

Toxicity-guided screening and identification of glycosides in extracts of dry leafless branches of *Nerium oleander* L. collected in Vietnam

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ABSTRACT

This study conducted toxicity-guided screening based on the *Allium cepa* L. root model in combination with chemical analysis. Accordingly, the crude ethanol extract (CEE) from dry leafless branches of oleander (*Nerium oleander* L.) collected in Vietnam was evaluated using three bioassay methods (A, B, and AB), all of which yielded Toxicity Unit (TU) values indicating low toxicity with a predominant effect on root growth inhibition. Among them, method AB produced the most stable response ($EC_{50} \approx 433 \mu\text{g/mL}$; $TU = 0.231$) and was therefore selected for subsequent toxicity screening of the derived fractions. Liquid–liquid partitioning of the CEE yielded HF, CF, and EAF fractions, of which the EAF exhibited the highest toxicity ($TU = 0.50$). The EAF fraction was further subjected to column chromatography, qualitative color reactions, and TLC–MS/MS analysis, leading to the identification of oleandrin and adynerin as the two major cardiac glycosides, with adynerin being predominant, consistent with the toxicity screening results. The integration of rapid root-based toxicity screening and TLC–MS/MS analysis demonstrates a promising approach for toxicity-guided identification of bioactive compounds from *Nerium oleander* L. extracts, providing a scientific basis for ecological toxin studies as well as plant waste management of this toxic species.

Keywords: *Nerium oleander* L.; *Allium cepa* L. model; Toxicity-guided screening; TLC–MS/MS; Cardiac glycosides.

1. INTRODUCTION

Nerium oleander L. (‘Trúc đào’ in Vietnamese) is a widely cultivated ornamental plant containing highly toxic cardenolide-type cardiac glycosides [1]. Pruning generates large amounts of plant waste, particularly dry leafless branches that become difficult to recognize and may be mistaken for firewood or other biomass, increasing the risk of accidental exposure, misuse, or improper integration into waste treatment chains (e.g., uncontrolled composting or burning), posing poisoning risks to humans, animals, and the environment [2]. Therefore, establishing a simple and rapid approach for early ecotoxicity detection to support the identification of toxicants in landscape pruning waste is essential for poisoning alerts, risk management, and the safe handling of biomass from this species. The *Allium cepa* L. (onion) root assay is widely used for rapid toxicity screening due to its simplicity, sensitivity, and low cost. Two main experimental approaches are commonly applied: (A) pre-rooting followed by extract exposure, and (B) direct exposure from the onset [3-5]. Approach B is typically used in environmental or field-related ecotoxicity studies, whereas approach A is more suitable for laboratory toxicity assessment and bioactivity-guided identification due to standardized initial root conditions [3-6]. Using approach A, early work by Tarkowska (1971) and more recent studies by Çilesizoğlu et al. (2022) demonstrated cytotoxic and genotoxic effects of aqueous, ethanol, or methanol extracts of *Nerium oleander* L. leaves and flowers in the *Allium cepa* L. model, with cellular alterations not fully reversible after transfer to pure water [4, 5]. These findings indicate the persistent cytotoxicity of water-soluble glycosides in

oleander and highlight the need for effective management of this waste category [2, 4, 5]. However, *Allium cepa* L. toxicity assays typically require long testing periods (> 72 h), and studies on extracts from dry leafless branches of *Nerium oleander* L. remain lacking. Moreover, an integrated approach combining the *Allium cepa* L. model with chemical analyses (e.g., TLC and LC–MS) to directly link biological responses with toxic compound identification has not yet been explored. Therefore, this study investigated the toxicity of extracts from dry leafless branches of *Nerium oleander* L. collected in Vietnam. The aims were to select a suitable assay to shorten toxicity detection time and to perform toxicity-guided screening coupled with TLC–LC/MS analysis to identify toxin-containing fractions, contributing to an integrated strategy for phytotoxicological assessment and risk management of biomass waste from *Nerium oleander* L. and other poisonous ornamental plants.

2. MATERIALS AND METHODS

2.1. Materials

Branches of *Nerium oleander* L. were collected in Bac Ninh province (formerly Bac Giang), Vietnam. After washing and drying below 45 °C to a constant weight, leaves were removed and the dry leafless branches were ground into a fine powder. A total of 100 g of the powder was used for extraction.

