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Assessment of anticoagulant potential of *Halocnemum strobilaceum* (Pall.) against black rat (*Rattus rattus* Lin.) from Algeria

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ABSTRACT

Background: Black rats (*Rattus rattus;* Rodentia: Muridae) cause significant damage to agricultural and human health, and they are considered a reservoir for a variety of diseases.
Objectives: The current study aimed to evaluate the toxicity of oral anticoagulants derived from *Halocnemum strobilaceum* (Pall.) M. Bieb. to these dangerous animals.
Method: Four selected doses (100, 150, 200 and 300 mg/day/kg of individual weight) and one control were utilized, with 10 repetitions of each dose (5 ♂ and 5 ♀). The extract was powdered on dates as bait.
Results: UPLC-ESI/MS-MS chromatography identified three distinct coumarins (coumaric acid, *p*-coumaric acid and 4-hydroxycoumarin). The results showed that the fourth dose used caused the highest number of deaths (100 % after three days of treatment). In contrast, after 48 h of therapy, all rats examined showed lethargy, anorexia and unconsciousness, followed by rapid death at 72 h in the treated individuals. The LD_{50} was recorded at 146.4 $mg.kg^{-1}$ with a LT_{50} of 59.37 h. At the end of the experiment, the autopsies of all deceased individuals allowed us to observe the bleeding of their internal organs. The analysis of PT and aPTT demonstrated that the halophyte under investigation possesses anticoagulant activity that increased with the concentration. Histological sections of the liver revealed cellular alteration and significant necrosis. As well, the kidneys had vascular occlusion with an inflammatory filtrate.
Conclusions: To reduce food losses and wastes caused by these pests, *H. strobilaceum* will be an important plant for use in protecting against invasive rodents, especially in environmental and stored product programs focused at eradicating rodent pests.

1. Introduction

Certain pest species, such as rats, constitute a severe threat to agriculture because they can damage crops, break husbandry rules, transfer diseases, and harm the environment (Mlik et al., 2018; 2022; Meddour et al., 2022; Mlik et al., 2024). Anthropization of natural ecosystems and the adaptability of certain euryetic species have caused the latter species to spread almost everywhere in the world (Hassell et al., 2021; Dalecky et al., 2023). The world's top three invasive rodent species include *Rattus norvegicus, R. rattus* and *Mus musculus* (Badou et al., 2023). Due to human migrations and international trade, especially maritime exchanges, they have expanded throughout the world (Badou et al., 2023; Skotnes-Brown, 2023). This has significant consequences for the spread of zoonotic pathogens e.g. *Yersinia pestis*, *Leptospira*, Hantavirus…etc. (Ganjeer et al., 2021; de Cock et al., 2023) and food destruction (Constant et al., 2020; Munawar et al., 2020; Jurišić et al., 2022; Jarzębowska, 2023). Rats and mice, along with their associated ectoparasites and rodent-borne diseases, are frequently found on large ships,

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Table 1
LC/MS detection parameters for three analysed compounds in *H. strobilaceum* (negative and positive ionization mode).

Compound	Charge (+/-)	Precursor (*m/z*)	Product (*m/z*)	Dwell time (msec)	Q1 Pre Bias (v)	CE (v)	Q3 Pre Bias (v)
Coumaric acid	+	165.100	101.200	100	−17	−14	−10
			69.150	100	−18	23	−27
4-hydroxy coumarin	−	160.800	117.100	100	18	22	20
			89.050	100	17	11	16
***P*-coumaric acid**	+	165.100	59.100	100	−12	−17	−22
			119.100	100	18	16	22
	−	162.800	93.150	100	19	31	17

from which they may be involuntarily introduced into seaports and eventually colonize new locations (Kuo et al., 2017; Rahelinirina et al., 2018). Nonetheless, black rats cause significant losses in several plant species, particularly cereals, where they can destroy crops like Meriones. In contrast, Mlik et al. (2017) and Alia et al. (2018) discovered that the black rat caused damage to spars and dates in southeastern Algeria, both in open fields and in storage. They also target domestic animals, particularly chickens and quails, and eat their eggs. Furthermore, they harbor a high community of parasitic arthropods capable of transmitting dangerous diseases to humans and animals (Mlik et al., 2022). Controlling these pests involves numerous strategies, including biological, mechanical, environmental, cultural, and chemical treatments (Constant et al., 2020). Other alternatives, such as plant-based natural extracts, have a diverse spectrum of bioactive compounds with various applications. When explored in a behavioral model, a range of plant extracts, either alone or in combination, can be tested to see if they could reduce rat infestation (Singh and Mirza, 2020). The primary anticoagulants containing coumarin derivatives are now utilized to manage commensal rodents in agricultural and urban environments (Mercer et al., 2022; Khidkhan et al., 2024), particularly in environmental programs to eradicate rodent pests on islands (Samaniego-Herrera et al., 2013; Wheeler et al., 2019), despite having the same mode of action; interference with coagulation factor synthesis, resulting in bleeding and death (Endepols et al., 2015). Moreover, it has been established that employing chemical anticoagulants (such as bromadiolone) to kill rodents has a deleterious influence on their predators-in this case, foxes (Jacquot et al., 2013; Rached et al., 2020).

