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Pharmacognostic characterization and biological activities of *Aponogeton crispus* and *A. rigidifolius*

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Abstract. *Sarveswaran R, Jayasuriya WJAB, Dias B, Kariyawasam I, Hettiarachchi P, Suresh S. 2025. Pharmacognostic characterization and biological activities of* Aponogeton crispus *and* A. rigidifolius*. Asian J Nat Prod Biochem 23: 1-10.* The potential anti-inflammatory effects of the plant family Aponogetonaceae have yet to be widely studied despite the existing ethnomedicinal evidence. This study aimed to investigate the pharmacognostic characteristics and pharmacological activities of *Aponogeton crispus* Thunb. and *Aponogeton rigidifolius* H. Bruggen plants. Anti-inflammatory and anti-hyperglycemia-related bioactivities were evaluated using well-established in vivo and in vitro methods. The safety profiles of the aqueous plant extracts were investigated in acute toxicity and chronic toxicity studies. Significance among different test groups in each assay was analyzed statistically. Reducing sugars, amino acids, alkaloids, flavonoids, and polyphenols were found in the preliminary phytochemical screening. In pharmacological assessments, aqueous extracts of *A. crispus* at 120 mg/kg showed the highest inhibition of carrageenan-induced paw edema, indicating its significant therapeutic potential. The percentage reduction in acetic acid-induced writhes was 46.48±3.46% for *A. crispus* and 54.22±2.10% for *A. rigidifolius*. Both extracts significantly reduced the rat body temperature at the third and fourth hour in brewer's yeast-induced pyrexia. Moreover, both plants showed appreciable safety profiles in 2,000 mg/kg dose. The study concludes that both *A. crispus* and *A. rigidifolius* plants possess explicit pharmacognostic characteristics, show significant therapeutic effects against the cardinal signs of inflammation and hyperglycemia, also exhibit no in vivo toxicities.

Keywords: Anti-hyperglycemic, anti-inflammatory, aquatic plants, in vivo, pharmacognosy

INTRODUCTION

The plant family Aponogetonaceae comprises only a single genus, *Aponogeton*, and consists of approximately sixty freshwater monocotyledons, including *Aponogeton natans* Engl. & K. Krause, *Aponogeton crispus* Thunb., *Aponogeton rigidifolius* H. Bruggen, *Aponogeton madagascariensis* (Mirb.) H. Bruggen, *Aponogeton jacobsenii, Aponogeton appendiculatus,* and *Aponogeton undulatus* Roxb. which are distributed mainly in the tropical or subtropical regions of the world (Manawaduge and Yakandawala 2018). *Aponogeton crispus* and *A. rigidifolius* are two interesting species found in Sri Lanka with the latter being native. *Aponogeton crispus* normally grows in freshwater tanks, lakes, and ponds, whereas *A. rigidifolius* habitats in slow or fast-running rivers, mostly in wet forests (Wijesundara and Shantha Siri 2004). The genus *Aponogeton* was found to be used in folkloric medicine to treat wounds, snake bites, diarrhea, acne, and jaundice (Chowdhury et al. 2011). Flowering spikes and young shoots of the plants from *Aponogeton* species are consumed as a popular green leafy vegetable (Misra et al. 2012). To alleviate itchiness caused by food allergies, *A. crispus* rhizome is ground with ghee, and the paste is applied over the skin. Flowers of *A. crispus* are specially used to treat *mhadhumehaya* (diabetes) in Ayurveda (Samarasekara 2002). It was found that the plant *A. rigidifolius* also has medicinal and nutritional benefits that are closely related to *A. crispus* (Weragoda 1994). In addition to the two species under the study, other *Aponogeton* species (e.g., *A. monostachyon*) are used in Siddha medical system to prepare Choorna medicines for skin diseases (Priyadharshini et al. 2019). Some related species, such as *A. appendiculatus*, *A. natans*, and *A. undulatus*, have demonstrated considerable antioxidant properties in various *in vitro* assays (Chougule et al. 2022). The extracts of *A. undulatus* and *A. madagascariensis* have further shown antitumor potential against various cancer cell lines (Islam et al. 2015; Gunawardena et al. 2021). The anti-diabetic properties of *A. natans* leaf extract were evaluated by Dash and co-workers using alloxan-induced diabetic rats; significant reduction in blood glucose levels have been recorded (Dash et al. 2014). *Aponogeton madagascariensis* has been extensively studied in botanical investigations of cell culture and genetic techniques due to its interesting anatomical and physiological characteristics (Rowarth et al. 2021).

Although the phytochemical composition of *A. natans* and *A. appendiculatus* has been extensively studied, the information on the phytoconstituents of *A. crispus* and *A.*

rigidifolius is inadequate in the literature (Chougule et al. 2022). The physicochemical and other pharmacognostic data on these plants were also described insufficiently despite the fact that these plant materials can be adulterated easily by adding either other plants or any foreign material to increase the mass or strength (Seethapathy et al. 2014). Inflammatory conditions involve the production of signaling biomolecules that will encourage the inflamed body area to receive more blood flow and immune cell activation. These inflammatory mediators, however, make pain receptors more sensitive and cause pain sensations. They will disrupt the physiological activity of insulin receptors in muscle and liver tissues and reduce cellular glucose uptake, which ultimately leads to hyperglycemia. Furthermore, body temperature and thrombolytic mechanisms may also be modulated through the inflammatory mediators (Soares et al. 2023). Acute and chronic assessment of toxicity is significant for the safety evaluation of herbal medicines as they have become major aspects in regulatory affairs of ethnomedical systems currently (Mukherjee et al. 2021). Therefore, elaborated investigations on the pharmacognostic characterization and pharmacology of these plant species are needed to promote their use as primary medicines as well as functional food. Considering this background, the present study focused on comparatively evaluating the pharmacognosy, biological effects, and safety profiles of *A. crispus* and *A. rigidifolius* to serve as potential medicinal agents, especially over cardinal signs of pathophysiological inflammation, using various in vivo and in vitro methods (Ribaldone et al. 2019). Additionally, an attempt has been made to scientifically validate the ethnomedicinal use of *A. crispus* as a remedy for diabetes.

