Pharmacological and phytochemical investigations of methanol extracts of leaves, bulb, and root of Cymbidium aloifolium (L.) Sw. (Orchidaceae): a promising ethnomedicinal plant

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Abstract

Background

Orchidaceae member *Cymbidium aloifolium* (L.) Sw. has been used as a medicine in numerous cultures for a range of ailments. This research evaluates the leaf, bulb, and root of *C. aloifolium* methanolic extract for pharmacological and phytochemical activities.

Methods: The DPPH free radical scavenging technique assessed antioxidant activity. Protein denaturation and brine shrimp mortality experiments were used to investigate in vitro anti-inflammatory and cytotoxic effects. In vivo analgesia was tested using acetic acid-induced writhing and a tail immersion test. Anxiolytic activity was assessed using elevated plus mazes (EPM) and head-dipping.

Results

The methanolic extract of *C. aloifolium* leaf, bulb, and root contained alkaloids, saponins, terpenes, steroids, and flavonoids. With an IC_{50} value of 51.141 µg/ml, the bulb extract of *C. aloifolium* showed strong antioxidant activity against DPPH free radicals. The leaf of *C. aloifolium* showed anti-inflammatory action in vitro with an IC_{50} value of 22.22 µg/ml, similar to Diclofenac-Na. During the cytotoxicity experiment, it was shown that the LC_{50} value for the methanol extract of the leaf (200.94 µg/ml) was much higher than that of the reference medicine (42.75 µg/ml). Significant ($p<0.001$) findings were observed in peripheral and central analgesic action for leaf, bulb, and root was found that the methanolic extract of *C. aloifolium* leaf, root, and bulb increased open arm time and entries in the elevated plus maze significantly ($p <0.05$) compared to the control group. This showed that the 200 and 400 mg/kg doses had anxiolytic activity. Our investigation found that a 400 mg/kg dosage of *C. aloifolium* bulb and root leads to considerable head lowering (24.00 ± 0.52*** and 37.83 ± 0.60***, respectively, $p<0.001$).

Conclusion

In this research, a methanol extract of *C. aloifolium* leaves, bulbs, and roots showed effects as an anti-inflammatory, cytotoxic, pain-relieving, and anxiety-reducing substance in the lab.

Background

Various countries across the globe extensively employ traditional medicines as herbal cures, dietary supplements, and alternative medical modalities. Over the past few years, there has been a substantial rise in the use of traditional medicine, with people across the country relying on it more and more as a primary source of treatment [1]. In the present day, individuals are actively investigating various methods to address, remedy, alleviate, and avert medical conditions and ailments [2]. Synthetic and semi-synthetic drugs cause hospitalizations and fatalities. Adverse medication reactions account for 8% of hospitalizations in the United States. According to a study [3], poisons cause the deaths of 100,000

individuals each year. Furthermore, these medications are expensive, which poses a significant obstacle for people seeking therapy. Scientists are currently conducting research on plant-based herbal remedies as a potential cure for this illness. Scientists are currently exploring novel herbal remedies to address pain, inflammation, diabetes, and various other illnesses. Plants possess phenols, glycosides, alkaloids, saponins, terpenoids, tannins, polysaccharides, flavonoids, plant lipids, resins, and essential oils [4, 5].

Oxidative stress is defined by elevated levels of reactive oxygen species (ROS) and insufficient antioxidant mechanisms. Cellular damage and tissue death can occur as a result of reactive stress [6]. Researchers have extensively studied SOD, epigallocatechin-3-O-gallate, CAT, lycopene, coenzyme Q10, ellagic acid, quercetin, indole-3-carbinol, genistein, vitamin E, and vitamin C as prophylactic and therapeutic drugs [7]. Rheumatoid arthritis (RA) affects around 0.5–1.0% of individuals in Western countries. It is a chronic, inflammatory, degenerative joint disease that can cause significant disability [8]. Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, have traditionally been the mainstay in the treatment of rheumatoid arthritis pain [9]. Newly introduced treatments encompass corticosteroids and disease-modifying anti-rheumatic medicines. The inflammatory response serves as a protective mechanism following the removal of detrimental external stimuli, allowing the body to initiate the process of self-restoration. Inflammation can manifest as either acute or chronic [10–12]. Common pharmaceuticals used as analgesics or anti-inflammatory drugs include paracetamol, diclofenac, ketorolac, and opioids. Aspirin, codeine, and morphine constitute a fundamental regimen for pain relief, but they each have their own set of undesirable side effects, which can impact the stomach, heart, kidneys, brain, and immune system [3]. Cancer is a lethal disease marked by abnormal cellular behavior, unregulated cell proliferation and expansion, and ultimately, cell death [13]. Existing conventional therapies, including surgical intervention, radiation, and chemotherapy, all possess certain limitations [14]. Natural cytotoxic chemicals have the potential to aid in cancer treatment by selectively damaging cancer cells, which is encouraging. Harm to body tissues causes pain, also known as algesia, an unpleasant sensation. The sensation of pain initially serves as a protective mechanism against potential harm, but it can have negative consequences in the future [15]. Analgesics are medications that specifically act on either the peripheral or central nervous systems in order to alleviate pain [16]. Common analgesic medications consist of nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids [17]. Classified as internalizing disorders, anxiety and depression are common mental disorders that frequently co-occur together. Treatment for anxiety disorders often involves the use of medicine. Some commonly prescribed alternatives include selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), pregabalin, buspirone, tricyclic antidepressants, monoamine oxidase inhibitors, and benzodiazepines [19]. Although these synthetic antidepressants and sedatives have proven to be effective, many patients discontinue their use due to adverse effects or a substantial delay in their effectiveness.

