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Article

Antibacterial and molluscicidal properties of *Agave salmiana* Otto ex Salm Dick and *Agave beaulerina* Jacobi

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ABSTRACT

The present work was undertaken to evaluate the phytochemical, antibacterial, and molluscicide properties of *Agave salmiana* and *Agave beaulerina* in the Matanzas, Cuba province. Leaves of both species were collected, cleaned, dried, and powdered. The extractions were carried out in 90 % ethanol and distilled water. The qualitative content of various secondary metabolites was determined, and reducing sugars and total soluble proteins were quantified. The antibacterial activity of the ethanolic extracts was assessed against *Staphylococcus aureus* and *Escherichia coli*. The molluscicide activity of the aqueous extracts was also evaluated against the snail *Praticollela griseola*. Terpenes, flavonoids, saponins, tannins, steroids, coumarins, and cardiac glycosides were observed in both plant species. The leaf ethanolic extracts presented an antibacterial effect against both pathogens, although the most significant results were obtained with *Agave beaulerina* extract. The higher molluscicidal activity was observed with the aqueous extract of *A. salmiana*, resulting in a 100% mortality after two hours of application. The data would suggest using these plants as a source of bioactive compounds with antibacterial properties. The aqueous extract of *A. salmiana* could be considered a promising biological molluscicide and an ecological alternative to control *Praticollela griseola* in vegetable production areas.

Keywords: Agavaceae; biopesticides; mollusks; saponin; *Staphylococcus aureus*.

INTRODUCTION

The excessive use of antibiotics in medical practice has led to the evolution of many pathogens developing resistance against conventional antibiotics. As such, treating infectious diseases has become a serious challenge and a constant concern of the World Health Organization [1]. The plant kingdom is an invaluable resource of chemical compounds with distinct biological activities, so many investigations have focused on searching for new bioactive compounds from plants with antimicrobial activity[2].

In intensive agricultural production systems, the systematic application of large amounts of chemical pesticides over the last decades has also concerned the scientific community and the general public. These agrochemicals contaminate the agroecosystems, represent a risk to human health, and enhance the emergence of new pest-resistant genotypes[3]. Biopesticides derived from plants, on the other hand, have been considered

eco-friendly and promising alternatives to those chemicals due to their effectiveness, low costs, rapid biodegradability, and low impact on the environment[4,5].

Of the extensive list of crop pests, mollusks are considered one of the most important and challenging to control. In Cuba, the low availability and number of commercial molluscicidal products affect the productivity of several crops, mainly vegetables cultivated under the semi-protected technology[6]. Furthermore, various species may host helminthic parasites, which can be transmitted to humans and cause several diseases[7].

Agave spp. has been traditionally used to obtain natural fibers and produce alcohol for certain beverages such as tequila, mezcal, etc. The genus also contains many metabolites, such as saponins, flavonoids, terpenes, tannins, and coumarins[8]. Those compounds are the biochemical base for various biological activities previously reported in this group of plants, such as cytotoxicity and anti-inflammatory[9], insecticidal[10], antibacterial[2,] and molluscicidal[11]. However, the phytochemical profile of a plant depends on many factors, including the genotype, plant physiological stage, soil and climate conditions, the method of extraction, etc. For this reason, phytochemical studies need to be done with native plants to make good use of the local flora. The objective of the present work was to evaluate the phytochemical, antibacterial, and molluscicidal properties of *Agave salmiana* Otto ex Salm-Dick and *Agave beaulerina* Jacobi present in the province of Matanzas, Cuba.

MATERIAL AND METHODS

Plant material

The experiments were carried out with Agave salmiana Otto ex Salm-Dick var plants. Ferox and Agave beaulerina Jacobi is in the Botanical Garden of Matanzas, province of Matanzas, Cuba. Plant specimens were identified and authenticated by specialists at this institution. Leaves (1.5 kg) without pests or mechanical injury were collected in October 2022 between 8:00 and 9:00 am.

Extract preparations

The fresh leaves were cleaned with distilled water and dried in an oven (Boxun) at 45 ºC. The leaves were pulverized using an electric grinder to obtain a powder with particles smaller than 0.2 mm. Ethanolic (90 %) and aqueous extracts were prepared. One hundred grams of dried and powdered leaves were mixed with 1 L of both solvents and kept on a rotary shaker (HDL® Apparattus) at 160 rpm for 24 hours. The extracts were filtered using a Whatman No. 1 paper filter and concentrated at 45 °C with a rotary vacuum evaporator. The residues were left to dry at room temperature and stored at 4 ºC for phytochemical and microbiological assays.

