design, combinatorial chemistry, and high throughput screening, natural products remain an essential element in modern drug discovery. As an indispensable source for lead generation and drug discovery, natural products have provided molecular diversity and structural novelty inaccessible by other means. The differences between the molecular properties of natural products and synthetic compounds had been investigated and natural products were found to be significantly different from synthetic compounds. As compared to synthetic compounds, natural products generally have higher molecular weights, fewer nitrogen, halogen, or sulfur atoms, more oxygen atoms, and more *sp*3-hybridized or bridgehead atoms. In addition, natural products typically have more rings and more chiral centers in their structures (Xiao et al., 2016).

In Cuba, cancer is the leading cause of death in the population aged 1–64 years, and the second cause of death after cardiovascular diseases in the population aged ≥65 years. It is the disease most reducing years of potential life, and its impact on disability-adjusted life years is growing (Galán et al., 2009).

The Cuban archipelago boasts of a broad variety of plants and animals (7,500 and 19,600 species, respectively), a very high number of which are endemic to the island (50% and 42%). In terms of biodiversity, Cuba ranks fourth among the world's islands and first in the Caribbean region (Calzadilla, 2013).

Despite the high endemism of plants in Cuba, less than 10% of them have been studied for their pharmacological properties. For this reason, they could be a very relevant source of new natural products and drugs discoveries (Crooker et al., 2010).

The brine shrimp lethality assay is the most convenient and versatile system for monitoring biological activities of plant species. It has been applied to screen the toxicity of plants extracts, detection of fungus toxin and cyanobacteria, toxicity of heavy metals and metal ions, toxicity of nanoparticle, screening of marine natural products, cytotoxicity of dental materials and pure

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compounds (Fernández et al., 2009). This method is very useful for preliminary assessment of toxicity of the plant extracts. Rapidity, simplicity and low expense are several advantages of this assay. The toxicity of herbal extracts using this assay has been determined in a concentration range of 10, 100 and 1000 μg/mL of the examined herbal extract (Arcanjo et al., 2012; dos Santos et al., 2017; Ngutaa et al., 2012). Most toxicity studies which use the brine shrimp lethality assay determine the toxicity after 24 hours of exposure to the tested sample. The median lethal concentration (LC_{50}) of the test samples is obtained by a plot of percentage of the dead shrimp against the logarithm of the sample concentration. LC_{50} values are estimated using a probit regression analysis and compared with either Meyer's or Clarkson's toxicity criteria. Furthermore, the positive correlation between Meyer's toxicity scale for *Artemia salina* and Gosselin, Smith and Hodge's toxicity scale for higher animal models (100% correlation) confirmed that the brine shrimp lethality assay is an excellent predictive tool for the toxic potential of plant extracts in humans (Hamidi et al., 2014).

Seed germination and plant growth bioassay are the most common techniques used to evaluate phytotoxicity (Mitelut and Popa, 2011). Inhibiting seed germination from tomato (*Solanum lycopersicum*) or lettuce (*Lactuca sativa*) has been used to detect secondary metabolites which inhibit plant growth, and is considered indicative of cytostatic properties, thereby providing a useful complement to the *Artemia salina* test (Ticona et al., 1998). These authors validated both tests with different commercial drugs using in the treatment of cancer, including cisplatin, cytarabine, fluorouracil, methotrexate and vincristine.

The objective of the present work was to evaluate the toxicity against *Artemia salina* and inhibiting on seeds germination of tomato and lettuce from different Cuban plant species prior to *in vitro* cell culture studies, validating our tests to detect plants with cytotoxic or cytostatic activities and study the phytochemistry of these plants.

