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A Coelomic Fluid of *Allolobophora caliginosa* as Novel Prospects for Medicinal Antioxidants, Anti-inflammatory, Antiproliferative, Analgesics, and Antipyretics

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Abstract: *Allolobophora caliginosa*, an indigenous earthworm of Egypt, has been widely utilized in the pharmaceutical industry because it has many therapeutic properties. These therapeutic properties may be attributed to the earthworm's coelomic fluid being a promising reservoir of bioactive secondary metabolites. Hence, the current study was intended to identify the bioactive constituents and the biological activities of *Allolobophora caliginosa* coelomic fluid (ACCF). The active constituents, total protein content, and amino acid profile of ACCF were identified using high-performance liquid chromatography, the Biuret method, and an amino acid analyzer. The antioxidant, anti-inflammatory, and cytotoxicity activities of ACCF were evaluated using *in vitro* assays. The analgesic effect was evaluated using the writhing and hot plate assays. The antipyretic potency was determined using Brewer's yeast-induced pyrexia assay. Various active phenolic and flavonoid constituents and a considerable amount of essential and non-essential amino acids, were found in ACCF. The current study clarified that ACCF exhibited dose-dependent radical scavenging and potent anti-inflammatory activities. Furthermore, the proliferation of HepG2 could be inhibited by ACCF with the IC50 value of 145.99 µg/ml. The present findings revealed that ACCF showed amazing peripherally and centrally analgesic and antipyretic potencies. ACCF has various secondary metabolites that were likely to act synergistically to produce the observed biological activities. Thus, it can be served as a promising alternative natural compound for the prophylaxis or treatment of infectious disorders.

Keywords: *Allolobophora caliginosa*; coelomic fluid; antioxidant; anti-inflammatory; cytotoxicity; analgesic

1. Introduction

Earthworms are famous eco-friendly terrestrial invertebrates that play a critical role in the soil ecosystem and plant growth medium [1]. Besides their ability to improve soil fertility, earthworms are gaining prominence as a potential source of novel therapeutic candidate compounds and nutraceutical resources because of the availability of various bioactive secondary metabolites possessing interesting biological activities [2, 3]. Also, earthworm metabolites are characterized by their stability and potency, making the earthworm a promising source for discovering new pharmaceuticals used for remedial medicine [4]. Over the world, diverse therapeutic molecules have been isolated and characterized, such as fibrinolytic, antiviral, antitumor, hepatoprotective, cytotoxic, and antioxidant from different species of

earthworms [5, 6]. Such potent bioactive molecules naturally reside mostly in intestinal fluids and subsequently in the tissue fluid of earthworms. So, earthworms' coelomic fluid is a promising reservoir of bioactive molecules possessing amazing biological properties that can be utilized as an antibacterial, anti-inflammatory, analgesic, and anticancer remedy [7, 8].

Naturally, earthworms are provided with strong innate immune responses against their predators via the secretion of their coelomic fluid. Coelomic fluid is a yellow-filled space between earthworms' body wall and gut that exudes rough handling and stress. Earthworms' coelomic fluid comprises specific coelomic cells called coelomocytes that play a significant role in defense reactions and immune responses against the invasion of microorganisms via destroying membranes of foreign cells [9]. Furthermore, coelomic fluid possesses plenty of bioactive compounds, which demonstrate a diversity of biological activities involving bacteriostatic, proteolytic, antifungal, and many other activities [10, 11]. Previous reports demonstrated that earthworms' coelomic fluid contains biologically several active proteins and peptide molecules that can inhibit the proliferation of cancer cells [10, 12]. Thus, more and more researchers' attention has been focused on investigating the different active constituents and biological activities of earthworm coelomic fluid to understand the pharmacological potential of earthworms in medicine. Among the variety of earthworms, *Allolobophora caliginosa* is one of the abundant earthworm species in Egypt that has amazing potency as an antioxidant, anti-inflammatory, and antipyretic [13]. However, the active secondary metabolites and the biological activities of *Allolobophora caliginosa* coelomic fluid (ACCF), such as anti-inflammatory, analgesic and antipyretic, and antitumor potency, have not been affected been adequately studied. Therefore, the current study was intended to identify the bioactive constituents and the nutritional value of ACCF. Also, the current study was designed to assess the antioxidant, anti-inflammatory, and antitumor activities of ACCF using *in vitro* methods and evaluate its analgesic and antipyretic potencies.

2. Materials and Methods

2.1. Allolobophora caliginosa collection.

Living earthworms, *Allolobophora caliginosa*, were collected from a commercial vermin culture at Giza Governorate and maintained in a plastic box containing decomposed organic matter till transported to the laboratory. Before collecting coelomic fluid, the healthy earthworms were washed with running tap water followed by phosphate buffer saline (0.01 M, pH 6.5) to remove any undesirable contents attached to the body surface and then placed on moist filter paper for 24 h. This permitted emptying their gut to avoid contamination during the coelomic fluid extraction.

2.2. Allolobophora caliginosa coelomic fluid (ACCF) extraction.

Coelomic fluid was extracted based on the heat shock method described by Dinesh *et al*. [14]. By using the heat shock method, ACCF can be directly collected from the body cavity of earthworms without causing any harm to them. Firstly, three to four healthy earthworms, each weighing 0.8-1.2 g, were placed in a sterile Petri dish and subjected to heat shock using hot water (45-50 ^{o}C) in a glass beaker. Then, due to heat shock drips, the coelomic fluid was extruded via the dorsal epidermal pore into the media and collected at the lower side of the Petri plate. The extruded fluid was collected in Falcon tubes using a sterilized pipette with a fine nozzle. Then, extruded coelomic fluid was centrifuged at 4000 RPM/ min for 30 min at 4

ºC. Finally, the resultant supernatant was divided into two parts, one part was used for ACCF protein concentration determination, while the second part was concentrated and dried using a lyophilizer (EDWARDS, Italy) for HPLC analysis and biological activities determination.

2.3. Biochemical characterization of ACCF.

2.3.1. Identification of bioactive constituents of ACCF.

The secondary metabolites of ACCF were determined by high-performance liquid chromatography (HPLC) on a 4.6 × 100 mm aKihetex C18 column (Millipore) connected to Agilent 1260 HPLC system. Firstly, 20 µl of the ACCF sample was injected with the flow rate of 0.5 ml/min into the HPLC system containing an isocratic solvent system that comprised phosphate buffer solution (pH 6.5) and acetonitrile in the ratio of 55:45. Then, the retention time was adjusted to 30 min. At wavelength 284 nm, the isolated peaks of secondary metabolites in the ACCF sample were identified by comparing their relative retention times with those of standards. Then, the concentration of each compound was calculated as peak area integration.

