

In vitro investigations of coelomic fluid of *Eisenia fetida*: protein analysis, antioxidant activities and antibacterial effects on diabetic wounds' bacteria

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ABSTRACT

Background and Objectives: Diabetes is a metabolic disorder characterized by elevated glucose levels, leading to complications such as infections and impaired wound healing. Diabetic wounds are prone to bacterial infections, with common pathogens including *Staphylococcus*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. Coelomic fluid of *Eisenia fetida* (CFEF) exhibits antimicrobial properties, making it a potential alternative to traditional antibiotics. This study aims to evaluate the in vitro antibacterial effects of CFEF on diabetic wound pathogens, alongside analyzing its protein content and antioxidant activities.

Materials and Methods: This study used bacterial strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 19659, and *Pseudomonas aeruginosa* ATCC 27853. CFEF was extracted using warm water and electric shock methods. Protein concentration was determined using the Bradford method, and protein analysis was conducted via Tricine SDS-PAGE. Antioxidant activities were evaluated using DPPH, FRAP, superoxide dismutase, and catalase assays. Antibacterial activities were tested by disc diffusion, MIC, and MBC methods.

Results: The study showed that CFEF exhibited significant antibacterial and antioxidant activities against common bacteria found in diabetic wound infections. The warm water shock method yielded superior results compared to the electric shock method.

Conclusion: CFEF demonstrates promising antibacterial and antioxidant properties, suggesting its potential as a natural alternative for treating diabetic wound infections. Further research is needed to evaluate its clinical application and safety.

Keywords: Anti-bacterial agents; *Staphylococcus aureus*; *Escherichia coli*; *Bacillus subtilis*; *Pseudomonas aeruginosa*

INTRODUCTION

Diabetes, a progressive metabolic disorder, arises from the body's inability to maintain normal homeostasis, leading to elevated glucose levels (1). As of 2017, the global prevalence of diabetes reached 451 million individuals (2). Common symptoms include persistent thirst, increased hunger, obesity, blurred

vision, and neuropathy, alongside impaired wound healing (3). Diabetic wounds often become infected due to compromised healing mechanisms, resulting in frequent progression to acute stages (4).

Immune dysfunction in diabetes exacerbates bacterial infections, fueled by elevated blood sugar levels and increased MMP activity, disrupting the

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wound healing process (5). Vascular complications further impede tissue perfusion, complicating wound recovery (6). While preventive measures encompass sugar control, foot examinations, and lifestyle modifications, treatment primarily involves antibiotics and surgery, lacking a preventive focus (7). Given antibiotic side effects, precise dosing and timing are crucial (8). Common antibiotics include mupirocin, dicloxacillin, cefalexin, cefazolin, doxycycline, and vancomycin (9). Infections predominantly involve *Staphylococcus*, *Enterococcus*, *Corynebacterium*, and *Enterobacteriaceae* species (10). *Escherichia coli* is another prevalent bacterium isolated from these wounds (18). A 2019 study by Thanganadar Appapalam Selvakumar et al., reported that the predominant microbial flora in the collected samples included *S. aureus* (38%), *P. aeruginosa* (23.2%), *B. subtilis* (21%), and *E. coli* (18%) (11).

Coelomic fluid (CF), with its immunological properties, plays a crucial role in the rapid tissue regeneration of earthworms (12). This fluid exhibits significant antimicrobial properties due to its active compounds, such as lysozyme and fetidin proteins, which can be utilized for isolating antimicrobial compounds and developing antibacterial drugs (13). The aim of this study is to investigate the in vitro antibacterial effects of coelomic fluid of *Eisenia fetida* (CFEF) on bacteria commonly found in diabetic wounds. This study will also analyze the protein content and assess the antioxidant activities of this fluid.

MATERIALS AND METHODS

Bacterial strains. In this investigation, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 19659, and *Pseudomonas aeruginosa* ATCC 27853 were the subjects of study. All reagents and chemicals utilized were of analytical grade and commercially sourced.

Extraction of CFEF. CFEF was extracted using the warm water (14) and electric shock (15) methods, following standardized protocols. Then, it was filtered through 0.22 micron filters for sterilization.