Materials and methods

Plants

Tabebuia angustata Britton (leaves and stems) (TA), *Tabebuia hypoleuca* (C. Wright) Urb. (leaves and stems) (TH), *Gymnates lucida* Sw. (leaves and stems) (GL), are endemic Cuban plants from the Bignoniaceae family*; Verbesina angustata* Urb. (leaves and stems) (VA), Asteraceae; *Castela lucida* (leaves and stems) (CL), Simaroubaceae; *Tithonia diversifolia* (Hemsl.) A. Gray

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(flowers) (TD), Asteraceae. All these species were collected in September 2009 in the Cuban National Botanical Garden, except *T. diversifolia* which was collected in Indio Hatuey, Matanzas. They were authenticated and deposited in the herbarium of the National Botanical Garden as voucher specimens as HFC-88204, HFC-88205, HFC-88206, HFC-8807, HFC-8808andHFC-8809, respectively.

Preparation of plant sample

Collected plant materials were separated into distinct plant parts. They were dried at room temperature for 72 h and then in an oven at 40 °C for 48 h. All were chopped in a grinding mill and stored in desiccators at room temperature.

Extraction procedure

100 g of each plant were extracted with 500 mL of methanol to obtain a total extract. In a separate experiment, 100 g of ground samples were extracted with organic solvents increasing in polarity (hexane, ethyl acetate and methanol) in a Soxhlet apparatus by 12 hour each. Extraction with different solvents guaranteed the total extraction of soluble compounds in each solvent. The samples were filtered using Whatman No. 1 filter paper and the solvents evaporated to dryness under reduced pressure at a maximum of 40 °C using a rotavapor-R (Büchi, France).

Brine shrimp cytotoxicity assay

A. salina encysted eggs were incubated in sterile seawater under light at 28 °C. After incubation for 48 h, nauplii were collected with a Pasteur pipette. The samples were prepared in a stock solution of 10 mg/mL using 1% DMSO. Next, the samples in triplicate to be assayed were dissolved and diluted serially (1000, 100, 10, 1 μg/mL) in seawater on 24 well plates. Ten nauplii were added to each set of tubes containing the samples. Controls containing 1% DMSO in the same serial dilution with seawater were included in each experiment. Twenty-four hours later, the number of survivors was counted. The criteria for mortality of the nauplii in the control group was established as when the percentage of dead *A. salina* larvae is below 10%.

Lethal concentration determination

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The lethal concentrations of plant extracts resulting in 50% mortality of the brine shrimp (LC_{50}) and confidence intervals at 95% were determined from the 24-h counts and the dose-response data were transformed into a straight line by means of a trend line fit linear regression analysis; the LC_{50} was derived from the best-fit line obtained, using Probit Analysis, STATGRAPHICS Plus 5 (Meyer et al., 1982). The toxicity grades from extracts were defined following the LC_{50} range shown: extremity toxic (LC_{50}<10 μg/mL), very toxic (10<LC_{50}<100), moderate toxic (100<LC_{50}<1000) and non-toxic (LC_{50}>1000 μg/mL) (Valdés- Iglesias et al., 2003).

Tomato and lettuce seed germination inhibition test

Seed germination: To six Petri plates with sterile filter paper with equal diameter as the plate were added 5 mL of distilled sterile water. After tomato seeds (~100) were put on each plate and germinated for four days at 25 °C, keeping the humidity in the plates. The same proceeding was made for lettuce seeds but the germination was only for one day. Roots of 0.5 cm of length were considered as germinating.

Biological Test: In each Petri plates with sterile filter paper were added plant extracts for evaluation and seven germinated seeds. All tests were made in triplicate and negative control were made using distillated sterile water and DMSO in work concentrations (1000-100-10-1). Finally, the plates were incubated at 25 °C for 4 days, at which time root length was measured.

Calculate of % germination: To define the activity of extracts we used the following formula:

$$\%\ growing = (Root\ length\ with\ the\ test\ extract / Root\ length\ in\ control) \times 100 \quad (I)$$

$$\%\ inhibition = 100 - \%\ growing \quad (II)$$

The activity in this test was interpreted as: slightly active (+) when 0<%I< 29%; active (++) when 30<%I<59% and very active (+++) when 60<%I<100%.

