Iranian Journal of Microbiology

Volume 17 Number 1 (*February 2025*) *163-170*
DOI: http://doi.org/10.18502/ijm.v17i1.17814

ORIGINAL ARTICLE

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

Negar Danafar, Marzieh Shokoohi*

Department of Life Sciences Engineering, School of New Sciences & Technologies, University of Tehran, Tehran, Iran

Received: September 2024**, Accepted:** January 2025

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

*Corresponding author: Marzieh Shokoohi, M.Sc, Department of Life Sciences Engineering, School of New Sciences & Technologies, University of Tehran, Tehran, Iran. Tel: +98-21-61113358 Fax: +98-21-66409348 Email: shokoohimarzieh@gmail.com

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 samples. 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 CFEF, a chloramphenicol antibiotic disc (positive control), and a distilled water disc (negative control). The average zone of inhibition of the extracted CFEF 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 CFEF 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 CFEF. The warm water shock method demonstrated a higher concentration of CFEF 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 CFEF extracted by each method.

Protein bands. Utilizing Image J software, analysis of protein band variations in CFEF 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 CFEF extracted by the warm water shock method increased in comparison with the CFEF 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 CFEF bands by either electric shock method or warm water shock method.

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

CFEF 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

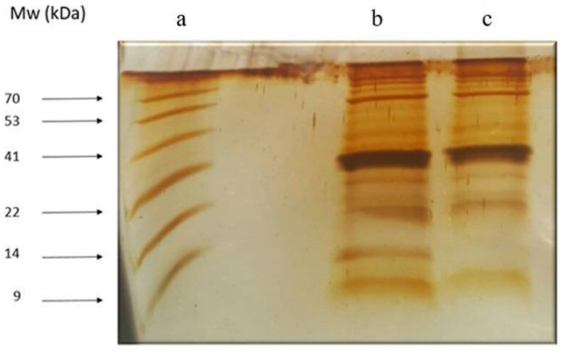

Fig. 1. Comparison of CFEF protein bands of two methods on SDS-PAGE, (a) Ladder, (b) CFEF extracted by warm water shock method and (c) CFEF extracted by electric shock method and.

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

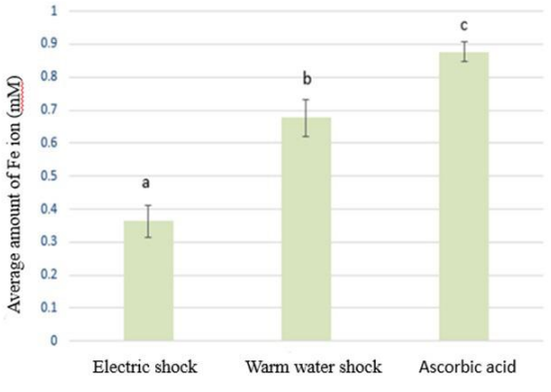

Fig. 2. Antioxidant capacity of total celomic fluids extracted by different methods. The data is the average of 3 repetitions ± standard error (indicates the absence of a significant difference between the CFEF of the two methods based on the comparison of the means with the Mann-Whitney test at the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

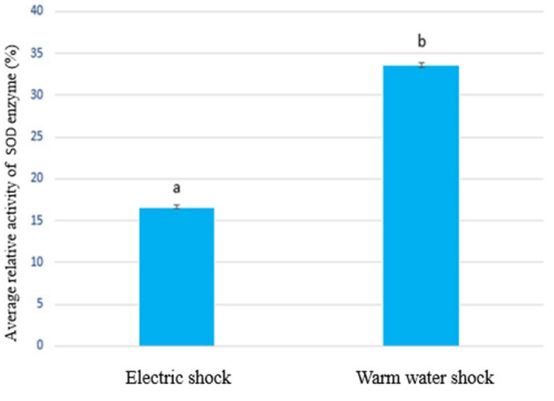

Fig. 4. Relative activity percentage of SOD enzyme of CFEFs extracted by different methods. The data is the average of 3 repetitions ± standard error (indicates the absence of a significant difference between the CFEF of the two methods based on the comparison of the means with the Mann-Whitney test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

