Vol. 1 / Issue 1 / 2024

FEZ MULTIDISCIPLINARY JOURNALS

Journal of Biology and Biomedical Research

ISSN: 3009-5522

Journal homepage: https://fmjpublishing.com/index.php/J2BR

Research article

Phytochemical characterization, acute toxicity and hemolytic activity of *Cotula cinerea* (Del.) aqueous and ethanolic extracts

Abdelkrim Agour [a*], Ibrahim Mssillou [a], Youness El Abdali [b], Amina Bari [b], Badiaa Lyoussi [a], Elhoussine Derwich [a]

[a] *Laboratory of Natural Substances, Pharmacology, Environment, Modeling, Health & Quality of Life (SNAMOPEQ), Faculty of Sciences Dhar El Mahraz, Sidi Mohamed Ben Abdellah University, Fez 30000, Morocco*

[b] *Laboratory of Biotechnology, Environment, Agri-food and Health, Faculty of Sciences Dhar El Mahraz, Sidi Mohammed Ben Abdellah University, Fez 30050, Morocco*

*Corresponding author: Abdelkrim Agour (abdekrimagour1@gmail.com)

Abstract

The toxicity of a plant extract is important in the design of medicinal plant-based drugs. A test of the hemolytic effect of a substance on red blood cells (RBC) is an essential starting point. The objective of this study is the phytochemical characterization of the aqueous extract (CCA) and the ethanolic extract (CCE) from the aerial parts of *Cotula cinerea* (*C. cinerea*), by LC-TOF-MS, to evaluate their acute toxicity at two different doses (1000 and 2000 mg/kg), and to assess their hemolytic activity against RBC of rat. For the acute toxicity assessment, statistical analysis revealed no significant differences in body weight between treated and control groups over a 14-day period. Liver and kidney weights also showed no significant variation between treated and control groups. Biochemical parameters such as ASAT, ALAT, urea, and creatinine showed similar levels in all groups, with slight variations observed for some parameters. In the hemolytic test, CCE concentrations showed a significantly higher percentage of hemolysis, whereas CCA had no hemolytic effect on RBC of rat. These data suggest the non-toxic effect of CCA, making it suitable for the preparation of medications involved in the treatment of various diseases. However, the CCE exhibits a concentration-dependent hemolytic effect. This underscores the dual nature of plant secondary metabolites. Further research is needed to understand the complex mechanisms behind these effects and to harness the therapeutic potential of plant extracts while mitigating undesirable hemolytic reactions.

Keywords: *Cotula cinerea*; LC-TOF-MS; Hemolytic activity; Acute toxicity; Erythrocytes

Citation: Agour, A.; Mssillou, I.; El Abdali, Y.; Bari, A.; Lyoussi, B.; Derwich, E. Phytochemical characterization, acute toxicity, and hemolytic activity of *Cotula cinerea* (Del.) aqueous and ethanolic extracts. *Journal of Biology and Biomedical Research*. 2024, 1(1), 70-79. https://doi.org/10.69998/j2br5

Edited by: Allali Aimad

1. Introduction

Between 35,000 and 70,000 plant species are utilized for medicinal purposes globally (Schippmann et al., 2002; Hamilton, 2004), with botanical families such as Asteraceae, Lamiaceae, Apiaceae, and Fabaceae being predominant (Gras et al., 2021; Fennane & Rejdali, 2016). Approximately 70 to 80% of the world's population relies on traditional medicine for healthcare needs (Sheng-Ji, 2001). Particularly in rural areas, this ancestral medical approach is extensively practiced for primary healthcare, subsistence, and livelihood (Elachouri et al., 2021; Amrati et al., 2021). The global demand for herbal medications is not only significant but also steadily rising (Srivastava, 2000). Estimation suggests that when utilized in appropriate forms and dosages, natural products are generally less harmful than synthetic ones, which frequently entail side effects (Duda-Chodaket al., 2023; Adisa et al., 2014). Despite their pharmacological properties, some plants extract can be toxic and have hemolytic activity.

Hemolysis is used to screen for oxidants or antioxidants, as well as to measure the effects of free radicals and their neutralization by antioxidants (Djeridane et al., 2007). Extracts of certain medicinal plants may contain secondary metabolites that can cause harmful effects, such as cellular toxicity, allergic reactions, and irritation of the gastrointestinal tract, and damage to vital organs such as the heart, liver, and kidneys, as well as the risk of cancer (Nondo et al., 2015; Cordier et al., 2012) reported that extracts of nine of the plants significantly increased haemolysis (p-value < 0.05) compared with phosphate-buffered saline (control). In another study, (Zohra &

Received: 20 April 2024; **Revised:** 27 July 2024; **Accepted:** 18 September 2024; **Published:** 29 September 2024

Fawzia, 2014) reported that at a concentration of 500 µg/mL, *Tamarix aphylla* and *Daphne gnidium* induced 6.568% and 7.060% haemolysis, respectively. Compared to *Morettia canescens*, *Daphne gnidium* and *Tamarix aphylla* exhibited lower toxicity, despite *Morettia canescens* causing a significant hemolytic effect on erythrocyte cell membranes (14.80% hemolysis).

On the other hand, there are medicinal plants that have no hemolytic activity. According to (Zubair et al., 2017), the hemolytic activity of Smilax macrophylla leaves against human erythrocytes was determined and found to be harmless. We also find that the hemolytic activity of four extracts (methanol, water, hexane, and dichloromethane) of *Juniperus oxycedrus* root bark against human erythrocytes was found to be safe, indicating that extracts of these plants can be safely used in pharmaceutical therapies and the food industry (Chaouche et al., 2015).

Cotula cinerea (Asteraceae family) has the synonym *Brocchia cinerea* and several vernacular names including Gartoufa, Chouihiya, Chihia and Robita. Traditionally, it is widely used to treat several of diseases such as colic, cough, diarrhea, and digestive disorders. It is administered through decoction, maceration, infusion, and inhalation. In traditional medicine, it functions as an antiseptic, antipyretic, analgesic, and anti-inflammatory agent, and is utilized in addressing rheumatism. Additionally, it is employed to relieve fever and cough, and applied as poultices for headaches and migraines (Lakhdar, 2018).

