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Research Article

IN VITRO ANTI-INFLAMMATORY AND CYTOTOXICITY OF *CRINUM X AMABILE* GROWN IN ECUADOR

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ABSTRACT

Objective: The aim of this research was to assess the *in vitro* anti-inflammatory activity and cytotoxicity of the methanolic extract and total alkaloid extract obtained from leaves and bulbs of *Crinum x amabile* (CA) growing in Ecuador.

Methods: Methanolic extracts of dry powered leaves and bulbs of CA obtained by cold maceration method were subjected to preliminary phytochemical screening. Total alkaloid extracts of leaves and bulbs of CA were obtained by conventional extraction of alkaloids base with an organic solvent. Furthermore, the anti-inflammatory activity and cytotoxicity of the four extracts were investigated by *in vitro* isolated neutrophils model using stable tetrazolium salt (WST-1).

Results: Phytochemical analysis of methanolic extracts revealed the major classes of phytochemicals such as alkaloids, flavonoids, tannins, triterpenes, and steroids, but no proteins or saponins could be detected on leaves extract. Extracts obtained from bulbs both methanolic and total alkaloids of CA show an interesting anti-inflammatory activity, although it was not significant compared to the standard anti-inflammatory drug, aspirin. Cytotoxicity of bulb alkaloid extract was lower compared with all of the other extracts.

Conclusion: Based on the results of this research, it could be concluded that CA is a very interesting source of natural anti-inflammatory compounds (especially alkaloids) which could be used to prevent many chronic disorders. Further, phytochemical studies are necessary to identify the chemical compounds responsible for the significant anti-inflammatory activity showed.

Keywords: *Crinum x amabile*, Anti-inflammatory activity, Cytotoxicity, Cell proliferation reagent, WST-1.

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INTRODUCTION

Since the beginning of civilization, people have used plants [1]. Medicinal plants have known for millennia as potential sources of pharmaceutical agents and/or as sources of lead compounds in drug discovery [2]. Between 70 and 80% of developing countries' population still is depending on medicinal plants because of the absence of both basic health-care facilities and the expensive cost of allopathic medicines [3]. In the plants, the secondary metabolites presence is responsible for the beneficial effects of plant materials [4]. Nature has been granted to Ecuador a variety of unique plant species due to its geographical, climatic, and altitude conditions; which makes it one of the countries with the highest biodiversity in the world and high endemism, documenting about 5400 plant species. At Ecuador, dry forests are mostly located in the center and south, west from the Andes, in the provinces of Imbabura, Esmeraldas, Manabí, Guayas, El Oro and Loja and are very important ecosystems [5]. Therefore, there is a need to explore these medicinal plants to find bioactive compounds that be as a basis for further pharmacological studies [6]. Scientific assessment and validation of the traditional therapeutic use of the medicinal plants may lead to the development of new and effective drugs as have occurred in the past [7,8].

The Amaryllidaceae family, containing ca. 85 genera and 1100 species, distribute widely on the tropical to temperate regions around the world. Many Amaryllidaceae species have been cultivated as ornamental plants for a long time because of their colorful and beautiful flowers; thus, making a big market and significant economic values. Not only as ornaments but also Amaryllidaceae plants have also been applied on the pharmaceutical purpose [9]. Plants belonging to the Amaryllidaceae family are well known for containing an exclusive group of alkaloids, which have been considered a distinctive taxonomic characteristic of this family [10].

Crinum is a pantropical genus that has extensive traditional use in Africa in a range of therapeutic applications, including antitumor and antimalarial, and treatment of rheumatism and kidney and bladder infections, among others [11]. Alkaloids purified from the *Crinum* species have identified as responsible for some of these properties [12]. Alkaloid-rich extracts from *Crinum angustum* have shown significant antibacterial and antifungal activities [13]. Approximately 130 species found throughout Africa, America, southern Asia, and Australia have classified within the *Crinum* genus [14]. Great efforts have been made to make clear the chemical structure of several alkaloids of indigenous *Crinum* species in America as *Crinum erubescens* [15].