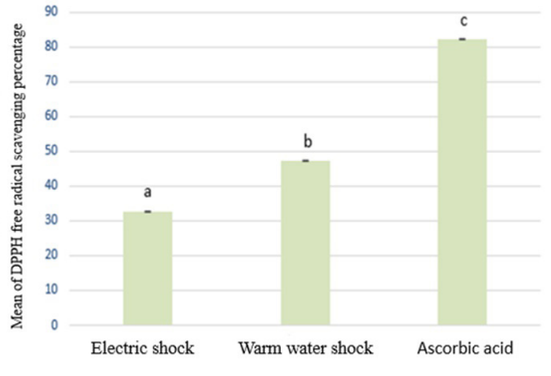

Fig. 3. DPPH free radical scavenging percentage of CFEFs extracted from different methods. The data is the average of 3 repetitions ± standard error (indicates the absence of a significant difference between the CFEF of the two methods based on the comparison of the means with the Mann-Whitney test at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

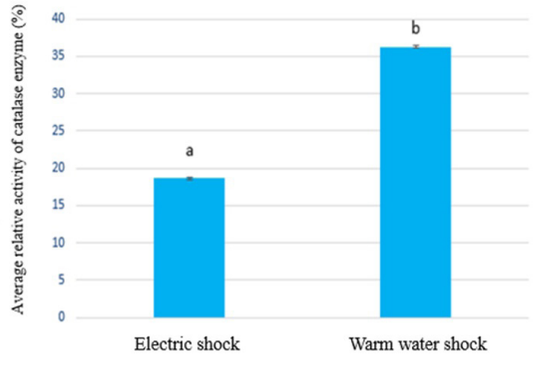

Fig. 5. Relative activity percentage of catalase enzyme of CFEFs extracted by different methods. The data is the average of 3 repetitions ± standard error (indicates the absence of a significant difference between the CFEF of the two methods based on the comparison of the means with the Mann-Whitney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

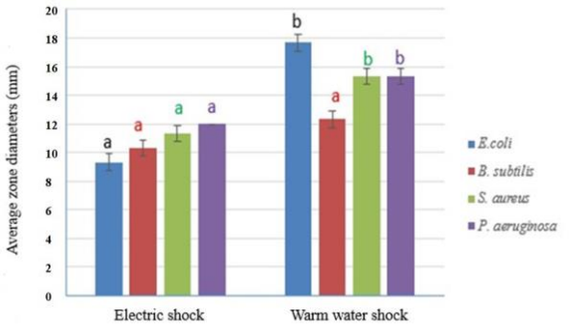

Fig. 6. Average zone diameters of inhibition of CFEF extracted by two methods on *E. coli*, *B. subtilis* bacteria. *S. aureus* and *P. aeruginosa*. The data is the average of 3 repetitions ± standard error (indicates the absence of a significant difference between the CFEF of the two methods based on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

methods on the tested bacteria. This comparison the 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 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.

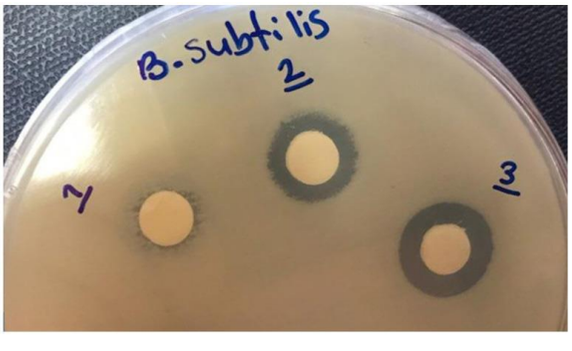

Fig. 7. Zone of inhibition of different discs: 1) distilled water (negative control), 2) CFEF extracted by the warm water shock method, 3) chloramphenicol antibiotic (positive control) on *B. subtilis* bacteria.