Phytochemical tests

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Phytochemical tests to detect the presence of phenols, saponins, tannins, flavonoids, steroids, triterpenes, coumarins, quinones, organic acids and alkaloids were performed following the method described by Rondina and Coussio (1969). Five grams of dried plant was extracted by maceration with 50 mL of methanol for 24 h at room temperature, after which it was refluxed 12 h, filtered and separated into different aliquots. Each aliquot was used for assays based on visual observation of color modification or precipitate formation after addition of specific reagents for chemicals (Ninhydrin, Gelatin, Ferric chloride, Lieberman, Borntrager, Shinoda and Rosenheim). The method was qualitative and semi-quantitative to detect the different functional chemical groups.

Results and discussion

The total methanol extracts of the different plants all have some cytotoxic or cytostatic activities (Table 1). For this reason, these two-alternative methods for antitumor activity prediction are available to distinguishthese properties. These methods are defined by several authors as economical and quick testing for the studies of antitumor drugs discovery. For example, Ticona et al. (1998) showed the importance of the combination of both tests to detect 100% of antitumor agent using in the tests the following known antitumor drugs: cytarabine, cisplatin, methotrexate, vincristine and fluorouracil, which were the base for the standardization and validation of the method in the previous application with plant extracts. The antitumor drugs including the different mode of action involved in its effect (alkylating drugs, antineoplastic antibiotic, antimetabolic and inhibition of protein synthesis). Reis Morais et al. (2007) reported that lethality in the brine shrimp assay has been correlated with in vitro antitumor activity in general and this assay is considered an important pre-screen for isolation of cytotoxic compounds and anticancer drug research. The significant correlation between the brine shrimp assay and in vitro growth inhibition of human solid tumor cell lines demonstrated by the National Cancer Institute (NCI, USA) is significant (Anderson et al., 1991). Therefore, this bioassay is useful for evaluating botanical product bioactivity leading to development of new drugs.

Table 1

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The activity of the fractions, obtained by the selective extraction of plants using solvent with increasing in polarity is described in Table 2. In the case of the leaves from *Gymnates lucida* (hexane extract) had a good cytotoxicity (LD_{50}=79 ppm, µg/mL), in this case the activity would be associated with the less polar compounds. Hexane and methanol extracts of *Tabebuia angustata* (leaves) shown a moderate cytostatic effect and a very toxic activity cells with LD_{50} of 18 ppm and 17 ppm, respectability. The ethyl acetate extract of *Tithonia diversifolia* (flowers) inhibited the seed germination 57% at 10 ppm, for this reason the fraction is classified as active cytostatic and has a moderate toxic effect in *Artemia salina* test. The methanol fraction from this plant is classified as active in seed germination with 57% of inhibition activity at 10 ppm and it is extremely cytotoxic (LD_{50}=14 ppm). The methanol fraction of *Castela lucida* (stem) was extremely toxic with a LD_{50} of 0.052 ppm. In the case of the leaves of *Castela lucida*, the ethyl acetate fraction is active as cytostatic (61% of seeds germinate inhibition) and it is very toxic (LD_{50}=78 ppm) and the methanol fraction inhibited tomato seeds germination by 66% at 10 ppm with an LD_{50} of 44 ppm.

Table 2

We considered the most promising plant in its anticancer effect to be *Tabebuia hypoleuca* (leaves) comparing its activity with other fractions in the present study. In this case we found enough cytotoxic and cytostatic effects from hexane, ethyl acetate and methanol fractions with the best results from ethyl acetate and methanol fractions (92% inhibition of seed germination at 10 ppm in lettuce and 53% in tomato and LD_{50} of 13 ppm with ethyl acetate and, 66% of inhibition with lettuce, 18% in tomato and LD_{50} of 16 ppm with methanol fraction).

However, fractions from other species with LD_{50} very low could be leaders in the isolation of new antitumor compounds, for example the methanol fraction of *Castela lucida* (stem) with a LD_{50} of 0.052 ppm associated with polar alkaloids.

