

Phytochemical Screening and Pharmacological Investigations of *Curculigo orchioides* Leaf Extracts

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Abstract

Curculigo orchioides Gaertn. (*C. orchioides*) is commonly known as Talamuli in Bangladesh. It is commonly distributed in Asian countries. The Indian (Ayurvedic) and Chinese traditional medicine systems use this plant frequently as an aphrodisiac herb for treating asthma and jaundice. Our study aimed to perform a comparative phytochemical analysis and evaluation of some biological effects using *in vitro* and *in vivo* approaches of its leaf's ethanol (ECO), n-hexane (HCO), and chloroform (CCO) fractions. After extraction and fractionation, a phytochemical analysis was followed by different tests for anti-bacterial, antioxidant, anti-inflammatory, anti-diabetic, anti-diarrheal, and anxiolytic effects. High intensity was observed for the phytochemical groups, including alkaloids, glycosides, tannins, and reducing sugars in the fractions. Crude ECO did not cause death in *Swiss* mice up to a 3000 mg/kg acute oral dose. HCO was inactive against the test bacteria. ECO and CCO exhibited zones of inhibition against the bacteria within 9.89 ± 3.56 and 13.00 ± 1.00 mm. HCO exhibited better radical scavenging, alpha-amylase inhibition, and anti-diarrheal effects, while CCO significantly and effectively inhibited protein denaturation and membrane lysis and had hypoglycemic and anxiolytic effects. Taken together, *C. orchioides* might be a good source of phytotherapeutic agents.

1. Introduction

To treat various ailments or to maintain sound health, the use of plant-based products has dramatically increased throughout the world [1]. More than 80% of people around the world use medicinal plants and their derivatives as self-medications for various health benefits [2]. Interestingly, the intake of herbal supplements is high among men and women of all ages. According to a survey, global consumption of herbal medicines was estimated to be worth around USD 230.03 billion in 2021. By 2028, the market is expected to grow to USD 430.05 billion [3]. This might be due to the few adverse effects and excellent safety of experimental animals and humans [4]. Plants have medical value because they contain certain chemicals that have a clear physiological impact on humans, such as alkaloids, glycosides, phenolics, flavonoids, tannins, lignins, and reducing sugars [5, 6].

Hypoxidaceae plant *Curculigo orchioides* Gaertn. (*C. orchioides*) is also known as "Talamuli" in Bangladesh, "Kali musli" in India, and "Xian mao" in China. It is widely distributed in large quantities in China, India, Nepal, Bangladesh, Japan, Malaya, and Australia [7]. Figure 1 shows plant's leaf and flower.

C. orchioides is essential in Indian and Chinese traditional medicine systems for its valuable bioactives. Its leaves are employed as Rasayana (anti-aging), Vrushya (aphrodisiac), Brimhana (weight loss), alterative, appetizer, fattening, and beneficial in piles, jaundice, menstrual irregularities, menstrual cramps, colic disorders, asthma, and amenorrhea. The leaf juice also strengthens the bones, spleen, and kidneys in humans [8, 9]. To date, a total of 61 phytoconstituents have been isolated under the gross phytochemical groups of glycosides, lignans, polysaccharides, alkaloids, saponins, triterpenes, and aliphatic compounds [10]. Different extracts of the plant have diverse biological effects, including antioxidant [11], anti-hypensive [12–25] anti-microbial [26], anti-inflammatory [27, 28], immunostimulant

[29], neuroprotective [30–33], hepatoprotective [34], aphrodisiac [35–39], anti-arthritis [16, 40], anti-asthmatic [8, 41], anticancer [11], antidiabetic [42], and hearing improvement [43] properties.

The current study aims to perform a preliminary comparative phytochemical screening and investigate some biological properties of the ethanol (ECO), n-hexane (HCO), and chloroform (CCO) leaf extracts of *C. orchioides*.

2. Materials and methods

2.1. Collection, identification, drying and grinding of plant materials

Fresh *C. orchioides* leaves were collected from the Bangladesh Forest Research Institute (BFRI), Chittagong, and were identified by a taxonomist from the same institution's Herbarium Section (Voucher specimen number: BFRIH-37928). The plant parts were collected, separated from adulterants (e.g., dust, other plant parts), and washed with running tap water. The leaves were cut into small pieces and dried for a week at room temperature in the shade (25–35 °C). Finally, the small pieces of rhizomes were put into an electric oven for 30 minutes at 35 °C and ground into a coarse powder using a mechanical grinder. The powder was kept in a dry, cool, and amber-colored airtight glass container until the extraction commenced.

2.2. Extraction and fractionation

Approximately 345 g of coarse powder was taken in a Soxhlet apparatus, and it was extracted with 800 ml of absolute ethanol for 8 h. A rotary evaporator was used to make the concentrate of the extract at a temperature below 40°C. The gummy concentrate was taken in a clean, airtight container, weighed, properly labeled, and kept in a refrigerator at 4°C. The solvent-solvent fractionation procedure was followed as below (Fig. 2).

2.3. Reagents and chemicals

Ethanol, chloroform, n-hexane, tween 80, ascorbic acid, and alpha amylase were purchased from Merck India, while nutrient agar medium was purchased from Himedia Laboratories Ltd., India. All the reference standards were kindly provided by different pharmaceutical industries in Bangladesh.

2.4. Experimental animals

The young *Swiss* albino male mice with an average weight of 24–30 gm were purchased from the animal research branch of the Bangladesh Council of Scientific and Industrial Research (BCSIR), Chattogram, Bangladesh. The animals were maintained in standard laboratory conditions (12 h light/dark cycle; room temperature 25 ± 2°C; relative humidity 55–60%) and fed a standard diet and water *ad libitum*. Prior to executing the studies, the animals were acclimatized to the laboratory environment for 7 days. Before the studies, the animals were starved for overnight (12 h). All experimental animals were cared for in

accordance with the *Swiss* Academy of Medical Sciences and the *Swiss* Academy of Sciences Ethical Principles and Guidelines for Scientific Experiments with Animals (1995). The Institutional Ethics Committee (SUB/IAEC/12.01) authorized all experimental protocols.

2.5. Phytochemical investigation

Preliminary phytochemical screenings were carried out according to the method described by [44] to investigate plant secondary metabolites present in the crude extract of the plant by adopting the standard procedure. Three types of solutions of extract were made first for this test: 5% w/v solution of extract was prepared for each fraction.