The current proposal is to evaluate the anticoagulant potential of coumarin derivative with rodent-killing ability caused by vitamin K inhibition (as an anticoagulant) using a fraction of a spontaneous halophyte from south-eastern Algeria, *Halocnemum strobilaceum*, on the roof rat *Rattus rattus*.

2. Materials and methods

2.1. Plant material

The plant material employed in this experiment was *Halocnemum strobilaceum* (Pall. 1819), a species of the Amaranthaceae family, also known as "Grina" locally. In May 2020, this plant's aerial part was collected in the province of Ouargla (32°23'49.22"*N*; 5°22'27.45"*E*) in Southeast Algeria. The plant was crushed after seven days of air-drying at room temperature (25–30 °C). 30 g of the plant's powder was then combined with 100 mL of MeOH/H_2O (20/80 mL: v/v) and macerated for five days. The resulting solution was filtered and evaporated using a rotary evaporator, then liquid–liquid extracted with ethyl acetate (EtOAc) and stored at 4 °C until further analysis and activities. Total phenols, flavonoids, and tannins were analyzed to identify their abundance in this halophyte's extracts.

2.2. High-performance liquid chromatography (HPLC)

The resulting ethyl acetate fraction was analyzed using a Shimadzu LC 20 AL HPLC with a universal injector (Hamilton 25l). The phenolic components of the selected fraction were detected using an analytical column Shim-pack VP-ODS C18 (4.6 mm, 250 mm, 5 m) with a UV–VIS detector type SPD 20A (Shimadzu). The mobile phase (acetonitrile/acetic acid 0.1 % v/v) used gradient elution and the reverse chromatographic phase analyses used non-polar aliphatic residues. The flow rate was 1 mL/min, whereas the injection volume was 0.45 l. The monitoring wavelength was 268 nm, and the sample and standard phase injection volumes were 20 l each. To identify the compounds, their retention times and UV absorbance were compared to the standards.

2.3. Ultra-performance liquid chromatography-mass spectrometry (UPLC/MS-MS)

To optimize standard polyphenols, three coumarins were separated on a SHIMADZU 8040 Triple Quad UPLC/MS with electrospray ionization (ESI). LC featured the binary bump (Nexera XR LC-20AD). Gradient elution was used with the mobile phase consisting of 30 % H_2O, 0.1 % HCOOH and 70 % MeOH with a total flow of 0.2 mL/min and an injection volume of 6 l and positive and negative ion (ESI) proven. In multiple reaction monitoring (MRM) modes. The ionization source was set up with the following parameters: 6000 V for the spray voltage, 250 °C for the source temperature, and 350 °C for the desolvation temperature. The nebulizer gas utilized was nitrogen, with a flow rate of 3 l/min. High-purity nitrogen was under 0.23 MPa of pressure during collision-induced dissociation (CID). Quantification was performed using multiple reaction monitoring (MRM) modes. A summary of MS/MS detection parameters is presented in Table 1.

2.4. Test animals

The current experiment used adult black rats (*Rattus rattus*) weighing between 150 and 180 g. All of the individuals included in the current study were captured by farmers as part of the pest management strategy. For three months, the animals were acclimatized at 25 ± 2 °C and 40 ± 10 % relative humidity, with a 12 h light/ 12 dark cycle to prevent mortality and abnormal behavior. Four doses (100, 150, 200 and 300 mg/day/kg of individual weight) and one control were chosen, with six replicates of each dose (5 ♂ and 5 ♀). The present experiment followed the OECD Guideline 423 (OECD 2001). This work has been authorized by the ethical committee of the University of Kasdi-Merbah in Ouargla.

2.5. Preparation of rodenticide baits

The bait was formulated by removing the pits of dates, adding the extract, and closing it with some crushed dates. Fasting for 24 h before testing was required to verify that these animals consumed treated dates daily. Following administration of the appropriate doses, the animals were monitored at least once during the first 30 min, regularly throughout the first 24 h following treatment, with special attention during the first 4 h, and then daily for the next four days (until individual death). This was done to analyze the animals' general physical state, including mortality rates, toxicological signs, and other symptoms including morphological, physiological, and behavioral changes. Lethal dose and time (LD_{50} and LT_{50}) were determined using the Probit

Table 2
Identified compounds and their retention time by seconds in the *H. strobilaceum* fraction (μg.mg^{-1} extract).

Compounds (μg.mL^{-1})	Retention time	EtOAc fraction (μg.mg^{-1})
Gallic Acid	5.29	0.23
Chlorogenic Acid	13.392	69.99
Caffiec Acid	16.277	8.14
Vanilin	21.46	0.45
***p*-Comaric Acid**	23.817	9.87
Rutin	28.37	2.73
Quercetin	45.047	23.55

method.