Phytochemical screening

The qualitative phytochemical study was carried out using standard procedures described by Chigodi *et al*. [12], to identify the presence of flavonoids, terpenoids, anthocyanins, tannins, saponins, steroids, coumarins, anthraquinones, cardiac glycosides and phlobatannins. The qualitative content was expressed by the non-parametric system: +++ (abundant), ++ (moderate), + (low), and – (absence).

Content of reducing sugars

The dinitrosalysic acid (DNS) method13 determined the reduced sugar content. D-glucose (1 mg mL^{-1}) was used as standard. Absorbance was recorded at 456 nm in a spectrophotometer Ultrospect 2000 (Pharmacia Biotech, Sweden).

Content of total soluble proteins

Total soluble protein concentration was determined as described by Lowry *et al*.[14], using bovine serum albumin (1 mg mL^{-1}) as standard. The absorbance values were recorded at 750 nm in a spectrophotometer Ultrospect 2000.

Calcium oxalate crystal detection

Calcium oxalate crystals were directly observed from the crude extracts using an Optical Microscopy (Olympus) with a magnification of 400X. Photographs were taken to record the presence of crystal structures.

Antibacterial assay

The antibacterial activity of the ethanolic extracts was evaluated against *Staphylococcus aureus* ATCC 25923 (Gram-positive) and *Escherichia coli* ATCC 25922 (Gram-negative). The assay was carried out using the agar well diffusion method15. The bacterial strains were previously cultivated on Brain-Heart-Infusion agar medium at 37 ºC for 24 hours. Isolated colonies were taken to prepare bacterial suspensions in saline solutions 0.85 % (v/v). Mueller-Hinton Agar medium was further inoculated with an equivalent cellular concentration of 0.5 in the Mc Farland scale. Wells was done with a sterile borer of 8 mm diameter, and 100 µL of each extract (200 mg mL^{-1}) was added. Plates were incubated overnight at 37 ºC. Cephalexin (50 µg) and amikacin (50 µg) were positive controls for Gram + and Gram – bacteria. The negative control consisted of a hydroalcoholic solution similar to that used to obtain the extracts. The antibacterial activity was obtained by measuring the diameter of the bacterial growth inhibition zone. Three replications were performed for each assay.

Molluscicidal activity assay

The molluscicidal activity was determined using aqueous extracts of fresh leaves. Before the experiment, the leaves were cut into small pieces, homogenized in distilled water and filtered with a Whatmann No. 1 filter paper, to obtain a concentration of 1 g mL-1, considered the initial solution (100 %). From it, five dilutions were prepared: 1/2 (50%), 1/4 (25%), 1/8 (12.50%), 1/16 (6.25%) and 1/32 (3.12%). The extracts were evaluated against *Praticolella griseola* (Pfeiffer) snails following the methodology described by Morales-Rabanales *et al*.[16]. Five animals were set in Petri dishes (20 cm diameter). The snails were adapted to laboratory conditions for seven days before being tested. Four replications (plates) per treatment (extract) were performed. Four milliliters of each extract and water (control) were applied all over the plates containing the animals and the food (fresh leaves of common bean). Mortality (%) was recorded at scheduled times over 24 hours.

Experimental design and statistics analysis

Molluscicidal assays were conducted using a completely randomized design. The antibacterial activity and the qualitative determination of secondary metabolites were carried out in triplicate, and the absorbance values were used to calculate the content of reducing sugars and total soluble proteins in the extracts.

Data was processed with the statistic pack SPSS version 18.0 for Windows. Data adjustment to a normal distribution was determined through the Kolmogorov-Smirnov test and variance homogeneity by Bartlett's tests. Antibacterial activity, content of reducing sugars, and total soluble proteins data were statistically analyzed by simple analysis of variance (ANOVA). Means were compared using Tukey's multiple ranges comparison test ($p<0.05$). The mortality percentages were compared using an analysis of proportions with the software CompaProp version 3.01 over Windows 17.

RESULTS AND DISCUSSION

Phytochemical screening

The phytochemical screening results are shown in Table 1. A higher content of reducing sugars was observed in the ethanolic extract of *Agave salmiana*. The quantification of these compounds in *Agave* species could be of interest because they may interfere with the extraction of saponins, which have been extensively used for pharmaceutical purposes[18]. The ethanolic extract of *Agave beaulerina* showed the highest concentration of total soluble proteins. Ethanol was a better solvent for the extraction of both metabolites than the aqueous counterpart, which results from the difference in polarity between these substances.