2.6. Acute toxicity analysis and dose determination

The test dose for this study of crude extracts was selected by the acute toxicity study following the OECD guidelines using *Swiss* albino mice. Briefly, the ECO was administered at doses of 500, 1000, 2000, and 3000 mg/kg orally. The animals were then frequently observed for behavioral changes, toxicological symptoms, and death for 2 days [45].

2.7. Antibacterial activity test (Disc diffusion assay)

This test was performed according to the method described by [46] with slight modifications. Briefly, the test samples were prepared by dissolving the extracts of the samples in ethanol (ECO), chloroform (CCO), and n-hexane (HCO). All the samples were tested at 250 µg/disc. Azithomycin (AZT) was taken as a reference antibiotic and was tested at 25 µg/disc. For this study, we used 5 gram (+) and 5 gram (-) bacteria (Table 2). After the inoculation of the test bacterial strain and after allowing the plates to solidify, the respective paper discs containing the test sample or standard drug were subsequently impregnated centrally into the agar gel separately with the help of sterile forceps to assure complete contact with the previously cultured medium surface. Finally, the plates were incubated at 37°C for 24 hours. The antibacterial sensitivities of *C. orchoides* leaf extarcts were determined by comparing the zone of inhibition (mm) with the standard drug azithromycin (25 µg/µl).

2.8. Free radical scavenging test (DPPH frre radical scavenging assay)

The free radical scavenging capacity of the extracts was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay described by [47] with slight modifications. Briefly, a 0.004% w/v DPPH solution was prepared in ethanol. Immediately, its absorbance was taken. To 1 ml of extract solution of different concentrations (20, 40, 60, 80, and 100 µg/ml) 2 ml of DPPH solution was added, mixed properly, and allowed to stand in a dark condition for 30 minutes to complete the reaction. The same concentrations of ascorbic acid (AA) were used as a reference radical scavenger, while ethanol was used as a blank. After the reaction time, the absorbance was measured at 517 nm using a UV spectrophotometer. Then the percent inhibition was calculated using the following formula:

$$\% \text{Radical scavenge} = [\text{Absorbance}_{\text{Before}} - \text{Absorbance}_{\text{After}}] \div \text{Absorbance}_{\text{Before}} \times 100$$

The half-minimal inhibitory concentration (IC₅₀) was determined using non-linear regression analysis using Graph Pad Prism software.

2.9. Anti-inflammatory activity test

2.9.1. Egg album assay

This test was performed by checking inhibitory effects on egg albumin using the method described by Banerjee and Chanda[48]. Briefly, 0.2 ml of egg albumin (from a fresh hen's egg) was mixed with 2.8 ml of isosaline (0.9% NaCl, pH 6.4) and 2 ml of a test sample or standard drug. Distilled water and diclofenac-Na served as control and positive controls, respectively. The sample and standard were tested at 20, 40, 60, 80, and 100 µg/ml. After incubation at 37 ± 2°C for 15 minutes, the reaction mixtures were then heated at 70°C for 5 minutes in a water bath. The mixtures were then cooled and filtered through Whatmann filter paper no. 1. The absorbance was measured at 660 nm using a colorimeter. The percentage inhibition of protein denaturation was estimated using the following formula:

$$\% \text{Inhibition of protein denaturation} = 100 \times ([V_t/V_c] - 1)$$

Where, V_t and V_c stand for the absorbance of the test sample and the control, respectively. The IC₅₀ values were determined as mentioned above.

2.9.2. Membrane stabilizing test (HRBC assay)

This test was done according to the method described by Gandhidasan and his coworkers [49] with slight modifications. Briefly, 0.5 ml of 10% RBC suspension was mixed with 5 ml of hypotonic solution (0.5% NaCl solution, pH 7.4) and 1 ml of different concentrations of test extracts or standard solution. After incubation at 37 ± 2°C for 10 min, all the reaction mixtures were centrifuged for 10 min at 3000 rpm. The mixtures were then filtered using Whatmann filter paper no. 1. Then, the absorbance was measured at 546 nm using a colorimeter. Hemolysis was compared with the control group, and the percentage inhibition of hemolysis was determined using the following formula:

$$\% \text{haemolysis inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test/Stanadr}}{\text{Absorbance of Control}} \times 100$$

2.10. Hypoglycemic test

2.10.1. Alpha-amylase inhibition assay (*in vitro*)

This test was performed according to the method described by [50] with slight modifications. Briefly, 80 µl of alpha amylase solution (0.050 mg/ml) was mixed with 5.2 ml of Tris-HCl buffer (0.01 M containing 0.006 M NaCl, pH 6.8) and 320 µl of different concentrations of test samples or standard solutions (20–100 µg/ml). Acarbose was used as a reference drug for this assay. After incubation at 37 °C for 20 min, 400 µl of the starch solution (0.1%) was added, and the mixture was re-incubated at 37°C for 20 min,

after which 8 ml of 0.01% acidic iodine solution was added to the reaction mixtures. After filtration, the absorbance of the filtrate was taken at 578 nm using a colorimeter. Alpha amylase inhibitory activity was determined as below.

$$\% \text{Amylase activity inhibition} = \left[\frac{\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Sample}}}{\text{Absorbance}_{\text{Control}}} \right] \times 100$$

The IC₅₀ values were determined as mentioned above.

2.10.2. Glucose tolerance test (*in vivo*)

For this study, we followed the method described by [51] with slight modifications. After 12 hours of fasting, the animals were randomly divided into different groups (n = 6). Baseline blood glucose levels were measured by using a glucometer. Blood was collected by cutting the tail aseptically with a surgical blade. The control group received 0.1% tween-80 dissolved in a 0.9% NaCl solution, while the positive control group received the standard hypoglycemic drug glibenclamide (GBC) at 10 mg/kg. The extracts were tested at 500 mg/kg. All the treatments were given orally (p.o.). After 30 minutes, the animals were treated with a 10% glucose solution at 10 g/kg body weight. Then, the blood glucose level was measured similarly at 30, 90, and 120th minutes after the standard glucose solution administration. A glucose solution, test sample, and controls were given orally.