Cymbidium aloifolium (L.) Sw. is a member of the Orchidaceae family, which is one of the biggest groupings of flowering plants and includes over 28,000 species of vulnerable medical orchids. The orchid family classifies Cymbidium within the subfamily Epidendroideae. This subfamily includes most orchid genera as well as several well-known orchid species [20]. *C. aloifolium*, commonly known as the

aloe-leafed cymbidium, is an orchid species found in Bangladesh and various parts of Asia, particularly China and Southeast Asia, spanning from Burma to Sumatra. People often observe it growing in crevices or on other vegetation [21]. People widely use the leaves of this species for their styptic effects in treating boils and fevers. The roots possess medicinal properties that are effective in treating paralysis and chronic illnesses. The entire plant can also serve as a tonic and be used in the treatment of vertigo, ocular debility, burns, and sores [22–25]. Traditional medicine uses the plant's leaf extract to treat earaches. The indigenous peoples of Bangladesh use leaf and root preparations for the treatment of asthma and paralysis [26–28]. In addition to its medical significance, this orchid also appeals to the floriculture industry due to its enduring and exceptionally captivating blossoms. This plant contains several biochemical compounds such as alkaloids, saponins, terpenoids, steroids, flavonoids, phenols, leucoanthocyanins, coumarins, anthocyanins, phlobatannins, cardiac glycosides, gums, mucilage, quinones, and oxalates. Diverse phytochemical investigations have identified these compounds [29, 30]. There are active chemicals in different parts of plants that can kill bacteria, relax muscles, kill cells, relieve pain, reduce inflammation, stop platelet aggregation, fight allergies, protect cells from damage, harm plants, and slow down the central nervous system [31, 30].

In this experiment, we assessed the pharmacological effects of the methanolic extract of the leaf, root, and bulb of *C. aloifolium*. This evaluation was based on the plant's traditional usage and other pharmacological activities. Further investigation is required to identify and separate the new molecule responsible for these pharmacological effects.

Methods

Collection and identification of the plant

The mature plant components were acquired in August 2022 under the guidance of a renowned indigenous healer. The taxonomist, renowned for their expertise, classified it as *C. aloifolium*, assigning it a specific herbarium number 1182 has been preserved at the Bangladesh National Herbarium (BNH).

Preparation of crude extracts

The plant material (leaf, bulb, and root) was thoroughly cleaned, precisely chopped, and then dried in the shade for a duration of eight days. A total of 700 grams of leaf, bulb, and root powder from the *C. aloifolium* plant were immersed separately in 3.5 liters of methanol for each part. The solution underwent filtration, and the resultant liquid was concentrated using a rotary evaporator under reduced pressure (Stuart, UK).

Qualitative phytochemical screening

A preliminary phytochemical screening was conducted using a standard procedure to qualitatively detect the presence of terpenoids, saponins, flavonoids, phenol, tannins, phlobatannins, steroids, glycosides, anthraquinones, alkaloids, resins, cardiac glycosides, carbohydrates, fat and oil, proteins, and coumarin

[32]. The intensity of color or the production of precipitates were used as analytical responses to these qualitative assessments.

Free radical scavenging activity [DPPH]

The radical scavenging properties of *C. aloifolium* leaf, bulb, and root's crude methanol extracts were quantitatively assessed using Blois' approach [33]. In summary, a 0.1 mM solution of 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) in methanol was prepared. Then, 1 ml of this solution was combined with 3 ml of extracts at different concentrations ranging from 500 to 0.49 µg/ml. As a reference or standard, ascorbic acid was used. The discoloration was observed at a wavelength of 517 nm after a period of 30 minutes in darkness. Measurements were acquired on three separate occasions. The equation provided was used to calculate the capacity to remove the DPPH free radical, which was then expressed as a percentage of inhibition.

% inhibition = {(Absorbance of control – Absorbance of test)/Absorbance of control} ×100

Using a regression equation developed from data collected at varying concentrations of methanol extracts, we were able to determine the IC_{50} values (the concentration of sample necessary to scavenge 50% of free radicals).

In vitro protein denaturation method

Sakat et al. [34] conducted *in vitro* protein denaturation. The protein denaturation suppression method was employed to investigate the anti-inflammatory capabilities of *C. aloifolium*. The reaction mixture consists of 5 ml of total volume, comprising 0.2 ml of freshly obtained hen's egg albumin, 2.8 ml of phosphate-buffered saline with a pH of 6.4, and 2 ml of various quantities of plant extracts. For control purposes, an equivalent volume of double-distilled water was employed. The mixtures were subsequently placed in an incubator set at a temperature of 37 ± 2°C for a duration of 15 minutes, followed by heating at 70°C for a period of 5 minutes. Following the cooling process, the absorbance of the sample at a wavelength of 660 nm was measured, with a vehicle serving as a reference. A reference medicine, diclofenac, was used at a final dosage of 1 mg/mL. It was handled in the same way for absorbance determination. The percentage inhibition of protein denaturation was computed.