Biological Material: Purple onion bulbs (*Allium cepa* L.) of uniform size were selected, ensuring they were non-germinated and free of mechanical damage.

Chemicals and Reagents: Ethanol (90%), n-hexane, chloroform, and ethyl acetate were purchased from Fisher Scientific. HPLC-grade methanol and acetonitrile were obtained from Merck (Germany). All other chemicals were of analytical grade.

Instrumentation: Ultrasonic bath, rotary evaporator (extraction); analytical balance (weighing); separatory funnels, silica gel columns, TLC plates (separation); glass beakers, precision caliper (bioassay and root measurement); Sciex Triple Quad 4500+ LC–MS/MS (compound identification).

2.2. Research methods

2.2.1. Extraction method

100 g of dried plant material was macerated in 600 mL of 90% ethanol for 24 h and ultrasonicated for 30 min at 30 °C, then filtered and evaporated under reduced pressure to obtain 20 mL of crude ethanol extract (CEE) [6].

2.2.2. Liquid-liquid partitioning

CEE was sequentially partitioned with n-hexane, chloroform, and ethyl acetate (3 × 20 mL each). The organic layers were concentrated under reduced pressure to obtain the n-hexane (HF), chloroform (CF), and ethyl acetate (EAF) fractions [5, 6].

2.2.3. Toxicity-guided screening on allium cepa roots

Onion bulbs (1.5 - 2.0 cm in diameter) were pre-germinated in distilled water at room temperature (25 ± 2 °C). After 2–4 days, five bulbs with uniform root growth were selected as biological replicates for each treatment and control group (n = 5), following the standardized *Allium cepa* L. assay protocol [3]. CEE was tested at 50, 100, 200, 400, and 800 µg/mL using three exposure methods: Method A (48 h pre-rooting in distilled water followed by 24 h exposure), Method B (continuous exposure for 72 h), and Method A+B (rooting in distilled water until roots reached 1–2 cm, then 48 h exposure) [4, 5]. The evaluated parameters included root length (cm) and growth inhibition rate (GI, %), calculated as $GI (\%) = (1 - L_{test}/L_{control}) \times 100$, where L_{test} and $L_{control}$ are the mean root lengths of the treated and control groups, respectively. The EC_{50} value (50% inhibition of root elongation) was determined by linear interpolation between the two concentrations surrounding the 50% inhibition level.

2.2.4. Toxicity classification based on toxic units

The Toxic Unit (TU) index was calculated as $TU = 100/EC_{50}$ (EC_{50} expressed in mg/L or $\mu\text{g/mL}$) according to the environmental toxicity classification proposed by Persoone et al. (2018). Toxicity levels were defined as: $TU < 0.4$ (Class I, non-toxic); $0.4 < TU < 1.0$ (Class II, low toxicity); $1.0 < TU < 10.0$ (Class III, toxic); $10.0 < TU < 100.0$ (Class IV, very toxic); and $TU > 100$ (Class V, extremely toxic) [7].

2.2.5. Purification and chemical analysis [6, 8]

Toxic constituents were purified by silica gel column chromatography with chloroform:methanol (98:2, v/v). Sub-fractions (9 mL) positive to glycoside reagents were pooled, concentrated, and further purified by preparative TLC. TLC was developed with chloroform:methanol (98:2, v/v) and visualized by dipping plates in 5% H_2SO_4 followed by heating at 100 °C. Cardiac glycosides appeared as brown, light purple, or deep pink spots and were recovered for LC–MS/MS analysis. LC–MS/MS analysis was performed on a Sciex Triple Quad 4500+ using a C18 column (100 × 2.1 mm, 2.7 μm) with mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in ACN), injection volume 5 μL , and flow rate 0.3 mL/min. MS conditions were MRM (ESI+), CUR 35, collision gas N_2 (High), IS 5500 V, TEM 550 °C, GS1/GS2 50. Gradient: 10% B (1 min) → 90% B in 3 min → hold 4 min → return to 10% B in 2 min.

2.2.6. Statistical analysis

Data are presented as mean \pm SD. Effects of treatment method (A, B, AB) or extract fraction (HF, CF, EAF) and concentration on root length were analyzed by two-way ANOVA. Significant effects of factors and their interaction were observed ($p < 0.001$). Tukey's test was used for multiple comparisons ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Crude extraction of dry leafless branches of *Nerium oleander* L. and bio-guided toxicity screening on *Allium cepa* L., root growth

The phytotoxic effects of CEE from dry leafless branches of *Nerium oleander* L., were evaluated through GI. The results are summarised in Table 1.