2.11. Antidiarrheal activity test (*castor oil-induced diarrheal model*)

For this study, we followed the method described by Awouters[52] with slight modifications. After 12 hours of fasting, the animals were randomly divided into different groups (n = 6). The control group received 0.1% tween-80 dissolved in a 0.9% NaCl solution, while the positive control group received the standard antidiarrheal drug loperamide (LOP) at 3 mg/kg. The extracts were tested at 500 mg/kg. All the treatments were given orally (p.o.). After 30 minutes, the animals were treated with 0.4 ml of castor oil. Then, each animal was placed in an individual cage, the floor of which was lined with blotting paper. The floor lining was changed every hour. The latency period (first diarrheal stooling time after the administration of castor oil) and the total number of feces (hard and soft) excreted by the animals were recorded during the observation period (4 hours). After the study, we also observe for an additional 20 hours to check for serious illness or mortality.

2.12. Anxiolytic activity study

2.12.1. Open-field test

This test was performed according to the method described by Molla [53] with slight modifications. The animals (n = 6) were grouped as per the previous study, and the treatments were given. In this case, we used diazepam (DZP) at 3 mg/kg as a reference drug. All the treatments were given orally (p.o.). After 25 minutes of sample or drug administration, the number of squares crossed by each mouse was counted for 5 minutes.

2.12.2. Hole-cross test

This study was performed according to the method described by Takagi [54]. The animals from the open-field test were given a 2-minute rest, and then submitted to this study. In this case, we counted the number of squares crossed by each mouse within 5 minutes.

2.13. Statistical analysis

Values are expressed as the mean $\pm$ standard error of the mean (SEM). One-way analysis of variance (ANOVA) was followed by Newman Keuls *post hoc* t-students test using the Graph Pad Prism software (version 6.5), considering $p < 0.05$ at 95% confidence intervals.

3. Results

3.1. Extraction and phytochemical investigation

The ECO was a gummy concentrate. The yield values of ECO, CCO, and HCO were 11.14, 0.87, and 2.03%, respectively. Preliminary phytochemical screening showed that the ECO contains alkaloids, glycosides, tannins, flavonoids, saponins reducing sugar, gums, and amides. On the other hand, CCO contains alkaloids, glycosides, steroids, tannins, saponins, reducing sugar, and amides. The HCO contains alkaloids, glycosides, steroids, tannins, flavonoids, saponins reducing sugar, gums, and amides (Table 1).

Table 1
Phytochemical groups detected in *Curculigo orchioides* leaf extracts

Chemical groups	Fractions and their color intensities		
	<i>ECO</i>	<i>CCO</i>	<i>HCO</i>
Alkaloids	+++	+++	+++
Glycosides	++	+	+
Steroids	-	+	+
Tannins	++	++	+++
Flavonoids	+	-	+
Saponins	+	+	+
Reducing sugar	++	++	++
Gums	+	-	+
Amides	+	+	+
+ = Positive (Present); - = Negative (Absent); multiplication of plus (+) sign means observed intensity; ECO: Ethanolic <i>Curculigo orchioides</i> extract; CCO: Chloroform <i>Curculigo orchioides</i> extract; HCO: n-Hexane <i>Curculigo orchioides</i> extract			

3.2. Pharmacological activities

3.2.1. Acute toxicity analysis and dose determination

The findings of the acute toxicity study demonstrate that the ECO did not cause serious illness or death in the *Swiss* mice up to an oral dose of 3000 mg/kg. Thus, we selected 1/6th, i.e., 500 mg/kg, as a test dose for *in vivo* studies.

3.2.2. Antibacterial activity

Table 2 suggests that ECO exhibited antibacterial effects against all the tested gram (+) bacteria (zone of inhibition: 9.90 ± 0.14 to 12.22 ± 2.04 mm) and 4 species of gram (-) bacteria (zone of inhibition: 9.89 ± 3.56 to 12.53 ± 1.50 mm). However, it remained inactive against *Salmonella typhi*. On the other hand, the CCO was found to act against *Lactobacillus casei*, *Lactobacillus coryniformis*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* between the zones of ingestion 11.33 ± 1.16 and 13.00 ± 1.00 mm. It remained insensitive at the test concentration (250 µg/disc) towards *Bacillus azotoformans*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Vibrio cholera*. The HCO fraction did not act against any test bacteria at 250 µg/disc. The standard drug AZT exhibited a zone of inhibition within the range of 17.33 ± 0.58 to 21.67 ± 0.58 mm at 25 µg/disc. It was found to act against all the test bacteria.

Table 2

Antibacterial activity of *Curculigo orchoides* leaf extracts and the reference antibacterial drug

Test bacteria	Zone of inhibition (mm)			
	Leaf extract (250 µg/disc)			AZT (25 µg/disc)
	ECO	CCO	HCO	
Gram (+)ve species				
<i>Lactobacillus casei</i>	11.50 ± 2.58	13.00 ± 1.00	NI	18.33 ± 0.58
<i>Lactobacillus coryniformis</i>	9.90 ± 0.14	11.33 ± 1.16	NI	17.33 ± 0.58
<i>Bacillus cereus</i>	12.22 ± 2.04	12.22 ± 0.75	NI	19.00 ± 1.00
<i>Staphylococcus aureus</i>	10.29 ± 2.47	11.50 ± 1.53	NI	20.00 ± 1.00
<i>Bacillus azotoformans</i>	10.33 ± 3.22	NI	NI	19.00 ± 1.00
Gram (-)ve species				
<i>Escherichia coli</i>	9.89 ± 3.56	12.17 ± 1.89	NI	19.67 ± 1.53
<i>Pseudomonas aeruginosa</i>	11.56 ± 1.50	NI	NI	18.67 ± 0.58
<i>Salmonella typhi</i>	NI	NI	NI	19.67 ± 0.58
<i>Klebsiella pneumoniae</i>	12.33 ± 2.52	12.00 ± 2.65	NI	18.33 ± 0.58
<i>Vibrio cholera</i>	12.53 ± 1.50	NI	NI	21.67 ± 0.58
Values are mean ± SEM (standard error of mean) (n = 3); NI = No inhibition; Zone of inhibition ≤ 7 mm were discarded; AZT: Azithromycin; ECO: Ethanolic <i>Curculigo orchoides</i> extract; CCO: Chloroform <i>Curculigo orchoides</i> extract; HCO: n-Hexane <i>Curculigo orchoides</i> extract				

3.2.3. Free radical scavenging capacity

Table 3 suggests that the control group exhibited negligible ($02.17 \pm 0.37\%$) inhibition of DPPH free radicals. Both the test sample (all fractions) and the standard drug AA exhibited concentration-dependent radical scavenging capacities. AA produced better activities than the test subjects. Among the extracts, ECO exhibited better radical scavenging capacity. The IC_{50} values calculated for the AA, ECO, CCO, and HCO are 16.11 ± 0.08 , 34.01 ± 0.11 , 17.82 ± 0.12 , and 54.01 ± 0.11 µg/ml, respectively.