Metabolites	*Agave beaulerina*		*Agave salmiana*	
	Ethanol	Water	Ethanol	Water
RS (mg g^{-1})	4.52 ± 0.08 ^{b}	0.74 ± 0.07 ^{d}	5.62 ± 0.13 ^{a}	2.84 ± 0.03 ^{c}
TSP (mg g^{-1})	29.60 ± 0.32 ^{a}	12.86 ± 0.12 ^{c}	24.63 ± 0.41 ^{b}	10.60 ± 0.17 ^{d}
Flavonoids	++	+	+	+
Terpens	+++	++	++	+
Steroids	+	-	+	-
Anthocyanins	-	-	-	-
Saponins	+++	+++	+++	+++
Tannins	+	-	+	-
Coumarins	-	+	-	+
C. Glycosides	++	+	++	+
Phlobatannins	-	-	-	-
Anthraquinones	-	-	-	-

Table 1. Phytochemical composition of ethanolic and aqueous leaf extracts of *Agave salmiana* var. Ferox and *Agave beaulerina* Jacobi. RS (reducing sugar), TSP (total soluble protein). Different letters mean statistical difference among extracts (Tukey Test, p<0.05).

Flavonoids, terpenes, saponins, and cardiac glycosides were observed in both aqueous and ethanolic extracts of *A. salmiana* and *A. beaulerina*. Steroids and tannins were only detected in ethanolic extracts of both species, while coumarins were found in the aqueous extracts. The obtained results are consistent with the literature reporting the presence of these compounds in *Agave* species such as *A. salmiana*[19] and *A. americana*[20]. However, to our knowledge, there are no reports of phytochemical screening studies on *Agave beaulerina* Jacobi. The presence of flavonoids, terpenes, and saponins in the extracts of *A. salmiana* and *A. beaulerina*, suggests a potential use of these plants as a source of bioactive compounds for developing agriculture and pharmaceutical industry[19]. Flavonoids and terpenes have been described as antioxidant compounds with anti-inflammatory activity, which may be used to treat different pathologies related to oxidative stress such as inflammatory process, atherosclerosis, Parkinson's disease, Alzheimer's disease, Diabetes mellitus, and cancer[21]. Saponins have been associated with antibacterial[22], molluscicidal4, and cytotoxic activities and are commonly used as a precursor for the synthesis of drugs[19].

Antibacterial activity

The ethanolic extracts of *A. beaulerina* and *A. salmiana* showed antibacterial activity against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 (Table 2). The best results were observed with the extract of *A. beaulerina* against *S. aureus*, where the inhibitory effect was similar to that of the control (cephalexin). Similarly, the antibacterial activity of *A. beaulerina* extract against *E. coli* was higher in comparison with the extract of *A. salmiana*.

Extracts	*S. aureus*		*E. coli*	
	DZI (mm)	**± EE**	**DZI (mm)**	**± EE**
Hydro-alcoholic solution	0.0 ^{e}	0.00	0.0 ^{e}	0.00
Cephalexin	22.7 ^{a}	0.57	-	-

Amikacin	-	-	19.0 [b]	0.33
A. salmiana	16.0 [c]	0.53	11.7 [d]	0.30
A. beaulerina	22.3 [a]	0.38	17.3[bc]	0.33

Table 2. Antibacterial activity of Agave salmiana Otto ex Salm-Dick and Agave beaulerina Jacobi's leaf ethanolic extracts against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. DZI= diameter of zone of inhibition. Values represent the average of three replications. Different letters indicate statistical differences among extracts and controls for the same pathogen (Tukey test, p<0.05).

The antibacterial activity of *Agave* extracts has been previously reported in *A. americana*[2] and *A. angustifolia*[23], against various pathogens such as *S. aureus*, *S. epidermidis,* and *E. coli*. Medina-Galván *et al.*[11] reported an inhibitory activity of flower aqueous extract of *Agave salmiana* against the gram-negative bacteria *Listeria monocytogenes*, *Escherichia coli*, *Shigella sonnei,* and *Salmonella typhimurium*. However, few studies have been carried out on Agave salmiana to determine its antibacterial properties, and no antibacterial reports have been found in the literature on *Agave beaulerina*.