Percentage inhibition = (Abs Control - Abs Sample)/Abs Control ×100

Evaluation of cytotoxic activity

Preparation of seawater

The solution was filtered to remove contaminants after dissolving 38 grams of iodine-free sea salt in 1 liter of distilled water. We could maintain a consistent pH range of 8.0 to 8.5 for the seawater by adding a 1 N NaOH solution.

Brine shrimp eggs hatching

Pet stores provided Artemia salina leach, also known as brine shrimp eggs, for the experiment. The little tank was filled with saline solution and subsequently populated with prawn eggs. The nauplii would hatch from the eggs after a period of two days. During the hatching process, the oxygen pump maintained a consistent and uninterrupted supply. Shrimp nauplii were removed from the illuminated section of the tank due to their attraction to light, a behavior known as phototaxis. The nauplii were extracted from the aquarium using a pipette. Research Framework A total of 10 nauplii were introduced into 5 ml of seawater and exposed to various concentrations (1000, 800, 500, 250, 125, 62.5, and 31.25 µg/ml) of a plant extract. After a duration of 24 hours, the Petri plates were inspected using a black light and a magnifying lens to count the quantity of surviving nauplii. In this bioassay, death was operationally defined as the absence of synchronized forward locomotion for a duration of 30 seconds. The equation [35] was used to compute the mortality percentage (%) of brine shrimp nauplii for each concentration.

% Mortality = Nd/N × 100

Here, Nd = Number of dead nauplii, and N = Number of nauplii taken.

Determination of median lethal concentration (LC_{50})

The LC_{50} value was determined by employing a linear regression model to estimate the concentration of the extract at which 50% of brine shrimp nauplii perished following exposure to the drug for a specific duration. A graph showed a linear correlation between the concentration and the percentage of deaths. A trend line fit linear regression analysis transformed the concentration-response data into a straight line. Microsoft Excel 2007. The LC50 results were determined by extrapolating the line of best fit.

In vivo studies

Experimental animals

This experiment included male Swiss albino mice weighing 20 to 30 grams. The animals were housed in plastic enclosures and adapted to controlled environmental conditions for a period of 14 days. These parameters included a temperature of 25 ± 2°C and a relative humidity ranging from 60–70%. Following the acclimatization process, we subjected the animals to a 12-hour alternating light and dark cycle and provided them with pellets as their food source. During the trial, the mice were given a well-balanced meal and access to water at all times. Scientists used chloroform anesthesia to euthanize the mice. The scientists used diethyl ether anesthesia to euthanize the mice after each session. This paper rigorously follows the ARRIVE Guidelines for reporting animal research. The ethical committee has evaluated and approved all experiments under the permit number.

Experimental design

Eight groups of mice, each consisting of five mice, were selected for the assessment of analgesic activity. The control group received a dose of 10 ml/kg of 1% tween-80. For the acetic acid writhing study, the standard group received 50 mg/kg of diclofenac sodium and 10 mg/kg of morphine sulfate for the tail immersion method. The remaining groups were given doses of 200 and 400 mg/kg of crude

methanol extract of the leaf, bulb, and root of *C. aloifolium*. In order to assess the anxiolytic effects, mice were selected, their weights were measured, and they were then divided into eight groups for individual testing. The control group was administered 10 ml/kg of a 1% solution of tween-80. The standard group received the typical medication diazepam at a dosage of 1 mg/kg. The treatment group was given methanol extract at dosages of 200 mg/kg and 400 mg/kg. Each experimental period ended with the chloroform-induced euthanasia of all treated animals.

Acute toxicity Study

A study was done to investigate the acute toxicity of the methanol extract of the leaf, bulb, and root of *C. aloifolium*. The purpose of the study was to identify the correct dosage based on the guidelines provided by the OECD [36]. The extract was administered orally at increasing dosages of 500, 1000, and 2000 mg/kg. The subjects were monitored for a duration of 48 hours in order to evaluate any alterations in behavior, toxicity, or mortality. There were no deaths or other immediate negative effects detected. Therefore, the doses administered in the present investigation were 200 and 400 mg/kg body weight in order to evaluate the analgesic and CNS depressive effects. The effective dose would be lower than the median lethal dose (LD_{50}).

Evaluation of analgesic activity

Acetic acid writhing method

This analgesic behavioral observation evaluation approach was used to demonstrate the painful stimulation in mice. The investigation utilized Dambisya and Lee's [37] adaptation of Koster's technique. Each mouse received an intraperitoneal injection of 0.7% glacial acetic acid at a dose of 10 ml/kg, 15 minutes after the usual administration and 30 minutes after the extract was administered. This injection was intended to induce discomfort, which was observed as abdominal constriction or writhing. Each mouse in all groups underwent a careful 20-minute observation after 5 minutes to record and count the number of writhes. Following each observation session, all mice that received treatment were euthanized using diethyl ether. The measurement of analgesia was determined by calculating the percentage reduction in abdominal writhing using the formula:

% of pain inhibition = (Nc – Nt)/Nc

Here, Nc = the number of writhings in the control group, and Nt = the number of writhings in the treatment group.