Table 3
DPPH free radical scavenging potential of *Curculigo orchioides* leaf extarcts and controls

Parameters		Percentage radical scavenging			
		AA	ECO	CCO	HCO
Conc. (µg/ml)	20	55.67 ± 0.27*	40.89 ± 0.36*	54.52 ± 0.27*	35.96 ± 0.29*
	40	68.97 ± 0.19*	58.46 ± 0.25*	61.41 ± 0.23*	43.02 ± 0.34*
	60	78.00 ± 0.14*	64.86 ± 0.21*	68.14 ± 0.19*	57.96 ± 0.26*
	80	85.55 ± 0.08*	78.65 ± 0.13*	72.91 ± 0.17*	67.98 ± 0.24*
	100	93.27 ± 0.04*	83.42 ± 0.11*	79.64 ± 0.12*	75.86 ± 0.18*
IC ₅₀ (µg/ml)		16.11 ± 0.08	34.01 ± 0.11	17.82 ± 0.12	54.01 ± 0.11
CI (µg/ml)		9.11 – 28.47	26.97 – 42.66	11.47 – 22.70	44.68 – 61.57
r ²		0.96	0.84	0.84	0.83
Control		02.17 ± 0.37			
Values are mean ± SEM (standard error of mean) (n = 3); *p< 0.05 when compared to the control (0.05% tween 780 dissolved in 0.9% NaCl solution) group; AA: Ascorbic acid; ECO: Ethanolic <i>Curculigo orchioides</i> extract; CCO: Chloroform <i>Curculigo orchioides</i> extract; HCO: n-Hexane <i>Curculigo orchioides</i> extract; IC ₅₀ : Half minimum inhibitory concentration; CI: Confidence of intervals; r ² : Co-efficient of determination					

3.2.4. Anti-inflammatory activity

Egg albumin denaturation inhibition assay

According to Table 4, the control group exhibited negligible (02.23 ± 0.29%) inhibition of egg protein denaturation capacity. All the fractions and the standard drug DCN exhibited concentration-dependent stabilization effects on the egg protein. DCN produced better activities than the test subjects. Among the extracts, CCO exhibited better protein protection capacity. The IC₅₀ values calculated for the AA, ECO, CCO, and HCO are 43.82 ± 0.05, 89.05 ± 0.07, 62.94 ± 0.04, and 109.65 ± 0.04 µg/ml, respectively.

Table 4
Egg protein protection capacity of *Curculigo orchoides* leaf extracts and controls

Parameters		Percentage inhibition of protein denaturation			
		DCN	ECO	CCO	HCO
Conc. (µg/ml)	20	31.37 ± 0.21*	27.86 ± 0.33*	15.44 ± 0.29*	09.80 ± 0.21*
	40	49.75 ± 0.13*	38.73 ± 0.23*	18.22 ± 0.27*	20.92 ± 0.31*
	60	69.04 ± 0.15*	40.36 ± 0.25*	49.59 ± 0.34*	23.53 ± 0.23*
	80	84.23 ± 0.18*	46.08 ± 0.17*	55.23 ± 0.36*	38.73 ± 0.33*
	100	97.06 ± 0.25*	62.91 ± 0.19*	63.56 ± 0.32*	46.08 ± 0.15*
IC ₅₀ (µg/ml)		43.82 ± 0.05	89.05 ± 0.07	62.94 ± 0.04	109.65 ± 0.04
CI (µg/ml)		33.04 – 59.65	78.99 – 97.93	52.62 – 63.31	97.68 – 119.42
r ²		0.93	0.82	0.94	0.95
Control		02.23 ± 0.29			
Values are mean ± SEM (standard error of mean) (n = 3); *p< 0.05 when compared to the control (0.05% tween 780 dissolved in 0.9% NaCl solution) group; DCN: Diclofenac-Na; ECO: Ethanolic <i>Curculigo orchoides</i> extract; CCO: Chloroform <i>Curculigo orchoides</i> extract; HCO: n-Hexane <i>Curculigo orchoides</i> extract; IC ₅₀ : Half minimum inhibitory concentration; CI: Confidence of intervals; r ² : Co-efficient of determination					

HRBC membrane stabilization assay

Table 5 shows that the control group exhibited a negligible (01.39 ± 0.11%) cytoprotective effect on the HRBC suspension. All the fractions and the standard drug ASA exhibited concentration-dependent stabilization effects on the HRBC suspension. ASA produced better activities than the test subjects. Among the extracts, CCO exhibited better membrane stabilizing capacity. The IC₅₀ values calculated for the AA, ECO, CCO, and HCO are 38.35 ± 0.03, 95.05 ± 0.04, 87.52 ± 0.05, and 107.38 ± 0.07 µg/ml, respectively.