2.6. Biochemical analysis

To assess the anticoagulant potential of this extract, biochemical analyses of the two primary factors responsible for blood clotting in the body were undertaken e.g. extrinsic (prothrombin time: PT) and intrinsic pathway (activated partial thromboplastin time: aPTT). Blood samples were brought to the laboratory for analysis. At the end of the experiment, the rats were sacrificed, and liver and kidney tissues were removed for histological examination.

2.7. Statistical data

Statistica v. 10.0 (StatSoft) software was used to interpret the data analysis. The results obtained were presented as mean ± SD and analyzed using parametric tests for normal results (ANOVA) and non-parametric tests (Kruskal-Wallis) for abnormal data when comparing doses. Linear regression was performed to correlate the coagulation factors (PT and aPTT).

3. Results

3.1. Chemical characterization

The obtained results indicated that the phenolic contents of *H. strobilaceum*'s ethyl acetate fraction were 17.1 ± 0.013 mg.g^{-1} DW. In contrast, flavonoids had a value of 12 ± 0.2 mg.g^{-1} DW and tannins had a value of 28.5 ± 0.01 mg.g^{-1} DW.

3.2. HPLC and UPLC/MS analyses

Table 2 lists the phenolic chemicals that HPLC identified in the *H. strobilaceum* fraction.

HPLC analyses showed seven phenolic components in the ethyl acetate fraction of *H. strobilaceum*: gallic acid, chlorogenic acid, vanillin, *p*-coumaric acid, rutin, and quercetin. (Table 2, Fig. 1).

Depending on the flavonoid content of the EtOAc fraction, a multiple reaction mode analysis (MRM) was used to identify coumarin derivatives having anticoagulant characteristics. Fig. 2 depicts the UPLC/MS chromatographic profiles for detecting and quantifying the three separated coumarins are shown in.

The analytical approach was used to evaluate the EtOAc fraction of *H. strobilaceum* coumarin derivatives. The MS spectra revealed the molecular weights of the discovered substances (Fig. 2). *P*-coumaric acid exhibited the largest molecular mass in both positive and negative ionization modes, followed by coumaric acid and 4-hydroxycoumarin. Fig. 3 depicts the chemical structures of the identified coumarins, as well as their associated MS spectra, to aid comprehension. The molecular weights of the compounds displayed in the spectra are as follows:

- 4-Hydroxycoumarin: Molecular weight: [162.14 g/mol], MS spectrum shown in Fig. 2A.
- *P*-coumaric acid: Molecular weight: [164.0473 g/mol], MS spectrum shown in Fig. 2B.
- Coumaric acid: Molecular weight: [164.0473 g/mol], MS spectrum shown in Fig. 2C.

The correlation between the chemical structures and their MS spectra is crucial for understanding the molecular characteristics and behaviors of these molecules. By clearly indicating the molecular weights in the spectra, they provide a comprehensive analysis that enhances the reproducibility and reliability of our findings.

3.3. Efficacy of H. strobilaceum fraction on the black rats

The current study showed that the EtOAc fraction of this halophyte has an important effect on black rats, with the fourth dose resulting in the maximum number of deaths (Fig. 4). None of the control rats died, implying that the expected effective dose of 300 mg.kg^{-1} for three days was sufficient to cause 100 % mortality in black rats. Statistical analysis revealed a very highly significant difference ($p = 0.0041$) between doses in the EtOAc fraction.

Overall and after 48 h of treatment, all rats examined showed lethargy, anorexia, and unconsciousness, followed by sudden death after 72 h of treatment. Females were mainly impacted by these treatments, and after the first dose, no mortality was observed for 72 h, with females always dying before males (Fig. 4). On the other hand, the third and fourth doses showed that females had a higher all-cause mortality after 72 h than males (Fig. 4). At the end of the experiment, an autopsy of all the dead individuals allowed us to observe that their internal organs were all bleeding (Fig. 5).

To establish the effect of the EtOAc fraction on internal organs, an anticoagulant factor analysis (PT and PTT) was performed. The analysis