Determination of protein concentration by Bradford method. The Bradford method, a rapid, precise, and sensitive colorimetric technique, was employed to ascertain protein concentration in biological sam-

ples. It operates on the principle of forming a complex between Coomassie blue dye G-250 and proteins in a stable solution (16). CFEF, extracted by warm water and electric shock methods at a concentration of 5 mg/ml, was utilized in the Bradford method, adhering to previously established procedures (17).

Tricine SDS-PAGE and silver nitrate staining. SDS-PAGE, a cost-effective and reproducible method for protein analysis, was conducted to assess purification, quantify protein levels, and determine molecular weights. Tricine SDS-PAGE and silver nitrate staining were performed following established protocols (18).

Protein precipitation by ammonium sulfate. Protein precipitation by ammonium sulfate was carried out using CFEF extracted via the electric shock method at a concentration of 5 mg/ml, following a previously reported protocol (19).

Total antioxidant properties. The antioxidant activity of CFEF (concentration: 1 mg/ml), extracted through electric shock and warm water shock methods, was assessed using the free radical scavenging assay (DPPH), following established procedures (20).

Free radical scavenging activity. The antioxidant activity of total CFEF (concentration: 1 mg/ml) was gauged using the FRAP method (21).

Superoxide dismutase enzyme activity. Superoxide dismutase enzyme activity in CFEF (concentration: 5 mg/ml) was measured using the method outlined (22).

Catalase enzyme activity. Catalase enzyme activity in CFEF (concentration: 1 mg/ml) was investigated employing the method established (23).

Disc diffusion method. To incorporate the CFEF into the discs, 20 µl of various CFEF samples (concentration: 1 mg/ml) were inoculated onto sterile discs prepared by Padtan Teb Co. using a sampler, and then dried in a 37°C incubator in preparation for the disc diffusion method. The antimicrobial efficacy of the CFEF samples was assessed using the disc diffusion method, a commonly used technique for sensitivity testing. This method was performed as described previously (24). Following the method of

Balouiri et al., the antimicrobial sensitivity of each bacterium was determined by measuring the zone of inhibition around each disc (measured with a millimeter ruler).

First, the disc diffusion method was evaluated on discs containing CFEEF, a chloramphenicol antibiotic disc (positive control), and a distilled water disc (negative control). The average zone of inhibition of the extracted CFEEF was then measured using two different extraction methods.

Minimum inhibitory concentration (MIC). MIC, denoting the lowest drug concentration inhibiting bacterial growth, was determined using the method outlined (25), with CFEEF concentration set at 5 mg/ml.

Minimum bactericidal concentration (MBC). MBC, the lowest drug concentration eliminating bacteria, was determined by culturing concentrations derived from MIC on Mueller Hinton Agar culture medium (25). Dilutions devoid of bacterial growth were deemed as MBC.

Statistical analysis. Statistical analysis was conducted with three repetitions using a completely randomized block design and SPSS 24 software. T-tests (with unequal variances), Mann-Whitney tests, and Duncan tests were employed for mean data comparison. Standard error indicated deviation from average data, and Excel 2017 software was utilized for graph plotting. Analysis of variance was performed using the ANOVA method.

RESULTS

Protein concentration in CFEEF. The warm water shock method demonstrated a higher concentration of CFEEF protein compared to the electric shock method. Furthermore, protein precipitation analysis indicated more than two-fold increase in protein concentration with the warm water shock method. Table 1 illustrates the protein concentration (mg/ml) of CFEEF extracted by each method.

Protein bands. Utilizing Image J software, analysis of protein band variations in CFEEF extraction methods revealed notable differences. According to Fig. 1, the size of protein band ranges of 9-14 kDa, 14-22 kDa, and 22-41 kDa, of CFEEF extracted by the warm water

Table 1. Protein concentration (mg/ml) of CFEEF extracted by two methods

CFEEF extraction method	Total protein concentration (mg/ml)
Warm water shock method	4.27
Electric shock method	2.14
Protein precipitation obtained from the precipitation method of celomic fluid (extraction by electric shock method) with ammonium sulfate salt.	4.83