2.3.2. Assessment of ACCF total protein content.

The crude ACCF filtrate was further processed by a series of precipitation steps according to the method adopted by Endharti *et al*. [7] to determine total protein content. Firstly, ACCF was precipitated by adding 2 mM ammonium sulfate slowly, and then the precipitated proteins were collected by centrifugation at 15000 RPM/min for 10 min. The resultant pellet was resuspended in 20 mM Tris HCl (pH 8.0), followed by centrifugation. Thereafter, the pellet was resuspended in cold acetone and incubated for 20 min. Following incubation, the remaining acetone was removed by centrifugation. The total protein concentration of ACCF was measured using the Biuret method.

2.3.3. Amino acid analysis of ACCF.

The amino acid composition of ACCF was evaluated as Csomós, and Simon-Sarkadi [15] described using the Automatic Amino Acid Analyzer (AAA 4INGOsNGOS Ltd.). 100 mg of ACCF was hydrolyzed with 10 ml of 6N HCl in a sealed tube at 110ºC in an oven for 24 h. Then, the acid was evaporated at 80ºC under vacuum with continuous addition of distilled water until dryness. The free HCl residue was dissolved in 2 ml of loading buffer (6.2 M, pH 2.2).

2.4. In vitro preliminary studies.

2.4.1. Evaluation of antioxidant potency of ACCF.

2.4.1.1. Free radical scavenging activity of ACCF.

The free radical scavenging potency of ACCF was assessed by DPPH (1,1- diphenyl-2-picrylhydrazyl) radical assay described by Brand-Williams *et al*. [16]. Briefly, various concentrations (50- 500µg/ml methanol) of ACCF and ascorbic acid as standard antioxidants were prepared. Then, DPPH was mixed with methanol to obtain a 0.1 mM solution and stored in the dark. A 2 ml of ACCF or ascorbic acid at different concentrations was added to a tube containing 2ml DPPH and shaken vigorously. All tubes were incubated in the dark at 37°C for

30 min before measuring the absorbance. The control tube (DPPH only) was prepared in the same manner. The absorbance of each solution was measured at λ = 517 nm against methanol as blank. The radical scavenging activity (%) was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{sample or standard}})/ A_{\text{control}}] \times 100$$

where A_{control} was the absorbance of the control and A_{sample} was the absorbance of the sample under the same conditions.

2.4.1.2. Total antioxidant capacity of ACCF.

The total antioxidant capacity of the ACCF was evaluated by the phosphomolybdenum assay according to Prieto *et al*. [17]. Concisely, 0.1 ml of ACCF at different concentrations (1.25-10 mg/ml DMSO) was mixed with 1 ml of molybdate reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Then, the tubes were incubated at 95 ºC for 90 min. After incubation, the tubes were cooled, and the absorbance of the reaction mixture at different concentrations of the sample was measured at 695 nm using a UV-VIS spectrophotometer against blank (0.1 ml DMSO and 1 ml of molybdate reagent solution). The total antioxidant activity is expressed as the number of grams equivalent to ascorbic acid.

2.4.2. Evaluation of *in vitro* anti-inflammatory potency of ACCF.

To evaluate *in vitro* anti-inflammatory potency of ACCF, the human red blood cell (HRBCs) membrane stabilization technique was incorporated according to Shinde *et al*. [18]. First, the blood was collected from a healthy volunteer in a heparinized tube and then centrifuged at 3000 rpm for 10 min. The resulting packed red blood cells were washed three times with an equal volume of saline; then, a 10% HRBC suspension was reconstituted with normal saline by measuring the volume of packed red blood cells. To 1 ml of HRBC suspension, an equal volume of ACCF or Aspirin (standard anti-inflammatory drug) was added at different concentrations (50– 400 µg/ml). All the reaction mixtures were incubated at 56 ºC for 30 min and then centrifuged at 2500 rpm for 5 min. The hemoglobin content in the supernatant solution was estimated using a spectrophotometer at 560 nm. The control sample consisted of 1ml HRBC suspension mixed with normal saline solution and tested similarly. The percentage of HRBCs membrane stabilization was calculated using the following equation:

$$\%\text{HRBCs membrane stabilization} = 100 - [(A_{\text{sample or standard}}/A_{\text{control}}) \times 100]$$

Then, half-maximal inhibitory concentration (IC_{50}) was calculated based on an equation from the plot graph between the percentage of HRBCs membrane stabilization and the various sample concentrations.

2.4.3. Evaluation of *in vitro* antiproliferative activity of ACCF.

The *in vitro* antitumor potential of ACCF was assessed using Sulphorhodamine-B (SRB) assay against human hepatoma cell line (HepG-2) [19]. Briefly, a total of 4×10^3 cells/well, suspended in fresh growth medium (200 µl), were seeded in a 96-well microliter plate. Then, the plate was incubated for 24 h to allow cell adhesion. Next, the cells were treated for 48 h with different concentrations of ACCF or doxorubicin as standard

antitumor agent (0–200µg/ml). Meanwhile, the control cells were treated with only 0.1% DMSO in the control group. After 48 h, the cells were fixed with ice-cold trichloroacetic acid (50 µl/ well, 10% w/v) for 1h at 4 ºC. Then, the plate was washed with distilled H_2O, stained with 50 µl SRB dye (0.2 % in 1% aqueous acetic acid), and kept in the dark at room temperature for 30 min. Following staining, the plates were quickly rinsed with 1% acetic acid to remove the unbound dye and then air-dried. The bounded dye was solubilized in a 10M tris base (pH 10.5), and the optical density of each well was measured spectrophotometrically at 570 nm with an enzyme-linked immunosorbent assay (ELISA) microplate reader (Sunrise Tecan reader, Germany). The control group was the cells that were not treated with ACCF or doxorubicin but with otherwise identical conditions. The percentage of proliferation inhibition was calculated as follows:

% Proliferation inhibition= [O.D. (treated cells)/ O.D. (control cells)] x 100.

Furthermore, the half-maximal inhibition concentration (IC50) was calculated based on an equation from the plot graph between the percentage of cell survival and the various sample concentrations.

2.5. In vivo studies.

2.5.1. Experimental animals.

Adult male Swiss male albino mice (*Mus musculus*) weighing 20–25 g were used in the analgesic and antipyretic studies. Animals were purchased from the National Research Center (NRC), Egypt. They were grouped and housed in spacious polypropylene cages (five animals/cage) bedded with sawdust and nesting (Kleenex tissues) material in a well-ventilated animal house at a temperature of 23 ± 2°C within a natural day/night cycle. They nourished standard chow pellets and drinking water ad libitum. Before the commencement of this study, animals were acclimatized to the animal house conditions for 7 days. All experimental procedures described were approved by the Cairo University Institutional Animal Care and Use Committee (IACUC) (Egypt) (CU/I/F/6/19). All the experimental procedures were carried out in agreement with international guidelines for the care and use of laboratory animals.