The pharmacological properties of *C. cinerea* extracts and essential oils offer diverse benefits. *C. cinerea* crude methanolic extract exhibits potent insecticidal activity against the larval and pupal stages of *Culex pipiens*, likely due to the synergistic or independent action of its components, including flavonoids, phenolic acids, and alkaloids (Demouche et al., 2023). Moreover, the antibacterial potential of *C. cinerea* essential oils demonstrates effective inhibition against *E. coli* and *Staphylococcus aureus*, with inhibitory diameters ranging from 16.70 to 14.64 mm (Mekhadmi et al., 2023). Additionally, *C. cinerea* essential oil shows pain-inhibiting properties (Lakache et al., 2023). Furthermore, the application of aqueous and ethanolic extracts of *C. cinerea* on rats and mice showcases the aqueous extract's ability to alleviate abdominal contortions and enhance the re-epithelialization rate of burn-induced wounds (Agour et al., 2023). Lastly, both *C. cinerea* essential oil and hexane extract exhibit notable cytotoxic effects against RD and Vero cell lines, respectively, indicating their potential as sources of novel antitumor agents (Guaouguaou et al., 2018).

The toxicity of a plant extract is important in the design of medicinal plant-based drugs. A test of the hemolytic effect of a substance on RBC is an essential starting point, as it provides the first indications of the interaction between biological entities at cellular level and active molecules extracted from plants (Ghosh, Biswas et al., 2018). In addition, RBC are directly exposed to any substance absorbed or injected intravenously, making them the main targets of toxic effects (Msengwa et al., 2023). In the sense of assessing the toxicity of plant extracts, we determined the objective of this study to be: to assess the acute toxicity of the aqueous and ethanolic extract of *C. cinerea* aerial parts and, for the first time, to assess their hemolytic effect using RBC of rat.

2. Materials and Methods

2.1. Plants material

During February 2021, a sample of *C. cinerea* (Figure 1) was collected in the province of Tata near the town of Akka, South-east Morocco (Latitude: 29.4053843° N (29N3253121.303m N), Longitude: 8.2705204° W (29N 570773.258m E), Elevation: 569. 000m). A specimen, designated (BC0019220211) is deposited in the herbarium of the Biotechnology, Environment, Agri-food and Health Laboratory of the Dhar El Mahraz Faculty of Sciences, USMBA, Fez.

Figure 1: Aerial parts of *C. cinerea* in flowering stage

2.2. Preparation of C. cinerea extracts

2.2.1. Preparation of CCA by infusion

The aerial parts of the plant under study are air-dried, then ground with a Waring® blender. 100 g of the powder is added to 1 L of boiling water. After 20 minutes, the mixture is filtered using Whatman n°1 paper. The filtrate is then dried in an oven at 37°C (Agour et al., 2022).

2.2.2. Preparation of CCE by maceration

To prepare CCE, 100 g of powder was macerated in 1 L of hydroethanolic solution (700 mL ethanol and 300 mL distilled water). Maceration is carried out for 48 hours at room temperature. Preparations are filtered using Whatman n°1 paper, then the solvent is evaporated at 37°C in an oven (Mssillou et al., 2022).

2.3. LC-TOFMS analysis of CCA and CCE

CCA and CCE are analyzed by CL-TOFMS using the method described by Ojanperä et al. (2006), with a few modifications. Samples are dissolved in 1 mL acetonitrile (ACN) and vortexed for 10 s. They are then left to stand for 30 min and vortexed again for 10 s. 200 µL of extract is taken and diluted to 2 mL with ACN. The resulting solutions are filtered (CLARIFY-PTFE 13 0.45 um syringe filter) into HPLC vials. A blank is treated in the same way as the sample. The ACN used in all these experiments is Honeywell/Riedel-de Haën Chromasolv HPLC Gradient. The instruments are Agilent 1200 HPLC with in-line diode array detector coupled to a Bruker Esquire 3000+ ion trap mass spectrometer with electrospray ionization.

HPLC eluent A was Milli-Q water with 0.1% (v/v) formic acid. Eluent B was acetonitrile with 0.1% (v/v) formic acid (AnalaR NORMAPUR). The gradient is: 1 min 1% B, 30 min linear gradient from 1% B to 99% B, maintain 99% B until 40 min. Injection volume was 20 µL. Eluent flow rate was 0.7 mL/min. Column temperature 30°C. The column is an Agilent C18, 4.6 mm ID, 250 mm long.

The mass analyzer is a Bruker Daltonics MicrOTOF MS with an electrospray ionization (ESI) source and a six-port bypass valve (Bremen, Germany). The instrument is controlled using HyStar 3.1 and micrOTOF Control 1.1 software (Bruker Daltonics). The nominal resolution of the instrument is 10,000. The instrument operated in negative ion mode at m/z 50-1000. Capillary voltage was 5000 V and capillary output was -90 V. Nebulizer gas pressure was 0.7 bar and drying gas flow rate was 6.0 L/min. The drying temperature is 250°C. Transfer time is 8.0 µs and hexapolar RF is 120 Vpp hexapolar is 120 Vpp.

2.4. Acute toxicity test

Figure 2: *C. cinerea* extracts prepared for oral administration. (BA1: CCA (1000 mg/mL); BE1: CCE (1000m g/mL); BA2: CCA (2000 mg/mL); BE2: CCE at 2000 mg/mL)

The acute toxicity of *C. cinerea* extracts is assessed in accordance with OECD guideline no 423. CCA and CCE are tested at two distinct doses (1000 and 2000 mg/kg. Figure 2) on fasted mice for 12 hours. Five groups of animals (males and females) are used (n = 7). Extracts were administered orally by gastric gavage for each group. The negative control group received 0.9% NaCl.

2.4.1. Handling and housing of animals

To perform the acute toxicity test, male and female mice (0.03-0.04 kg) were obtained from the Department of Biology, Faculty of Sciences Dhar El-Mahraz, Sidi Mohamed Ben Abdellah University, Fez, Morocco. Animal housing conditions were as follows: Temperature (28-32 °C); humidity (50-55%); day/night photoperiod (~12/12 h). Mice used in this test had free access to food and handled with the approval of the ethics committee (LBEAS-February 2021).