Table 5
RBC membrane stabilizing capacity of *Curculigo orchioides* leaf extarcts and controls

Parameters		Percentage inhibition of membrane lysis			
		ASA	ECO	CCO	HCO
Conc. (µg/ml)	25	39.17 ± 0.23*	18.71 ± 0.31*	20.39 ± 0.31*	08.78 ± 0.29*
	50	69.36 ± 0.26*	38.73 ± 0.23*	40.36 ± 0.27*	20.92 ± 0.11*
	100	90.20 ± 0.19*	55.23 ± 0.29*	63.56 ± 0.23*	49.75 ± 0.13*
IC ₅₀ (µg/ml)		38.35 ± 0.03	95.05 ± 0.04	87.52 ± 0.05	107.38 ± 0.07
CI (µg/ml)		20.20 – 48.76	87.35 – 101.50	76.58 – 98.6	97.51 – 119.70
r ²		0.98	0.98	0.97	0.94
Control		01.39 ± 0.11			
Values are mean ± SEM (standard error of mean) (n = 3); * <i>p</i> < 0.05 when compared to the control (0.05% tween 780 dissolved in 0.9% NaCl solution) group; ASA: Acetyl salicylic acid; ECO: Ethanolic <i>Curculigo orchioides</i> extract; CCO: Chloroform <i>Curculigo orchioides</i> extract; HCO: n-Hexane <i>Curculigo orchioides</i> extract; IC ₅₀ : Half minimum inhibitory concentration; CI: Confidence of intervals; r ² : Co-efficient of determination					

3.2.5. Hypoglycemic activity test

Alpha-amylase inhibitory effect

Table 6 shows that the control group exhibited a negligible (02.39 ± 0.08%) alpha amylase inhibitory effect. All the fractions and the standard drug ACB exhibited significant (*p* < 0.05) and concentration-dependent alpha amylase inhibitory effects as compared to the control group. ACB produced better activities than the test subjects. Among the extracts, HCO exhibited a better alpha-amylase inhibitory effect. The IC₅₀ values calculated for the AA, ECO, CCO, and HCO are 43.63 ± 0.06, 67.69 ± 0.23, 268.94 ± 0.06, and 121.82 ± 0.03 µg/ml, respectively.

Table 6
Alpha amylase inhibition capacity of *Curculigo orchoides* leaf extracts and controls

Parameters		Percentage amylase activity inhibition			
		ACB	ECO	CCO	HCO
Conc. (µg/ml)	25	42.31 ± 0.14*	21.43 ± 0.27*	10.16 ± 0.23*	12.50 ± 0.27*
	50	57.69 ± 0.21*	41.43 ± 0.17*	20.31 ± 0.21*	28.13 ± 0.33*
	100	69.23 ± 0.23*	58.57 ± 0.13*	32.01 ± 0.17*	46.88 ± 0.31*
	200	80.77 ± 0.34*	72.14 ± 0.29*	46.88 ± 0.14*	62.50 ± 0.27*
	400	96.15 ± 0.28*	85.71 ± 0.31*	63.28 ± 0.11*	78.13 ± 0.24*
IC ₅₀ (µg/ml)		43.63 ± 0.06	67.69 ± 0.23	268.94 ± 0.06	121.82 ± 0.03
CI (µg/ml)		31.74 – 52.01	58.56 – 82.83	246.51 – 289.18	111.67 – 134.45
r ²		0.96	0.98	0.96	0.99
Control		02.39 ± 0.08			
Values are mean ± SEM (standard error of mean) (n = 3); * <i>p</i> < 0.05 when compared to the control (0.05% tween 780 dissolved in 0.9% NaCl solution) group; ACB: Acarbose; ECO: Ethanolic <i>Curculigo orchoides</i> extract; CCO: Chloroform <i>Curculigo orchoides</i> extract; HCO: n-Hexane <i>Curculigo orchoides</i> extract; IC ₅₀ : Half minimum inhibitory concentration; CI: Confidence of intervals; r ² : Co-efficient of determination					

Glucose tolerance test

Table 7 suggests that the standard hypoglycemic drug GBC reduced peripheral blood glucose levels effectively after 30 minutes. In comparison to the control group, all the extracts also significantly (*p* < 0.05) reduced peripheral blood glucose levels after 30 minutes. CCO at 500 mg/kg exerted strong hypoglycemic effects in comparison to the control group.

Table 7

Glucose level (mmol/L) of peripheral blood at observation time for *Curculigo orchioides* leaf extracts and controls

Treatments (p.o.)		Glucose level (mmol/L)			
		0 min	30 min	60 min	120 min
Control (10 ml/kg)		22.25 ± 0.18	18.35 ± 1.09	15.35 ± 1.08	12.55 ± 1.02
GBC (10 mg/kg)		14.65 ± 0.07*	6.15 ± 1.48*	3.75 ± 0.49*	3.33 ± 0.45*
500 mg/kg	ECO	15.20 ± 1.56*	7.35 ± 1.91*	4.20 ± 1.27*	4.05 ± 0.64*
	CCO	16.75 ± 0.64*	7.85 ± 1.20*	5.15 ± 0.07*	3.55 ± 0.07*
	HCO	18.25 ± 0.07*	8.90 ± 0.28*	5.55 ± 0.78*	4.35 ± 0.07*

Values are mean ± SEM (standard error of mean) (n = 5); One-way ANOVA followed by *t*-student test; **p* < 0.05 when compared to the control group; GBC: Glibenclamide; ECO: Ethanolic *Curculigo orchioides* extract; CCO: Chloroform *Curculigo orchioides* extract; HCO: n-Hexane *Curculigo orchioides* extract

3.2.6. Antidiarrheal activity

According to Table 8, the standard drug LOP at 3 mg/kg significantly (*p* < 0.05) augmented the latency period and reduced the total diarrheic secretions in animals. In comparison to the control group, all the extracts significantly (*p* < 0.05) increased the latency period and reduced the total diarrheic secretions in animals. ECO and HCO at 500 mg/kg exerted better antidiarrheal effects than CCO.

Table 8

Latency and diarrheic secretions observed in *Curculigo orchioides* and controls

Treatment groups (p.o.)		Observed parameters	
		Latency (min)	Diarrheic secretions
Control (10 mL/kg)		28.00 ± 3.00	22.33 ± 2.52
LOP (3 mg/kg)		50.33 ± 1.53*	6.33 ± 2.52*
500 mg/kg	ECO	42.67 ± 2.89*	8.00 ± 1.00*
	CCO	39.33 ± 2.52*	9.67 ± 1.53
	HCO	40.67 ± 2.08*	8.33 ± 2.08*

Values are mean ± SEM (standard error of mean) (n = 5); One-way ANOVA followed by *t*-student test; **p* < 0.05 when compared to the control group; DZP: Diazepam; p.o.: Per oral; ECO: Ethanolic *Curculigo orchioides* extract; CCO: Chloroform *Curculigo orchioides* extract; HCO: n-Hexane *Curculigo orchioides* extract

3.2.7. Anxiolytic activity

Table 9 suggests that the standard drug DZP at 3 mg/kg significantly ($p < 0.05$) reduced the number of square crossing and hole crossing behaviors in animals. Among the test extarcts CCO was found to reduce the square crossing and hole crossing behaviors in animals significantly. However, in comparison to the control group ECO and HCO groups also exerted significant ($p < 0.05$) calming effects effects in animals.