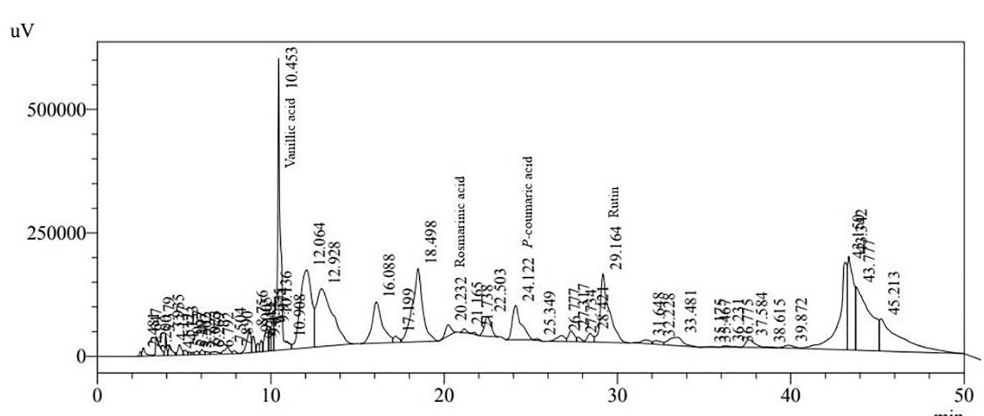

Fig. 1. HPLC profile of the ethyl acetate fraction of *H. strobilaceum.*

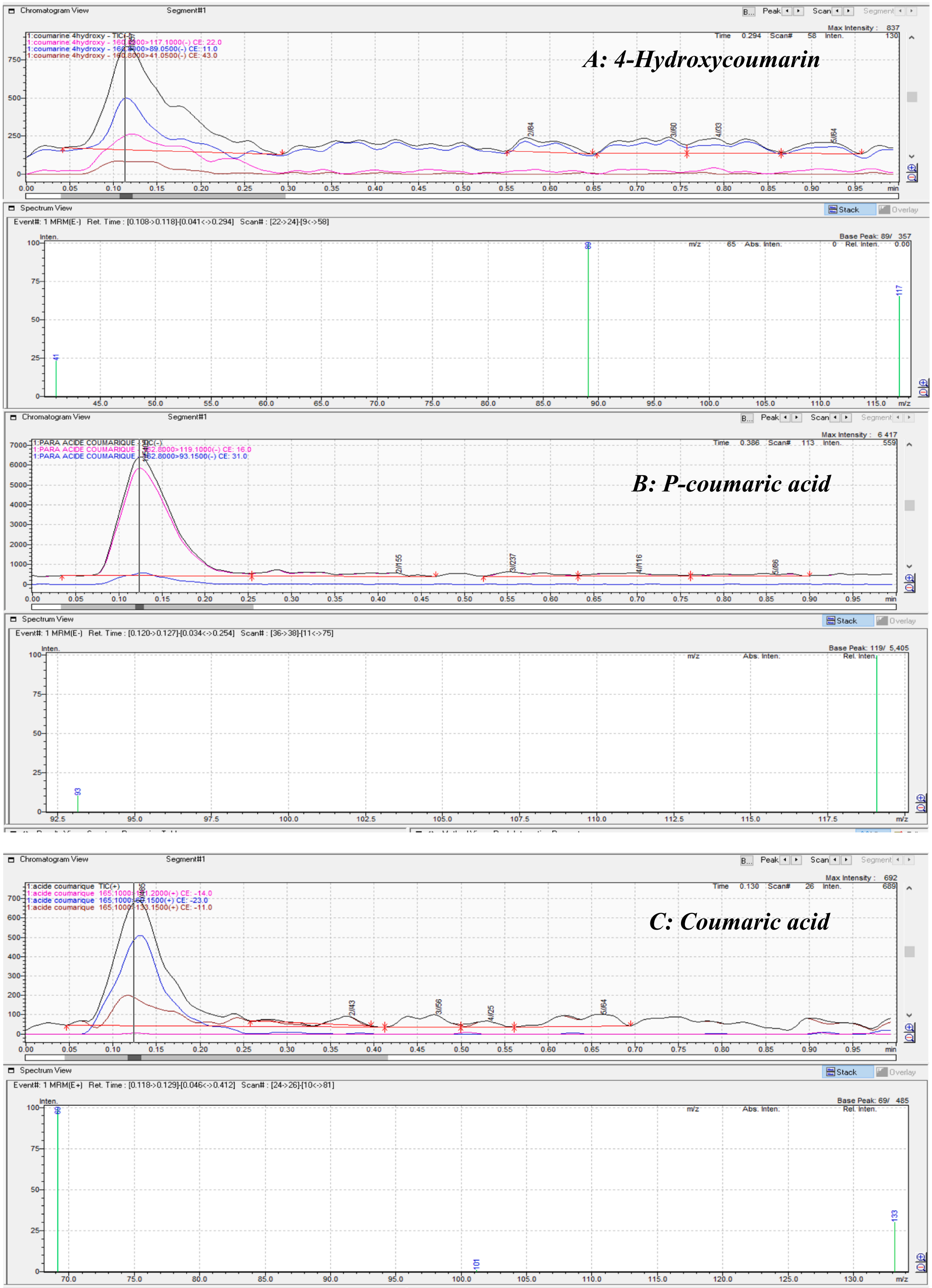

Fig. 2. UPLC/MS chromatography profiles of coumarins detected in *H. strobilaceum* fraction.