2.4.2. Body weight measurement and signs of toxicity

Groups of mice are observed prior to oral administration of different extracts of *C. cinerea*, and then examined every hour for five hours, then at 24 hours, and finally every day for 14 days. Body weight measurements were taken every 3 days during the test period. All observations are meticulously documented, maintaining individualized records for each animal. Cage assessments encompass evaluation of skin and fur condition, ocular health, respiratory parameters, autonomic responses such as salivation, diarrhea, and urination, as well as central nervous system manifestations including tremors, convulsions, tail curling, ptosis, relaxation, behavioral alterations, gait and posture variations, response to handling, changes in strength, and stereotypical behaviors (Demma et al., 2007; Nair et al., 2009).

2.4.3. Relative organ weights (ROW)

At the end of the 14-day acute toxicity period, the relative kidney and liver weights of each mouse are calculated according to Kifayatullah et al. (2015) as follows:

$$ROW = \frac{Organ\ weight(g)}{Animal\ body\ weight(g)} \times 100$$

ROW: Relative organ weights

2.4.4. Analysis of biochemical parameters

After anaesthetizing the mice used in this test with sodium pentobarbital intraperitoneally at a dose of 30 mg/kg and sacrificing them, blood samples were taken by cardiac puncture into heparinized tubes, then centrifuged at 1500 rpm for 10 min. Animal sera were recovered for analysis of the following biochemical parameters: creatinine, urea, aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) (Ramadan et al., 2012).

2.5. Red blood cell haemolysis test

2.5.1. Red Blood Cell Preparation

Freshly obtained rat blood samples were combined with a heparin anticoagulant solution. To obtain a purified erythrocyte suspension, the blood sample underwent three successive washes with sterile NaCl saline (0.9%). After

each wash, the cells were separated by centrifugation (3500 rpm for 10 minutes at 4°C) and the supernatant carefully aspirated. Finally, the erythrocytes were reconstituted in saline to form a 3% solution for use in the hemolytic assay.

2.5.2. Hemolytic Assay

The evaluation of the hemolytic activity of the *C. cinerea* extracts was conducted following the modified protocol described previously by Saleh et al., 2021. In hemolysis tubes, 100 µL of various initial concentrations of the extracts (1.556, 3.125, 6.25, 12.5, 25 and 50 mg/mL) was combined with 1900 µL of the prepared erythrocyte suspension. The mixtures obtained were then incubated at 37 °C for 45 min in water bath. 500 µL from each tube was extracted and mixed with 1.5 mL of a phosphate buffered saline (PBS) solution. Following this, the tubes underwent centrifugation at 3000 rpm for 10 minutes. Finally, the absorbance of supernatants was measured at 540 nm (representing hemoglobin absorbance after erythrocyte lysis) using a UV-visible spectrophotometer against a blank containing PBS. A negative control tube was prepared under identical experimental conditions, comprising 500 µL of erythrocyte suspension and 1500 µL of PBS buffer, without extract.

The percentage of hemolysis was determined relative to a total hemolysis tube prepare with distilled water. The hemolysis rate for the extract samples was calculated as a percentage (%) of total hemolysis after 45 minutes of incubation, using the following equation.

$$\text{Hemolysis rate (\%)} = [(A - A0)/(At - A0)] \times 100$$

A, A0, and At were the absorbance of the sample, the absorbance of the negative control, and the absorbance of the positive control (total hemolysis), respectively

2.6. Statistical analysis

GraphPad Prism software (version 8) was used to perform the analysis using one-way analysis of variance (ANOVA) followed by Tuckey-test. Differences at $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Yield of extracts and their chemical composition obtained by LC-TOFMS.

The yield of CCE obtained by maceration (7.44%) is slightly higher than that of CCA obtained by infusion (5.51%). Analysis of the two *C. cinerea* extracts by LCTOFMS shows the presence of fatty acids in this species. Two acids were identified: palmitic acid and stearic acid. These two acids are found in both *C. cinerea* extracts (Figure 3, Figure 4). LC-TOF-MS analysis also revealed the presence of other compounds in CCE, which is known for its high concentration of phenolic compounds. These include rosmarinic acid, kaempferol and luteolin (Figure 5). However, these compounds are not detected in CCA.

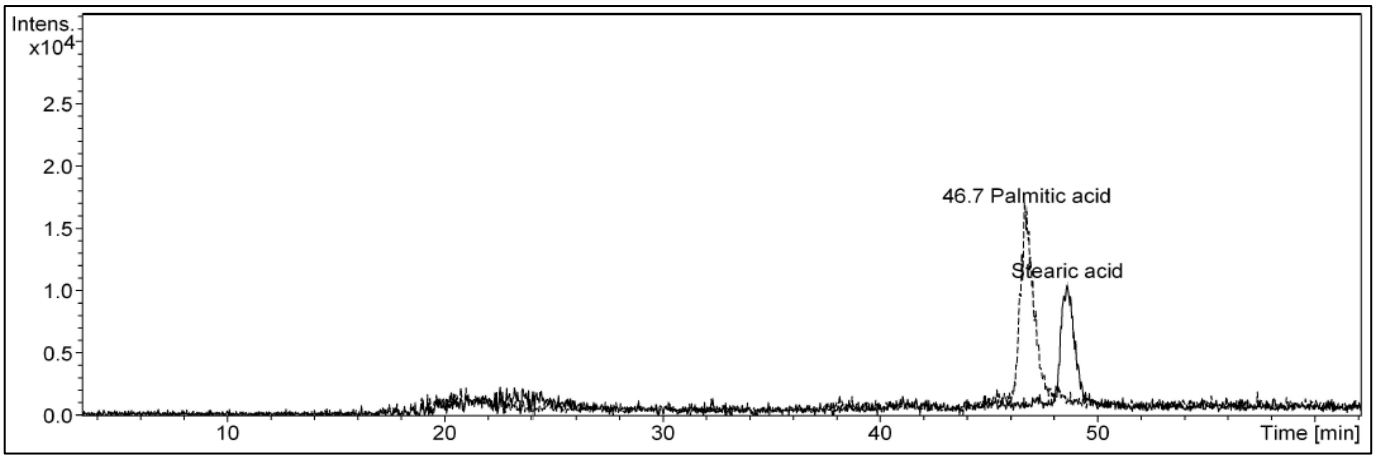

Figure 3: CCA chromatogram by LC-TOF-MS.