MATERIALS AND METHODS

Ethical approval

Ethical clearance was obtained from the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka, under approval certificate no. 43/17 and 46/18 for animal studies and human-involved investigations, respectively. All the information sheets and consent forms were reviewed and approved.

Plant materials

Aponogeton crispus and *A. rigidifolius* plants were collected from the wet zone (6.9061N, 79.9630E and 6.3731N, 80.3575E, respectively) of Sri Lanka and were prepared into herbarium specimens. Then these were authenticated by National Herbarium, Peradeniya, Sri Lanka under the voucher numbers 6/01/H/03 and NH/BOT/4/2019-59, respectively.

Pharmacognostic analysis

The analytical methods described in the literature were used in the pharmacognostic standardization (Kumar et al. 2018). In the macroscopic study, morphological descriptions of different plant parts of *A. crispus* and *A. rigidifolius* were studied through direct visual observation or with the assistance of a magnifying lens (5x). Freehand cross sections of plant tissues (leaf and petiole) of both plants were stained with safranin. Detailed anatomy was observed under the compound microscope (Olympus, Japan). Structures and features in the cut sections of both plants were recorded using photomicrographs. The petiole parts of both of the plants were powdered separately and then treated with chloral hydrate solution for the microscopic powder analysis performed under the compound microscope. Structures and features in the powders of both plants were recorded using photomicrographs.

Physicochemical analysis

Standard analytical methods were used in the physicochemical standardization (moisture content, total ash, water-soluble ash content, acid-soluble ash content, extractable matter content) of fresh parts of *A. crispus* and *A. rigidifolius* (Sebastian and Nirmal 2020).Where necessary, the hot air oven (Memmert, Germany), muffle furnace (Hobersal, Spain), and analytical balance (Precista, Switzerland) were used.

Phytochemical screening

Qualitative phytochemical screening

A 20.0 g aliquot of air-dried, coarsely powdered flower and stalk samples of both *A. crispus* and *A. rigidifolius* were separately refluxed with dichloromethane for 1.5 hours. The contents were filtered, and the remaining solid residue was refluxed again with ethanol and distilled water successively for the same duration of time. Qualitative phytochemical screening was then carried out on the syrupy material left after evaporating each extract.

In brief, Benedict's test for reducing sugars (1 mL of the sample + 3 drops of Benedict's reagent and heated for 3 minutes), ninhydrin test for amino acids (1 mL of the sample + 3 drops of 0.25% ninhydrin solution and heated for 3 minutes), biuret test for proteins (1 mL of the sample + 3 drops biuret solution and heated for 3 minutes), alcoholic ferric test for tannins (1 mL of the sample + 10% alcoholic ferric chloride), Keller-Kiliani test for cardiac glycosides (2 mL of the sample + ferric chloride in glacial acetic acid + concentrated sulphuric acid), frothing test for saponins (2 mL of the sample was vigorously shaken with 2 mL of distilled water for 60 seconds), Mayer's and Wagner's tests for alkaloids (2 mL of each sample + 3 drops of Mayer's and Wagner's reagents separately), cyanidin test for flavonoids (2 mL of the sample + hydrochloric acid and magnesium), Liberman-Burchard test and Salkowski test for steroids and triterpenoids, sulphuric acid test for quinones (1 mL of the sample + 1 mL of sulphuric acid), hydroxide test for coumarins (1 mL of the sample + 2 drops of 1% NaOH and heated for 3 minutes), and hydrochloric acid test for leucoanthocyanins (1 mL of the sample + 0.5 mL of concentrated hydrochloric acid and heated for 3 minutes) were performed as per the established methods (Morsy 2014).

Quantitative determination of total phenolic content

A 20.0 g aliquot of air-dried, coarsely powdered flower and stalk samples of both *A. crispus* and *A. rigidifolius* were separately refluxed with distilled water for 1.5 hours. The contents were filtered, and the remaining solid residue was removed. The total phenolic content of this crude aqueous extract was assessed by the Folin-Ciocalteu method described by Perera and co-workers with some modifications (Perera et al. 2017). In brief, 500.0 µL of aqueous extract of A. crispus, A. rigidifolius, and standard gallic acid (n=4) at concentrations of 31.25, 62.5, 125, 250, 500 ppm were mixed with 2.5 mL of Folin-Ciocalteu reagent (diluted ten-fold of initial concentration with deionized water) and left to stand for 5 minutes at room temperature after mixing with 2.5 mL of sodium carbonate solution. The final volume was made up to 10.0 mL using deionized water. The absorbance was measured at 765 nm. The phenolic contents of plant materials were expressed as mg of gallic acid equivalent per gram of extract.

Pharmacological studies

Animals

Both male and female adult Wistar rats (age 6-8 weeks) were purchased from the Medical Research Institute (MRI), Sri Lanka. Animals were housed at room temperature (27±2°C) and a 12-hour light/ dark cycle while feeding with food pellets formulated using MRI standards. Clean, fresh water was provided *ad libitum*.