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

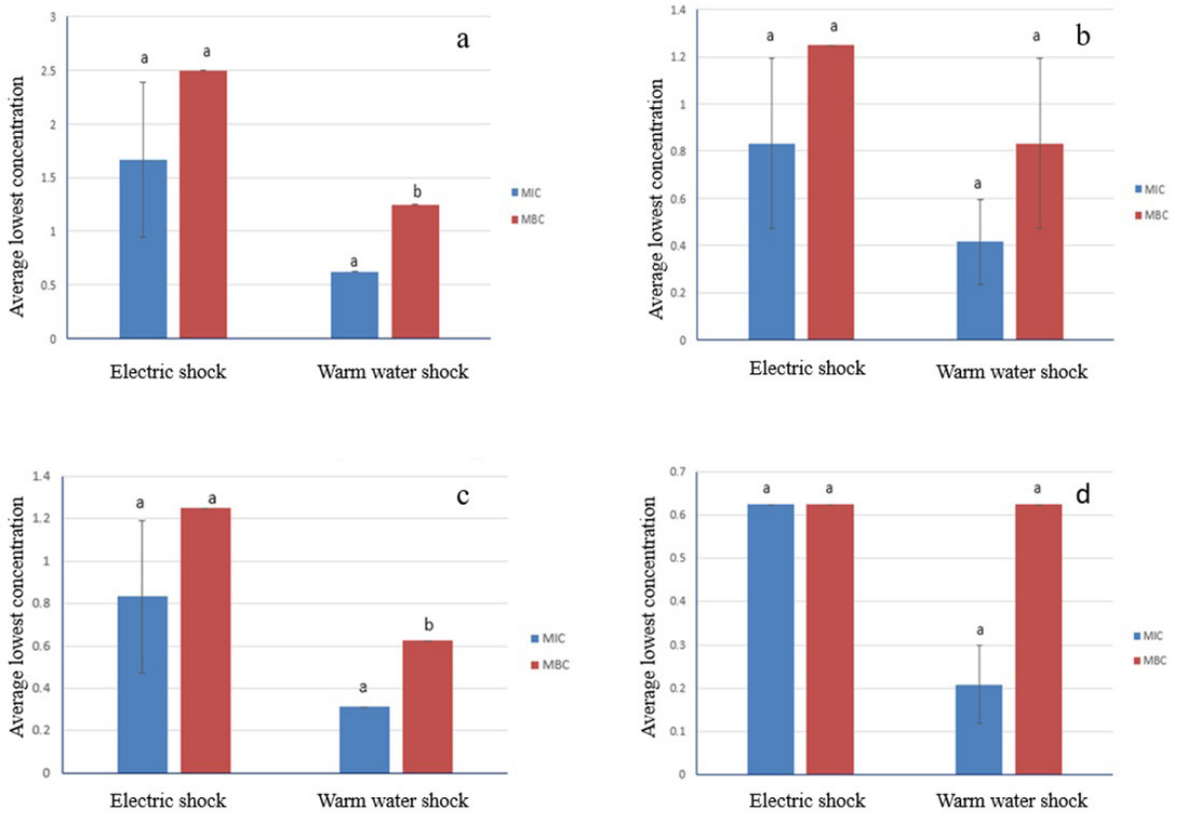

Fig. 8. The value of MIC and MBC (mg/ml) of CFEF extracted by different methods on (a) *E. coli*, (b) *B. subtilis*, (c) *S. aureus* and (d) *P. aeruginosa*. The data is the average of 3 repetitions ± standard error (indicates the absence of a significant difference between the CFEF of the two methods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

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 lysenin, lysozyme, and eiseniapore), antimicrobial peptides, coelomic cytolytic factors (CCF and CCF-I), lysenin, 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 CFEF, 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 CFEF against bacteria isolated from diabetic patients. Additionally, any future clinical applications will require rigorous ethical evaluations to ensure safety and efficacy.

CONCLUSION

In summary, CFEF 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.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the support of the University of Tehran for this study. We also extend our gratitude to Dr. Mahdi Zarabi, Dr. Javad Mohammadnejad Arough, Dr. Mehran Habibi-Rezaei and Dr. Ashraf Sadat Hatamian Zaremi for their invaluable assistance.