Tail immersion method

The thermal approach was employed to determine the central analgesic activity of the extracts being studied. This investigation adhered to the methodology described by Di Stasi et al. [38]. Prior to the 30-minute treatment session, the duration for each mouse to remove its tail from water heated to 50 ± 1°C was measured and documented. The tail of each mouse was immersed to a depth of approximately 2–3 cm. The animals were chosen for the study based on their ability to react to a rapid and repetitive

movement within a timeframe of three to five seconds. The time limit for tail protection was established at 15 seconds. The mice that received medicine were assessed after 30, 60, 90, and 120 minutes after the start of treatment. The animals were gently restrained to temporarily immobilize them in order to facilitate the collection of measurements. All mice that received treatment were euthanized using diethyl ether anesthesia at the conclusion of each observation period. The equation used to calculate the analgesic efficacy of the extract resulted in a percentage of the maximum possible effect (% MPE).

MPE (%) = (post-drug latency-pre-drug latency) / (cut-off time-pre-drug latency) × 100

The percentage of time elongation was calculated from the following equation:

Elongation (%) = (Latency of Test-Latency of Control)/Latency of Test × 100

Anxiolytic test

Elevated plus-maze test

The plus maze device, comprising two open arms (16 × 5 × 12 cm) and two closed arms (16 × 5 × 12 cm) with an elevated open roof 50 cm above the floor, is used for observing anxiolytic behavior in animals [39]. After the dose is administered, each fresh group of mice is placed on the elevated plus-maze equipment for 30 minutes. Each mouse was positioned in the center of the elevated plus-maze, with its head oriented towards the open arms. The behavioral impacts of the mice were assessed for a duration of 5 minutes using various parameters, including the time spent in open arms, time spent in closed arms, number of entries into open arms, and number of entries into closed arms.

Hole-board test

The apparatus consisted of a rectangular wooden box measuring 40 cm × 40 cm × 25 cm. The box had sixteen evenly spaced holes on the floor, each measuring 3 cm in diameter [40]. The distance from the box's nearest wall to the center of each hole was 10 cm. The box's floor was elevated 15 cm above the ground and marked with a water-resistant marker to create squares measuring 10 cm by 10 cm. An animal is positioned in the center of the hole board and given 5 minutes to investigate the contraption. The cumulative count of head movements downward has been documented. The head dip is scored when both eyes completely vanish into the hole.

Statistical analysis

The mean and standard error of the mean (SEM) represent the data. The statistical analysis involved a one-way analysis of variance and Dunnett's t-test. A significance level of *P < 0.05 was deemed significant. In addition, SPSS was used to do the statistical analysis. The version is 20, and it is developed by IBM Corporation, located in New York, USA. Excel 2007 utilized linear regression methods to estimate LC50 values. Microsoft is located in Redmond, Washington, USA.

Results

Qualitative phytochemical analysis

The methanol extract of *C. aloifolium* leaf, roots, and bulb contains several compounds such as alkaloids, glycosides, saponins, tannins, phenols, and flavonoids, as shown in Table 1.

Table 1
Qualitative test of eleven other phytochemicals of *C. aloifolium*

Plant parts used	Phytochemicals (% of Coloration)										
	Flv.	Sap.	Tan.	Phl.	Ter.	Str.	Gly.	Pro.	Car. gly.	Qui.	Cou
Leaf	+++	+++	+++	-	-	++	+++	+++	-	+++	+++
Root	+	+++	+++	-	++	-	+++	+	+++	+++	+
Bulb	+++	+++	+++	-	+++	-	+++	-	++	+++	+++

Note: Flv. = Flavanones, Sap. = Saponins, Tan.= Tannins, Phl.= Phlobatannins, Ter.= Terpenoids, Str.= Steriods, Gly.= Glycosides, Pro.= Protein, Car. gly. = Cardiac glycosides, Qui.= Quinine and Cou.= Coumarin. Sample was express by (+) lowest; (++) moderate; (+++) highest.

Antioxidant activity

Table 2 presents the degree to which the scavenging activity of free radicals (DPPH) is inhibited, expressed as a percentage. The conventional medicine ascorbic acid had the highest percentage inhibition, reaching 96.63% with an IC_{50} value of 21.166 µg/ml. The maximum percentage of inhibition was seen in the leaf, root, and bulb at a dose of 500 µg/ml, with values of 77.127%, 82.093%, and 82.624%, respectively.

Table 2
DPPH free radical Scavenging assay of methanolic crude extract of C. *aloifolium* leaf, bulb and root.

Concentration (µg/ml)	Ascorbic acid % Scavenging activity)	Leaf % Scavenging activity	Root % Scavenging activity	Bulb % Scavenging activity
500	96.63	77.127	82.093	82.624
250	95.57	58.156	77.304	80.319
125	94.85	39.184	53.900	58.865
62.25	93.97	10.008	27.127	36.170
31.252	91.31	9.574	10.815	17.730
IC_{50}	21.16	91.47	81.310	51.141

In vitro Anti-inflammatory

When using the protein denaturation method, the drug Diclofenac-Na exhibited the highest level of inhibition, reaching 90.89%. At a concentration of 250 g/ml, the IC_{50} value, which represents the concentration required for 50% inhibition, was found to be 15.63 µg/ml. At a concentration of 250 µg/ml, the leaf extract exhibited the highest percentage of inhibition among the leaf, root, and bulb extracts, with a value of 96.79% (Table 3). The IC_{50} value for this extract was determined to be 22.22 µg/ml.