Table 1. GI and EC_{50} values of CEE in relation to different exposure methods.

Method	Parameter	Concentration ($\mu\text{g/mL}$)						EC_{50} ($\mu\text{g/mL}$)	TU
		0	50	100	200	400	800		
A	Root length (cm)	4.32 \pm 0.21 ^{aA}	3.95 \pm 0.17 ^{aB}	3.62 \pm 0.19 ^{aC}	3.11 \pm 0.15 ^{aD}	2.61 \pm 0.14 ^{aE}	2.22 \pm 0.13 ^{aF}	> 800	< 0.125
	GI (%)	–	8.6	16.2	28.0	39.6	48.6	–	–
B	Root length (cm)	4.28 \pm 0.18 ^{aA}	3.52 \pm 0.20 ^{bB}	3.05 \pm 0.16 ^{cC}	2.46 \pm 0.14 ^{cD}	1.92 \pm 0.12 ^{cE}	1.48 \pm 0.11 ^{cF}	319	0.313
	GI (%)	–	17.8	28.7	42.5	55.1	65.3	–	–
AB	Root length (cm)	4.30 \pm 0.19 ^{aA}	3.71 \pm 0.18 ^{abB}	3.29 \pm 0.15 ^{bc}	2.74 \pm 0.13 ^{bd}	2.18 \pm 0.11 ^{bE}	1.82 \pm 0.10 ^{bf}	433	0.231
	GI (%)	–	13.7	23.5	36.3	49.3	57.7	–	–

Data are expressed as mean \pm SD ($n = 5$). Different lowercase and uppercase letters indicate significant differences among methods and concentrations, respectively (two-way ANOVA, Tukey's test, $p < 0.05$).

According to the toxicity classification system based on TU, all exposure protocols yielded TU values below 0.4 (0.125–0.313), corresponding to Class I (non-toxic). Two-way ANOVA revealed that root elongation was significantly affected by concentration, exposure method, and their

interaction ($p < 0.001$), indicating that the inhibitory response was both dose-dependent and method-dependent. Post hoc comparisons confirmed that no significant differences were observed among methods under control conditions, whereas from 100 $\mu\text{g/mL}$ onward, significant differences emerged among protocols, with a consistent ranking of inhibitory strength (Method B > Method AB > Method A). Within each method, all tested concentrations differed significantly from one another, confirming a clear concentration-dependent reduction in root length. Thus, CEE does not induce acute phytotoxicity but rather exerts a statistically significant, concentration-dependent inhibitory effect on root growth. This conclusion is generally consistent with the findings of Çilesizoğlu et al. (2022), who also reported a dose-dependent reduction in root length and growth parameters in *Allium cepa* L. exposed to *N. oleander* extracts. Therefore, both studies demonstrate that ethanol extracts of *N. oleander* affect root development in the *Allium cepa* L. model.

Although the same CEE sample was used, different exposure protocols generated distinct EC_{50} and TU values. Method B was identified as the most sensitive, with an EC_{50} of approximately 319 $\mu\text{g/mL}$ and a TU of 0.313, and was associated with pronounced root stunting and morphological alterations at higher concentrations. In contrast, Method A exhibited minimal impact ($\text{EC}_{50} > 800 \mu\text{g/mL}$; $\text{TU} < 0.125$) and showed no evident structural abnormalities. The modified integrated protocol (Method AB) displayed an intermediate response ($\text{EC}_{50} \approx 433 \mu\text{g/mL}$; $\text{TU} = 0.231$), characterized primarily by reduced root elongation without signs of necrosis even at the highest tested concentration. Although Method B demonstrated the highest sensitivity, Method AB was selected for subsequent phytotoxicity screening in the *Allium cepa* L. model because it provides a balanced and comprehensive assessment, capturing measurable growth inhibition while avoiding excessive morphological damage that could confound further fraction-based evaluations.

3.2. Comparative phytotoxicity of *N. oleander* fractions using method AB

Liquid–liquid partitioning of CEE produced three fractions: HF (0.25 g), CF (0.45 g), and EAF (0.62 g), reflecting polarity-dependent phytochemical distribution in *N. oleander* branches. These fractions were evaluated using Method AB (Table 2).

Table 2. Phytotoxic activities of *N. oleander* fractions evaluated by method AB.