We found the cytotoxic effect in *Tithonia diversifolia* growing in Cuba associated with alkaloids. Other authors report the cytotoxic activity of this species; however, this activity was associated with the presence of other chemical groups. For example, Kuroda et al. (2007) isolated sesquiterpenoids and flavonoids from the aerial part ethanol extract of *T. diversifolia* and tested its cytotoxic activity against HL-60 leukemia cells. Lee et al. (2011) reported antiproliferative activity

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against human glioblastoma U373 cells, with an IC_{50} value of 59.2±3.7 μg/mL from a methanolic extract of *T. diversifolia* and the activity associated with tagitinin C, the major sesquiterpenoid compound in the extract. Aqueous, methanol and dichloromethane extracts of the leaves of this plant have exhibited interesting health promoting properties, resulting both from its free radical scavenger capacity and also by induction of protective cellular systems involved in cellular stress defenses and in adipogenesis of mesenchymal cells (Di Giacomo et al., 2015).

Other activities are reported for this plant: anti-inflammatory properties, especially related to nuclear factor-kappa B inhibition and on human neutrophils (Abe et al., 2015).

The other endemic Cuban plants tested in the present study had not been reported in the literature. It was the first biological evaluation as cytotoxic and cytostatic properties and it was the first screening of their chemical compounds.

In Table 3 we show the secondary metabolites (chemical groups) present in the plants in the phytochemical study.

Table 3

Lee et al. (2011) and Chagas-Paula et al. (2012) reported the presence of terpenoids and flavonoids in aerial parts of *Tithonia diversifolia* and anti-inflammatory, analgesic, antimalarial, antimicrobial and antidiabetic activities associated with its constituents. However, we did not find flavonoids in our study. Li et al. (2013) found monoterpenes in the aerial part of this plant and reported the anti-hyperglycemic activity of these compounds. The difference in secondary metabolites present in the Cuban species may be associated with other chemical compounds different from literature reports or perhaps may be a result of synergic/additive effects of the mixture of compounds including alkaloids.

Tabebuia species have been reported to possess flavonoids, cyclopentene dialdehydes, benzoic acid and benzaldehyde derivatives, quinones, furano naphthoquinones and, most importantly, naphthoquinones and anthraquinones. From the 18 most relevant quinones registered so far, lapachol and β-lapachone are of some clinical importance since they have been related to the pharmacological activity of Red Lapacho tea (*Tabebuia impetiginosa*) (Gómez et al., 2009).

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Reis de Morais et al. (2007) reported the cytotoxicity towards the brine shrimp larva species *Artemia franciscana* of the methanol extract from bark of *Tabebuia incana* with moderate lethality and the detection of two furano naphthoquinones by HPLC.

However, the two Cuban endemic *Tabebuia* species studied in the present work have not been associated with quinones. In our case the activity was associated with the presence a high content of triterpenes.

In general, it is known that the secondary metabolite presence and concentrations in plants are influencing by genetic and ambient factors and that extraction method may alter the compounds polarity due to extractive solvent polarity. Some of these factors are geographic distribution, climate, station, precipitation, altitude, etc. Our work is the first report of cytostatic and cytotoxic activity from extracts of endemic Cuban plants. Though *Verbesina angulata* and *Castela lucida* without any chemistry reported for these genera, could be sources of new lead antitumor compounds for the future development of new anticancer drugs.