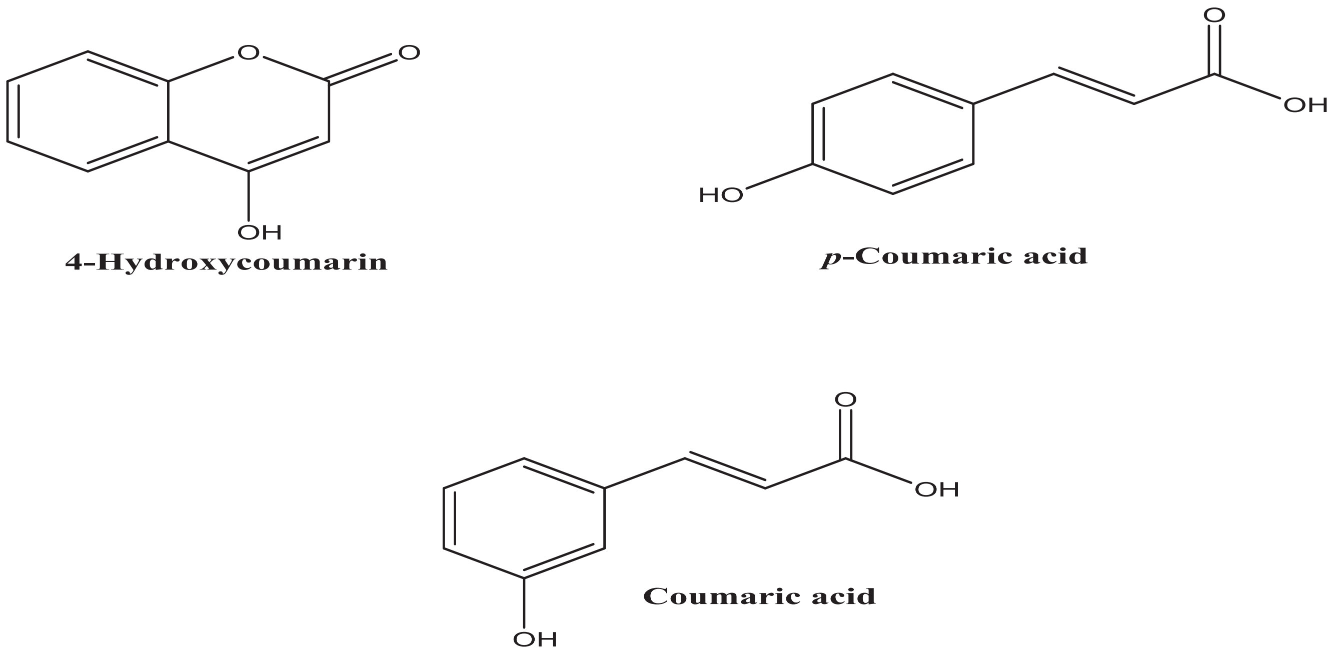

Fig. 3. Chemical structures of the detected coumarins in *H. strobilaceum* fraction.

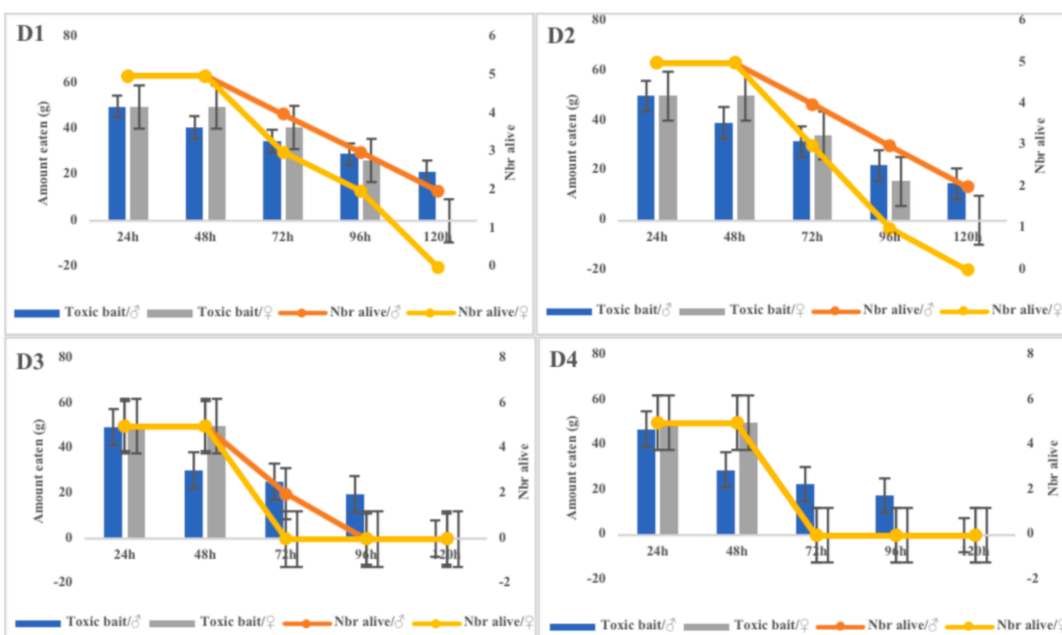

Fig. 4. Efficacy of EtOAc fraction of *H. strobilaceum* in black rats.

of intrinsic and extrinsic pathway factors (PT and aPTT) revealed that this plant's anticoagulant activity increased with concentration. The fourth dose had the highest values (PT=144.0 ± 0.62″ and aPTT=125.3 ± 0.80″), indicating that the aPTT increased with the PT (Fig. 6). There was a correlation between these two factors (Fig. 6). This indicated that increasing or reducing the PT value influenced the aPTT. The Probit method indicates that the EtOAc fraction had a lethal dose of 146.4 mg. kg^{-1}. Similarly, the lethal time (LT_{50}) indicated that 50 % of the black rat population could be eradicated in 59.37 h.