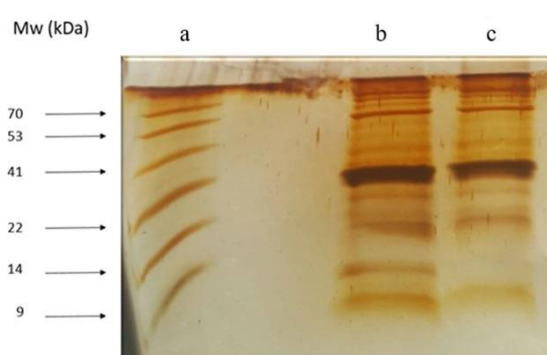


Fig. 1. Comparison of CFEEF protein bands of two methods on SDS-PAGE, (a) Ladder, (b) CFEEF extracted by warm water shock method and (c) CFEEF extracted by electric shock method and.

shock method increased in comparison with the CFEEF extracted by the electric shock method. When extracted by the warm water shock method, the size of the protein bands of 41-53 kDa, increased as well. Some protein bands were not observed in the electric shock extraction method. In the protein range of 53-70 kDa, no significant difference was observed in the number or width of CFEEF bands by either electric shock method or warm water shock method.

Total antioxidant properties. FRAP method was done on CFEEF extracted with electric shock and warm water shock methods and the results are shown in Fig. 2. The reducibility of iron in the CFEEF extracted by the warm water shock method was significantly higher than of the electric shock method.

Free radical scavenging activity. Antioxidant activity was measured based on DPPH method. The results of comparing DPPH free radical scavenging activity related to CFEEFs are shown in Fig. 3. The scavenging

activity of CFEF of warm water shock is significantly higher than electric shock. The average collection percentage of DPPH free radical of the sample obtained from warm water and electric shock was reported as 47.28 and 36.603, respectively.

Superoxide dismutase enzyme activity. The superoxide dismutase enzyme activity of CFEF extracted by the warm water shock method was significantly higher than the electric shock method. The average percentage of relative activity of this enzyme in CFEF extracted by electric shock and warm water shock was

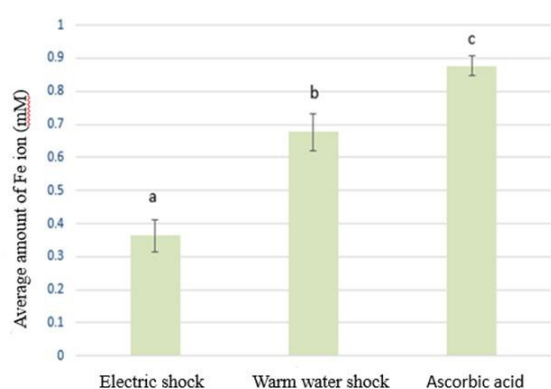


Fig. 2. Antioxidant capacity of total celomic fluids extracted by different methods. The data is the average of 3 repetitions ± standard error (indicates the absence of a significant difference between the CFEF of the two methods based on the comparison of the means with the Mann-Whitney test at the probability level of $P \leq 0.05$).

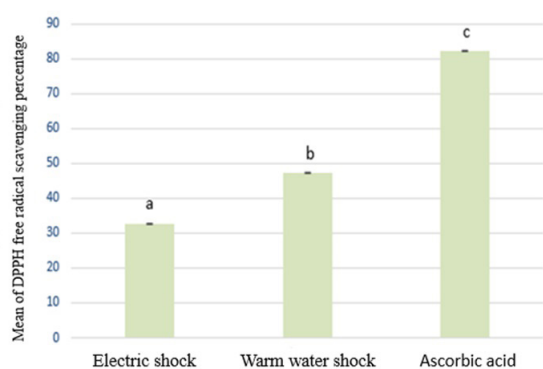


Fig. 3. DPPH free radical scavenging percentage of CFEFs extracted from different methods. The data is the average of 3 repetitions ± standard error (indicates the absence of a significant difference between the CFEF of the two methods based on the comparison of the means with the Mann-Whitney test at the probability level of $P \leq 0.05$).

16.63 and 33.616, respectively (Fig. 4).