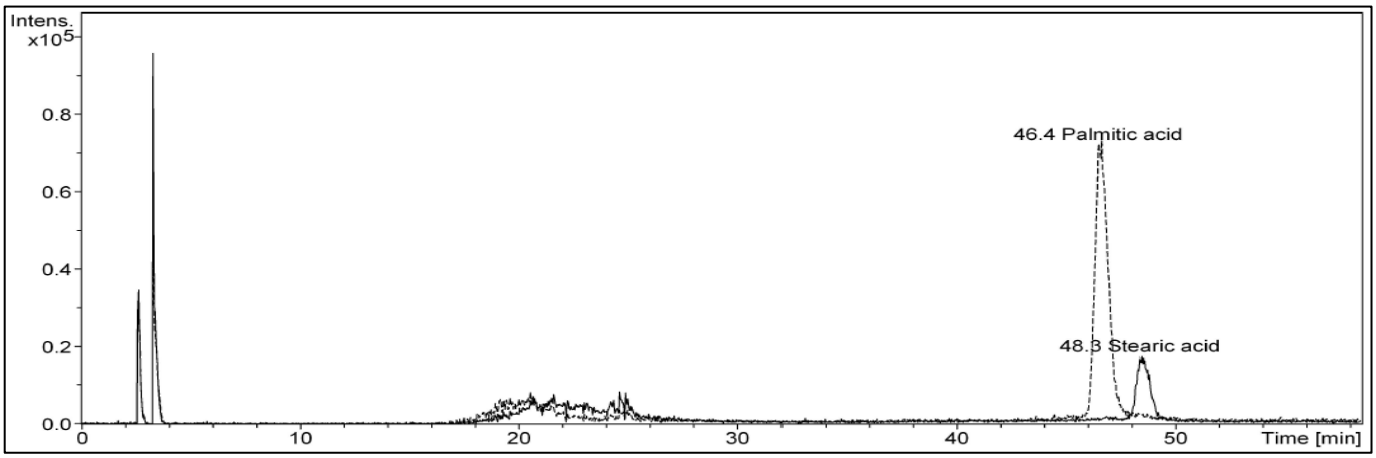

Figure 4: CCE chromatogram by LC-TOF-MS (Fatty acids).

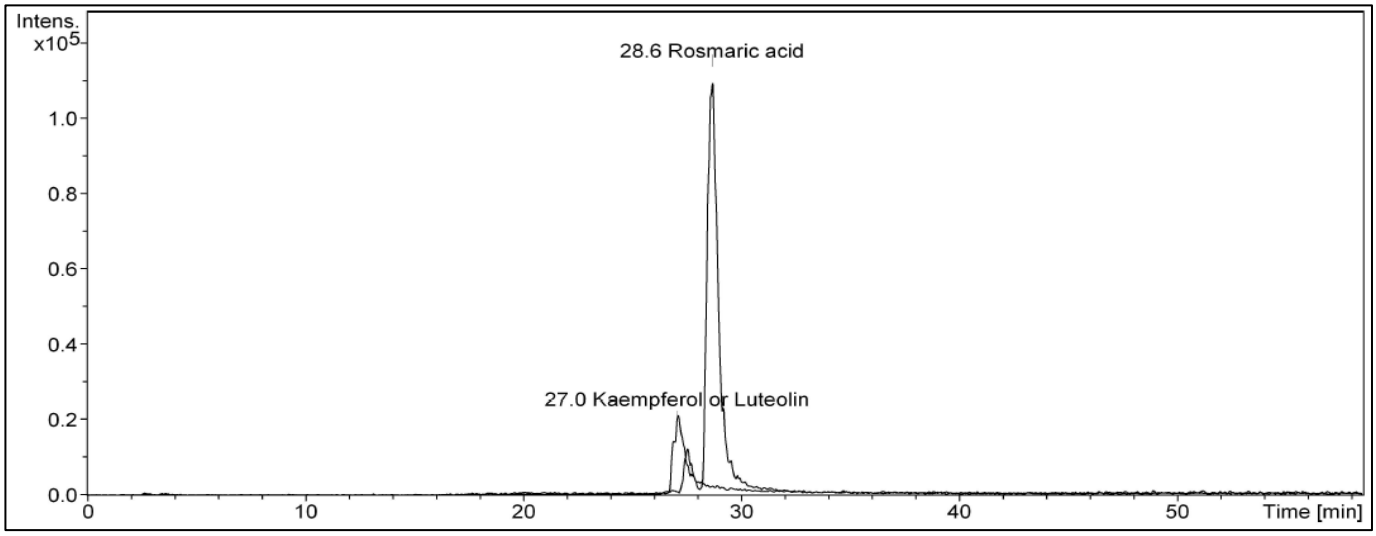

Figure 5: CCE chromatogram by LC-TOF-MS (Phenolic compounds)

The phytochemical composition of *C. cinerea* extracts has been reported in previous studies, which identified several polyphenolic compounds in different plant organs. The study of (Radjai et al., 2022) reported that the identification of compounds by LC/MS/QTOF in *C. cinerea* extract showed the presence of: Ethylmethanethiosulfonate, Glycoaldehyde, N Butylbenzenesulfonamide, Hydroxyhexanoic acid, Proline, Perillylic alcohol, Pipecolic acid, 7-Hydroxy-2-methoxyflavone, N,N-Dimethyldodecylamine N-oxide, Pecueloic acid, Octene, and Dodecalactone. Similarly, the main phenolic compounds identified in a methanolic extract were 4,5-dicaffeoylquinic acid, luteolin-4'-O-glucoside, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and cryptochlorogenic acid. In addition, another methanolic extract of *C. cinerea* contains echinocoids: dicaffeoylquinic acid isomers and chlorogenic acid isomers (Khallouki et al., 2015; Bouziane et al., 2013).