Table 3
% Inhibition of albumin denaturation (anti-inflammatory activity) for methanolic crude extract leaf, root and bulb of *C. aloifolium.*

Conc (µg/ml)	ASA(Standard)	Leaf	Root	Bulb
50	48.55	81.07	33.21	50.36
100	67.88	90.71	46.79	66.43
150	75.56	95.36	60.00	75.71
200	82.18	96.42	75.00	79.64
250	90.89	96.79	77.86	81.79
IC_{50}	15.63	22.22	42.02	37.70

Cytotoxicity assay

The LC_{50} value for vincristine sulfate, determined using the brine shrimp lethality assay, was 42.75 µg/ml. The LC_{50} values for leaf, root, and bulb were 200.94 µg/ml, 160.80 µg/ml, and 120.81 µg/ml, respectively (Table 4).

Table 4
Cytotoxic activity of the different extract of *C. aloifolium*

Sample	Equation	R^2	LC_{50}(42.75)
Vincristine sulfate	y = 10.441x + 75.689	R^2 = 0.4939	42.75
Leaf	y = 22.571x + 46.359	R^2 = 0.9178	200.94
Root	y = 29.347x + 26.751	R^2 = 0.7606	160.80
Bulb	y = 26.949x + 32.019	R^2 = 0.9109	120.81

Acute toxicity study

Based on the procedures stated above, a study on acute toxicity was carried out. Each group of mice, comprising five Swiss albino mice, was administered extracts following an overnight fast. Different

animal groups were administered oral doses of 1000, 2000, 3000, and 4000 mg/kg of body weight for each extract. After the administration of the plant extract, a fasting interval of three to four hours was enforced. After the initial 30 minutes, the animals were monitored for the first 24 hours and then for the following three days. Every day, the mice had comprehensive inspections to identify any changes in their skin, hair, mucous membranes, eyes, respiration rate, heart rate, central nervous system, and autonomic nervous system. The effective dose would be a fraction of the LD_{50} (median lethal dosage).

In vivo Analgesic activity

Acetic acid writhing method

In the acetic acid writhing method, all parts of the plant exhibited a high level of statistically significant results ($p < 0.001$) in both the 200 and 400 mg/kg body weight doses when compared to the control (Table 5). The medicine Diclofenac-Na demonstrated highly significant results ($p < 0.001$) in the acetic acid writhing method, which is the standard drug used.

Table 5
Effect of methanolic crude extract of *C. aloifolium* leaf, bulb and root on acetic acid induced writhing in mice.

Treatment group	Dose (mg/kg)	Average number of writhing	% of inhibition
Control (Saline Water)	10	60.33 ± 0.98	-
Standard (Diclofenac Na)	10	28.67 ± 0.70***	82.47
Leaf	200	22.00 ± 0.471***	63.53
Leaf	400	20.00 ± 0.471***	66.85
Root	200	26.5 ± 0.61***	56.07
Root	400	23.17 ± 0.67***	61.59
Bulb	200	32.67 ± 0.70***	45.85
Bulb	400	30.00 ± 0.82***	50.27

Note: Values are represented as mean ± SEM; (n = 5). *p < 0.05, **p < 0.01 and ***p < 0.001 as compared with control. Data was analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett's test for multiple comparisons

Tail immersion method

The methanolic extract of the leaf, root, and bulb increased the percentage delay time when the tail immersion method was used in a way that was statistically significant ($p < 0.05$), as shown in Table 6. At a dosage of 400 mg/kg, all parts of the plant (leaf, root, and bulb) exhibited extremely significant results ($p < 0.001$) between 30 and 60 minutes after administration. Over time, reactivity diminishes with MPE.

Table 6
Analgesic activity by tail immersion method of the methanolic extract of leaf, root and bulb the plant

Test Samples	Dose (mg/kg)	Pretreatment	Reaction times in seconds (Mean ± SEM) and %MPE			
			30 minutes	60 minutes	90 minutes	120 minutes
Control	10ml/kg	2.22 ± 0.109%	4.93 ± 0.83 1.86%	2.12 ± 0.16 1.66%	2.42 ± 0.15 2.7%	2.75 ± 0.18 1.7%
Standard (Morphine sulfate)	50	2.19 ± 0.77%	7.96 ± 0.41*** 22%	5.64 ± 0.92*** 22.5%	4.16 ± 0.87 16.18%	2.97 ± 0.21 8.03%
Leaf	200	2.27 ± 0.83%	7.22 ± 0.23* 9.2%	6.51 ± 0.37* 7.23%	2.56 ± 0.15 5.12%	2.47 ± 0.15 3.19%
Leaf	400	2.30 ± 0.42%	8.5 ± 0.24*** 21.01%	8.32 ± 0.26*** 16.9%	2.5 ± 0.14* 12.67%	2.31 ± 0.14 10.71%
Root	200	2.17 ± 0.18%	7.12 ± 0.23 9.6%	4.41 ± 0.47 6.23%	2.66 ± 0.16 4.22%**	1.97 ± 0.25 2.15%
Root	400	2.40 ± 0.27%	5.5 ± 0.24*** 19.06%	4.12 ± 0.16* 15.8%	1.9 ± 0.12* 11.62%	2.32 ± 0.17 9.61%
Bulb	200	2.77 ± 0.311%	8.10 ± 0.33 10.6%	5.51 ± 0.37 8.23%	4.76 ± 0.26* 5.92%	2.97 ± 0.15 4.19%
Bulb	400	2.90 ± 0.48%	15.5 ± 0.14**** 22.07%	10.62 ± 0.26**** 17.2%	9.1 ± 0.22 14.12%	6.12 ± 0.27 10.91%