Catalase enzyme activity. The catalase enzyme activity of CFEF extracted by the warm water shock method was significantly higher than the electric shock method. The average percentage of relative activity of this enzyme in CFEF extracted by electric shock and warm water shock was 18.636 and 36.283, respectively (Fig. 5).

Disc diffusion. Fig. 6 displays the average zone of inhibition of the CFEF extracted by two different

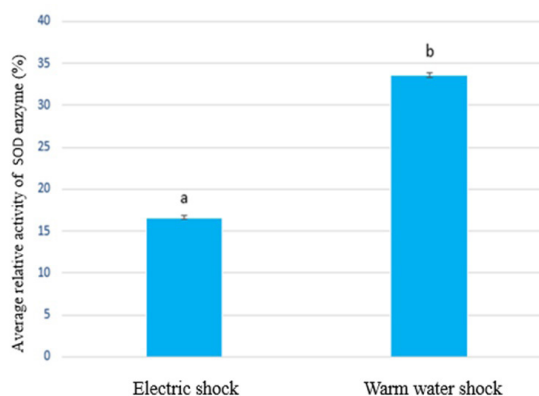


Fig. 4. Relative activity percentage of SOD enzyme of CFEFs extracted by different methods. The data is the average of 3 repetitions ± standard error (indicates the absence of a significant difference between the CFEF of the two methods based on the comparison of the means with the Mann-Whitney test at the probability level of $P \leq 0.05$).

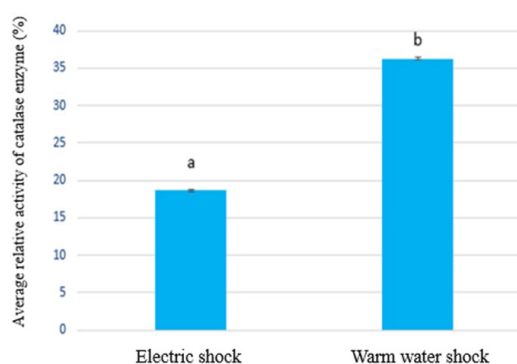


Fig. 5. Relative activity percentage of catalase enzyme of CFEFs extracted by different methods. The data is the average of 3 repetitions ± standard error (indicates the absence of a significant difference between the CFEF of the two methods based on the comparison of the means with the Mann-Whitney test at the probability level of $P \leq 0.05$).

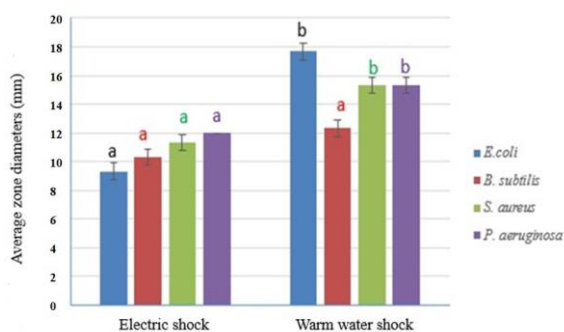


Fig. 6. Average zone diameters of inhibition of CFEF extracted by two methods on *E. coli*, *B. subtilis* bacteria. *S. aureus* and *P. aeruginosa*. The data is the average of 3 repetitions \pm standard error (indicates the absence of a significant difference between the CFEF of the two methods based on the comparison of the means with the Mann-Whitney test at the probability level of $P \leq 0.05$).

methods on the tested bacteria. This comparison showed that the antibacterial effect of CFEF of the warm water shock method on *E. coli*, *S. aureus*, and *P. aeruginosa* bacteria was significantly higher than that of CFEF of the electric shock method. The average zone of inhibition of CFEF extracted by two methods against *B. subtilis* bacteria did not show any significant difference. The highest average zone of inhibition of CFEF of the electric shock method was observed on *P. aeruginosa*, *S. aureus*, *B. subtilis*, and *E. coli* bacteria, respectively. Also, the most effective CFEF of the warm water shock method was on *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis* bacteria, respectively.