3.2. Acute toxicity

3.2.1. Change in body weight over 14 days

The data in figures 6 and 7 represent changes in the body weight of the animals used in the acute toxicity test. An appropriate statistical analysis (Student's t-test) was used to compare the means of the different groups, with a significance threshold of $p < 0.05$. The mice were examined for 14 days, and at the start of the study, their initial weight varied between 27.96 ± 3.97 g (CCA) and 30.60 ± 5.21 g (CCE). After this period, on day 15, body weight increased in all groups, reaching between 32.73 ± 4.67 g (CCA) and 34.74 ± 2.86 g (CCE). Mice treated with CCE showed a significant increase from day 6 ($p < 0.05$). No significant difference was observed for the CCA group compared with the control group. In general, these results indicate that the plant extracts tested did not have a significant impact on the body weight of the mice during the 14-day experiment.

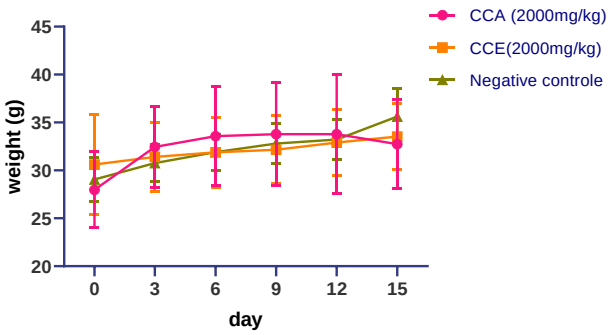

Figure 6: Variation in animal body weight over 14 days (dose of 2000 mg/kg).

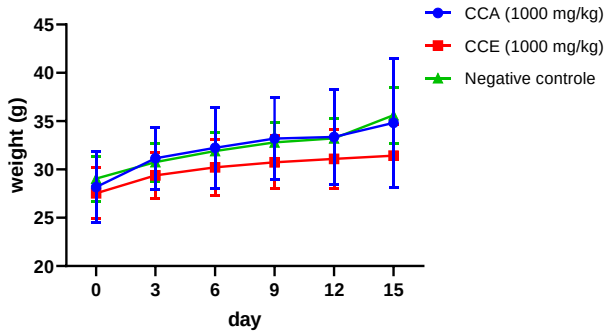

Figure 7: Variation in animal body weight over 14 days (dose of 1000 mg/kg).

3.2.2. Relative liver and kidney weights 14 days after treatment

The results presented in Figure 8 show the relative liver and kidney weights in mice used for acute toxicity assessment. These results show that the mean relative liver weight ranged from 4.13 ± 0.98% to 4.71 ± 0.79% for the treated groups, with no significant difference from the control group (-) ($p < 0.05$). Relative kidney weight varied from 1.36 ± 0.13 % to 1.64 ± 0.24 % for the groups treated with

the different extracts, with no significant difference compared with the control group (-). Thus, plant extracts had no significant effect on relative liver and kidney weights in treated mice.

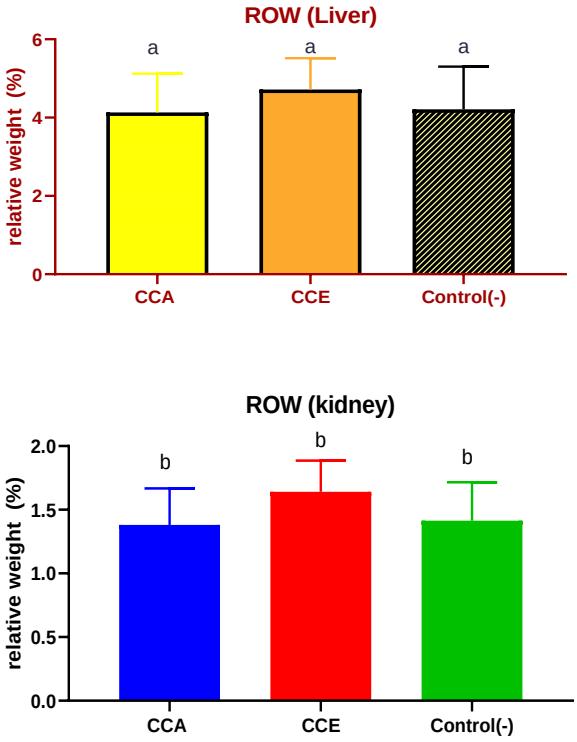

Figure 8: Relative weight of liver and kidney 14 days after treatment with *C. cinerea* extracts (2000 mg/mL); ROW: relative organ weight. For each parameter, bars with different letters represent statistically different values ($p < 0.05$)

3.2.3. Effect of C. cinerea extracts on biochemical parameters of animals 14 days after treatment

The Figure 9 presents the results of the analysis of the biochemical parameters of the serum of the animals used in the acute toxicity test (dose 2000 mg/mL). We note that no significant difference was observed between the treated groups and the control group in terms of effects on the various biochemical parameters measured. For Aspartate aminotransferase (ASAT), CCA and CCE extracts showed lower average levels, with 291 ± 37.40 and 296 ± 35.04 IU/I respectively. With regard to Alanine aminotransferase (ALAT), similar mean levels were observed for all groups. The control group had a mean level of 43.46 ± 5.06 IU/I. For urea, mean levels were similar for all groups, although the CCE had slightly higher mean levels of 0.37 ± 0.11 g/L than the other groups.

For urea, mean levels were similar for all groups, although the CCE had slightly higher mean levels of 0.37 ± 0.11 g/L than the other groups. The control group had a mean level of 0.28 ± 0.04 g/L. With regard to creatinin, mean levels were also comparable for all groups, although the CCE had slightly higher mean levels of 5.04 ± 1.50 mg/L than the other groups. The control group had a mean level of 3.56 ± 0.40 mg/L. However, it should be noted that these differences are not statistically significant.

In this acute toxicity test of orally administered extracts of *C. cinerea* (CCA and CCE), changes in body weight, relative liver and kidney weights, and serum biochemical parameters of the animals were examined. Statistical analysis revealed no significant differences in body weight between treated and control groups over a 14-day period. Liver and kidney weights also showed no significant variation between treated and control groups. Biochemical parameters such as AST, ALT, urea and creatinine showed similar levels in all groups, with slight variations observed for some parameters, although these were not statistically significant. Overall, the plant extracts tested did not significantly affect the parameters measured in mice tested for acute toxicity.