The tested extracts of *A. beaulerina* and *A. salmiana* showed a higher antibacterial activity against *S. aureus*, when compared with the inhibitory effect against *E. coli*. This result may be attributed to the complexity of the gram-negative *E. coli* cell wall, which reduces the entrance of bioactive compounds with antibacterial activity into the cell[24]. However, comparable studies with extracts of *Agave salmiana* showed the maximum inhibitory activity against *E. coli* compared with the gram-positive *S. aureus*[11]. These differences may be associated with the bacterial strains' intrinsic resistance and the plants' phytochemical profile, which depend on their physiological stage, environmental conditions, and the metabolites extraction procedure.

The extracts' antibacterial activity should be related to the presence of secondary metabolites previously identified in the phytochemical screening. Tannins, for instance, may react with proteins rich in proline to form irreversible complexes, inhibiting enzymes' catalytic activity and the biosynthesis of proteins[25]. Saponins are surfactant substances that alter cell membranes' physical and chemical properties, leading to cellular lysis[21]. Flavonoids and terpens have also shown antibacterial properties with a noticeable inhibitory effect against several pathogens[26].

Molluscicidal activity

Figures 1 and 2 show the results of the molluscicidal activity of *Agave salmiana* and *Agave beaulerina* aqueous extracts against *Praticolella griseola* (Pfeiffer), respectively. *A. salmiana* evidenced the best results of all the tested concentrations with 100% mortality after 4 hours of extract application, except for the lower concentration (3.1%), in which the activity was statistically similar to that of the control. In the case of *Agave beaulerina*, the extracts 100%, 50%, and 25% showed similar results (100% mortality) after 24 hours of assay. The application of the extract at 12.5% still reported high effectiveness, identical to those of the extracts mentioned above. However, mortality decreased to 60% with the application of the extract of 6.25%, and the lower concentration (3.12%) showed no difference with the control.

The molluscicidal activities of the assessed *Agave* extracts are in agreement with other reports and demonstrate the potential of this genus as a source of biocontroling agents for mollusks. The leaf extract of *Agave americana* was effective against *Praticolella griseola* Pfeiffer[6] and *Melanoides tuberculata*[27]. Similarly, Morales-Rabanales *et al.*[16] reported a molluscicidal activity of *Agave angustifolia* Haw extracts against *Fossaria obrussa*.

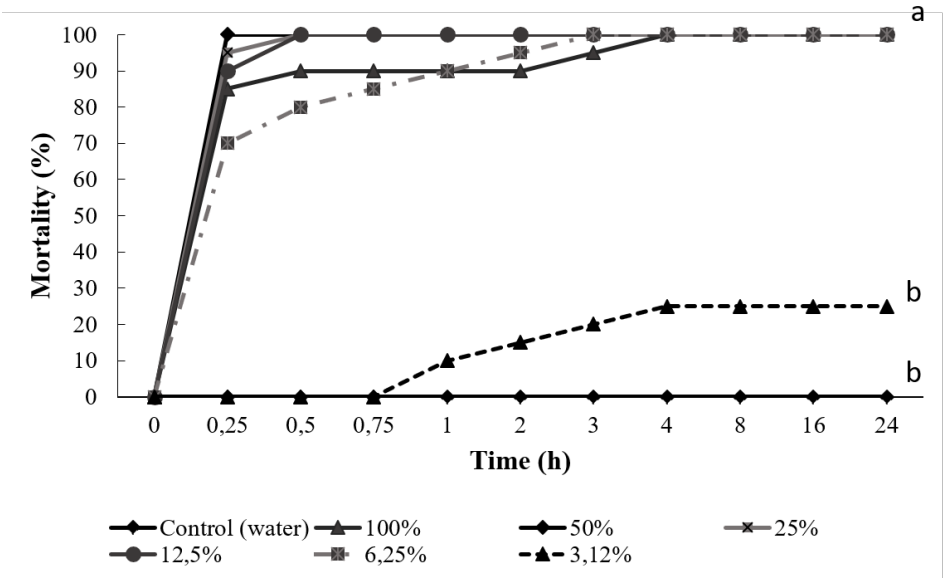

Figure 1. The molluscicidal activity of Agave salmiana Otto leaf aqueous extracts ex Salm-Dick against *Praticolella griseola* (Pfeiffer). Different letters mean statistical differences among the extract concentrations after 24 hours of application.