Fig. 7 shows the zone of inhibition of the CFEF disc (extracted by the warm water shock method), the chloramphenicol antibiotic disc, and the distilled

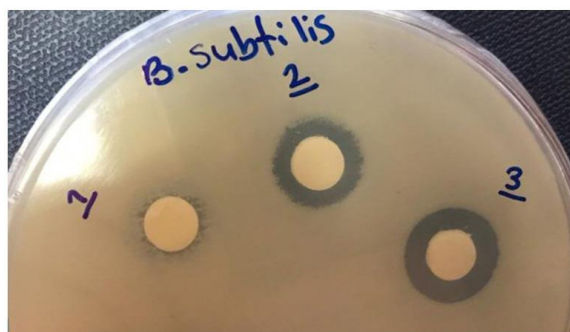


Fig. 7. Zone of inhibition of different discs: 1) distilled water (negative control), 2) CFEF extracted by the warm water shock method, 3) chloramphenicol antibiotic (positive control) on *B. subtilis* bacteria.

water disc on *B. subtilis* bacteria. Bacterial growth around the disc of distilled water was considered as a negative control. However, inhibition zone was observed around the chloramphenicol disc (positive control) and the disc of CFEF, which confirms the antibacterial effect of CFEF.

MIC and MBC. According to Fig. 8, MIC and MBC values of CFEF extracted from two methods of electric shock and warm water shock are shown on *E. coli*, *B. subtilis*, *S. aureus*, and *P. aeruginosa* separately. The average values of MIC of CFEF extracted by the electric shock method on *E. coli*, *B. subtilis*, *S. aureus*, and *P. aeruginosa* were reported as 1.66, 0.83, 0.83, and 0.625, respectively, while these values were reported for CFEF extracted by the warm water shock method as 0.625, 0.416, 0.312, and 0.208, respectively.

The average values of MBC of CFEF extracted by the electric shock method on *E. coli*, *B. subtilis*, *S. aureus*, and *P. aeruginosa* were reported as 2.5, 1.25, 1.25, and 0.625, respectively. While the values were reported for CFEF extracted by the warm water shock method were 1.25, 0.833, 0.625, and 0.625, respectively. The statistical analysis showed that MBC values of the CFEF extracted by the two methods on *B. subtilis* and *P. aeruginosa* did not have a significant difference, but there was a significant difference in MBC values of CFEFs on *E. coli* and *S. aureus*.

According to the results of MIC and MBC tests, *P. aeruginosa* and *S. aureus* are more sensitive to the CFEF compared to *E. coli* and *B. subtilis*. In addition, MIC and MBC showed that the antibacterial activity is higher in CFEF protein precipitation. The statistical analysis indicated that the values of MIC and MBC of the CFEF of the two methods on 4 bacteria were not significantly different.

DISCUSSION

In this research, we investigated the antibacterial properties of CFEF against four prevalent bacteria associated with diabetic wound infections. Our comparison of two the extraction methods revealed that the warm water shock method, compared to the electric shock method, yielded CFEF with significantly greater antibacterial and antioxidant activities.

According to previous studies, CF can serve as a natural alternative to synthetic antibiotics (26). Although the findings showed that CF contains many