Table 9		
Number of square crossings and hole crossings observed in <i>Curculigo orchioides</i> leaf extracts and control groups		
Treatment groups		Observed parameters
		Square crossing Hole crossing
Control (10 mL/kg, p.o.)		90.00 ± 2.00 10.00 ± 1.00
DZP (3 mg/kg, i.p.)		49.00 ± 4.58* 3.67 ± 0.58*
500 mg/kg (p.o.)	ECO	74.67 ± 2.52* 5.33 ± 1.53*
	CCO	64.67 ± 2.52* 4.00 ± 3.60*
	HCO	81.33 ± 3.06* 6.00 ± 2.00*
Values are mean ± SEM (standard error of mean) (n = 5); One-way ANOVA followed by <i>t</i> -student test; * $p < 0.05$ when compared to the control group; DZP: Diazepam; p.o.: Per oral; ECO: Ethanolic <i>Curculigo orchioides</i> extract; CCO: Chloroform <i>Curculigo orchioides</i> extract; HCO: n-Hexane <i>Curculigo orchioides</i> extract		

4. Discussion

The traditional use of a plant provides indirect hints to investigators that the plant is the source of targeted phytochemicals. This information has been passed down from generation to generation, and in recent decades, scientists have been taking these local usage practices into great consideration. It has been estimated by the World Health Organization (WHO) that herbal remedies are relied upon by roughly 80% of people worldwide for some aspects of their primary healthcare requirements. The WHO has stated that there are around 21,000 plant species that possess medicinal properties. Based on available data, it has been concluded that over three-quarters of the global population primarily depends on plants and plant extracts for their healthcare needs [55, 56].

Curculigo orchioides, the origin of this plant, is traced back to India, where it is recognized as a powerful adaptogen and aphrodisiac in the Ayurvedic medical tradition. It is widely used in various Ayurvedic remedies and is believed to possess hepatoprotective, anticancer, antioxidant, antidiabetic, aphrodisiac, and immunostimulatory properties [57]. Its rhizomes are well used traditionally rather than leaf. The rhizomes of this plant have been utilized for numerous purposes in traditional practices, such as treating skin ailments, promoting wound healing, providing a cooling effect, combating bacterial infections, enhancing sensual experiences, managing jaundice and respiratory conditions like asthma and

bronchitis, preventing cancer, and serving as a tonic for leucorrhea, hemorrhoids, pruritis, and sweetness, among others [58].

Plants contain a wide range of components that possess various bioactivities in both plant and animal systems. These components can regulate biological signaling pathways and are therefore potentially useful in treating and managing a wide range of diseases and disorders [59]. *C. orchioides* contains a diverse range of phytoconstituents, as indicated by qualitative analyses of extracts obtained from both the plant's rhizomes and the whole plant. These extracts contain saponins, phenolics, alkaloids, steroids, flavonoids, triterpenes, and polysaccharides [3, 60]. Major glycosides identified from *C. orchioides* plant includes syringic acid; glucopyranoside; curculigosaponins E, F, G, H, I, and J; curculigoside B; curculigines P and Q; curculigine B and C; orcinosides I-J; 2-beta-D-glucopyranosyloxy-5-hydroxybenzyl-2'-methoxy-6'-hydroxybenzoate; 2,6-dimethoxy benzoic acid; orcinol-β-D-glucoside) [61–65].

In our study, we found that *C. orchioides* contains flavonoids, sugar moieties, and glycosides in its ECO, CCO, and HCO extractions. The issue of antibiotic resistance is a significant challenge in the contemporary era, resulting in the failure of conventional antibiotics, chronic ailments, treatment complexities, and escalated costs. Multidrug-resistant bacteria can cause severe health consequences for both humans and animals. Phytochemicals offer a promising alternative by providing a diverse range of bioactive groups such as alkaloids, glycosides, polyphenols, and flavonoids, which are capable of combating various pathogens, including multidrug-resistant ones, by targeting their efflux pumps, biofilms, membrane-protein dysregulation, interrupting cellular communications, and other biological processes [66]. It has evidence that *C. orchioides* has an anti-*Streptococcus pyogenes* effect with a minimum inhibitory concentration (MIC) value of 49 µg/ml [67]. Our research findings indicate that at a dosage of 250 µg/disc, the herb's CCO exhibited antibacterial properties against a range of gram-positive and gram-negative pathogenic bacteria. This confirms earlier reports of the plant's antibacterial effects.

Unbound radicals are chemical compounds containing atoms, molecules, or ions that contain one or more unpaired electrons in their outer shells, rendering them extremely responsive. Usually, these molecules play a double duty in our organisms: on one hand, they can inflict oxidative harm and tissue malfunction as poisonous byproducts of oxygen metabolism, while on the other hand, they also act as molecular signaling agents that promote advantageous stress cascades [68]. An overabundance of unbound radicals can inflict harm on a living system. Antioxidants counteract these radicals in a living system via various means. Medicinal plants and their byproducts, boasting a range of chemical groups, have the potential to act against an excess of hazardous unbound radicals that may affect our bodies [69].

As an antioxidant, *C. orchioides* is believed to possess the ability to scavenge radicals, according to previous reports. Furthermore, another study indicated that the antioxidant impact of *C. orchioides* is associated with its ability to protect the liver in experimental animals [34, 70]. In our investigation, it was discovered that every fraction of *C. orchioides* leaf could effectively and proportionally remove DPPH free radicals at a concentration range of 20 – 100 µg/ml. The overproduction of harmful free radicals is

related to the destruction of important cellular macromolecules such as carbohydrates, proteins, lipids, and genetic molecules inside an organism. This phenomenon can stimulate both pro-inflammatory and inflammatory reactions, which may trigger the liberation of various mediators [71].