Crinum x amabile (CA) is native from India, and it is a hybrid between *Crinum asiaticum* x *Crinum zeylanicum*. This widely grown species is not only a decorative plant but also it has been applied to a long time in Vietnamese folk medicine as an emetic and as a remedy for rheumatism and earache [16]. Afro-Ecuadorians living in the province of Esmeraldas have used use CA to treat inflammations and particularly hemorrhoids for more than 500 years.

The present study was conducted with the goals (1) to find the major classes of phytochemicals present in the leaves and bulbs of CA variety grown in Ecuador and (2) to test anti-inflammatory activity and cytotoxicity of CA leaves and bulbs by *in vitro* isolated neutrophils model using stable tetrazolium salt (WST-1) to confirm its use in traditional medicine.

METHODS

Drugs and chemicals

Ficoll plaque, modified Hank's solution, Zimosan A, and Triton X-100 were purchased from Sigma-Aldrich, S.L. (USA), water-soluble tetrazolium salt (WST-1) from Roche (USA), aspirin from J.T. Baker (USA), dimethyl sulfoxide (DMSO), and ammonium chloride from Merck (Germany), deionized water was used in all experimental procedures. All other reagents were of analytical or high-performance liquid chromatography grade as appropriate.

Collection of plant material

CA, Amaryllidaceae, it was collected in Ecuador, Esmeraldas province, Muisne town, sector N 0° 36' 09.5'' W 080° 01' 30.1'', at 5 meters above sea level. The plant material was taxonomically identified by Professor Alan Meerow. CA leaves and bulbs were collected, dried at 50°C in a forced convection oven for 24 h, and they were ground in a knife mill until the particle size of 2–3 mm.

Extraction of the plant material

Methanolic extracts obtention

20 g of CA bulbs or leaves were separately macerated with 400 ml of methanol for 48 h at room temperature with occasional shaking. Then, the extracts were filtrates, and the process was repeated on the marc until material exhausting. The collected filtrates concentrated under reduced pressure (45°C) to produce the dry extracts (5.02% and 6.28% for yield of leaves and bulbs, respectively). The solids were stored at 4°C and vacuum until use [17].

Alkaloidal extracts obtention

The dried samples were left in methanol for 48 h. Alternatively, for a proper maceration, ultrasonic baths of 2 h per day were make, with solvent replacement every 24 h. After the maceration time, the methanolic extract was filter, and the solvent was evaporate using a rotary evaporator under reduced pressure at 50°C. The dried crude extract was then dissolved with H_2SO_4 (2%v/v) to remove the neutral material (waxes, chlorophylls, mucilage, etc.) with ethyl ether (4 times). The acidic aqueous phase was then subjected to basification with NH_4OH (25% v/v) to pH~10 for alkaloid extraction with ethyl acetate. The extract was concentrated under reduced pressure at 45°C [18]. The yield of dry alkaloidal extracts was 0.41% and 0.62% to leaves and bulbs, respectively.

Preliminary phytochemical screening test for CA

The phytochemical screening on crude extracts obtained from CA leaves and flowers was made to investigate secondary metabolites presence such as alkaloids, flavonoids, terpenoids, saponins, tannins, and phenols using standard procedure [19,20].