The results obtained regarding acute toxicity align with findings from previous studies on the toxicity of *C. cinerea* extracts. Both fresh and dry aerial parts of *C. cinerea*, administered at doses ranging from 200 to 800 mg/kg, did not induce any acute oral toxicity effects or mortality in Wistar rats (Chlif et al., 2022). Similarly, Markouk et al. found that oral administration of ether, ethyl acetate, and n-butanol extracts of *B. cinerea* up to 10 mL/kg to Wistar rats did not result in acute toxicity (Markouk et al., 1999). Furthermore, acute oral toxicity testing at a dose of 2000 mg/kg in mice, utilizing steam distillation for essential oil extraction and maceration using solvents (hexane, ethyl acetate, n-butanol) for non-volatile compounds, demonstrated non-toxicity even at the highest dose (Guaouguaou et al., 2020). Additionally, examination of essential oil extracts from the leaves and flowers revealed LD50 values of 1131.37 mg/kg and 1264.91 mg/kg, respectively, indicating low toxicity (Bettayeb et al., 2022). These collective findings underscore the safety profile of the tested extracts, reinforcing their potential for various pharmacological applications.

3.3. *Hemolytic activity*

The hemolytic activity of C. cinerea extracts was assessed using RBC of rat, and the results are shown in Figure 10. To analyze the results, we performed a one-way analysis of variance (ANOVA) to assess the differences between CCA and CCE concentrations in terms of percentage hemolysis of rat RBC. The results revealed a significant difference between CCA and CCE concentrations in terms of percentage hemolysis ($p < 0.05$). In particular, CCE concentrations showed a significantly higher percentage of hemolysis than the corresponding CCA concentrations. For example, at the highest concentration (625 µg/mL), the percentage of hemolysis for CCE was 98.81% ± 0.27, while for CCA it was 0.97% ± 0.62.

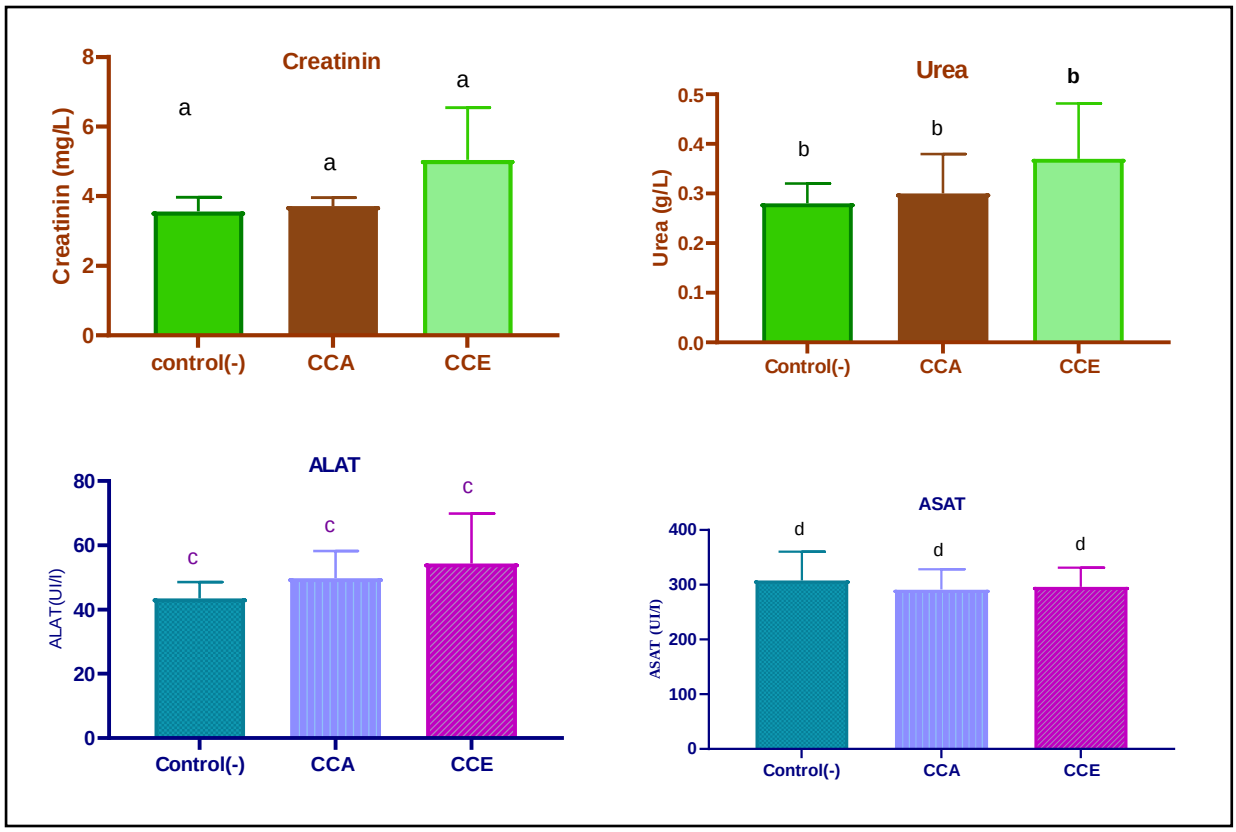

Figure 9: Effect of CCA and CCE on biochemical parameters in treated animals (2000 mg/mL); ALAT: Alanine Aminotransferase; ASAT: Aspartate Aminotransferase. For each parameter, bars with different letters represent statistically different values ($p < 0.05$)

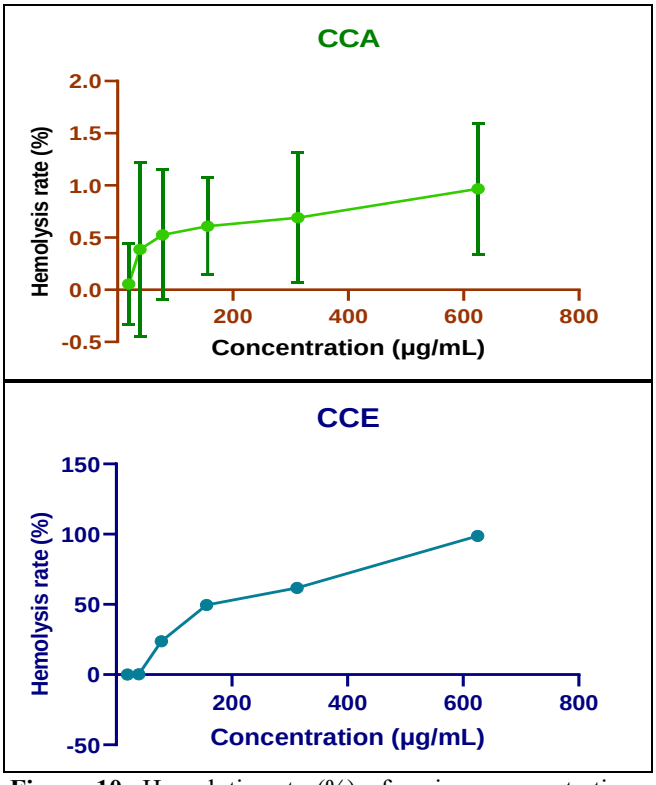

Figure 10: Hemolytic rate (%) of various concentrations CCA and CCE after 45 min of incubation.