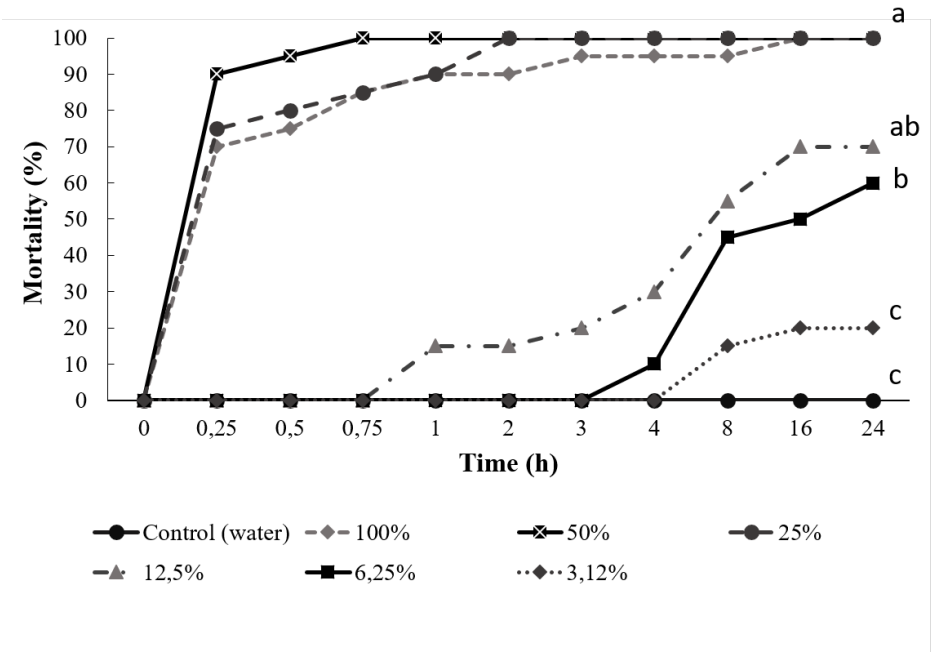

Figure 2. The molluscicidal activity of leaf aqueous extracts of *Agave beaulerina* Jacobi against *Praticolella griseola* (Pfeiffer). Different letters mean statistical differences among the extract concentrations after 24 h of application.

The ability of *A. salmiana* and *A. beaulerina* to kill mollusks may be related to the presence of metabolites such as saponins, tannins, terpenes, and flavonoids. Saponins, in particular, are well-known to have molluscicidal properties. These compounds may irritate the soft skin and digestive tube of the snails[4]. Saponins are also toxic over muscles, intestines, hepatopancreas, and hemolymph and may increase the solubility of biological membranes, which results from their amphiphilic nature. This chemical characteristic allows the saponins to interact with sterols present in cell membranes, causing cell rupture and the loss of internal fluids, leading to dehydration of the animal[7]. In addition, its absorption at high concentrations into the blood circulation may increase cardiac frequency and heart failure[28].

The presence of insoluble calcium oxalate crystals in the crude extracts of *A. salmiana* and *A. beaulerina* may be involved with the molluscicidal activity of this species[29] (Figure 3). Those substances were reported to have

a highly irritant effect and could cause the violent reaction observed in the animals when they immediately come into contact with the extracts.