Nonetheless, microbial invasions can cause both oxidative stress and inflammatory responses within our bodies [72]. Though leaf extract was not previously studied, in a previous study conducted by [28], rhizome extracts of the plant, when administered at 150, 300, and 600 mg/kg doses, had a significant impact on reducing the levels of tumor necrosis factor alpha (TNF- α), interleukin (IL)-1 β , and IL-6 when compared to the control groups in rats. Our investigation revealed that all the *C. orchoides* leaf extracts exhibited dose-dependent inhibition of egg protein denaturation and safeguarded HRBC within the range of 20 to 100 μ g/ml. The improper management of blood sugar levels continues to pose a significant health challenge globally, leading to a surge in the prevalence of diabetes mellitus and related fatalities. Although conventional antidiabetic medications have been successful, they often come with inevitable side effects. Hence, alternative options, such as the development of novel and safe antidiabetic drugs, may be necessary to address this issue [73].

As per several prior studies, *C. orchoides* possesses antidiabetic characteristics [39, 74]. According to Umar [74], *C. orchoides* is capable of inhibiting alpha-glucosidase. In diabetic patients, enzymes such as alpha-amylase and alpha-glucosidase decompose carbohydrates and lead to elevated glucose levels in the blood after meals. Thus, compounds that can restrict these enzymes could regulate postprandial hyperglycemia, minimizing the likelihood of diabetes development [75]. Our research indicates that all the segments of *C. orchoides* leaves were able to dose-dependently restrain alpha-amylase. In addition, they also reduced the oral glucose level in experimental animals 30 minutes after extract administration at a 500 mg/kg oral dose. Diarrhea is a medical condition in which a person experiences frequent bowel movements that are unusually watery or loose in texture. The condition is triggered by a combination of factors, including osmotic and active secretion, impaired peristalsis, and exudation [75]. It has been estimated that diarrhea leads to about 1.7 billion cases annually in children and is responsible for almost 5.5 million child deaths below the age of 5. In South Asia, diarrhea causes nearly 2.75 million deaths among children under the age of 5. Luckily, diarrhea can be prevented and cured. Nevertheless, traditional antidiarrheal medications are hazardous, develop resistance, become ineffective, and are not recommended for numerous pathological conditions and therapeutic approaches [76].

The use of local medicinal plants for diarrhea treatment has a long history in many countries across the globe. As far as we know, this study represents the first antidiarrheal activity test conducted using the castor oil-induced diarrhea model in *Swiss* mice. Castor oil prevents water reabsorption, leading to an increase in the volume of intestinal contents and causing diarrhea in animals. The ricinoleic acid present in castor oil mediates irritation and inflammation of the intestinal mucosa, resulting in prostaglandin secretion that alters the transport of mucosal fluid and electrolytes, thereby preventing the reabsorption of NaCl and water. In experimental animals, ricinoleic acid was found to significantly increase the PGE2 content in the gut lumen, leading to the release of excessive water and electrolytes into the small intestine. Additionally, castor oil has the ability to synthesize nitric oxide (NO), which increases the

permeability of the epithelial layer to calcium ions, resulting in increased intracellular Ca^{2+} levels and stimulation of NO synthase activity. As a result, both prostaglandins and NO can induce intestinal secretion and trigger inflammatory cascades [77].

Bacterial diarrhea is commonly caused by specific pathogens, including *E. coli* and *Salmonella* sp. According to Marasini et al.'s [67] study and our own findings, there may be a correlation between the antibacterial effects observed and the efficacy against *E. coli* and *Salmonella* sp. In our research, we found that administering CCO at a dose of 500 mg/kg and the standard antidiarrheal drug LOP at 3 mg/kg resulted in similar latency and diarrhea episodes in the castor oil-induced diarrhea model in mice. According to Islam [44], there are more than 600 known neurological diseases and disorders. Anxiety is a common mood disorder caused by stress that can cause disability and even premature death. Around 20% of adults experience anxiety at some point in their lives [78, 79]. The neurotransmission systems involving gamma-amino butyric acid (GABA) and serotonin play a role in anxiety. Although SSRIs are effective in treating anxiety, they are mainly used as antidepressants [80].

Benzodiazepines are commonly used to treat anxiety, but they have many unwanted effects like muscle dependence, relaxation, memory problems, sedation, and interaction with other treatments. Therefore, plant-based compounds can be a safe and effective source of anxiolytic agents. Our research indicates that CCO can be used as an alternative to benzodiazepines to treat anxiety [81]. The study found that CCO showed similar anxiolytic effects as the positive control group (DZP 3 mg/kg, i.p.) in *Swiss* mice at 500 mg/kg (p.o.). All the extracts significantly reduced the number of square and hole-crossing behaviors in animals compared to the control group ($p < 0.05$). Various solvents are used for extracting compounds from plants, such as ethanol, chloroform, n-hexane, methanol, and ethyl acetate, depending on the type of compound. Ethanol, methanol, and ethyl-acetate are used for extracting hydrophilic compounds, while chloroform, dichloromethane, or a mixture of dichloromethane and methanol are used for lipophilic compounds. Moreover, n-hexane is used to remove chlorophyll from plant extracts [82]. To obtain initial extraction, dried plant materials are usually extracted with chloroform. This solvent is often utilized to extract glycosidic contents from crude samples as well [83]. A preliminary phytochemical study suggests that CCO exhibited a higher intensity of glycoside contents in the leaf extract, which is consistent with previous studies that isolated glycosides from the plant. Furthermore, according to a study, the polysaccharides of this herb are evidently enhancing the rate of osteogenic mineralization in MC3T3-E1 cells. Osteoarthritis is related to oxidative stress and inflammation, which are promising factors [60, 84].

C. orchoides has been previously reported to have anti-arthritis effects [25, 85, 86]. Gong [85] attributed this effect to orcinol glucoside, present in *C. orchoides*, which attenuated oxidative stress and autophagic cascades in SAMP6 mice. The development of arthritis is associated with chronic inflammation of the synovial membrane and bone erosion, caused by autoantibodies and proinflammatory cytokines like TNF- α and IL-6 [87]. *C. orchoides* rhizome extract has been found to be active against TNF- α and IL-6 [28], and it also exhibits immunostimulatory effects in mice [29]. The CCO

and HCO fractions of the plant contain bioactive sugars that might be responsible for their observed free radical scavenging and anti-inflammatory effects.