2.5.2. Animals grouping and dosing.

Swiss albino mice were randomly divided into four groups (5 animals/group) as follows: Group I was assigned as negative control and received a vehicle. Group II was served as positive control and treated with standard drugs (Sodium diclofenac or aspirin). Groups III and VI were treated with ACCF at 10 and 20 mg/kg body weight, respectively. ACCF doses were selected based on the acute toxicity study done previously [20].

2.5.3. Evaluation of analgesic potency of ACCF.

In the current assay, two different methods were performed for assessing the potential peripheral analgesic (acetic acid-induced writhing test) and central analgesic (hot plate latency assay) potency of ACCF.

2.5.3.1. Assessment of peripheral analgesic potency of ACCF.

The acetic acid-induced writhing assay was carried out to evaluate the peripheral analgesic activity of ACCF in comparison with sodium diclofenac as a standard analgesic drug according to the method previously described by Hijazi *et al*. [21]. Briefly, mice were treated with vehicle, sodium diclofenac (20 mg/kg body weight, i.p) and ACCF at two doses (10 and 20 mg/kg body weight, orally) 30 min before intraperitoneal injection of 0.1 ml of 1% acetic acid solution to induce a writhing response. After 5 min of acetic acid injection, the mice were then placed in an observation box, and the number of writhing movements (full extension of the hind limb) for each group was recorded for 15 min (Figure 1a). The inhibition percentage of writhing frequency of treated groups as an index of analgesia was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(Wc - Wt)}{Wc} \times 100$$

where Wc = Number of writhing in the control group; Wt = Number of writhing in treated groups.

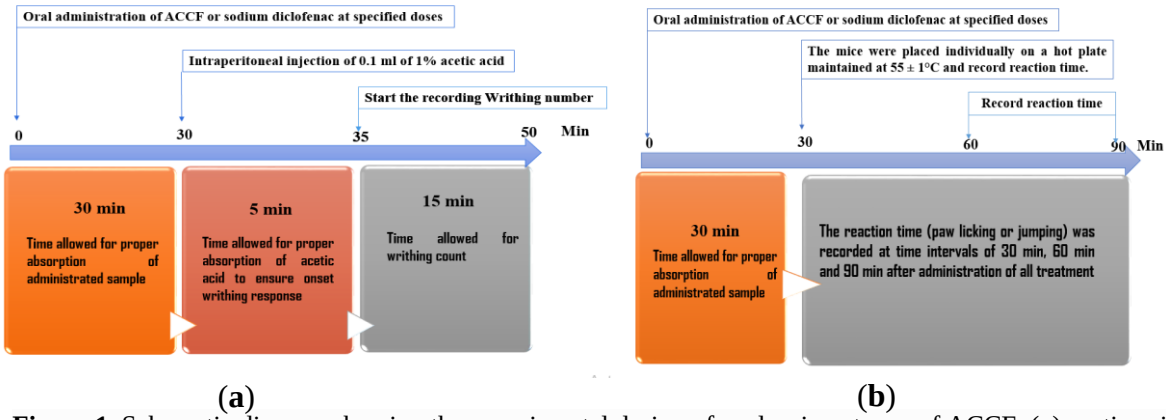

Figure 1. Schematic diagram showing the experimental design of analgesic potency of ACCF. (**a**) acetic acid-induced writhing response; (**b**) Hot plate latency assay.

2.5.3.2. Assessment of central analgesic potency of ACCF.

The central analgesic potency of ACCF was evaluated by Eddy's hot plate method. Mice were treated with vehicle (distilled H_2O, orally), sodium diclofenac (20 mg/kg body weight, i.p.), and ACCF (10 and 20 mg/kg body weight, orally). After 30 min of administration, the mice were placed individually on a hot plate maintained at 55 ± 1°C to induce pain stimulus. The reaction time (paw licking or jumping) was recorded for each mouse at time intervals of 30 min, 60 min, and 90 min after administration of all treatments, with a cutoff time of 15 sec to prevent tissue damage (Figure 1b).

2.5.4. Evaluation of antipyretic potency of ACCF.

The antipyretic potency of ACCF was determined by using Brewer's yeast-induced pyrexia assay, according to Brune *et al*. [22]. Briefly, Swiss albino mice were randomly divided into four groups (5 mice/group) as described previously and fasted overnight with free water access. Each mouse's initial basal rectal temperature was measured using a well-lubricated digital thermometer (RossmaxTG380, Taiwan, China) by inserting a thermistor probe about 3 cm into the rectum. Hyperthermia was induced in all mice by subcutaneous injection of 20 ml/kg body weight of 20% Brewer's yeast suspension into the back near the nape of the neck. The rectal temperature was recorded using a thermometer immediately before and 18 h after Brewer's yeast injection. After 18 h of yeast injection, mice were

treated with vehicle (distilled H_2O, orally), aspirin (200 mg/kg body weight, orally), and ACCF (10 and 20 mg/kg body weight, orally). Finally, the rectal temperature of each mouse was periodically measured at 30, 60, and 90 min after the administration of various treatments (Figure 2). Percentage reduction in rectal temperature was calculated using the following equation:

$$\% \text{ Reduction of pyrexia} = \frac{(B - Cn)}{B - A} \times 100$$

where A is the normal temperature, B is the rectal temperature after 18 h of yeast injection, and Cn is the rectal temperature after 30, 60, and 90 min.

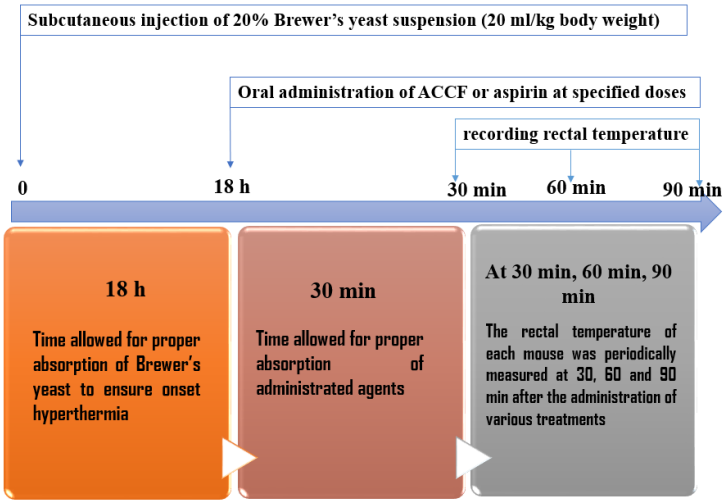

Figure 2. Schematic diagram showing the experimental design of antipyretic potency of ACCF.

2.6. Statistical analysis.

All results were expressed as means ± SEM (n= 5) for analgesic and antipyretic potencies. For variances evaluation between the groups, one-way analysis of variance (ANOVA) followed by the Duncan post hoc test was used to compare between two group means using IBM SPSS statistic 22 software (SPSS Inc., Chicago, IL, USA) software. Values of $P < 0.05$ were regarded as statistically significant.