REFERENCES

1. Association AD. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2010; 33(Suppl 1): S62-S69.
2. Cho NH, Shaw JE, Karuranga S, Huang Y, da Rocha Fernandes JD, Ohlrogge A, et al. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Res Clin Pract* 2018; 138: 271-281.
3. Drivsholm T, de Fine Olivarius N, Nielsen AB, Siersma V. Symptoms, signs and complications in newly diagnosed type 2 diabetic patients, and their relationship to glycaemia, blood pressure and weight. *Diabetologia* 2005; 48: 210-214.
4. Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, et al. Diagnosis and treatment of diabetic foot infections. *Plast Reconstr Surg* 2006; 117(7 Suppl): 212S-238S.
5. Falanga V. Wound healing and its impairment in the diabetic foot. *Lancet* 2005; 366: 1736-1743.
6. Lipsky BA, Berendt AR, Cornia PB, Pile JC, Peters EJ, Armstrong DG, et al. 2012 Infectious Diseases Society of America clinical practice guideline for the diagnosis and treatment of diabetic foot infections. *Clin Infect Dis* 2012; 54(12): e132-e173.
7. Ousey K, Chadwick P, Jawień A, Tariq G, Nair HKR, Lázaro-Martínez JL, et al. Identifying and treating foot ulcers in patients with diabetes: saving feet, legs and lives. *J Wound Care* 2018; 27(Sup5): S1-S52.
8. Lipsky BA. Medical treatment of diabetic foot infections. *Clin Infect Dis* 2004; 39 Suppl 2: S104-S114.
9. Cunha BA. Antibiotic selection for diabetic foot infections: a review. *J Foot Ankle Surg* 2000; 39: 253-257.
10. Bowler PG, Duerden BI, Armstrong DG. Wound microbiology and associated approaches to wound management. *Clin Microbiol Rev* 2001; 14: 244-269.
11. Thanganadar Appapalam S, Muniyan A, Vasanthi Mohan K, Panchamoorthy R. A study on isolation, characterization, and exploration of multiantibiotic-resistant bacteria in the wound site of diabetic foot ulcer patients. *Int J Low Extrem Wounds* 2021; 20: 6-14.
12. Molnar L, Pollak E, Skopek Z, Gutt E, Kruk J, Morgan AJ, et al. Immune system participates in brain regeneration and restoration of reproduction in the earthworm Dendrobaena veneta. *Dev Comp Immunol* 2015; 52: 269-279.
13. Schenk S, Hoeger U. Annelid coelomic fluid proteins. *Subcell Biochem* 2020; 94: 1-34.
14. Permana S, Putri Fityanti R, Norahmawati E, Iskandar A, Anggraini Mulyadi ED, Tri Endharti A. Coelomic fluid of Eisenia fetida Ameliorates Cetuximab to reduce K-Ras and Vimentin expression through promoting RUNX3 in an AOM/DSS-Induced Colitis Associated Colon Cancer. *Evid Based Complement Alternat Med* 2020; 2020: 9418520.
15. Chellathurai Vasantha N, Rajagopalan K, Selvan Christyraj JD, Subbiahanadar Chelladurai K, Ganesan M, Azhagesan A, et al. Heat-inactivated coelomic fluid of the earthworm Perionyx excavatus is a possible alternative source for fetal bovine serum in animal cell culture. *Biotechnol Prog* 2019; 35(4): e2817.
16. Ku H-K, Lim H-M, Oh K-H, Yang H-J, Jeong J-S, Kim S-K. Interpretation of protein quantitation using the Bradford assay: comparison with two calculation models. *Anal Biochem* 2013; 434: 178-180.
17. Kielkopf CL, Bauer W, Urbatsch IL. Bradford assay for determining protein concentration. *Cold Spring Harb Protoc* 2020; 2020: 102269.
18. Schägger H. Tricine–sds-page. *Nat Protoc* 2006; 1: 16-22.
19. Burgess RR. Protein precipitation techniques. *Methods Enzymol* 2009; 463: 331-342.
20. Miliauskas G, Venskutonis PR, Van Beek TA. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chem* 2004; 85: 231-237.
21. Hsieh C, Rajashekaraiah V. Ferric reducing ability of plasma: a potential oxidative stress marker in stored plasma. *Acta Haematol Pol* 2021; 52: 61-67.
22. Weydert CJ, Cullen JJ. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nat Protoc* 2010; 5: 51-66.
23. Shangari N, O'Brien PJ. Catalase activity assays. *Curr Protoc Toxicol* 2006; Chapter 7: Unit 7.7.1-15.
24. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. *J Pharm Anal* 2016; 6: 71-79.
25. Patton T, Barrett J, Brennan J, Moran N. Use of a spectrophotometric bioassay for determination of microbial sensitivity to manuka honey. *J Microbiol Methods*

2006; 64: 84-95.
26. Hussain M, Liaqat I, Hanif U, Sultan A, Ara C, Aftab N, et al. Medicinal perspective of antibacterial bioactive agents in earthworms (Clitellata, Annelida): A comprehensive review. *J Oleo Sci* 2022; 71: 563-573.
27. Zhu Z, Deng X, Xie W, Li H, Li Y, Deng Z. Pharmacological effects of bioactive agents in earthworm extract: A comprehensive review. *Animal Model Exp Med* 2024; 7: 653-672.
28. Mustafa RG, Dr Saiqa A, Domínguez J, Jamil M, Manzoor S, Wazir S, et al. Therapeutic values of earthworm species extract from Azad Kashmir as anticoagulant, antibacterial, and antioxidant agents. *Can J Infect Dis Med Microbiol* 2022; 2022: 6949117.
29. Hua Z, Wang Y-H, Cao H-W, Pu L-J, Cui Y-D. Purification of a protein from coelomic fluid of the earthworm Eisenia foetida and evaluation of its hemolytic, antibacterial, and antitumor activities. *Pharm Biol* 2011; 49: 269-275.
30. Li C, Chen M, Li X, Yang M, Wang Y, Yang X. Purification and function of two analgesic and anti-inflammatory peptides from coelomic fluid of the earthworm, Eisenia foetida. *Peptides* 2017; 89: 71-81.