Anti-inflammatory assay

Neutrophils isolation is necessary for the performance of anti-inflammatory and cytotoxicity tests. Heparinized fresh venous blood sample was drawn from healthy volunteers and neutrophils were isolated [21]. Whole blood was added to Ficoll paque, and the mixture was centrifuged for 30 min at 1500 rpm. After discarding the supernatant, red blood cells traces lysis was made by mixing with hypotonic ammonium chloride solution (0.83% w/v). It was centrifuged again, and the neutrophils were washed with MHS (modified Hank's solution, pH 7.4) and suspended at a concentration of 10^7 cells/mL in an MHS suitable volume [22]. Anti-inflammatory activity was determined using modified assay [23]. This *in vitro* assay is based on the reduction of WST-1 in the presence of activated neutrophils, because during the inflammatory response it is known that white cells produce reactive oxygen species [24]. Anti-inflammatory assay was determined in a total volume of 250 µL MHS (pH 7.4) containing 10^4 neutrophils/µL, 500 µM WST-1 and various concentrations of the test extracts. Control contained buffer, neutrophils, and WST-1. All compounds were equilibrated at 37°C, and the reaction was initiated by adding opsonized Zymosan A (15 mg/mL), which was prepared by mixing it with human pooled serum, followed by centrifugation at 3000 rpm and the pellet was suspended in phosphate buffer solution. Absorbance was measured at 450 nm [22]. Aspirin was used as a positive control that is widely used as nonsteroidal anti-inflammatory drugs for the treatment of several inflammatory diseases [25,26]. DMSO was used as blank, and the anti-inflammatory activity was expressed as produced superoxide anions inhibition percent.

Cell viability assay (cytotoxicity)

Metabolically active cells reduce tetrazolium salts into colored formazan compounds. Therefore, tetrazolium salt-based colorimetric assays detect viable cells only. These sensitive assays can readily be performed in a microtiter plate with relatively few cells using a modified method [27]. In this study, the human isolated neutrophils (10^7 cells/mL) were incubated with test extracts for 30 min then WST-1 (250 µM) was added and incubated in shaking water bath at 37°C for 3 h. The absorbance was measured at 450 nm. Triton X-100 (0.1% v/v) was used as negative control showed 0.00% cell viability [22]. DMSO (5% v/v) was used as positive control exhibited 100.00% cell viability. The OD is the mean of three replicates. Cell viability percentage was calculated using the following formula:

$$\% \text{ Cell viability} = (OD_{test}/OD_{DMSO}) \times 100 \quad (1)$$

Data analysis

The results are expressed as a mean± standard deviation. Student's t-test and one-way analysis of variance were applicable and were used to analyze the level of statistical significance between groups. $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Phytochemical screening

According to the results of preliminary phytochemical screening of both the extracts showed the presence of alkaloids, flavonoids, carbohydrates, and glycosides. Proteins and cardiac glycosides were absent in both the extracts (Table 1).

Evaluation of *in vitro* anti-inflammatory activity

The inhibitory effect on inflammation derived from different concentrations of the CA extracts on isolated neutrophils model using stable tetrazolium salt (WST-1) is summarized in Table 2.

Both the bulb methanolic extract and bulb alkaloids extract showed appreciable inhibition of inflammation on isolated neutrophils (activated with opsonized zymosan A) in a dose-dependent way. The *in vitro* anti-inflammatory activity was not comparable to the aspirin, a reference anti-inflammatory drug at the same dose levels of extracts. A significant difference in the inflammatory inhibition was observed in case of bulb methanolic extract when is compared with the bulb alkaloids extract at concentrations of 12.5 µg/mL and 200 µg/mL.

Cytotoxicity

The cytotoxicity was evaluated in terms of cell viability using freshly isolated neutrophils as a cellular model. The results of the cytotoxicity are shown in Table 3.

Major classes of phytochemicals were revealed from the results of phytochemical screening of both leaves and bulbs of CA, especially alkaloids, follow by flavonoids, glycosides, and carbohydrates, but no protein was detected. In addition, the bulb of CA showed saponins while the leaves tannin, steroids, and terpenes.