3. Results and Discussion

3.1. Biochemical characterization of ACCF.

3.1.1. Bioactive constituents of ACCF using HPLC.

Identifying the bioactive compounds of natural products is the foremost issue in the drug discovery process [23]. Based on HPLC results, Figure 3 demonstrates that ACCF has various bioactive constituents as confirmed by the chromatogram obtained at different retention times (3.39, 5.47, 7.96, 9.02, 10.68, 12.98, 13.96, 15.11, 16.61, 18.22, 20.10, 21.33). The obtained HPLC chromatogram demonstrated a total of twelve active constituents found in ACCF as shown in Table 1. Furthermore, gallic acid as a phenolic compound represented the highest concentration, where its percentage was found to be 69.85% of total bioactive constituents in ACCF. Meanwhile, syringic acid signified as the minor phenolic compound in ACCF with a percentage of 0.10% when compared with other phenolic compounds such as p-hydroxybenzoic acid, *o*- coumaric acid, benzoic acid, cinnamic acid, *p*- coumaric acid and ferulic acid (6.10%, 1.35%, 0.44%, 0.26%, 0.22% and 0.16%, respectively), revealing its

therapeutic importance (Table 1). The phenolic profile of ACCF was similar to those found in previous studies on the coelomic fluid of different earthworm species and other invertebrates [2 , 25], respecting that there are no previous reports correlating the phenolic profile of ACCF. Naspolini *et al*. [26] demonstrated that phenolic acids, especially gallic, *p*-coumaric, and ferulic acids, are secondary aromatic natural products that possess diverse physiological and pharmacological properties. Remarkably, gallic acid is the most abundant phenolic compound in ACCF, which have various amazing biological properties, including antioxidant, anti-inflammatory, and antineoplastic activities as well; as it can be utilized as a natural remedy for the treatment of various diseases such as gastrointestinal, neuropsychological, metabolic, and cardiovascular diseases [27, 28]. Likewise, *p*-hydroxybenzoic, *p*-coumaric, and ferulic acids are also found in ACCF, which have various biological benefits, especially their ability to inhibit tumor growth as a fibroblast growth factor inhibitor providing chemoprevention of cancer [29, 30]. Besides phenolic compounds, the present HPLC findings showed the predominance of various flavons such as rutin represented the highest concentration (16.98%), followed by catechol, catechin, and quercetin (4.53%, 0.33%, and 0.21%, respectively) as shown in Table 1. Overall, the present data corroborate some previous reports on the chemical variability of the flavonoids profile in other invertebrate species [31, 32]. As previous studies reported, rutin, a glycoside of the flavonoid quercetin, has extraordinary biological activities, especially anti-inflammatory and anti-carcinogenic activities [33, 34]. Furthermore, quercetin is a potent antioxidant and has other biological activities such as anti-inflammatory and analgesic effects [35, 36]. Finally, the identifying phenolic and flavonoids prospect of ACCF suggests its therapeutic significance due to diverse secondary metabolites of ACCF with different biological activities that can be utilized as pharmaceuticals, which could permit future to relate the studied biological activities with the presence of secondary metabolites.

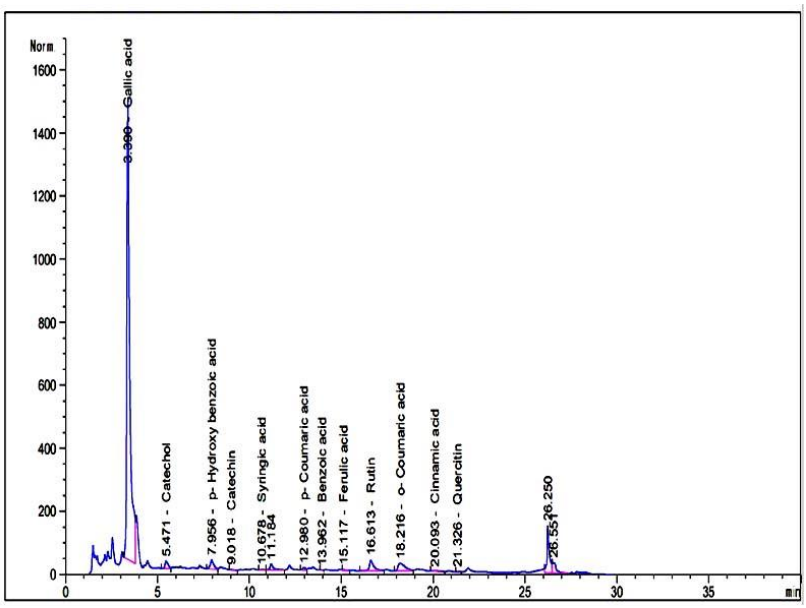

Figure 3. HPLC chromatogram of phenolic and flavonoid constituents of ACCF detected at 284nm.

Table 1. Different bioactive constituents of ACCF.

Sample	Bioactive constituents	Concentration (mg/kg dry powder)	Percentage (%)
	Gallic acid	1904.37	69.85
	Rutin	463.06	16.98
	p-Hydroxy-benzoic acid	166.36	6.10
	Catechol	123.44	4.53
	o- Coumaric acid	36.79	1.35
	Benzoic acid	11.90	0.44

Sample	Bioactive constituents	Concentration (mg/kg dry powder)	Percentage (%)
ACCF	Catechin	9.00	0.33
	Cinnamic acid	7.10	0.26
	p- Coumaric acid	5.88	0.22
	Quercetin	5.61	0.21
	Ferulic acid	4.48	0.16
	Syringic acid	2.80	0.10

3.1.2. Total protein content and amino acid profile of ACCF.

The nutritional inquiry of indigenous natural products provides their therapeutic potential in the food and pharmaceutical industries. Considering the high nutrient value of earthworms, the current study demonstrated that ACCF had an appreciable amount of protein where the total protein content of ACCF was found to be 496.8 mg/dl, suggesting its amazing potential as an economical alternative source of proteins for humans and animals, confirming its nutritional value (Table 2). This finding is confirmed by amino acid analysis of ACCF, which revealed the presence of both essential and non-essential amino acids (Table 2). Table 2 demonstrates that non-essential amino acid content is higher than comparable essential amino acids in ACCF, where the non-essential amino acids constitute 54.70 % of the total amino acids while the essential amino acids constitute 45.30% of the total amino acids in ACCF. Furthermore, Table 3 demonstrates that leucine is an essential amino acid and represents the highest concentration, followed by lysine and isoleucine in ACCF. Alternatively, the non-essential glutamic acid disclosed the highest concentration comparable to alanine as well as aspartic acid in ACCF. Overall, the present study demonstrated that ACCF serves as a source of non-essential amino acids and essential amino acids, especially lysine, limiting in many basic foodstuffs, suggesting its high nutritional value. The amino acid composition of ACCF with the dominance of two essential amino acids, namely leucine and lysine, and non-essential amino acid, namely tyrosine, was consistent with the previous result from the earthworm, *Perionyx excavatus*, coelomic fluid [37].