Conc. ($\mu\text{g/mL}$)	<i>N. oleander</i> fractions			GI - HF (%)	GI - CF (%)	GI - EAF (%)
	HF (cm)	CF (cm)	EAF (cm)			
Control	4.30 \pm 0.19 ^{aA}	4.30 \pm 0.19 ^{aA}	4.30 \pm 0.19 ^{aA}	100.0	100.0	100.0
50	3.98 \pm 0.17 ^{aB}	3.76 \pm 0.16 ^{bB}	3.28 \pm 0.15 ^{cB}	93.1	88.2	76.4
100	3.85 \pm 0.16 ^{ac}	3.31 \pm 0.15 ^{bc}	2.84 \pm 0.14 ^{cc}	90.4	78.5	65.8
200	3.61 \pm 0.15 ^{aD}	2.89 \pm 0.13 ^{bD}	2.32 \pm 0.12 ^{cD}	84.7	63.6	52.3
400	3.28 \pm 0.14 ^{aE}	2.44 \pm 0.12 ^{bE}	1.88 \pm 0.11 ^{cE}	71.2	46.8	24.6
800	2.98 \pm 0.13 ^{af}	2.07 \pm 0.11 ^{bf}	1.41 \pm 0.10 ^{cf}	58.9	33.5	15.8
EC_{50} ($\mu\text{g/mL}$)	598.2	372.6	201.4	–	–	–
TU (100/ EC_{50})	0.17	0.27	0.50	–	–	–

Data are presented as mean \pm SD ($n = 5$). Different lowercase (rows) and uppercase (columns) letters indicate significant differences among fractions and concentrations, respectively (two-way ANOVA, Tukey’s test, $p < 0.05$).

Two-way ANOVA showed that root elongation was significantly affected by fraction type, concentration, and their interaction ($p < 0.001$). Post hoc analysis confirmed significant differences among fractions at most concentrations ($p < 0.05$), revealing a consistent activity gradient of EAF > CF > HF, in agreement with GI and EC_{50} values.

In accordance with the calculated TU, the fractions derived from the dry leafless branches of *N. oleander* yielded values of 0.17 for HF, 0.27 for CF, and 0.50 for EAF. Following the

established toxicity classification scale, the HF and CF fractions, each characterized by a TU < 0.4, were assigned to Class I (Non-toxic), reflecting a negligible biological impact on the *Allium cepa* L. model. In contrast, the EAF fraction, with a TU = 0.50 falling within the 0.4 < TU < 1.0 range, was categorized as Class II (Slightly toxic). This indicates a more pronounced inhibitory effect on root growth compared to the other two fractions, although it has not yet reached the 'toxic' threshold defined by the TU criteria [7]. Consequently, based on the TU assessment, the HF and CF fractions from *N. oleander* branches are categorized as non-toxic. In contrast, the ethyl acetate (EAF) fraction exhibited the most significant toxic effect and is therefore proposed as the priority candidate for subsequent studies.

3.3. Isolation and TLC-MS analysis for the characterization of glycosidic constituents in the EAF fraction

The ethyl acetate fraction residue (EAF, 0.62 g) was subjected to silica gel column chromatography using chloroform: methanol (98:2, v/v) as the eluent, yielding 23 sub-fractions (9 mL each). Sub-fractions were monitored by TLC using a glycoside-specific reagent. Fractions 6–17 showed characteristic color reactions, confirming glycosides, and were pooled and concentrated to yield 0.12 g of a cardiac glycoside-rich residue for further analysis.

Preparative TLC of the concentrated residue under the same conditions showed glycoside spots ranging from brown to deep pink. A prominent deep pink band at $R_f \approx 0.5$ was identified as the target glycosidic toxin region. The silica gel corresponding to $R_f \approx 0.5$ was collected, desorbed, and prepared for LC-MS/MS analysis to identify cardiac glycosides in the EAF fraction (Figure 1, Table 3).