The rate of hemolysis increased with increasing concentration of CCE, whereas CCA had almost no hemolytic effect on rat RBC, although the variation in the rate of hemolysis was not significant between the different concentrations of CCA. In addition, the lowest hemolysis rates obtained by applying CCE were 0.027% and 0.30% for concentrations of 19.53 and 39.06 µg/mL, respectively, and 50% of hemolysis is obtained with a concentration of 176.86 µg/mL. Thus, these results suggest that the ethanolic extract (CCE) may have a more pronounced effect on hemolysis compared to those of CCA. The latter could have important implications in their potential use as therapeutic agents without the danger of hemolysis in the erythrocyte encounter state.

To date and to our knowledge, this work is the first to report the hemolytic activity of *C. cinerea* extracts. Taking into account bibliographical data, may help to explain the results obtained in this test. We have already reported in a previous study that CCA is characterized by its high polyphenol and flavonoid content and its ability to trap free radicals. CCA is highly active against the DPPH radical, with an IC_{50} = 0.049 mg/mL (Agour et al., 2023). Moreover, among the secondary plant metabolites that can protect cells from oxidative damage are flavonoids. The scavenging of free radicals by secondary plant metabolites has been shown to reduce lipid peroxidation and stabilize red blood cell membranes (Khalili et al., 2014). Additionally, it can be said that instead of having a hemolytic activity against RBC, CCA can exhibit an anti-

hemolytic activity due to its significant antioxidant capacity.

In some cases, plant extracts can exert a greater anti-haemolytic effect than standard compounds. Nine extracts were more effective than vitamin C in inhibiting haemolysis, including extracts of Galium verum L. (IC_{50}=1.32 µg mL^{-1}) and *Scutellaria tournefortii* Benth (2.08 µg mL^{-1}) (Khalili et al., 2014). Extracts of other species, such as *Paronychia chlorothyra* and *Arthrophytum schmittianum*, are non-toxic to human erythrocytes at concentrations below 500 µg/mL (Zohra & Fawzia, 2014).

On the other hand, CCE has been shown to have a hemolytic effect that varies with extract concentration, and 50% hemolysis was achieved at a concentration of 173.00 mg/mL. This extract is not acutely toxic at a dose of 2000 mg/mL. Hemolytic effect of CCA may be attributed to its saponin content, with a higher concentration of saponins than CCA (Figure 2; BE2).

Plant saponins are characterized by their biological activities such as antitumor, antiviral, antiprotozoal, antibacterial, and anti-inflammatory effects. However, they can also cause hemolytic effects. The addition of saponins to a blood suspension causes alterations in the membranes of RBC, leading to the release of hemoglobin. Previous research has shown that the damage inflicted by saponins on the lipid bilayer of RBC is irreversible (Sparg et al., 2004; Baumann et al., 2000; Karou et al., 2011; Gnoula et al., 2008).

The ethanolic extract of Calotropis procera does not show significant acute toxicity, but is toxic to rat RBC. The opposite case can be obtained for extracts of other plant species, such as Calotropis procera. Costa-Lotufo et al., (2005) reported that extracts of this species showed low hemolytic activity on human erythrocytes, compared with other non-toxic species.

4. Conclusion

This study reported the acute toxicity and, for the first time, the hemolytic activity of the aqueous and ethanolic extracts of the aerial parts of *C. cinerea* (CCA and CCE) harvested in Southeast Morocco. Oral administration of these extracts in mice showed no signs of toxicity in terms of animal body weight, relative weight of the liver and kidneys, as well as serum biochemical parameters including ALAT, ASAT, urea, and creatinine. On the other hand, the red blood cell hemolysis test in rats confirmed the non-toxicity of the aqueous extract, it showed no cytotoxic effect on erythrocytes, and these data suggest the non-toxic effect of the extract, making it suitable for the preparation of medications involved in the treatment of various diseases. However, the CCE exhibits a concentration-dependent hemolytic effect. This underscores the dual nature of plant secondary metabolites. Further research is needed to understand the complex mechanisms behind these effects and to harness the therapeutic potential of plant extracts while mitigating undesirable hemolytic reactions. Moreover, caution is required in the administration of the plant orally or by other means, and determining the hemolytic effect of the extracts becomes necessary but not sufficient.

Funding

This research received no external funding

Conflicts of Interest

The authors declare no conflicts of interest.

Data availability statement

Data will be available upon request from the corresponding author

References

Agour, A., Mssillou, I., El Barnossi, A., Chebaibi, M., Bari, A., Abudawood, M., Al-Sheikh, Y.A., Bourhia, M., Giesy, J.P., Aboul-Soud, M.A.M., Lyoussi, B., Derwich, E., 2023. Extracts of *Brocchia cinerea* (Delile) Vis Exhibit In Vivo Wound Healing, Anti-Inflammatory and Analgesic Activities, and Other In Vitro Therapeutic Effects. Life 13. https://doi.org/10.3390/life13030776

Agour, A., Mssillou, I., Es-safi, I., Conte, R., Mechchate, H., Slighoua, M., Amrati, F.E.Z., Parvez, M.K., Numan, O., Bari, A., Lyoussi, B., Derwich, E., 2022. The Antioxidant, Analgesic, Anti-Inflammatory, and Wound Healing Activities of *Haplophyllum tuberculatum* (Forsskal) A. Juss Aqueous and Ethanolic Extract. Life 12. https://doi.org/10.3390/life12101553

Bettayeb, Z., Fawzia Toumi Benali, Abdelfattah Benyamina, Sara Ouldchikh, Said Mohammed El-Amin, Hanane Salem, Youcef Benaissa Keddar, 2022. Acute toxicity and anti-inflammatory activity of essential oils of *Cotula cinerea* from southwest Algeria. South Asian Journal of Experimental Biology 12, 609–615. https://doi.org/10.38150/sajeb.12(5).p609-615

Bouziane, M., Badjah-Hadj-Ahmed, Y., Hadj-Mahammed, M., 2013. Chemical composition of the essential oil of *Brocchia cinerea* grown in south eastern of Algeria. Asian Journal of Chemistry 25, 3917–3921.