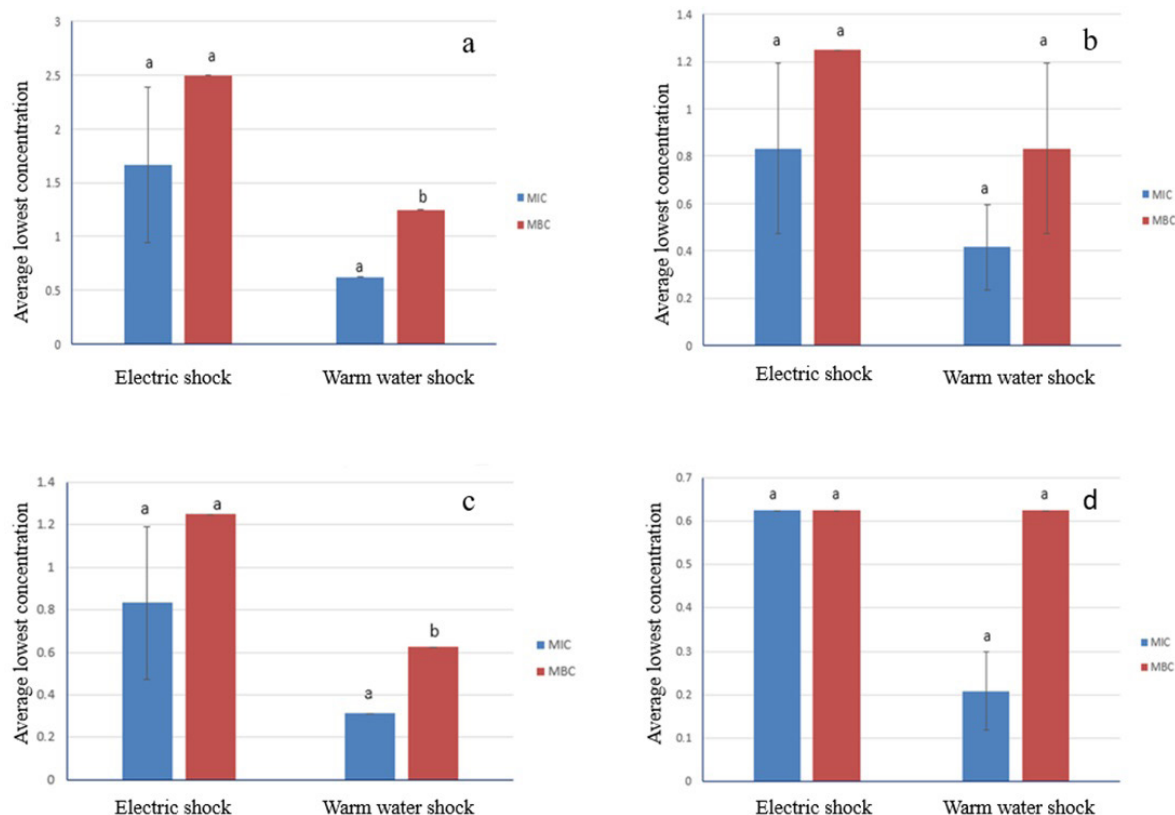


Fig. 8. The value of MIC and MBC (mg/ml) of CFEEF extracted by different methods on (a) *E. coli*, (b) *B. subtilis*, (c) *S. aureus* and (d) *P. aeruginosa*. The data is the average of 3 repetitions \pm standard error (indicates the absence of a significant difference between the CFEEF of the two methods based on the comparison of the means with the Mann-Whitney test at the probability level of $P \leq 0.05$).

bioactive agents which are able to control various biological activities and may act as antibacterial agents (27), the precise therapeutic effects of its antibacterial activity remain uncertain and require further investigation. CF contains various bioactive agents, including proteases (proteins or peptides), metabolites, metal-binding proteins, active proteins (such as lysozyme, lysozyme, and lysozyme), antimicrobial peptides, coelomic cytolytic factors (CCF and CCF-I), lysozyme, fetidin, lumbricin complex, organic acids (i.e., fatty acids), and some organic compounds (such as vitamin D, purines, and vitamin D) (28), which require further investigation to explore their antibacterial effects. In a study, Hua et al. investigated the antibacterial activity of a protein extracted from CF. The protein (ECFP) was isolated and purified using ultrafiltration, gel chromatography, and ion exchange chromatography. The results showed that ECFP exhibited significant antibacterial effects against *E. coli* and *S. aureus* (29). Some peptides identified and isolated from CFEEF, such as VQ-5 and

AQ-5, could serve as candidates for the development of new drugs (30).

This study focused on in vitro assessments rather than direct application to diabetic wounds. Future research should explore the antibacterial properties of CFEEF against bacteria isolated from diabetic patients. Additionally, any future clinical applications will require rigorous ethical evaluations to ensure safety and efficacy.

CONCLUSION

In summary, CFEEF demonstrates notable antibacterial and antioxidant activities against bacteria commonly implicated in diabetic wound infections, highlighting its potential as a natural therapeutic option. The warm water extraction method was particularly effective, producing a higher-quality fluid compared to the electric shock method. However, addressing concerns about antibiotic resistance emphasizes the

need for further studies on the clinical applications of CFEF and its role in managing diabetic wounds.

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