Conclusion

All of the plants studied showed some activity as indicators of antitumor effect in the two alternative methods associated with their chemical composition. The leaves of *Tabebuia hypolecuca* had the most promising activity in the design of a new antitumor drug.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Резиме

Инхибиција на `ртење на семе, токсичност на *Artemia salina* и фитохемиска анализа на растенија од Куба како индикатор за антитуморна активност

Луз Марија Санчез Перера, Бети Манцебо Дорвигнијанд, Ада Ивис Регаладо Велоз

Оддел за развој на биофармацевтски производи,
Националниот центар за здравје на животните, CENSA, Apdo. 10, КП 32 700,
Сан Хозе де лас Лајас, Мајабек, Куба

Клучни зборови: кубански растенија, цитотоксичен; *Artemia salina*, цитостатик, `ртење на семе, хемиски соединенија

Куба има висок биодиверзитет и многу растенија се нашироко познати и се користат во народната медицина, како и за комерцијално производство на фитопрепарати. Сепак, многу растенија, особено ендемските растенија, сеуште не се детално проучени од аспект на нивните фармаколошки својства.

Две едноставни, ефтини и брзи биолошки анализи и тоа инхибиција на `ртење на семето на доматот и зелената салата и токсичност врз ракчиња *A. salina,* беа иницијално евалуирани во однос на можна антитуморна активност.

Екстрактите од шест растителни видови собрани во Хавана, Куба, беа подложени на тест за леталност од ракчиња и инхибиција на `ртење на семето, со цел да се откријат потенцијалните извори на нови цитотоксични и цитостатички антитуморни соединенија, соодветно. Ларвицидната активност, базирана на процентот на смртност на ларва, беше проценета по 24 часа изложеност на третманите. Во случај на инхибиција на `ртење на семето, отчитувањата беа направени 48 часа по изложувањето. Семиквантитативната фитохемиска анализа беше направена со реакции на боја и преципитација на хемиски функционални групи. Сите тестирани видови покажаа некои цитотоксични и цитостатички ефекти. Два екстракти покажаа висока цитотоксичност во тестот на *Artemia salina,* метанолниот екстракт од цветовите на *Tithonia diversifolia* (TD) со IC_{50} (инхибиторна

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концентрација 50) или LD_{50} (Летална доза 50) од 1,14 μg/mL и метанолниот екстракт од изданок на *Castela lucida* (CL) со LD_{50} од 0,052 μg/mL. Сепак, екстрактот од лист од *Tabebuia hypoleuca* (TH) покажа супериорен цитостатски ефект од 65% и добар цитотоксичен ефект, при што најзначајни класи на соединенија се тритерпените, танините, фенолите и алкалоидите.

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Table 1. Cytotoxicity (*Artemia salina*) and cytostatic (inhibition of seed´s germination) effects of methanol total extract from vegetal species

Vegetal extract (Total Methanol)	LETTUCE % Inhibition				TOMATO % Inhibition				LD_{50} (ppm) *Artemia salina* (Confidence interval)
	1000	100	10	1 ppm	1000	100	10	1 ppm	
	++	++	++	++	-	+	-	+	202 (3.5 - 354) moderate toxic
GL (stem)	-	-	+	-	+	-	-	-	1417 (220 - 3054) not toxic
TA (leaves)	-	++	-	+	-	-	+	+	201 (13 - 415) moderate toxic
TA (stem)	-	-	+	-	-	-	-	-	811 (19 -) moderate toxic
TH (leaves)	+	+	-	+	-	-	-	-	436 (73 - 798) moderate toxic
TH (stem)	-	-	-	+	-	+	-	+	358 (15 - 831) moderate toxic
VA (leaves)	-	-	-	+	-	-	-	-	391 (221 - 561) moderate toxic
VA (stem)	-	+	+	+	-	+	+	+	227 (54 - 398) moderate toxic
TD (flowers)	+	-	+	++	-	+	-	+	na

- no active (na), few active (+) when 0<%I<29%; actives (++) when 30<%I<59% and very actives (+++) when 60<%I<100%.na: no assignable

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Table 2. Cytotoxicity (*Artemia salina*) and cytostatic (inhibition of seed´s germination) effects from fractions of plants, using successive extraction with solvents with increasing of polarity