Preparation of extracts for pharmacological studies

The air-dried and powdered flowers and stalks of each plant (*A. crispus* and *A. rigidifolius*) were combined to get two final powder samples separately. Sixty grams of each sample was boiled with 1.9 L of distilled water in separate clay pots, and the final volume was reduced to about 240.0 mL by gentle boiling. Concentrated crude extracts were freeze-dried separately. The required concentrations of the aqueous extracts of *A. crispus* and *A. rigidifolius* were prepared by dissolving freeze-dried extracts in distilled water suitably.

In vivo anti-inflammatory activity in Wistar rats

Healthy, adult male Wistar rats were randomly divided into 7 groups (n=6 per group). The animals in group 1 received 1.0 mL of distilled water; this group served as the baseline for comparison. Animals in groups 2 and 3 were orally administered with aqueous extracts of *A. crispus* at doses of 90 mg/kg and 120 mg/kg body weight (b.w.), respectively. Groups 4 and 5 were orally administered with the same doses of *A. rigidifolius,* respectively. Animals in groups 6 and 7 were treated with standard drugs, indomethacin (10 mg/kg) and diclofenac sodium (15 mg/kg), respectively. After 1 hour of administration of the test samples, inflammation underneath the plantar tissue of the rat's left hind paw was induced by subcutaneous injection of 0.1 mL of carrageenan (1%) aqueous suspension under mild anesthesia. The anti-inflammatory activities of test substances were determined by the paw edema method described elsewhere (Jayasuriya et al. 2020).

In vivo antipyretic activity in Wistar rats

Healthy, adult male Wistar rats were randomly divided into 4 groups (n=6 per group). The basal rectal temperature of each rat was recorded using a flexible digital thermometer. Pyrexia in rats was induced by injecting a 10.0 mL/kg b.w. dose of brewer's yeast suspension (20% w/v) into the rat dorsum. Eighteen hours after the injection, the rise in rectal temperature was recorded. Since all the subject animals had ≥ 0.7°C (or 1°F) increase in body temperature, all of them were employed in the investigation. The animals in group 1 served as the control group and received 1.0 mL of distilled water. Groups 2 and 3 rats were administered with aqueous extracts of *A. crispus* and *A. rigidifolius* at a dose of 90 mg/kg b.w., respectively. Acetylsalicylic acid (100 mg/kg) was given orally to test animals in group 4. Then, the rectal temperature of the rats was recorded at hourly intervals for 4 hours to determine the antipyretic activity of the test materials (Bhowmick et al. 2014).

In vivo anti-nociceptive activity in Wistar rats

Healthy, adult male Wistar rats were randomly divided into 4 groups (n=6 per group). Animals in group 1 served as the control group and received 1.0 mL of distilled water orally. Groups 2 and 3 rats were treated with aqueous extracts of *A. crispus* and *A. rigidifolius* at a dose of 90 mg/kg b.w., respectively. Group 4 was administered with a 100 mg/kg dose of acetylsalicylic acid and considered as the positive control group. After 30 minutes of oral administration of the test substances, writhing was induced by intraperitoneal 0.6% acetic acid injection (10.0 mL/kg). The anti-nociceptive activity was then assessed by cumulatively counting the number of abdominal constrictions produced for 20 minutes after a latency period of 5 minutes from the injection. The percentage reduction of writhes was determined by the method described in the literature. Anti-nociceptive activities of the test materials and controls were thereby reported as the analgesic index calculated by the methods given in the literature (Rahaman et al. 2020).

In vitro thrombolytic activity on human blood

Healthy human volunteers (n=10) who had no history of smoking or taking lipid-lowering drugs, oral contraceptives, or anticoagulant medications were selected, and informed consent was obtained for the study. Then, 6.0 mL of venous blood was withdrawn from each of them, and 2.5 mL of blood volume was transferred to pre-weighed sterile microcentrifuge tubes. Tubes were incubated at 37°C for 45 minutes, and the serum was completely removed from the tube after that. Then, 100.0 µL quantities of aqueous extracts of *A. crispus* and *A. rigidifolius* in different concentrations (45 mg/mL, 90 mg/mL, 135 mg/mL, and 180 mg/mL) were added to each of the tubes (n=4). As the positive control, 100.0 µL of streptokinase (30,000 IU) was used, and the same amount of sterile distilled water was added to the control. After incubating all tubes at 37°C for another 90 minutes, the supernatant liquid was removed without disturbing the clot. These tubes were weighed again to determine the clot

weight. Results were produced as a percentage of clot lysis (Bhowmick et al. 2014).

In vivo hypoglycemic activity in healthy Wistar rats

The hypoglycemic activity was studied using only the different concentrations of the aqueous extract of *A. crispus.* Healthy, adult male Wistar rats were fasted overnight and randomly divided into 6 groups (n=6 per group). The animals that received 1.0 mL of distilled water were included in group 1, the control group. Animals in groups 2-5 were orally administered with an aqueous extract of *A. crispus* at respective doses of 22.5, 45, 90, and 180 mg/kg b.w. The animals in group 6 were treated with the standard drug metformin (15 µg/kg). After 30 minutes of administration of the test substance, serum glucose levels were raised by loading glucose orally at a dose of 3 g/kg b.w. in all groups. After 90 minutes, blood was collected from the lateral tail vein, and the serum glucose levels were determined using the glucose reagents kits (Biolabo reagents, France). The optimum time of the hypoglycemic activity of the aqueous extract of *A. crispus* (90 mg/kg b.w.) was determined by evaluating serum glucose levels of a separate group of rats at 30-minute intervals from the glucose loading (Jayasuriya et al. 2012).