Furthermore, this finding is supported by the amino acid profile of *Allolobophora caliginosa* extract, where protein/amino acid contents are dominated by acidic amino acids such as aspartic and glutamic acids, followed by neutral amino acids such as glycine [13]. Interestingly, amino acids are the main precursors for synthesizing secondary metabolites such as alkaloids, which provide chemical defense for coelomic fluid that confer valuable physiological potencies in consumers [38]. Moreover, amino acids have diverse potential biological activities within the body, including antioxidant, antineoplastic, and anti-inflammatory, which provide specific health benefits. Regarding antioxidant amino acids, the hydrophobic amino acids group, including alanine, valine, leucine, and isoleucine, can inhibit free radicals by proton donation and exhibit a high antioxidant potency in natural products [39]. Additionally, glutamic acid and glycine are precursors of glutathione, which leads to an increase in the antioxidant capacity of ACCF [40].

Table 2. Total protein content and total amino acid profile of ACCF.

Sample	Total protein (mg/dl)	Total amino acids	
		Essential amino acids (%)	Non-essential amino acids (%)
ACCF	496.8	45.30	54.70

Hence, the obtained amino acid profile of ACCF in the current study is recommended that ACCF contribute significantly to the nutrient requirement of humans and represent good

candidates for a practicable, sustainable, and ameliorative intervention strategy for the endemic protein source in nutrition.

Table 3. Essential and non-essential amino acid content of ACCF.

Essential amino acids	Essential amino acids concentration (mg/g)	Non-essential amino acids	Non- essential amino acids concentration (mg/g)
Leucine	49.20	Glutamic acid	68.00
Lysine	26.70	Alanine	51.40
Isoleucine	26.00	Aspartic acid	50.20
Arginine	20.10	Glycine	47.10
Histidine	15.00	Serine	20.14
Valine	37.00	Tyrosine	9.00
Threonine	20.00		
Methionine	7.30		
Phenylalanine	2.20		

3.2. The antioxidant potency of ACCF.

Identifying biological activities of natural products is societal demand and increasing interest to characterize their health-promoting properties. Hence, the ongoing investigation tended to evaluate various bioactivities of ACCF. Oxidative stress is considered to be substantial in the initiation and development of chronic degenerative diseases. Figure 4a reveals that ACCF has a strong antioxidant potency as it could scavenge free radical (DPPH) to some extent comparable to ascorbic acid (standard antioxidant agent). Also, it was noticed that the free radical scavenging activity of ACCF was positively correlated to the concentration of ACCF as it increased with an increase in its concentration. ACCF was able to scavenge more than 80% of the DPPH radical at a concentration of 50 µg/ml, whereas the standard ascorbic scavenged 92.53% of the DPPH at the same concentration.

Furthermore, ACCF exhibited highly free radical scavenging potency with a high percentage of 90.12% at a high concentration, approximately as ascorbic acid that scavenged DPPH by 95.24% at the same concentration (Figure 4 a). Concerning total antioxidant capacity, the present study revealed that ACCF possesses a considerable amount of antioxidants at various concentrations, as evidenced by ACCF possessing significant total antioxidant capacity equivalent to 1931.81mg/g ascorbic acid at low concentration (1.25 mg/ml) as shown in Figure 4b. At high concentrations, the total antioxidant capacity of ACCF was found to be 2370.45 mg/g of ascorbic acid. This indicates that the total antioxidant capacity of ACCF was concentration-dependent as it increased with an increase in its concentration (Figure 4b). This result was consistent with the highest DPPH scavenging potency of ACCF. These findings may be revealed the ability of ACCF to inhibit the autoxidation of lipids and, consequently, its valuable ability to treat various chronic diseases where lipid peroxidation is a principal mechanism for their pathogenesis. The antioxidant efficacy of ACCF was reliable to the previous report, which confirmed the antioxidant potency of coelomic fluid from different earthworm species (*Eisenia fetida*) [24]. The high antioxidant potency of ACCF is extremely related to its phenolic and flavonoid compounds, particularly gallic acid and quercetin, which are found in great quantities in ACCF, as Badhani *et al*. [41] suggested. Gallic acid is characterized by reducing properties that can absorb and neutralize free radicals by donating hydrogen atoms to free radicals and reducing the reactive oxygen species [42].

Furthermore, Dehghan *et al*. reported that quercetin is a potent antioxidant due to the presence of considerable hydroxyl groups, which can donate hydrogen atoms to free radicals and neutralize them [43]. Besides the phenolic and flavonoids compound, the potent

antioxidant potency of ACCF may be attributed to its hydrophobic amino acid group, especially alanine, valine, leucine, and isoleucine which can inhibit the serious effect of free radicals by proton donation, so these amino acids are responsible for a high antioxidant potency of natural products ads Sonklin *et al*. [39] postulated. Thus, ACCF is a good source of antioxidants, and it might be helpful for the treatment of diseases associated with oxidative stress, such as cancer.

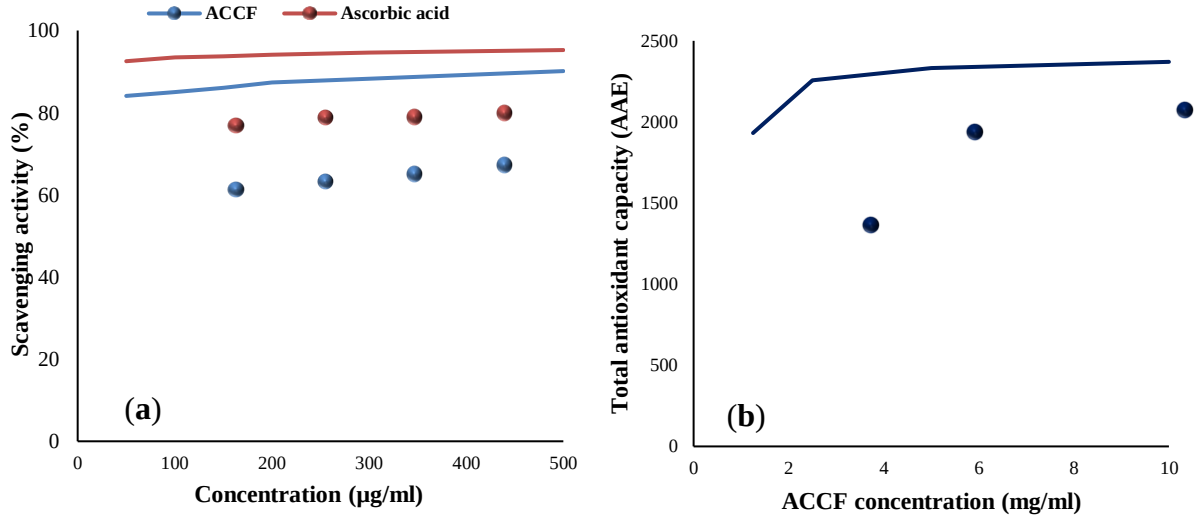

Figure 4. The antioxidant potency of ACCF. (**a**) DPPH radical scavenging activity of ACCF; (**b**) Total antioxidant capacity of ACCF.