No	Fraction	Lettuce % Inhibition				Tomato % Inhibition				LD50 (ppm) *Artemia salina* (Confidence interval)
		1000	100	10	1 ppm	1000	100	10	1 ppm	
1	GL (leaves) hexane	35.7% (++)	-	-	-	-	-	-	-	**79 (0.01 - 164) VERY TOXIC**
2	Ethyl acetate	45 (++)	40.7 (++)	40.1 (++)	48.5 (++)	-	29.2 (+)	27.5 (+)	22.3 (+)	NO TOXIC
3	Methanol	52.4 (++)	28 (+)	22.1 (+)	9.6 (+)	-	13.9 (+)	27.5 (+)	22.3 (+)	NO TOXIC
4	TA (leaves) hexane	44.2 (++)	9.05 (+)	-	-	-	23.6 (+)	25.6 (+)	20.8 (+)	**18 (0.08 – 36.4) VERY TOXIC**
5	Ethyl acetate	39.1 (++)	-	-	-	-	50.1 (++)	13.6 (+)	-	140 (0.2 – 955) MODERATE TOXIC
6	Methanol	32.3	14.2	-	-	-	48.8	16.8	8.1	**17 (1.9 – 149) VERY TOXIC**
7	TH (stem) hexane	-	-	-	-	-	-	-	-	NO TOXIC
8	Ethyl acetate.	35 (++)	-	-	-	-	52.64 (++)	47.2 (++)	50.4 (++)	801 (328- 1274) MODERATE TOXIC
9	Methanol	36	-	-	-	3.03	52.8	45	14.2	No toxic
10	TH (leaves) hexane	40.8 (++)	24.6 (+)	30.2 (++)	44.4 (++)	-	54.7 (++)	42 (++)	8.9 (+)	120 (3.7 – 386) MODERATE TOXIC
11	Ethyl acetate	**89.3** (+++)	**89.4** (+++)	**92.2** (+++)	-	-	**53.9** (++)	**52.9** (++)	**14.9** (+)	**13 (2.7 – 63)** VERY TOXIC
12	Methanol	**56.79 (++)**	**40 (++)**	**66 (+++)**	**19.5 (+)**	**64.3 (+++)**	**34.3 (++)**	**18.5 (+)**	-	**16 (1.9 – 149) VERY TOXIC**
13	TD (leaves) hexane	34.6 (++)	-	34.7 (++)	-	64.4 (+++)	20.2 (+)	23.4 (+)	-	3623 (1040 – 6206) NO TOXIC
14	Ethyl acetate	-	10.7 (+)	-	37.5 (++)	**64.3 (+++)**	**70.4 (+++)**	57.2 (++)	38 (++)	592 (NA) MODERATE TOXIC
15	Methanol	16	17.3	27.3	-	**64.3**	**68.2**	57.2	42	**1.14 (0.0054 – 239) EXTREME TOXIC**

-no active, few active (+) when 0<%I<29%; actives (++) when 30<%I<59% and very actives (+++) when 60<%I<100%. na: no assignable.

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Table 3. Chemical compounds detecting in the plants

Plant	Chemical group						
	Phenols	Tannins	Antra-quinones	Triterpenes	Glycosides	Alkaloids	Flavonoids
GL (leaves)	(+)	(+)	(++)	(++)	-	(+)	-
GL (stem)	(+)	(+)	-	(+)	-	(+)	-
TA (leaves)	(+)	(+)	-	(++)	-	(++)	-
TA (stem)	(+)	(+)	-	(+)	-	(+++)	-
TH (leaves)	(+)	(+)	-	(+++)	-	(++)	-
TH (stem)	(+)	(+)	-	-	-	(++)	-
VA (leaves)	(+)	(+)	-	(++)	-	(++)	-
VA (stem)	(+)	(+)	-	-	-	(++)	-
TD (flowers)	-	-	-	(+++)	-	(+++)	-
CL (leaves)	(+)	(+)	-	(+)	-	(++)	-
CL (stem)	(+)	(+)	-	-	-	(++)	-

+++: high quantity; ++: notable quantity; +: slight quantity

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