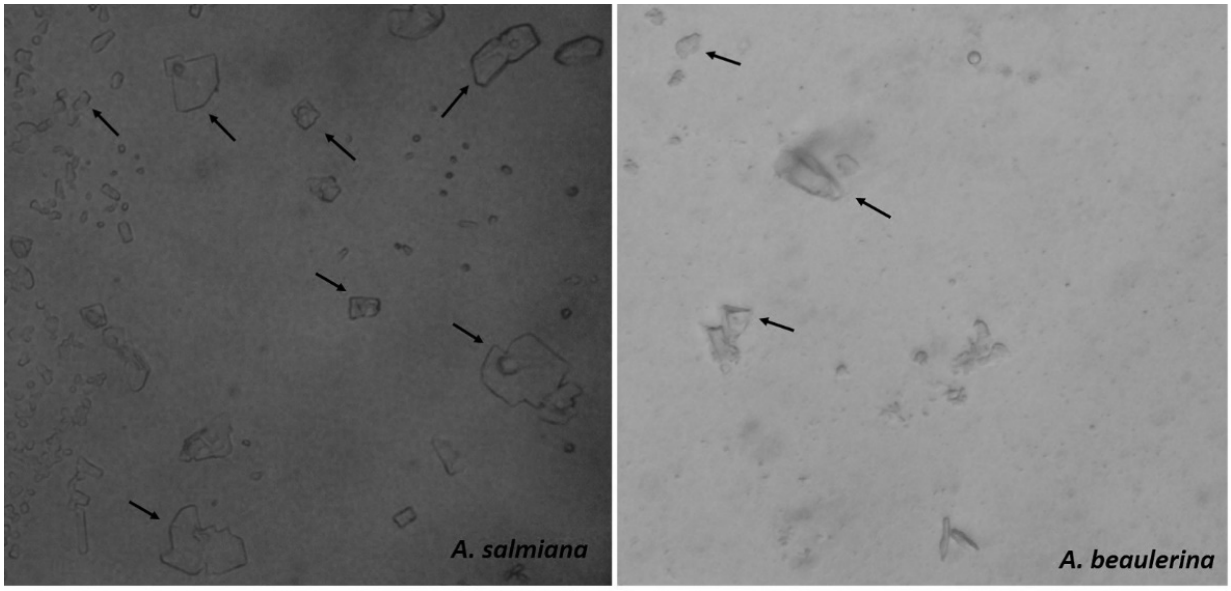

Figure 3. Calcium oxaloacetate crystals in leave juice of *Agave salmiana* Otto ex Salm-Dick var. Ferox (left) and *Agave beaulerina* Jacobi (right). Black arrows indicate the presence of crystal structures. Magnifications of 400X.

Some spontaneously retreated inside the shell, while others were vigorously expelled out of the shell by themselves. Oxalate crystals have the potential to inflict mechanical injury to the skin and mucosal linings of the alimentary canal[30]. The oxalate toxicity involves mechanical abrasion and does not depend on absorption into the body[31].

The present investigation has shown that the extracts of *Agave salmiana* and *Agave beaulerina* possess antibacterial and molluscicidal properties. The phytochemical, antibacterial, and molluscicidal properties of the extracts of these two species of Agave were evaluated. The phytochemical analysis revealed the presence of various secondary metabolites, including terpenes, flavonoids, saponins, tannins, steroids, coumarins, and cardiac glycosides, in both species. The ethanolic extracts of both plants were found to have an antibacterial effect against *Staphylococcus aureus* and *Escherichia coli*, with the Agave beaulerina extract showing the most significant results. Additionally, the aqueous extract of *A. salmiana* exhibited high molluscicidal activity against the snail *Praticollela griseola*, resulting in 100% mortality after two hours of application.

The results of this study suggest that *Agave salmiana* and *Agave beaulerina* could be promising sources of bioactive compounds with antibacterial and molluscicidal properties. The presence of these compounds in the extracts could be responsible for the observed biological activities. The antibacterial activity of the extracts could be attributed to the presence of saponins, tannins, flavonoids, and terpenes, which are known to have antibacterial properties. The molluscicidal activity of the *A. salmiana* extract could be due to the presence of saponins, which are known to have molluscicidal properties. The presence of insoluble calcium oxalate crystals in the crude extracts of both species may also contribute to their molluscicidal activity.

Further studies are needed to evaluate the extracts' toxicity and determine the optimal concentrations and application methods for their use in integrated pest management programs. Additional research is required to isolate and identify the specific bioactive compounds responsible for the observed biological activities.

Overall, the findings of this study suggest that *Agave salmiana* and *Agave beaulerina* could be potential sources of naturally antibacterial and molluscicidal agents. Using these plants as biopesticides could be an eco-friendly and promising alternative to synthetic pesticides, which are known to negatively impact the environment and human health.

CONCLUSIONS

The leaf extracts of *Agave salmiana* Otto ex Salm-Dick and *Agave beaulerina* Jacobi showed the presence of important secondary metabolites such as terpenes, flavonoids, saponins, tannins, steroids, cardiac glycosides and anthraquinones, which are responsible for many biological activities described in these plants. The ethanolic extract of *A. beaulerina* revealed a high antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. In contrast, the aqueous extract of *A. salmiana* was highly toxic against the gastropod *Praticolella griseola* (Pfeiffer) and a promising biological molluscicide. This is the first investigation and use of *Agave beaulerina* as a potential antibacterial and molluscicidal candidate. Other studies regarding the toxicity of the extracts should be carried out before their use in integrated pest management programs.

Author Contributions: A short paragraph specifying their contributions must be provided for research articles with several authors. The following statements should be used: "Conceptualization, YP; LC and MM; methodology, YP; RL and DS; software, CC and MM; validation, YP; LC and DS; formal analysis, YP, LC, RL and DS; investigation, YP, YR, CC; AV and RL; resources, MM; data curation, YP; YR and MM; writing—original draft preparation, YP and AV; writing review and editing, YP; LC; AV and MM; visualization, RL; supervision, DS; project administration.

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