3.4. Histological section

The liver is the primary tissue target for rodenticide persistence since all anticoagulants work by inhibiting the synthesis of blood clotting factors. The obtained PT and aPTT analysis results were then validated by histological sections. While the sections of the liver mentioned below showed cellular change, significant necrosis, and cellular infiltration, inflammation of the portal vein space was associated with peliosis in the blood vessels, and the hepatocytes increased in size and their cytoplasm was cleared, as viewed during the destruction process (Fig. 7). Furthermore, all treated rats kidneys showed vascular blockage with an inflammatory filtrate (Fig. 8).

4. Discussion

As the first report of *H. strobilaceum* as a rodenticide, the current

Fig. 5. Effect of *H. strobilaceum* fraction on blood coagulation of *R. rattus* (**A:** control, **B:** treated rat (bleeding internal organs).

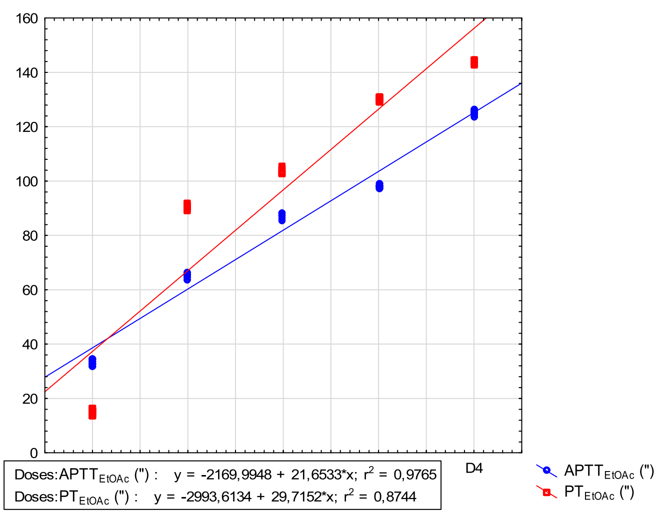

Fig. 6. Correlation between PT and aPTT of EtOAc fraction of *H. strobilaceum.*

experiment enabled us to detect three distinct coumarins using UPLC-ESI/MS-MS chromatography (coumaric acid, *p*-coumaric acid, and 4-hydroxycoumarine). The rodenticide tests revealed that the fourth dose caused the highest number of deaths (100 % after three days of treatment). In contrast, after 48 h of therapy, all rats evaluated displayed lethargy, anorexia, and unconsciousness, followed by sudden death at 72 h. The LD_{50} was 146.4 mg.kg^{-1}, with a LT_{50} of 59.37 h. At the end of the experiment, the autopsies on all deceased individuals allowed us to observe the bleeding of their internal organs. Our findings on the composition of this plant were comparable to those reported by Abdel-Razek et al. (2020) and Gheraissa et al. (2023). Furthermore, various research has demonstrated the phenolic compounds richness of this halophyte, with Handoussa et al. (2019) showing that the ethyl acetate extract of the aerial part of *H. strobilaceum* has a phenolic content of 29.42 mg GAE/g DW. Furthermore, Gheraissa et al. (2023) reported that the hydro-methanolic extract of this plant has 24.97 ± 0.09 μg GAE/g DW as polyphenol content, and 12.17 ± 0.16 and 5.43 ± 0.06 μg QE/g DW as total flavonoid and flavanols content, respectively. On the other hand, coumarins are benzopyrone molecules that belong to the flavonoid group of phytochemicals, with over 1300 discovered in plants, bacteria, and fungi (WHO, 2020; Tsivileva et al., 2022). Coumarins have distinct antiviral, antimicrobial, antioxidant, anti-inflammatory, antiadipogenic, cytotoxic, apoptosis, antiproliferative, cytotoxic, antituberculous, and anticoagulant properties (Umashankar et al., 2015; Bouhaoui et al., 2021). Coumarins and their derivatives have grown in importance in synthesis and production because of their diverse pharmacological properties, and they have later been employed in rodenticide formulations. As bait avoidance or aversion, rodents, particularly rats (*Rattus* spp.), are smart enough to link the emergence of uncomfortable health symptoms with the new food source. Consequently, anticoagulant rodenticides have a long history of effective use because of their beneficial properties, which include a significant delay between the lethal dose and the onset of symptoms after ingestion (Howard et al., 2020). The current study demonstrated the interesting potential of this halophyte to inhibit Vit-K of the tested rodents due to its