Evaluation of safety profile

The safety profiles of *A. crispus* and *A. rigidifolius* were assessed according to the guidelines of the Organization for Economic Co-operation and Development (OECD 2002, 2018).

Acute toxicity

Fifteen male and fifteen female Wistar rats were randomly divided into three groups (n=10 per group). Group 1 animals were served as the controlled group and received distilled water. Groups 2 and 3 were treated with 2,000 mg/kg b.w. single doses of aqueous extracts of *A. crispus* and *A. rigidifolius,* respectively. At the end of 14 days, biochemical parameters of the animal, i.e., serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total protein, and creatinine were assessed. All the animals were then sacrificed to determine the relative organ weight percentages.

A histopathological study was conducted to determine whether there were alterations in tissue samples of the heart, kidney, and liver. In brief, rat tissues were cleaned with normal saline and fixed in a 10% formalin solution. Then, the tissues were cleaned thorough with distilled water and processed (Microm STP, United Kingdom) over 24 hours. Tissues were sliced into 4-5 µm thick sections using a microtome, dewaxed with xylene and hydrated through a descending series of ethanol to water. After staining with hematoxylin and eosin, the animal tissues were cover slipped using dibutylphthalate polystyrene xylene (DPX) as the mountant. Finally, slides were microscopically examined for histopathological changes.

Sub-chronic toxicity

Wistar male and female rats were randomly divided into three groups (n=10 per group). The first group was the control and received distilled water. In comparison, the other two groups were orally treated with aqueous extracts of *A. crispus* and *A. rigidifolius* at 90 mg/kg b.w. daily doses for 90 consecutive days. After 90 days, the same biochemical parameters mentioned above were evaluated along with hematological parameters using the rat serum. Then, the animals were sacrificed, and relative organ weights were determined.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) where necessary. The data obtained from each experiment were analyzed in Microsoft Excel 2016 and SPSS version 25 software. Analysis of variance (ANOVA) was conducted at the significance levels of $p<0.001$, $p<0.01$, and $p<0.05$ using Bonferroni and Tukey post hoc tests.

RESULTS AND DISCUSSION

Pharmacognostic evaluation

Macroscopic assessment

This study on *A. crispus* and *A. rigidifolius* revealed that both plants lack floating leaves. Mature plants have single or several inflorescences, whereas younger plants do not have any inflorescence. The main distinctive character is their presentation of rootstock, i.e., *A. crispus* has a non-branching tuber, whereas *A. rigidifolius* has a branched rhizome. In addition to that, *A. crispus* has dense flowers, and *A. rigidifolius* has a slightly dense flower pattern. The flowers of both species are white with two tepals. Further, these flowers contain three ovaries and six stamens.

Microscopic anatomy

According to the microscopic assessment of different parts of *A. crispus* and *A. rigidifolius*, monocot-type vascular bundles were found in the petiole of both plants. The basal part of *A. crispus* leaf is triangular in a cross-sectional view. Towards the inner side of the epidermal layer, two to three layers of compact parenchyma cells were observed (Figure 1).

Powder microscopy

The powder microscopy of the petiole of *A. crispus* and *A. rigidifolius* resulted in fragments of large vessels, parts of epidermal cell layers, calcium carbonate crystals, and sclereids in both plants. Furthermore, the powder microscopy of *A. rigidifolius* petioles presented a striking image of the parenchymal cell layer, the four-cell thickness of the epidermal cell layer, fragments of large vessels with pitted zones, idioblast crystals, and sclereids (Figure 2).

Physicochemical properties

In the physicochemical assessment of plant materials, it was observed that *A. crispus* had slightly higher ash values compared to *A. rigidifolius.* In comparison, the acid-soluble ash content in *A. crispus* was found to be about two-fold

greater than in *A. rigidifolius.* Although fresh materials of both plants had almost similar moisture contents, powdered *A. crispus* showed a greater moisture level compared to powdered *A. rigidifolius* sample (Table 1).

Phytochemical analysis

Qualitative phytochemical screening

According to the results of the preliminary phytochemical screening of *A. crispus* and *A. rigidifolius*, cardiac glycosides were not present in any of the test extracts. Aqueous extracts of both plants yielded positive results for higher levels of alkaloids, phenols, flavonoids, tannins, proteins, and sugars. The dichloromethane extract of *A. rigidifolius* was positive only for a few phytochemicals, including phenolics and leucoanthocyanins (Table 2).

Table 1. Physicochemical properties of *Aponogeton crispus* and *A. rigidifolius*

Physicochemical properties	Plant sample (%)	
	A. crispus	***A. rigidifolius***
Weight loss on drying		
Moisture content of fresh sample	95.10±0.04	94.55±0.12
Moisture content of powdered sample	12.83±0.03	7.52±0.05
Ash values		
Total ash	18.74±0.05	17.69±0.03
Water soluble ash	7.16±0.28	6.51±0.22
Acid soluble ash	7.19±0.08	3.76±0.10
Extractive values		
Water	20.48±0.31	20.66±0.18
Ethanol	5.30±0.08	5.48±0.11
Dichloromethane	2.49±0.11	1.75±0.05

Note: Data values are expressed as mean of percentages ± SEM (n=3)

Table 2. Qualitative phytochemical screening of *Aponogeton crispus* and *A. rigidifolius*