3.3. Anti-inflammatory potency of ACCF.

Inflammation is a self-defensive response of vascular tissues to harmful stimuli that are frequently associated with pain and involve various critical circumstances, including membrane alteration and the increase of vascular permeability and protein denaturation. In the present study, ACCF efficiently stabilized HRBCs membrane against hemolysis induced by heat comparable to aspirin as a standard anti-inflammatory drug in a concentration-dependent manner, indicating a promising anti-inflammatory potency of ACCF. As illustrated in Figure 5, ACCF could effectively stabilize the RBCs membrane with maximum stabilization of 51% at 400 µg/ml, whereas the standard aspirin stabilized only 40.10% at the same concentration. Furthermore, the IC_{50} of ACCF and aspirin were found to be 376.76 and 1128.00 µg/ml, respectively, suggesting that ACCF could be utilized as a potent anti-inflammatory agent (Table 4). The current findings revealed that ACCF exhibited amazing anti-inflammatory potency, and this was confirmed by its ability to resist the lysis of HRBC membrane comparable to the standard anti-inflammatory drug (aspirin), proving its anti-inflammatory potency. The present study suggested that ACCF stabilizes the HRBC membrane via binding with the HRBC membrane and subsequently alters the surface charge of the cells, which may be prevented physical interaction with aggregating agents or promote dispersion by related repulsion of identical charges. This postulation is supported by Ruiz-Ruiz *et al*. [44]. The present study attributed the anti-inflammatory potency of ACCF to its phenolic and flavonoid compounds, especially gallic acid and quercetin, which either could inhibit the activity of cyclooxygenase (COX) and lipoxygenase or prevent the release of lysosomal constituents by stabilizing the lysosomal membrane as previous studies recorded [45, 46]. Furthermore, the obtained stabilizing effect of ACCF may be due to its rutin compound, which is known as a potent anti-inflammatory agent [47]. Also, the dominance of amino acids in ACCF, especially glycine and histidine, exhibited an anti-inflammatory effect which helps in stabilizing the

HRBC membrane by ACCF [48]. Interestingly, the *in vitro* anti-inflammatory activity exhibited by the ACCF is consistent with its antioxidant potential. Previous findings demonstrated that natural extracts with antioxidant activity also display remarkable membrane stabilization properties concentration-dependent [49].

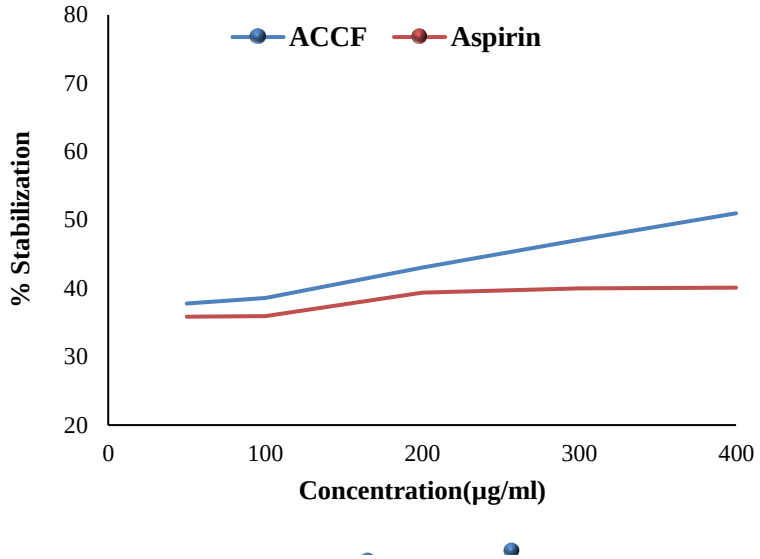

Figure 5. Stabilization percentage of HRBC membrane by ACCF and Aspirin.

Table 4. Half maximal inhibitory concentration (IC_{50}) values of anti-inflammatory and antiproliferative potencies.

Sample	IC_{50} value (µg/ml)	
	Anti-inflammatory potency	Antiproliferative potency
ACCF	376.76	145.99
Aspirin	1128.00	----
Doxorubicin	----	31.25

3.4. Antiproliferative potency of ACCF.

The current finding revealed that ACCF exhibited a promising antiproliferative potency on HepG-2 cell line comparable to doxorubicin (standard anticancer drug) due to its ability to inhibit the growth of HepG-2 cell line in a dose-dependent manner as indicated by reducing the cell survival with the increase in a dose ranging from 25–200 µg/ml (Figure 6 a). Additionally, ACCF was more effective than the standard doxorubicin by reducing cell survival up to 41.20% at a 200 µg/ml concentration, whereas doxorubicin inhibited only 54.50% of HepG-2 at the same concentration (Figure 6 a&b). Furthermore, the IC_{50} values revealed that ACCF could be the most promising antitumor remedy, with an IC_{50} value of 145.99 µg/ml (Table 4). The current result conforms with the results obtained by Fiołka *et al*. [50], who confirmed the antitumor activity of the coelomic fluid from the earthworm *Dendrobaena veneta* against A549 human lung cancer cells. Furthermore, Fiołka *et al*. [50] demonstrated that coelomic fluid exerted its cytotoxic effect via apoptosis and subsequently induced the death of cancer cells.

Interestingly, the antineoplastic activity of ACCF is attributed to its antioxidant and anti-inflammatory activities, where oxidative stress and inflammation processes are related to cancer development. So, the ability to prevent any of the processes will certainly lead to the inhibition of the others. Moreover, the current study suggested that the strong antitumor potency of ACCF may be due to its numerous phenolic and flavonoid compounds especially gallic acid, quercetin, and rutin, that exerted anticancer activity on different cancer cell lines

such as human hepatoma HepG2 and human carcinoma HeLa cells [51]. Besides the phenolic and flavonoid compounds, the potential antitumor potency of ACCF is related to its non-essential amino acid, L-glutamic acid, which exerts a cytotoxic effect on malignant tumors Budniak *et al*. [52] reported.