anticoagulant-killing activity, with all-cause mortality observed at 72 h at the highest doses. Bates (2016) validated our finding, stating that anticoagulant toxicoses develop progressively, causing anorexia, hemorrhage, shock, unconsciousness, and eventually death. Furthermore, Aleksandrov et al. (2024) highlighted that anticoagulant rodenticides (ARs) compete with vitamin K (VK), which is essential for the synthesis of blood clotting factors in the liver, resulting in blood coagulation inhibition and, in many cases, animal mortality due to hemorrhage. Moreover, PT and aPTT analysis confirmed the influence of the ethyl acetate fraction of *H. strobilaceum* over the control, whereas blood coagulation increased with treatment concentration. Additionally, the prolongation of PT and aPTT implied inhibition of both the extrinsic and intrinsic coagulation pathways. All anticoagulant rodenticides work in the same way, the active anticoagulant stops the epoxide reductase enzyme from being active when a rodent eats the bait, which stops activated vitamin K from being recycled (Silverman, 1980). Nevertheless, reducing the biological availability of vitamin K prevents these vital components from being synthesized, which in turn prevents the coagulation process. Given that prothrombin is essential to both the intrinsic and extrinsic coagulation cascades, this suggests that the chemical is a highly effective anticoagulant medication (Boron and Boulpaep, 2016). Thus, the presence of coumarin derivatives in this plant, i.e. 4-hydroxycoumarin, *p*-coumaric acid, and coumaric acid, explains the anticoagulant activity reported in the rats tested. Furthermore, coumarin derivatives are known for their anticoagulant properties (inhibiting vitamin K), as are 4-hydroxycoumarin and its derivatives, which are commonly employed in anticoagulant rodenticides and antithrombotic medicines). These anticoagulants, which target vitamin K 2,3-epoxide reductase in liver microsomes, are antagonists of vitamin K (Kasperkiewicz et al., 2020; Ramsis et al., 2023). This explains the severe damage observed in the black rats' internal organs such as the liver and kidney, as well as the sudden death of treated rats, given that the liver is vital in maintaining the balance between thrombosis and hemorrhage. Moreover, a high PT indicates significant liver diseases or cirrhosis. It could also mean that there is a higher danger of internal bleeding. In addition, Flores-Morales et al. (2023) demonstrated that coumarins are quickly absorbed, dispersed throughout the body, and found in the liver and kidneys in high concentrations.

5. Conclusions

The actual experiment and the acquired data revealed that the EtOAc fraction of *H. strobilaceum* had strong anticoagulant activity. These findings were supported by the extension of clotting times (extrinsic and intrinsic pathways) and substantial damage to internal organs such as the liver and kidney of black rats. Consequently, *H. strobilaceum* emerges as a significant plant for protecting native fauna against invasive rodents, making it particularly useful in environmental and stored product programs aimed at eradicating rodent pests. Future studies should include detailed mechanistic investigations to understand the specific biochemical pathways involved in comprehensive toxicological assessments to ensure the safety of non-target species, large-scale field trials to assess practical effectiveness, and comparative studies with other