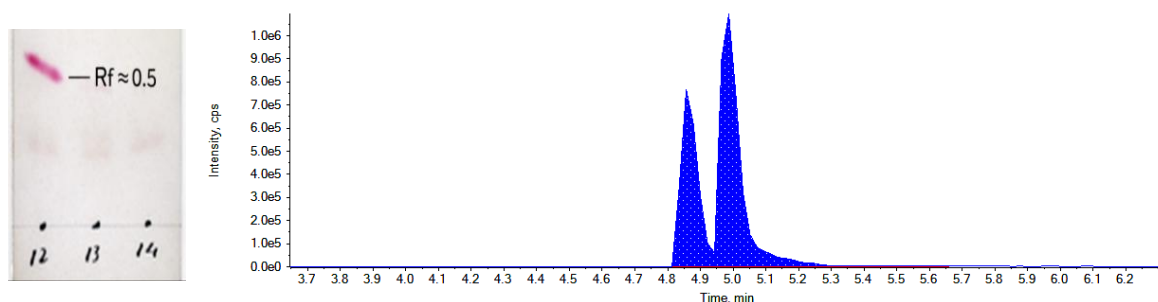


Figure 1. TLC-MS/MS analysis of glycosides:
Left: TLC of sub-fractions 6–17 showing a pink band ($R_f \approx 0.5$);
Right: LC-MS/MS chromatogram with corresponding ions).

Table 3. Tentative LC-MS/MS identification of glycosides in the EAF fraction.

Cardiac glycosides	RT (min)	Precurs or ion (Q1, m/z)	Product ions (Q3, m/z)	Declustering potential (V)	Collision energy (eV)	Intensity (cps)	Analysis Type
Olenandrin	4.98 - 4.99	577.2	373.2	50	20	8.38 x 10 ⁶	Qualitative
			433	50	15		Qualitative
			145	50	25		Qualitative
Adynerin	5.01-5.03	534	372.8	50	15	1.90 x 10 ⁷	Qualitative
			485.4	50	15		Qualitative
			112.5	50	15		Qualitative

The mass spectral data obtained from the analysis of characteristic TLC spots are detailed in Table 4. The parameters for precursor ions (Q1) and product ions (Q3) allow for the precise characterization of the two primary toxins, namely olenandrin and adynerin.

The LC-MS/MS analysis of EAF confirmed the presence of two major cardiac glycosides: oleandrin and adynerin. As shown in the chromatogram (Figure 1), two primary consecutive peaks appeared within the retention time (RT) range of approximately 4.9-5.1 minutes. These peaks align perfectly with the RT values of oleandrin (4.98-4.99 minutes) and adynerin (5.01 - 5.03 minutes) as determined in the MRM (Multiple Reaction Monitoring) data. Specifically, oleandrin was detected at an RT of 4.98-4.99 minutes, exhibiting a characteristic molecular ion at 577.2 m/z and several stable fragment ions (373.2, 433.0, and 145.0 m/z), which is fully consistent with established cardenolide fragmentation databases. Adynerin was identified by its molecular ion at 534.0 m/z and product ions at 485.4, 372.8, and 112.5 m/z at an RT of approximately 5.02 minutes.

Accordingly, the detected cardiac glycosides were tentatively assigned based on MS/MS spectral congruence with established literature data. The observed molecular ion clusters and characteristic fragment ions are in high agreement with the mass spectral descriptions reported in the study by Ying et al (2018), who defined HPLC-MS/MS identification criteria for oleandrin and adynerin [8]. The results presented in Table 3 and Figure 1 show that these compounds are present at significant and stable levels within the ethyl acetate extract (EAF). In this analysis, the second peak (RT \approx 5.02 minutes) exhibited a significantly higher intensity, corresponding to adynerin- the compound with the highest peak area (1.90×10^7 cps). The first peak (RT \approx 4.99 minutes) corresponded to oleandrin, which showed a lower intensity (8.38×10^6 cps). This evidence demonstrates that adynerin is the predominant cardiac glycoside in the EAF derived from dry leafless branches of *Nerium oleander* L. The results, summarized in Figure 1 and Table 3, confirm that the consistency observed in retention times, fragment ion clusters, and high peak intensities provides chemical evidence that aligns perfectly with the toxicity screening and TLC results presented in the preceding sections. Overall, this integrated analytical approach- combining biological screening, chromatographic separation, and LC/MS/MS- proves to be a highly effective and reliable strategy for the tentative identification of toxins from *Nerium oleander* L.