Note: Values are represented as mean ± SEM; (n = 5). *p < 0.05, **p < 0.01 and ***p < 0.001 as compared with control. Data was analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett's test for multiple comparisons

Table 7
Percent elongation of latency time after administration of all test samples

Test Samples	Dose (mg/kg)	% elongation of latency time			
		30 minutes	60 minutes	90 minutes	120 minutes
Standard (Morphine sulfate)	50	83.1%	71.221%	51.12%	41.17%
Leaf	200	39.15%	29.95%	19.22%	11.34%
Leaf	400	48.19%	39.29%	24.21%	14.15%
Root	200	29.19%	19.45%	10.02%	8.14%
Root	400	42.13%	37.24%	26.91%	11.13%
Bulb	200	35.13%	30.12%	20.09%	14.51%
Bulb	400	50.32%	41.21%	32.21%	26.19%

Anxiolytic test

Elevated plus maze

There was a statistically significant ($p < 0.05$) increase in the time spent in the open arms and the number of times animals entered the open arm of the elevated plus maze when the methanolic extract from the leaf, root, and bulb of *C. aloifolium* was used instead of the control group. This indicates the presence of anxiolytic activity in both the 200 mg/kg and 400 mg/kg doses, as shown in Table 8. In comparison to the animals treated with the extract, administering diazepam at a dosage of 1.0 mg/kg body weight significantly ($p < 0.001$) increased both the number of entries and the length of time spent in the open arms.

Table 8
Anxiolytic effect of methanolic crude extracts of leaf, bulb & root of *C. aloifolium*

Treatment group	Dose (mg/kg)	% of time spent in open arms	% of entry in open arms
Control (1% tween 80 in saline water)	10ml/kg	45.75 ± 1.32	51.63 ± 0.91
Standard (Diazepam)	1(mg/kg)	75.00 ± 1.08***	72.25 ± 1.12***
Leaf	200	30.50 ± 1.04	42.25 ± 1.11*
Leaf	400	37.00 ± 0.91***	51.50 ± 1.32*
Root	200	43.75 ± 1.10**	53.5 ± 1.32**
Root	400	44.25 ± 1.88*	66.25 ± 1.38**
Bulb	200	24.50 ± 1.25	33.50 ± 1.71**
Bulb	400	30.50 ± 0.65**	42.75 ± 1.75***

Note: Values are represented as mean ± SEM; (n = 5). *p < 0.05, **p < 0.01 and ***p < 0.001 as compared with control. Data was analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett's test for multiple comparisons.

Hole board test

During the hole board test, the conventional drug diazepam demonstrated a highly statistically significant effect ($p < 0.001$) on the head-dipping behavior. The leaf extract administered at a dosage of 200 mg/kg demonstrated highly significant statistical findings ($p < 0.001$) (Table 9). The bulb and root extract demonstrated a very significant outcome ($p < 0.001$) solely at a dosage of 400 mg/kg.

Table 9
Anxiolytic effect of methanolic crude extracts of leaf, bulb & root of *C. aloifolium* using hole board test

Treatment group	Dose (mg/kg)	No. of head dipping
Control (1% tween 80 in saline water)	10 ml/kg	25.17 ± 0.95
Standard (Diazepam)	1 (mg/kg)	35.00 ± 0.58***
Leaf	200	21.30 ± 0.49***
Leaf	400	26.50 ± 0.56**
Root	200	30.50 ± 0.76*
Root	400	37.83 ± 0.60***
Bulb	200	18.83 ± 0.47
Bulb	400	24.00 ± 0.52***

Note: Values are represented as mean ± SEM; (n = 5). *p < 0.05, **p < 0.01 and ***p < 0.001 as compared with control. Data was analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett's test for multiple comparisons

Discussion

Since ancient times, people have used herbal treatments to treat various ailments. In this study, we conducted a thorough analysis of the chemical compounds included in the methanolic extract of the leaf, root, and bulb of *C. aloifolium* and examined their impact on the body's physiological processes. The methanolic extracts of the leaf, root, and bulb of *C. aloifolium* were tested to determine the presence of various phytochemical substances. The findings revealed the presence of alkaloids, carbohydrates, flavonoids, terpenoids, phenols, saponins, and other chemicals. Early phytochemical screening assays may assist in identifying powerful phytochemicals that have the potential to contribute to the discovery and development of novel medications.