Secondary metabolites	*A. crispus*			*A. rigidifolius*		
	Water	**Ethanol**	**DCM**	**Water**	**Ethanol**	**DCM**
Alkaloids	+++	++	+	++	+	-
Phenols	+++	++	+	+++	++	+
Flavonoids	++	+++	+	++	-	-
Tannins	+++	+	-	+++	-	-
Cardiac glycosides	-	-	-	-	-	-
Steroids	-	+	+	-	-	-
Terpenoids	+	-	-	++	-	-
Saponins	-	+	-	-	+	-
Leucoanthocyanins	-	++	+	+	-	+
Reducing sugar	+++	++	-	++	-	-
Protein and amino acids	++	++	+	+++	+	-
Coumarins	-	+++	+	++	-	-
Quinones	++	-	-	+	-	-

Note: DCM: Dichloromethane, '-': not detected, '+': trace, '++': moderate, '+++': appreciable amount

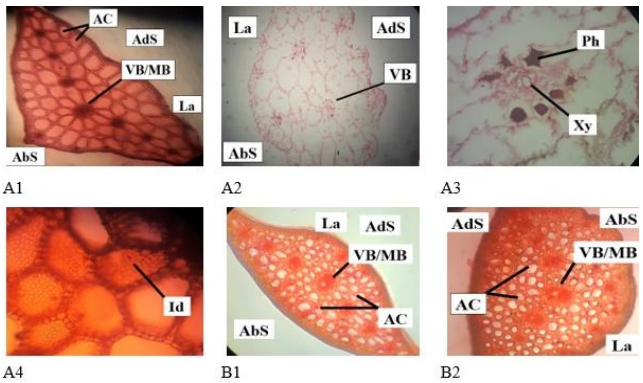

Figure 1. Microscopic view of transverse sections of A. *Aponogeton crispus*; B. *A. rigidifolius*, showing 1. Leaf, 2. Petiole, 3. Vascular system of the midrib, 4. Petiole with idioblasts. AbS: abaxial side, AdS: adaxial side, La: lamina, VB: vascular bundle, MB: median bundle, AC: aerenchyma cells, Ph: phloem, Xy: xylem, Id: idioblasts

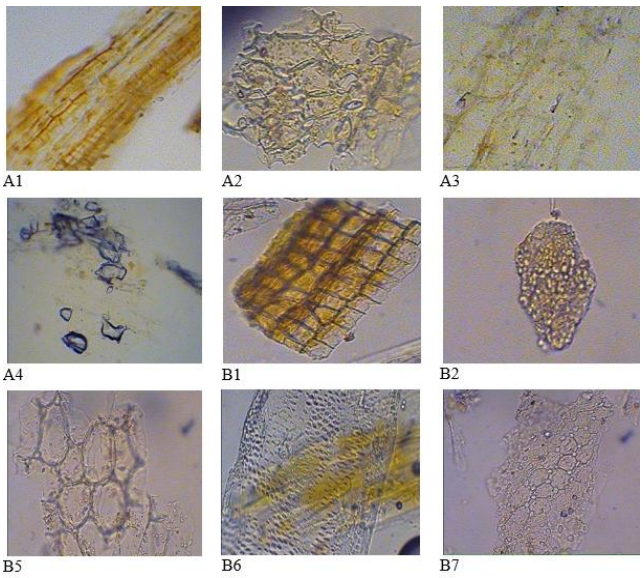

Figure 2. Photomicrographs of the plant powders of A. *Aponogeton crispus*; B. *A. rigidifolius*, showing 1. Epidermal cells, 2. Sclereids, 3. Fragments of large vessels, 4. Calcium carbonate crystals, 5. Parenchymal cells, 6. A fragment of large vessels with pitted zones, 7. idioblast crystals. 10×10

Table 3. Effect of aqueous extracts of *Aponogeton crispus*, *A. rigidifolius*, and acetylsalicylic acid on yeast-induced pyrexia

Test group	Rectal temperature after 18 hours of yeast injection (°C)	Rectal temperature (°C)			
		1 h	2 h	3 h	4 h
Distilled water	38.41±0.05	38.27±0.05	38.37±0.07	38.32±0.05	38.25±0.06
Acetylsalicylic acid 100 mg/kg	38.24±0.12	37.96±0.23	37.61±0.16*	37.38±0.12**	37.21±0.09***
A. crispus 90 mg/kg	38.39±0.12	38.29±0.16	38.16±0.12	37.53±0.15*	37.74±0.10*
A. rigidifolius 90 mg/kg	38.39±0.09	38.24±0.10	38.02±0.09	37.69±0.11*	37.32±0.09***

Note: Data values are expressed as mean ± SEM (n=6). Significant *p<0.05, **p<0.01, ***p<0.001 as compared to the control; ANOVA followed by Tukey's post-hoc test

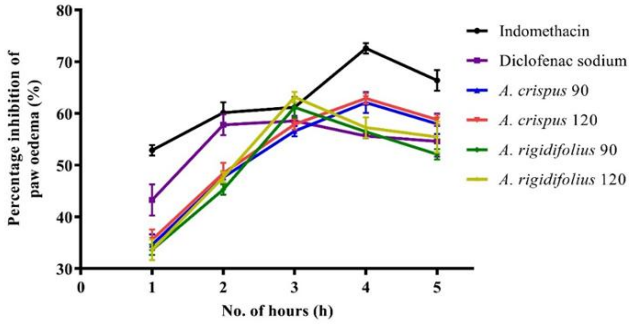

Figure 3. Effect of aqueous extracts of both *Aponogeton crispus* and *A. rigidifolius* (at 90 and 120 mg/kg b.w.), diclofenac sodium, and indomethacin on carrageenan-induced paw edema; data values are expressed as percentage mean ± SEM (n=6), all the data are significant (p<0.05) when compared to the distilled water control; ANOVA followed by Tukey's post-hoc test