In summary, the results demonstrate that the ethanol extract from dry leafless branches of *Nerium oleander* L. exhibits low phytotoxicity in the *Allium cepa* L. root model, with biological effects primarily expressed as concentration-dependent growth inhibition rather than acute cytotoxic damage. Although cardiac glycosides from *Nerium oleander* L. are well known to induce marked toxicity in animal systems via Na^+/K^+ -ATPase inhibition, the present plant-based assay indicates a milder response, likely associated with oxidative stress-mediated modulation of root elongation, consistent with previous phytochemical-toxicity correlations reported by N. B. Çilesizoğlu et al., [5]. The observed bioactivity was strongly influenced by the exposure protocol, with Method AB providing stable and reproducible responses that accurately reflected the toxic characteristics of the samples, thereby supporting its selection as the most suitable approach for bioassay-guided screening. Among the tested fractions, the ethyl acetate fraction (EAF) exhibited the highest toxicity (TU = 0.50) and was enriched in cardiac glycosides, particularly adynerin, as confirmed by LC-MS/MS analysis. The predominance of adynerin over oleandrin aligns closely with the toxicity ranking and GI/EC₅₀ results. Taken together, the integration of TU-based evaluation, standardized bioassays, and targeted chromatographic-mass spectrometric identification validates the *Allium cepa* L. model as an effective preliminary filter in bioactivity-guided fractionation. This strategy streamlines toxin discovery by limiting costly LC-MS/MS analyses to the most biologically relevant fractions while enhancing the reliability of tentative cardiac glycoside identification.

4. CONCLUSIONS

The study demonstrated that the ethanol extract from dry leafless branches of *Nerium oleander* L. exhibited low toxicity on the *Allium cepa* L. root model, with biological effects primarily characterized by growth inhibition and a clear dependence on the exposure method. The combined

testing method (AB) provided stable responses, accurately reflecting the toxic nature of the samples, and was identified as the most suitable approach for bioassay-guided screening. Among the obtained fractions, the ethyl acetate fraction (EAF) showed the highest toxicity ($TU = 0.50$) and was found to be enriched with cardiac glycosides. In this fraction, adynerin was predominant compared to oleandrin, which is highly consistent with the toxicity screening and LC/MS/MS analysis. These results indicate that using the *Allium cepa* L. model as a preliminary filter is an effective strategy for guiding bioactivity-based fractionation. This approach makes the process of finding toxic compounds faster and more cost-effective by reducing the number of samples that need to undergo expensive LC/MS/MS analysis. Furthermore, combining rapid biological screening with TLC-MS/MS increases the reliability of the tentative identification of cardiac glycosides in *Nerium oleander* L. extracts.

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TÓM TẮT

Sàng lọc độc tính dẫn hướng và nhận diện glycosid trong cặn chiết xuất cành khô rụng lá từ cây trúc đào (*Nerium oleander* L.) thu hái ở Việt Nam

Nghiên cứu thực hiện sàng lọc độc tính dẫn hướng dựa trên mô hình rễ *Allium cepa* L. kết hợp phân tích hóa học. Theo đó, cặn chiết ethanol thô (CEE) từ cành khô rụng lá của cây trúc đào (*Nerium oleander* L.) thu hái tại Việt Nam được đánh giá bằng ba phương pháp (A, B và AB) cho giá trị TU phản ánh độc tính thấp với tác động chủ yếu là ức chế sinh trưởng rễ; trong đó phương pháp AB cho đáp ứng ổn định nhất ($EC_{50} \approx 433 \mu\text{g/mL}$; $TU = 0,231$), được lựa chọn để sàng lọc các phân đoạn tiếp theo. Phân bố lỏng-lỏng cao CEE thu được các phân đoạn HF, CF và EAF, trong đó EAF thể hiện độc tính cao nhất ($TU = 0,50$). Phân đoạn EAF này tiếp tục được sắc ký cột, thử định tính màu và phân tích TLC-MS/MS, qua đó xác định oleandrin và adynerin là hai glycosid tim chủ yếu, với adynerin chiếm ưu thế, phù hợp với kết quả sàng lọc độc tính. Sự kết hợp giữa sàng lọc độc tính nhanh trên rễ hành và phân tích TLC-MS/MS cho thấy đây là cách tiếp cận tiềm năng trong nhận dạng định hướng độc chất từ dịch chiết *Nerium oleander* L., góp phần làm cơ sở cho các nghiên cứu về độc tố sinh thái cũng như quản lý rác thải thực vật từ loài cây độc này.

Từ khóa: Trúc đào *Nerium oleander* L.; Mô hình *Allium cepa* L.; Sàng lọc dẫn hướng độc tính; TLC-MS/MS; Glycosid tim.