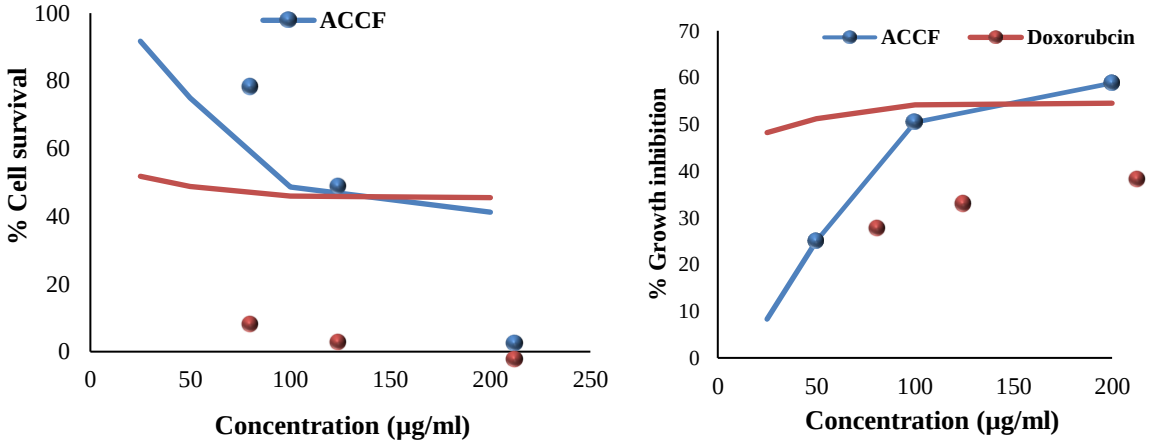

Figure 6. Cytotoxicity potency of ACCF on hepatoma cell line (HepG-2) using SRB assay. (**a**) Cell survival percentage of HepG-2 in the ACCF and doxorubicin using SRB assay; (**b**) Growth inhibition percentage of HepG-2 in the ACCF and doxorubicin using SRB assay.

3.5. Analgesic potency of ACCF.

Pain is a defensive response against dysfunction of an organ or imbalance in its function, which ultimately leads to actual or potential tissue damage [53]. The analgesic potency of ACCF was evaluated using two different assays that could act on either the peripheral nervous system or central nervous system [54]. Interestingly, the experimental evidence obtained from the current study strongly suggests that ACCF possesses peripherally and centrally mediated analgesic properties.

3.5.1. Peripheral analgesic potency (acetic acid-induced writhing response).

Regarding peripheral analgesic property, acetic acid injection-induced peripheral pain by impelling capillary permeability and activating the synthesis of prostaglandins and leukotrienes that induce abdominal constrictions and enhance inflammatory pain in the peritoneal cavity via the activation of peripheral nociceptors [55]. The current finding demonstrated that ACCF exhibits peripherally analgesic potency comparable to that produced by diclofenac, indicating inhibition of writhe response in the mice. Table 5 demonstrates that the writhing number of mice treated either with ACCF at 10 and 20 mg/kg body weight or the standard sodium diclofenac (20 mg/kg body weight) significantly ($P< 0.05$) decreased compared to that of control mice. Furthermore, ACCF treatment at 20 mg/kg body weight exhibited higher peripheral analgesic potency with a maximum inhibition percentage of 67.42% in the writhing reflex comparable to that of the standard sodium diclofenac with an inhibition percentage of 56.82%. Previous studies demonstrated that conventionally used therapeutic drugs such as sodium diclofenac could inhibit the peripheral pain mediated by acetic acid via inhibition of synthesis of prostaglandins and other inflammatory mediators or inhibition of pain responses mediated by nociceptors peripherally [56]. Therefore, perhaps the

peripherally acting analgesic effect of ACCF, similarly to sodium diclofenac, reduces the pain perception in nociceptors by inhibiting the production of prostaglandins.

Table 5. Peripheral analgesic potency of ACCF on acetic acid-induced writhing response.

Experimental groups	Peripheral analgesic potency (Acetic acid-induced writhing)	
	No of writhing	% Inhibition
Control	24.25± 0.66^{a}	---
$ACCF_{10}$	11.00± 0.32^{b}	54.63
$ACCF_{20}$	7.90± 0.84^{c}	67.42
Sodium diclofenac	10.47± 0.81^{b}	56.82

Values are mean ± SEM (n= 5). Values with different superscript letters are significantly different ($P < 0.05$). $ACCF_{10}$: *Allolobophora caliginosa* coelomic fluid (10 mg/kg bodyweight); $ACCF_{20}$: *Allolobophora caliginosa* coelomic fluid (20 mg/kg bodyweight).

3.5.2. Central analgesic potency (hot plate latency).

ACCF treatment at the two selected doses (10 and 20 mg/kg body weight) exhibited a significant ($P < 0.05$) decline in latency time for the thermal stimulus at 30 min, 60 min, and 90 min when compared to the control group (Table 6). Also, the standard analgesic drug (sodium diclofenac) showed a significant ($P < 0.05$) decrease in latency time at all observation times as compared to the control group. Interestingly, ACCF treatment at 20 mg/kg body weight exhibited higher central analgesic potency with a maximum inhibition percentage of 61.83% at 90 min, comparable to that of the standard sodium diclofenac with an inhibition percentage of 28.75% at the same observation time (Table 6). In the current study, ACCF significantly reduced the pain induced by thermal stimulus. This finding potentially suggested that ACCF possesses the potential centrally acting analgesic activity, and the probable mechanism may be the blockage of opioid receptors at the spinal cord level. Additionally, ACCF exerts a central anti-nociceptive effect via a reduction in Ca^{2+} influx at the axon termini of the afferent nerves and, consequently, may induce a decrease in adenylate cyclase activity as Lee *et al*. [57] suggested. The reduction in adenylate cyclase activity may be leading to a decrease in the levels of cyclic AMP and K^{+} efflux and result in nerve hyperpolarization and an apparent anti-nociceptive effect [57]. These results concur with other research studies on evaluating the analgesic activity of coelomic fluid from different earthworm species [58]. The centrally or peripheral mediated analgesic property of ACCF could be partly attributed to its anti-inflammatory activity.

Table 6. Central analgesic potency of ACCF on hot plate latency.

Experimental groups	Central analgesic potency Latency time (second)			% Inhibition at 90 min
	30 min	60 min	90 min	
Control	6.67± 0.29^{a}	5.60± 0.19^{a}	3.93± 0.34^{a}	---
$ACCF_{10}$	4.75± 0.34^{b}	2.63± 0.24^{b}	2.43± 0.36^{bc}	38.17
$ACCF_{20}$	3.10± 0.28^{c}	2.10± 0.19^{b}	1.50± 0.16^{c}	61.83
Sodium diclofenac	5.40± 0.19^{b}	3.73± 0.19^{c}	2.80± 0.37^{b}	28.75

Values are mean ± SEM (n= 5). Values with different superscript letters are significantly different ($P < 0.05$). $ACCF_{10}$: *Allolobophora caliginosa* coelomic fluid (10 mg/kg bodyweight); $ACCF_{20}$: *Allolobophora caliginosa* coelomic fluid (20 mg/kg bodyweight).