Plants belonging to the Amaryllidaceae family have alkaloids in their chemical composition. Alkaloids have been reported in *C. asiaticum* a species of the *Crinum* genus of which CA is hybrid [28]. The alkaloids of the CA bulb have been investigated, among which the most representative are licorine, bufanisine, Augustine, Ambelin, flexinin, crinamabine [16], amabiloside [29], and small amounts of galantamine, narvedine, galantine, hypeastrin, crinidine, and crinamine [30]. It has also been

Table 1: Results of phytochemical analysis of CA leaves and bulbs methanolic extracts

Phytochemical test	Name of the test	CA leaf extract	CA bulb extract
Tannins	$FeCl_3$ test, Lead acetate test	+	–
Steroids	Salkowski test	+	–
Flavonoids	Ammonia test, Alkaline reagent test	+	+
Saponins	Frothing test	–	+
Proteins and amino acids	Ninhydrin test	–	–
Alkaloids	Dragendorff's, Hager's, Meyer's, and Wagner's test	+	+
Carbohydrates	Molisch's test	+	+
Glycosides	Nitroprusside test	+	+
Terpenoids	Salkowski test (modified)	+	–

+: Present, –: Absent. CA: *Crinum x amabile*

Table 2: *In vitro* anti-inflammatory effect of CA methanolic extracts and alkaloids extracts by isolated neutrophils model using tetrazolium salt (WST-1)

Concentration (µg/ml)	Inflammatory inhibition (%)				
	Methanolic extract		Alkaloids extract		Aspirin
	Leaves	Bulbs	Leaves	Bulbs	
12.5	29.91±0.19	43.07±0.15*	4.04±0.78	42.36±0.10	54.09±0.21
25	30.75±0.15	47.52±0.19	10.62±0.15	46.92±0.34	57.96±0.33
50	32.17±0.29	51.13±0.15	15.18±0.34	51.48±0.24	61.51±0.18
100	33.79±0.24	53.97±0.19	18.96±0.24	54.30±0.14	65.79±0.25
200	35.66±1.16	56.35±0.24	22.93±5.57	57.12±0.14*	69.80±0.11

Values are mean±SD, n=3. *Significant values, $P<0.05$, using Student's t-test, bulbs alkaloids extract versus bulbs methanolic extract. SD: Standard deviation, CA: *Crinum x amabile*

Table 3: % Cell viability of CA methanolic extracts and alkaloid extracts at different concentrations

Concentration (µg/ml)	Cell viability (%)				
	Methanolic extract		Alkaloids extract		Aspirin
	Leaves	Bulbs	Leaves	Bulbs	
12.5	60.25±0.31	53.55±0.23	65.41±0.31	65.71±0.31*	45.22±0.24
25	56.95±0.23	45.21±0.23	61.22±0.31	63.05±0.23*	41.37±0.33
50	52.73±0.23	40.27±0.46	54.39±0.15	57.01±0.23*	37.53±0.18
100	46.55±0.46	35.94±0.23	50.92±0.38	52.92±0.15*	32.62±0.27
200	41.40±0.31	32.85±0.54	43.88±0.38	47.80±0.31*	29.84±0.37

Values are mean±SD, n=3. *Significant values, $P<0.05$, using Student's t-test, bulbs alkaloids extract versus bulbs methanolic extract. Triton X-100 (0.1%) was used as negative control, showed 0.00% cell viability. SD: Standard deviation, CA: *Crinum x amabile*

shown that the type and proportion of alkaloids can differ between the species of the *Crinum* genus, hence the difference in biological activity that the various species show [31]. Terpenes and steroids in the leaves of CA contrast with the total absence of these components in the CA bulbs. The absence of steroids has been proved in the leaves of *Crinum giganteum* [32] and *C. asiaticum* [33]. There are no references to respect for alkaloids composition of CA leaves. Nevertheless, since to a large amount of these compounds exist in the extracts tested; the structural elucidation of the reliable components for the biological activity of the extracts is necessary. Various gas chromatography-mass spectra methods are available for that purpose [34].

The cell viability decreased by increase of CA extracts concentration. The CA cytotoxicity leaves and bulbs alkaloids extracts on isolated neutrophils are remarkable.