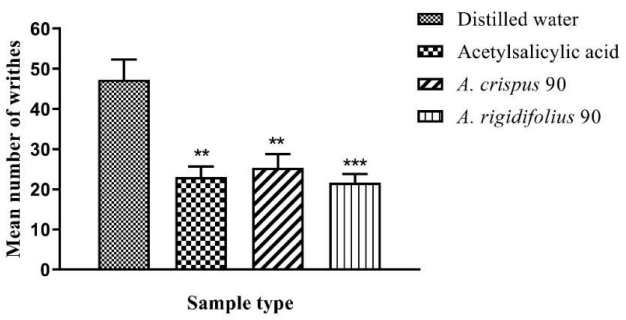

Figure 4. Effect of aqueous extracts of both *Aponogeton crispus* and *A. rigidifolius* (at 90 mg/kg b.w.), and acetylsalicylic acid on number of writhes produced; data values are expressed as mean ± SEM (n=6). Significant *p<0.05, **p<0.01, ***p<0.001 as compared to the control; ANOVA followed by Tukey's post-hoc test

Quantitative determination of total phenolic content

Crude aqueous extracts of *A. crispus* and *A. rigidifolius* contain total phenolics of 72.69±1.87 mg GAE/g and 49.25±1.81 mg GAE/g, respectively.

Pharmacological studies

In vivo anti-inflammatory activity

As per the results of the carrageenan-induced hind paw edema assay, the maximum inhibition of edema by *A. crispus* was observed at the 4th hour with 90 mg/kg (62.09±2.21%) and 120 mg/kg doses (62.90±3.45%) following oral administration, whereas 61.18±3.42% and 63.15±5.26% of inhibitions were observed at the 3rd hour with the same doses of *A. rigidifolius,* respectively. Furthermore, different doses of the same test plant demonstrated similar patterns in anti-edemic activity interestingly (Figure 3).

In vivo antipyretic activity

Subcutaneous injection of yeast caused elevated rectal temperatures (38.24-38.41°C) in all rats used in this study. None of the test plant extracts were able to exhibit significant antipyretic activities (p>0.05) during the first 2 hours of the dose administration; however, both plant extracts (90 mg/kg) demonstrated significant body temperature reduction effects (p<0.05) during the 3rd and 4th hours (Table 3).

In vivo anti-nociceptive activity

Aqueous extracts of *A. crispus* and *A. rigidifolius* showed 25.33±3.50 and 21.67±2.10 mean number of writhes, respectively, where the mean percentage reductions of writhes were 46.48% (p<0.01) and 54.22% (p<0.001) in the respective test group. Moreover, acetylsalicylic acid resulted in a 23.00±2.71 mean number of writhes (Figure 4).

In vitro thrombolytic activity

According to the percentage of clot lysis demonstrated by test groups, *A. crispus* dose of 135 mg/kg exhibited the highest activity (27.96±3.37%), which was comparable (p<0.05) to the standard drug, streptokinase (32.14±2.46%). Meanwhile, 180 mg/kg dose of the same extract demonstrated the lowest activity. *Aponogeton rigidifolius* doses of 90 mg/kg and 135 mg/kg demonstrated >20% of thrombolytic activity; however, it did not exhibit a significant effect (p>0.05) compared to the control. The statistical comparison confirmed that a negligible amount of clot lysis occurred with distilled water (Figure 5).

In vivo hypoglycemic activity

The aqueous extracts of *A. crispus* at the doses of 90 mg/kg and 180 mg/kg significantly (p<0.05) reduced the serum glucose levels in normal healthy rats. The postprandial serum glucose levels of the respective test groups were 3.64±0.35 and 3.87±0.32 mmol/L after 90 minutes of glucose loading. Among the four doses evaluated, the dose of 90 mg/kg appeared to be the most effective in reducing postprandial serum glucose levels in test animals (Figure 6.A). According to the serum glucose reduction determined in healthy Wistar rats following oral administration of 90 mg/kg dose of *A. crispus* against time,

the minimum serum glucose level of 3.64±0.35 mmol/L was observed at 90 minutes from the administration of the test substance (Figure 6.B).

Toxicity studies

All of the test rat groups survived after being treated with a single dose of 2,000 mg/kg of aqueous extracts of *A. crispus*, *A. rigidifolius*, and distilled water for 14 days in the acute toxicity study. The animals showed no significant changes ($p>0.05$) in their serum biochemical parameters. The results of histomorphological studies done at 14 days (as presented in Figure 7) also manifest no significant variations in the histology of heart, kidney, and liver tissues of the animals of the treatment group after the acute toxicity study.

All the animals tested in the sub-chronic toxicity study also survived without any significant abnormalities in tested serum biochemical or hematological parameters ($p>0.05$). All rats gained weight through the study but this observation was comparable with the respective control groups. The relative organ weight percentage of the animals used in the sub-chronic toxicity assay (as indicated in Table 4) did not show any significant alterations in test groups at any time point compared to the respective control group ($p>0.05$).