The inhibition of the acute inflammation by ACCF leads to its inhibitory impact on pain development [59]. Also, pain is accompanied by ROS generation during the release of prostaglandins, so the analgesic potency of ACCF could probably be due to the scavenging of

ROS because of its antioxidant activity [60]. The analgesic potency of ACCF may be related to the presence of various bioactive compounds, especially flavonoids and a phenolic compound which are well-known potent analgesics [61]. Flavonoids such as quercetin play a role in creating analgesic and anti-inflammatory effects of ACCF as they can prevent the metabolism of arachidonic acid and subsequently inhibit the synthesis of prostaglandins that are involved in the late phase of peripheral pain perception [62, 63]. Moreover, quercetin exerts centrally acting analgesic potency by acting on opioid receptors, leading to pain relief [64].

3.6. Antipyretic potency of ACCF.

Table 7 demonstrates that Brewer's yeast administration markedly increased the rectal temperature, and the mean increment recorded was 1.45ºC after 18h of administration. Brewer's yeast induced pathogenic fever in experimental animals via a significant activation of the synthesis of pro-inflammatory cytokines, crucial prostaglandin-producing enzymes in the periphery and brain, as Dangarembizi *et al*. [65] reported. Interestingly, treatment with ACCF at the two selected doses as well as aspirin (standard antipyretic agent) effectively attenuated hyperthermia as indicated by a significant ($P< 0.05$) reduction in the rectal temperature at 30, 60, and 90 min after drug administration, as compared to control group. Furthermore, ACCF at 20 mg/kg body weight exhibited highly antipyretic potency, which effectively reduced the rectal temperature a with the percentage of 162.50% after 30 min, comparable to that of ACCF at 10 mg/kg body weight and aspirin with a reduction percentage of 86.21% and 128.98%, respectively at the same observation time (Figure 7). Meanwhile, after 90 min of drug administration, ACCF at 10 mg/kg body weight showed the maximum reduction (220.68%) in the rectal temperature when compared with the reduction percentage of ACCF (20 mg/kg body weight) and aspirin (Figure 7). The current study considerably implies that the hypothermic potency of ACCF occurs similarly to aspirin via interfering with the release of prostaglandins and other pyrogenic cytokines. Comparable hypothermal potencies were reported from different species of earthworms *Lampito mauritii* and *Pheretima hawayana* [66]. The reversal of yeast provoked pyrexia after ACCF administration may be related to its active compounds, especially gallic acid, rutin, and quercetin are potent antipyretic agents acting on inhibition of prostaglandins synthesis [67, 68]. Additionally, suppression in elevating body temperature by ACCF might be due to its anti-inflammatory activity, as Balamurugan *et al*. [66] suggested.

Table 7. Antipyretic potency of ACCF.

Experimental groups	Rectal temperature (ºC)				
	Normal	Initial temperature (after 18h)	After administration of drugs		
			30 min	60 min	90 min
Control	36.46± 0.16^{a}	38.14± 0.10^{a}	37.90±0.10^{a}	38.10±0.14^{a}	37.20±0.16^{a}
$ACCF_{10}$	36.86± 0.22^{a}	38.02±0.12^{a}	37.02±0.17^{b}	36.30±0.10^{b}	35.46±0.29^{b}
$ACCF_{20}$	36.78± 0.10^{a}	38.38±0.05^{a}	35.78±0.40^{c}	36.16±0.12^{b}	35.42±0.39^{b}
Aspirin	36.72±0.10^{a}	38.10±0.13^{a}	36.32±0.11^{c}	36.20±0.34^{b}	36.08±0.54^{b}

Values are mean ± SEM (n= 5). Values with different superscript letters are significantly different ($P < 0.05$). $ACCF_{10}$: *Allolobophora caliginosa* coelomic fluid (10 mg/kg bodyweight); $ACCF_{20}$: *Allolobophora caliginosa* coelomic fluid (20 mg/kg bodyweight).

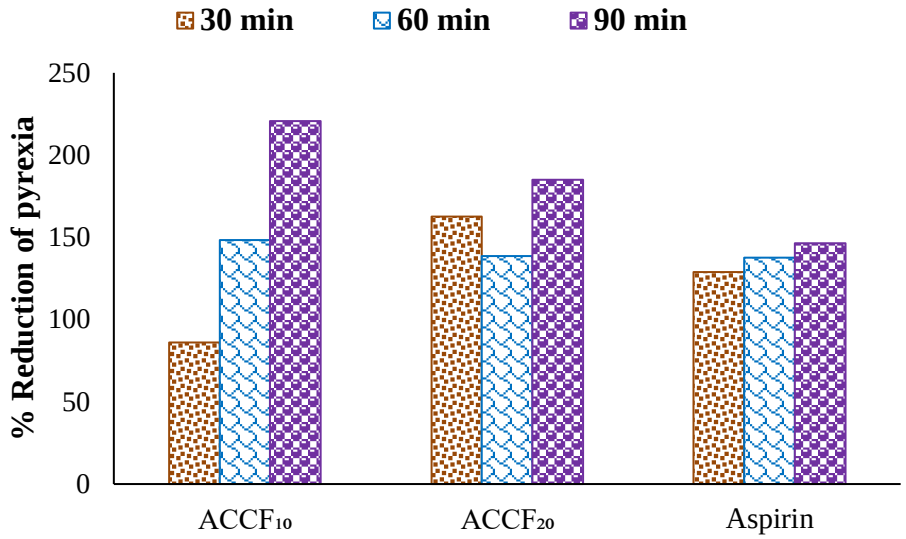

Figure7. The inhibition percentage of ACCF and aspirin on Brewer's yeast induced pyrexia. $ACCF_{10}$: *Allolobophora caliginosa* coelomic fluid (10 mg/kg body weight); $ACCF_{20}$: *Allolobophora caliginosa* coelomic fluid (20 mg/kg body weight).

4. Conclusions

The current study revealed that the coelomic fluid of *Allolobophora caliginosa* (ACCF) possessed various potential pharmacological properties, namely antioxidant, anti-inflammatory, antiproliferative, anti-nociceptive, and antipyretic activities. Biochemical screening of ACCF revealed the presence of various phenolic and flavonoid secondary metabolites, which were likely to act synergistically to produce the observed activities. Also, amino acid profiling of ACCF demonstrated the nutritional value of ACCF, which was signified as a good candidate for sustainable intervention strategy for the endemic protein source in nutrition. Therefore, the present findings confirmed the folklore use of earthworms to treat various diseases. However, further detailed studies are essential to explore the underlying mechanisms and isolate the active compound(s) responsible for these pharmacological properties of ACCF.

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Conflicts of interest

The authors declare no conflict of interest.

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