Antioxidants, which are chemicals that scavenge free radicals, shield cells in the body from harm by merging with and neutralizing these harmful molecules [41]. The data in Table 2 show that the bulb extract of *C. aloifolium* was very good at protecting against DPPH free radicals in this study, with an IC_{50} value of 51.141 µg/ml. In comparison, the IC_{50} value of ascorbic acid was 21.16 µg/mL. The maximum percentage of inhibition was seen in the leaf, root, and bulb at a dosage of 500 µg/ml. Therefore, we can conclude that the methanolic extract from the leaf, root, and bulb of our experimental crude substance possesses significant antioxidant properties. Functional phytochemicals, such as flavonoids, phenolic acids, and tannins, aid plants in neutralizing harmful free radicals and function as antioxidants [41]. Polyphenolic substances may assist in the extraction process by eliminating free radicals. The results we obtained were consistent with the findings reported by Arshad et al. (2017) [42].

Inflammation is an intricate biochemical reaction of blood vessels to damaging stimuli, pathogens, and allergens. It results in redness, warmth, swelling, and discomfort [43]. In this study, we are comparing the IC_{50} values of the standard medicine Diclofenac-Na (15.63 µg/ml) with those of the methanolic extract obtained from the leaf, root, and bulb of C. aloifolium. The leaf of *C. aloifolium* had an IC_{50} value of 22.22 µg/ml, which is comparable to the standard medicine Diclofenac-Na. In contrast, the root and bulb had IC_{50} values of 37.70 µg/ml and 42.02 µg/ml, respectively. Therefore, we can infer that the leaf extract of *C. aloifolium* exhibits more anti-inflammatory action compared to the root and bulb extracts, as well as the standard medication. A. capitiformis's methanolic extract contains powerful anti-inflammatory compounds such as 3-furaldehyde, phenol, catechol, and hydroquinone. These chemicals specifically target proteins involved in the inflammation process, as indicated by the study [44]. Foyet et al. (2014) conducted a study that further supports the anti-inflammatory and anti-arthritic benefits of Vitellaria paradoxa stem bark extract, in line with our own investigation [45].

The brine shrimp lethality test is a comprehensive method used to assess the cytotoxicity and other pharmacological effects of chemicals and plant preparations. These effects include antimicrobial, pesticidal, antiviral, anticancer, and others. [46]. The LC_{50} value of the methanol extract of the leaf (200.94 µg/ml) was much higher than that of the reference medication (42.75 µg/ml), suggesting that the plant extract is safe for use at therapeutic doses. The LC_{50} values for the methanolic extract of the root and bulb were 160.80 µg/ml and 120.81 µg/ml, respectively. Cell death was associated with secondary plant metabolites [47]. A first qualitative phytochemical analysis indicated that the presence of alkaloids, flavonoids, and tannins is responsible for the anti-cancer properties [48, 49]. This link is appropriate for our current study because our analysis of plant phytochemicals has demonstrated the existence of these molecules. Research from the past [50] has shown that some bioactive compounds, like quercetin, trans-cinnamic acid, o-coumaric acid, apigenin-7-o-glucoside, luteolin, and protocatechuic acid, can help fight cancer. The results of our study show that the methanol extract of leaf, root, and bulb from *C. aloifolium* has a notable cytotoxic effect. This discovery emphasizes the need for further investigation into the impact of this extract on these specific cell lines.

Pain is the innate reaction of the body to harmful stimuli, serving as a cautionary system and triggering a defensive reaction. While it has its benefits, it may also cause significant discomfort and provoke negative responses [51]. Acetic acid-induced pain in mice manifests as abdominal contractions (writhing) and hindlimb stretching. Pain stimulation induces a specific inflammatory response by liberating arachidonic acid from phospholipids in the affected tissue [52, 53]. A smaller number of writhings and a higher percentage of writhing inhibition in the acetic acid-induced writhing procedure indicate a greater peripheral analgesic effect in mice. This study used the acetic-acid writhing method to evaluate the analgesic effects of *C. aloifolium*. It was found that the methanolic extract of the leaf, root, and bulb of *C. aloifolium* greatly decreased the number of stomach constrictions that acetic acid caused in mice when given 200 mg/kg and 400 mg/kg ($p < 0.001$). If a leaf is involved, among the two doses of 200 and 400 mg/kg, the dose of 400 mg/kg exhibited a greater percentage of inhibition, namely 66.85%. This value is close to the percentage inhibition of the standard medicine diclofenac Na, which is 82.47%.

Scientific research has shown that the presence of flavonoids, phenols, terpenoids, and polyphenols in plants has a relaxing effect and may alleviate pain. Previous studies on various plant extracts [54, 55] support this conclusion. *C. aloifolium* contains abundant amounts of phenolics, carbohydrates, flavonoids, alkaloids, and tannins.

The central analgesic activity of tail immersion was assessed using heat stimuli. Sensory neurons sensitize nociceptors, which decreases the involvement of prostaglandins and increases the threshold for experiencing pain [56]. This study looked at how well *C. aloifolium* relieved pain by measuring the rise in either the MPE, PRT, or latency time at 200 and 400 mg/kg of leaf, root, and bulb. The variables PRT (reaction time), MPE (maximum permissible exposure), and latency duration all exhibited a dose-dependent increase up to 120 minutes. We observed the highest response times 30 and 60 minutes after administering the extract at both the 200 and 400 mg/kg doses. The dose of 400 mg/kg had a more significant effect than the dose of 200 mg/kg. The methanolic extract of the leaf, root, and bulb increased the percentage delay time when the tail immersion method was used in a way that was statistically significant ($p < 0.05$) (Table 8). At a dosage of 400 mg/kg, all parts of the plant (leaf, root, and bulb) exhibited extremely significant results ($p < 0.001$) between 30 and 60 minutes after administration. Over time, the response diminishes in magnitude with MPE. The administration of the usual medicine, morphine sulfate, had a very significant ($p < 0.001$) effect at both 30 and 60 minutes, as shown by reaction times of 7.96 ± 0.41*** seconds and 5.64 ± 0.92*** seconds, respectively. Therefore, we can infer that our extract possesses potent central analgesic properties. This excerpt may have influenced the analgesic pathways in the spinal cord and brain, similar to the effects of morphine sulfate. These findings are consistent with previous studies [57]. Therefore, the presence of saponins in the extract may be responsible for its analgesic activity [58].