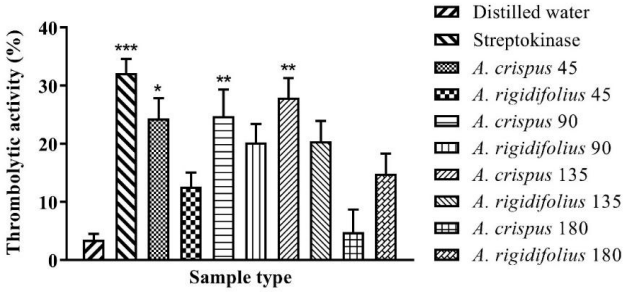

Figure 5. Effect of aqueous extracts of both *Aponogeton crispus*, *A. rigidifolius* (at 45, 90, 135, 180 mg/mL), and streptokinase on percentage clot lysis; data values are expressed as mean ± SEM (n=4). Significant *p<0.05, **p<0.01, ***p<0.001 as compared to the control; ANOVA followed by Tukey's post-hoc test

Table 4. The relative organ weight percentage of Wistar rats after receiving a single dose of test substances for 14 days and consecutive doses for 90 days

	Relative organ weight (%) at 14th day			Relative organ weight (%) at 90th day		
	A. crispus	***A. rigidifolius***	**Distilled water**	***A. crispus***	***A. rigidifolius***	**Distilled water**
Liver	3.73±0.09	3.95±0.04	3.76±0.06	4.28±0.03	4.25±0.04	4.26±0.05
Kidney	0.46±0.01	0.48±0.01	0.44±0.02	0.55±0.01	0.56±0.01	0.54±0.02
Heart	0.49±0.01	0.47±0.01	0.49±0.01	0.43±0.01	0.43± 0.01	0.42±0.01

Note: Data values are expressed as mean ± SEM (n=10). ANOVA followed by Tukey's post-hoc test

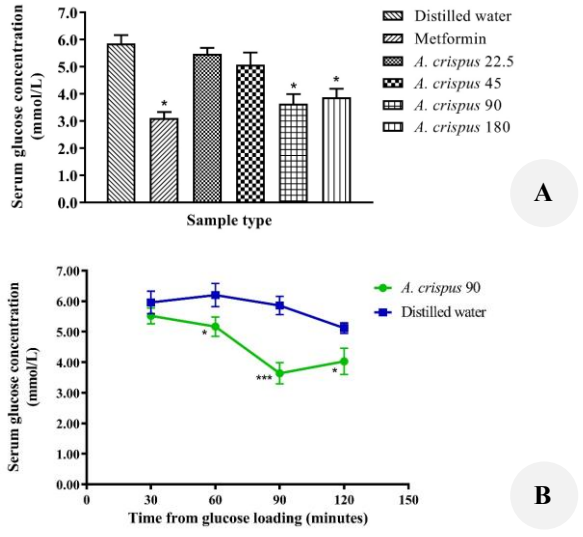

Figure 6. A. Effect of different doses of aqueous extract of *Aponogeton crispus* (at 22.5, 45, 90, 180 mg/kg b.w.) on serum glucose levels in healthy Wistar rats; B. Serum glucose reduction in healthy Wistar rats following oral administration of *A. crispus* aqueous extracts (90 mg/kg b.w.) with time; data values are expressed as mean ± SEM (n=6). Significant *p<0.05, **p<0.01, ***p<0.001 as compared to the control; ANOVA followed by Bonferroni's post-hoc test

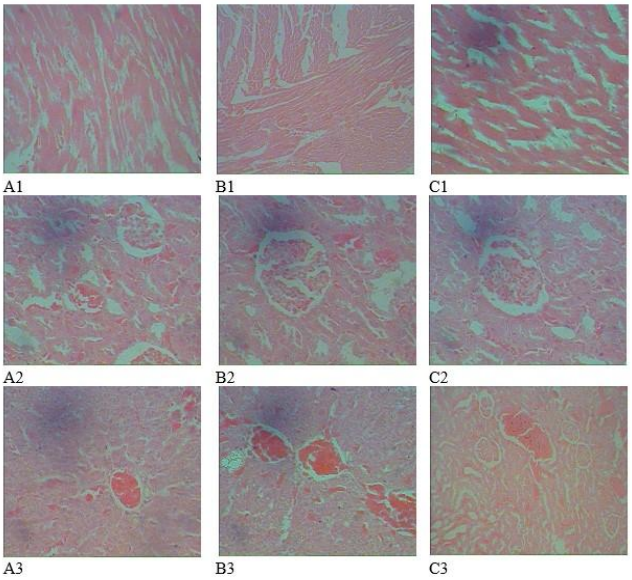

Figure 7. Effects of different *Aponogeton* plant extracts on histomorphology of various rat organs in acute oral toxicity study. A. Control group; B. Group treated with *A. Crispus*; C. Group treated with *A. rigidifolius*. 1. Heart tissues, 2. Kidney tissues, 3. Liver tissues. All the sections were hematoxylin and eosin (H & E) stained. 10×10

Discussion

As observed in our study, the morphology of both *A. crispus* and *A. rigidifolius* aligns with the initial monographs provided by Dassanayake and Fosberg in general (Dassanayake and Fosberg 1987). However, it was also observed that the length of the studied *Aponogeton* leaves vary in mature plants, depending on the depth of the habitat water. In pharmacognostic analysis, both of the plants yielded comparable levels of extractable matter, and the extractable contents in more polar solvents were found to be higher. This observation is supported by another study done on *A. natans,* where higher levels of extractable matter were observed in more polar solvents, compared to non-polar solvents (Dash et al. 2015). The absence of cardiac glycosides reported in the present phytochemical screening has also been observed in other *Aponogeton* species, making it a unique characteristic of *Aponogeton* genus (Nandagoapalan et al. 2016). In a separate study, the ethanolic extract of *A. natans* had resulted in a higher content of total phenolics compared to the less polar chloroform extracts. High phenolic contents in these test plants are critical since a number of bioactivities are thought to be mediated through the free-radical scavenging action of phenolic compounds (Janarny et al. 2021). Variation in phytochemical composition due to internal and external botanical factors influences the biological activities of the plant materials. Since the present study furnishes only preliminary evidence for medicinal use of *Aponogeton* species, further studies should incorporate samples from diverse geographical locations, environmental conditions, and herbal varieties to assess the impact of these variables in phytochemistry and pharmacological activity (Biondi et al. 2021).