Chlif, N., Bouymajane, A., Oulad El Majdoub, Y., Diouri, M., Rhazi Filali, F., Bentayeb, A., Altemimi, A.B., Mondello, L., Cacciola, F., 2022. Phenolic compounds, in vivo anti-inflammatory, analgesic and antipyretic activities of the aqueous extracts from fresh and dry aerial parts of *Brocchia cinerea* (Vis.). Journal of Pharmaceutical and Biomedical Analysis 213. https://doi.org/10.1016/j.jpba.2022.114695

Costa-Lotufo, L.V., Khan, M.T.H., Ather, A., Wilke, D.V., Jimenez, P.C., Pessoa, C., de Moraes, M.E.A., de

Moraes, M.O., 2005. Studies of the anticancer potential of plants used in Bangladeshi folk medicine. Journal of ethnopharmacology 99, 21–30. https://doi.org/https://doi.org/10.1016/j.jep.2005.01.041

Demma, J., Gebre-Mariam, T., Asres, K., Ergetie, W., Engidawork, E., 2007. Toxicological study on *Glinus lotoides*: a traditionally used taenicidal herb in Ethiopia. Journal of ethnopharmacology 111, 451–457. https://doi.org/10.1016/j.jep.2006.12.017

Duda-Chodak, A., Tarko, T., 2023. Possible side effects of polyphenols and their interactions with medicines. Molecules 28, 2536. https://doi.org/10.3390/molecules28062536

Gras, A., Hidalgo, O., D'ambrosio, U., Parada, M., Garnatje, T., Valles, J., 2021. The role of botanical families in medicinal ethnobotany: A phylogenetic perspective. Plants 10, 163. https://doi.org/10.3390/plants10010163

Guaouguaou, F.E., Bebaha, M.A.A., Taghzouti, K., Es-Safi, N.E., 2020. Phytochemical investigation, acute toxicity, central analgesic and antioxidant activities of extracts and essential oil of *Cotula cinerea* Del (Asteraceae). Current Bioactive Compounds 16, 164–173.https://doi.org/10.2174/1573407214666180821115826

Khalili, M., Ebrahimzadeh, M.A., Safdari, Y., 2014. Antihaemolytic activity of thirty herbal extracts in mouse red blood cells. Arhiv za Higijenu Rada i Toksikologiju 65, 399–406. https://doi.org/10.2478/10004-1254-65-2014-2513

Khallouki, F., Sellam, K., Koyun, R., Ricarte, I., Alem, C., Elrhaffari, L., Owen, R.W., 2015. Phytoconstituents and in vitro evaluation of antioxidant capacities of *cotula cinerea* (Morocco) methanol extracts. Records of Natural Products 9, 572–575.

Kifayatullah, M., Mustafa, M.S., Sengupta, P., Sarker, M.M.R., Das, A., Das, S.K., 2015. Evaluation of the acute and sub-acute toxicity of the ethanolic extract of *Pericampylus glaucus* (Lam.) Merr. in BALB/c mice. Journal of Acute Disease 4, 309–315. https://doi.org/10.1016/j.joad.2015.06.010

Markouk, M., Lazrek, H.B., Jana, M., 1999. Analgesic effect of extracts from *Cotula cinerea* (L). Phytotherapy Research 13, 229–230.

Mssillou, I., Agour, A., Slighoua, M., Chebaibi, M., Amrati, F.E.Z., Alshawwa, S.Z., Al Kamaly, O., El Moussaoui, A., Lyoussi, B., Derwich, E., 2022. Ointment-Based Combination of *Dittrichia viscosa* L. and *Marrubium vulgare* L. Accelerate Burn Wound Healing. Pharmaceuticals 15, 1–13. https://doi.org/10.3390/ph15030289

Nair, R., Shukla, V., Chanda, S., 2009. Effect of single dose administration of *Polyalthia longifolia* (Sonn.) Thw. var. pendula leaf on gross behavioral assessment in mice. Indian Drugs 46, 116–123.

Pelander, A., Pelzing, M., Krebs, IlmariOjanperä, S., Vuori, E., Ojanperä, I., 2006. Isotopic pattern and accurate mass determination in urine drug screening by liquid chromatography/time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry 20, 1161–1167. https://doi.org/10.1002/rcm.2429

Radjai, I., Chakali, G., And Blida, A., 2022. Extraction and identification of some metabolites from three Algerian Sahara medicinal plants in Agricultural and Natural Environments Against Crop Pests in the Regions of. Ukrainian Journal of Ecology 12, 57–65. doi: 10.15421/2022_354

Ramadan, A., Soliman, G., Mahmoud, S.S., Nofal, S.M., Abdel-Rahman, R.F., 2012. Evaluation of the safety and antioxidant activities of *Crocus sativus* and Propolis ethanolic extracts. Journal of Saudi Chemical Society 16, 13–21. https://doi.org/10.1016/j.jscs.2010.10.012

Saleh, E.A.M., Khan, A.U., Tahir, K., Almehmadi, S.J., Al-Abdulkarim, H.A., Alqarni, S., Muhammad, N., Dawsari, A.M.A.L., Nazir, S., Ullah, A., 2021. Phytoassisted synthesis and characterization of palladium nanoparticles (PdNPs); with enhanced antibacterial, antioxidant and hemolytic activities. Photodiagnosis and Photodynamic Therapy 36, 102542. https://doi.org/10.1016/j.pdpdt.2021.102542

Zohra, M., Fawzia, A., 2014. Hemolytic activity of different herbal extracts used in Algeria. International Journal of Pharma Sciences and Research 5, 495–500.