Anxiety disorders and severe symptoms can need many treatments. Psychotherapy and anxiolytics address the issue of concern [59]. Benzodiazepines have been the preferred medication for treating anxiety for a span of 40 years. These substances induce drowsiness, memory loss, muscle relaxation, and addiction [60, 61]. The EPM test presumes that an EPM triggers a much more severe approach-avoidance conflict than a limited arm [62]. The open-arm behaviors of mice in the elevated plus maze (EPM) suggest a conflict between the animal's natural tendency to stay in a secure area (such as the closed arms) and their desire to explore a new environment. The anxiolytic components in the open arm encourage the mice to engage in exploratory activity [63]. The methanolic extract of the leaf, root, and bulb of *C. aloifolium* showed a statistically significant ($p < 0.05$) increase in the time spent in the open arms and the number of entries in the open arm of the elevated plus maze, compared to the control. This indicates anxiolytic activity at both the 200 and 400 mg/kg doses. In comparison to the groups treated with the extract, the administration of diazepam at a dosage of 1.0 mg/kg body weight significantly ($p < 0.001$) enhanced both the frequency of entering the open arms and the length of time spent in them. Diazepam, a common drug, has a statistically significant effect when compared to the control group.

The hole-board test is a straightforward method for evaluating the anxiolytic effects by measuring the pattern of mouse reactions in a new setting. This approach facilitates the monitoring and quantification

of behavioral responses. However, the emotional condition of the animal strongly correlates with its head-dipping habit [64]. Mice's head-dipping frequency and length are considered markers of neophilia, a term for purposeful exploration. This behavior is distinct from their total locomotor activity. Neophilia is often associated with high levels of head-dipping, while low levels may indicate a lack of neophilia or a significant anxiety-related state in the animal [65]. Our study found that administering a dose of 400 mg/kg of *C. aloifolium* bulb and root significantly increased head lowering, with measurements of 24.00 ± 0.52*** and 37.83 ± 0.60***, respectively ($p < 0.001$). This supports the previous findings of an anxiolytic-like effect observed in the light-dark test. However, when it came to the leaf extract, the 200 mg/kg dose yielded a more statistically significant result compared to the 400 mg/kg dose. Diazepam, a commonly used medication, exhibited statistically significant findings when compared to the control group. Chemical and pharmacological investigations have demonstrated the sedative effect of saponins [66]. Previous research has shown that plants with alkaloids, flavonoids, terpenes, and saponins can help with sleep, anxiety, and seizures because they bind to the GABAergic system and release benzodiazepines [67]. These phytoconstituents may serve as ligands for neurotransmitter receptors, mimicking neuroactive hormones such as GABA. Our study has shown the presence of terpenes, glycosides, alkaloids, flavonoids, phenols, and saponins in our plant.

Conclusion

The study found that the methanolic extracts of the leaf, root, and bulb of *Cymbidium aloifolium* included a combination of phytochemicals and antioxidants. These extracts have also shown *in vitro* anti-inflammatory properties, cytotoxicity, *in vivo* analgesic effects, and CNS inhibitory action. While it is possible that it has contributed to the field of herbal medicine, further sophisticated investigation is required to ascertain the precise biological effects of this medicinal plant.

Abbreviations

DPPH: Diphenyl picrylhydrazyl; **BSA**: Bovine Serum Albumin; **ROS**: Reactive oxygen species; **MPE**: Maximal possible effect; UGO, **PRT**: Pain reaction time; **EPM**: Elevated plus maze; **CNS: Central Nervous System.**

Declarations

Ethics approval and consent to participate

All authors hereby declare that “Principles of Laboratory Animal Care” (NIH publication No. 85 – 23, revised 1985) were followed, as well as specific national laws where applicable. Ethical approval has been obtained from the Animal Ethics Review Board, Faculty of Biological Sciences, University of Chittagong, Chittagong, Bangladesh.

Consent for publication

All authors have given their consent to publish this article.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

Authors' contributionsMd. Mahbubur Rahman designed the experiments, the conception, and supervised the research work Mohammed Mozammel Hoque, Md. Sabbir Khan, Traya Chakma collected the plant material, performed the pharmacological assays and statistical analysis, and drafted the manuscript. BC contributed to perform pharmacological analysis. Animesh Biswasand and Mohammed Mozammel Hoque helped in the statistical analysis and write-up of the manuscript, critically revised the manuscript, provided punctual assistance, and gave the final approval for the submission of a revised version. Finally, all authors gave their final consent for the submission. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available upon reasonable request from the relevant author, and they were also discovered online by searching Google Scholar, PubMed, websites, book chapters, etc.

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