Ethnopharmacology often bridges traditional uses and scientific usage of medicinal plants. Research has shown that many plants used in traditional medical systems possess pharmacological properties that can be scientifically validated by established methodologies (Xing et al. 2021). The study of different doses of the same *Aponogeton* species showed no significant changes in the pattern of anti-inflammatory effect in the carrageenan-induced paw edema assay, other than dose-dependent variation. Carrageenan can release inflammatory and pro-inflammatory mediators such as histamine, prostaglandins, TNF-α, and bradykinin. Much evidence has been brought forward to prove that polar phytochemicals such as phenolics, triterpenoids, and flavonoids possess anti-inflammatory and antipyretic activities, especially by their interaction with the above-mentioned inflammatory mediators (Mansouri et al. 2015). Therefore, the present study suggests that the presence of such phytochemicals might be the underlying cause of the anti-inflammatory activity produced by *A. crispus* and *A. rigidifolius*. In a similar paw edema study, methanolic *A. natans* extract had showed a significant ($p<0.01$) inhibition of edema at the 4^{th} and 5^{th} hours of the experiment. In contrast, the petroleum-ether and benzene extracts of the plant had showed lesser activity (Dash et al. 2013), which justifies the greater anti-inflammatory activity demonstrated by the aqueous extracts of *A. crispus* and *A. rigidifolius* in the present rat paw edema model.

Studies have suggested that the antipyretic properties in plant extracts are likely linked with the interference of eicosanoids biosynthesis and inhibition of neutrophil degranulation in animals. These actions, when combined, result in the suppression of inflammatory mediators such as prostaglandins and lipoxygenase (Roe 2021). Furthermore, the sequence and pattern of body temperature lowering by test plants were almost similar to the standard drug, establishing that the antipyretic property of these plants may be mediated through the inhibition of prostaglandin synthesis similar to acetylsalicylic acid. The acetic acid-induced writhing test is recognized as a common in vivo method to evaluate compounds with peripheral analgesic effects. The injection of acetic acid sensitizes the analgesic receptors to prostaglandins or endogenous opioids (Rege et al. 2021). Therefore, it is suggested that the herbal extracts tested in the present study may provide analgesic activity by antagonizing the activity of prostaglandins and/or other inflammatory mediators in the body.

The thrombolytic activity of any substance is associated with the disruption of blood clots formed in an inflammatory condition. A previous study carried out on methanolic extract of *A. undulatus* has shown moderate thrombolytic activities (Chowdhury et al. 2011). According to the same study, saponins, alkaloids, and tannins were presumed to be responsible for the thrombolytic activity exerted by the plant. Especially, saponins have been repeatedly proven for their hemolytic activity through the disruption of blood cell membranes (Bissinger et al. 2014). Since the same type of phytochemicals was found in both of the test plants of the present study, it could be highlighted as the most probable reason for the in vitro thrombolytic activity. Apart from the biological effects of *A. crispus* and *A. rigidifolius* against the classical signs of inflammation, an appreciable anti-hyperglycemic activity was further demonstrated by *A. crispus* aqueous extract. Chloroform extracts of *A. natans* have also reported a significant ($p<0.05$) anti-hyperglycemic activity in a previous study (Dash et al. 2014).

Toxicity studies are essential for herbal materials with potential for drug development (Chen et al. 2022). The absence of statistically significant differences in toxicity parameters between the test and control animal groups of the present study justifies the use of *Aponogeton* spp. as a traditional food in different South Asian ethnic groups (Jayaweera and Senaratna 2006; Misra et al. 2012). Previous studies suggest that *A. crispus* and *A. rigidifolius* contain various phytochemicals, including quercetin, gallic acid, cyanidin, stigmasterol, p-hydroxybenzoic acid which are known for their anti-inflammatory and anti-hyperglycemic properties (Abdelkhalek et al. 2024). Specially, no specific phytochemical compound has been isolated or characterized from *A. rigidifolius* so far in any reported study. Isolation and characterization of compounds in a future study will provide solid evidence on its medicinal role. Results of the present study urge the need for an extensive study to demonstrate the molecular mechanisms in aqueous extracts of *A. crispus* and *A.*

rigidifolius with respect to their in vivo and in vitro biological activities evidenced here.

It is concluded by this study that the aqueous extract of both *A. crispus* and *A. rigidifolius* demonstrated significant acute anti-inflammatory activity by acting against the key signs of the inflammation, i.e., edema, pain, fever, and thrombosis. Interestingly, *A. rigidifolius*, which is native to Sri Lanka exerts higher analgesic and antipyretic effects in comparison to *A. crispus.* The hypoglycemic effect of *A. crispus* was greatest at 90 minutes, and a time-dependent activity was also evident. Moreover, both plants were found to be safe for consumption in acute or chronic settings, as per the findings of the toxicity study; therefore, they could be recommended as functional foods. The pharmacognostic findings of this study could be used as standard parameters to differentiate and standardize *A. crispus* and *A. rigidifolius* in ethnomedicine.

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