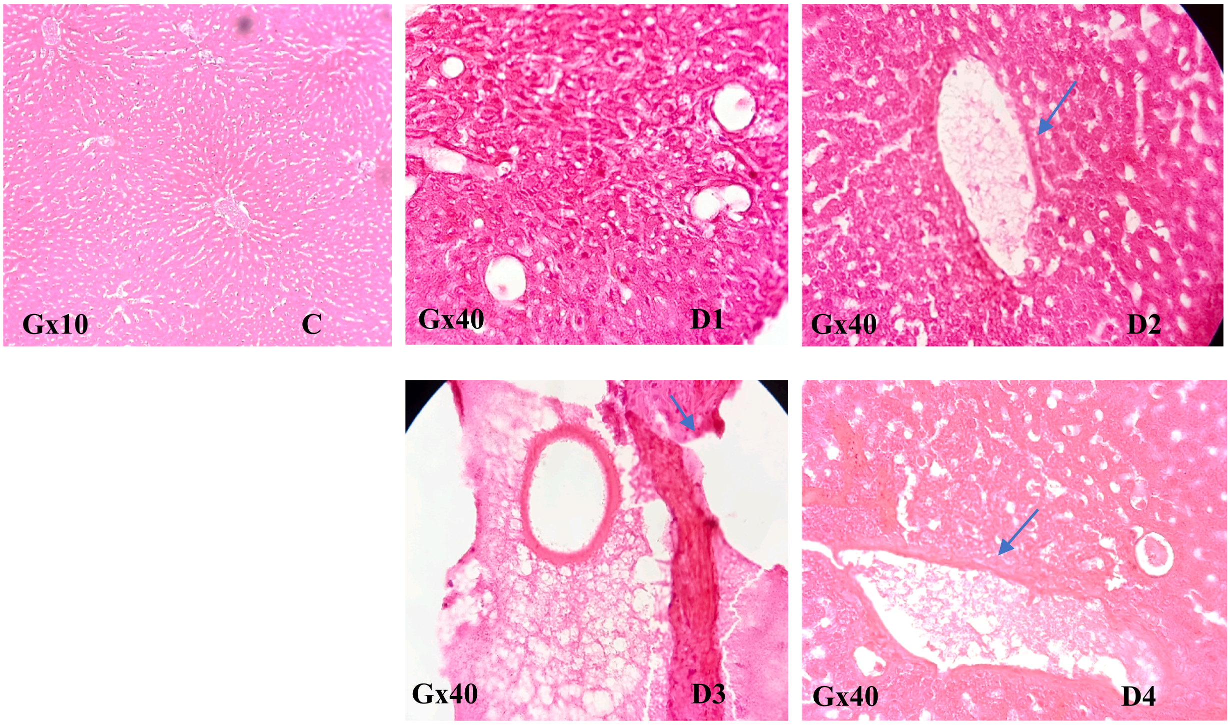

Fig. 7. Effect of EtOAc fraction of *H. strobilaceum* on the liver of treated black rats compared to control.

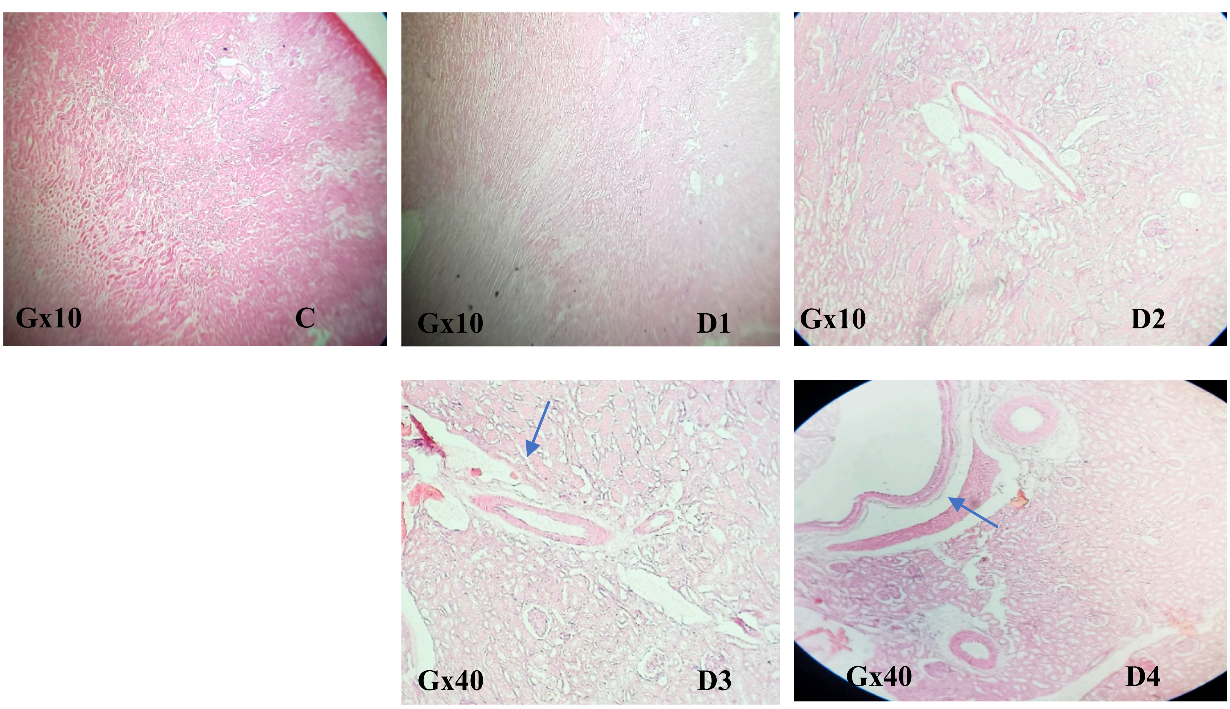

Fig. 8. Effect of EtOAc fraction of *H. strobilaceum* on the kidney of treated black rats compared to control.

anticoagulant plants and commercial rodenticides. Additionally, isolating and characterizing individual active compounds within the EtOAc fraction, as well as investigating the long-term impact on rat populations and resistance development will help to understand *H. strobilaceum's* potential as an eco-friendly rodent control agent.

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CRediT authorship contribution statement

Randa Mlik: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Salim Meddour:** Writing – review & editing, Validation, Methodology, Data curation. **Nour Elhouda Mekhadmi:** Writing – review & editing, Methodology. **Abdellah Henni:** Writing – review & editing, Methodology, Formal analysis. **Walid Boussebaa:** Methodology, Formal analysis, Data curation. **Asma Abid:** Writing – review & editing, Methodology, Formal analysis. **Amar Eddoud:** Writing – review & editing, Validation, Methodology, Formal analysis. **Makhlouf Sekour:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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