According to earlier reports [25, 85, 86], *C. orchoides* has anti-arthritis effects. Recently, Gong [85] found that the phenolic glycoside orcinol glucoside present in *C. orchoides* exerts an anti-arthritis effect by reducing oxidative stress and autophagic cascades in SAMP6 mice. Arthritis is a chronic inflammatory condition caused by autoantibodies and proinflammatory cytokines, such as TNF- α , and IL-6, which can lead to bone erosion and deformity. The *C. orchoides* extract is active against TNF- α , and IL-6 and has been shown to have immunostimulatory effects in mice. The observed free radical scavenging and anti-inflammatory effects of CCO and HCO may be due to the presence of bioactive sugars in these fractions[28, 29, 85, 87].

5. Conclusion

All the fractions showed high intensity for the phytochemical groups alkaloids, glycosides, tannins, and reducing sugars. Only ECO and CCO showed sensitivity against the test bacteria at 250 $\mu\text{g}/\text{disc}$. HCO exhibited better radical scavenging, alpha-amylase inhibition, and anti-diarrheal effects. On the other hand, CCO showed better inhibition of protein denaturation and membrane lysis, hypoglycemic, and anxiolytic effects. We suppose that *C. orchoides* leaf extract-mediated biological activities might be linked to the presence of high amounts of alkaloids, glycosides, tannins, and reducing sugars. The *in vitro* results (e.g., antioxidant, anti-inflammatory, and alpha-amylase inhibitory) were significant and concentration-dependent. All the extracts also showed significant hypoglycemic, antidiarrheal, and anxiolytic effects in *Swiss* mice at 500 mg/kg. Further studies are required to isolate and characterize its active principles and elucidate their bioactivities with possible molecular mechanisms.

Declarations

Ethical statement

The Institutional Ethics Committee (SUB/IAEC/12.01) authorized all experimental protocols.

Conflict of interest

None declared

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Author contribution

Investigation, Data Curation, Writing – original draft - TMNA, AAF and IS; Formal analysis, Writing – original draft – MSB, TI and RC; Writing – original draft, Project administration, Resources - SAA, IAA and NS; Conceptualization, Methodology, Validation, Supervision, Writing – review & editing – MTI and MMUC.

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Figures



Curculigo orchioides (leaf)



Curculigo orchioides (flower)

Figure 1

Curculigo orchioides (leaf and flower)

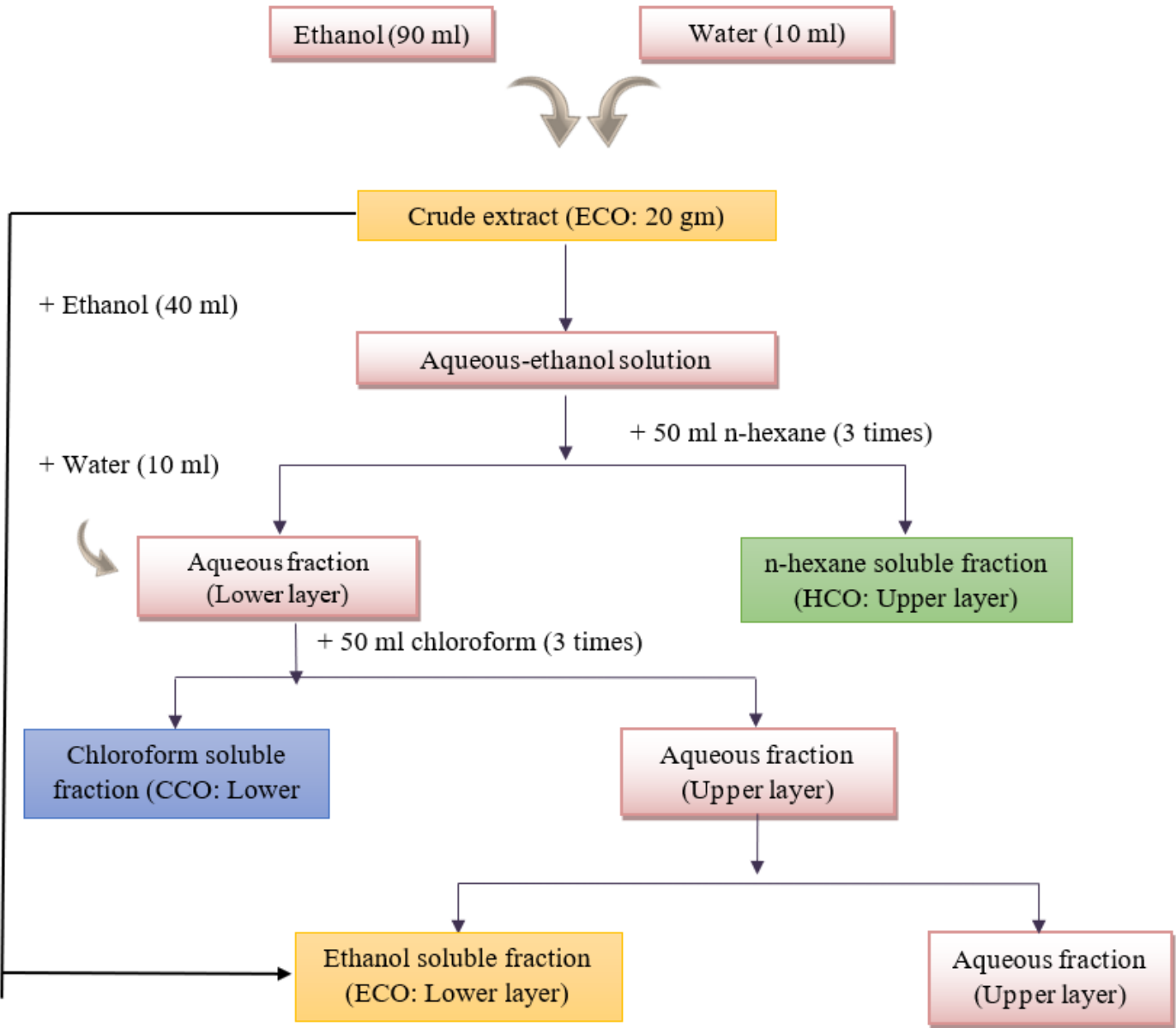


Figure 2

Schematic representation of fractionation of the crude extract of *Curculigo orchioides*