The results of the anti-inflammatory activity percent of CA leaves and bulbs extracts demonstrated a proportional relation between extract concentrations and anti-inflammatory activity. Amaryllidaceae family has not been widely studied for its anti-inflammatory activity but in its chemical composition (isoquinolinic type alkaloids). In this research, appreciable amounts of alkaloids would associate these components as responsible for the anti-inflammatory activity of CA.

Alkaloids have diverse biological activities, which are mainly related to its ability to inhibit enzymes [35], i.e., acetylcholinesterase [36] and its effects to induce apoptosis [37]. The pharmacological effects of isoquinolinic alkaloids such as its anti-Alzheimer [38], anti-inflammatory, antiprotozoal [39], antimicrobial, and anticancer activities [40]. Lycorine has been shown to be effective at inhibiting iNOS (inducible nitric oxide synthase) in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages [41]. Kang *et al*. highlighted that lycorine inhibited LPS-induced iNOS and COX-2 upregulation in RAW264.7 cells through the suppression of p38 and STATs activation [42]. LPS-induced tumor necrosis factor-α (TNF-α) is an important pro-inflammatory cytokine involved in the regulation of inflammation and related disorders. Lycorine turned out to inhibit TNF-α production in LPS-stimulated murine macrophages (IC_{50} 0.2 µg/mL) [43]. Furthermore, Liu *et al*. demonstrated that TNF-α signal transduction pathway and p21-mediated cell cycle inhibition were involved in the apoptosis of HL60 cells induced by lycorine [44].

When comparing the anti-inflammatory activity of the CA bulb extracts respect to anti-inflammatory activity of aspirin we found a statistically significant difference. Nevertheless, the potential of CA bulb extracts as an anti-inflammatory is measurable (56–57% inflammatory inhibition). Cell viability comparison between CA bulb alkaloids extract and CA bulb methanolic extract, and aspirin differs from each other.

Although the anti-inflammatory percentage of aspirin differs from the CA bulb extracts, it is not difficult to deduce that CA bulb extracts have interesting potential as an anti-inflammatory. All of the doses of CA bulb extract having an anti-inflammatory efficacy similar to that exhibited by aspirin.

The CA extracts have an anti-inflammatory activity like that of aspirin with a significant advantage over cytotoxicity, since the aspirin cytotoxicity is higher than that of leaves and bulbs extracts, which implies an important safety of use of this part of the plant and makes it a relatively safe alternative for its use as an anti-inflammatory. It is essential to make greater efforts focused on the isolation and structural elucidation of the functional components of CA, as well as studies to find other biological activities of this promising plant species like cholinesterase inhibition (Alzheimer *in vitro* experimental model). Finally, the research carried out is a contribution to appreciate the forgotten ancient use of this resource by Afro-Ecuadorians West Coast (of Muisne town especially) that uses it on hemorrhoids treatment. The results of this study could become an opportunity for the regional development of the communities through the planting, conservation, and rational use of this species; and, in this way, improve their economy.

CONCLUSION

Major classes of phytochemicals such as alkaloids and flavonoids followed by carbohydrates and glycosides were identified in the leaves and bulbs of CA variety grown in Ecuador. In addition, tannin, terpenoids, and steroids were identified in the leaves; and in the bulb only saponins. Although the anti-inflammatory activity of the CA bulbs did not reach results comparable to the control (aspirin), it has demonstrated under experimental conditions a considerable activity. CA leaves exhibited anti-inflammatory activity but did not achieve the efficacy denoted by CA bulbs. The research developed has established that the ethnobotanical use of CA is valid and that the use of this plant resource would be harmless in light of the results obtained.

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AUTHORS' CONTRIBUTIONS

Vinueza Diego conceived and designed the experiments as well as wrote the paper. Portero Santiago, Acosta Karen, and Pilco Gisela performed the analyses. García Marlene made a statistical analysis of the data. Abdo Susana made critical reading and contributions to the paper.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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