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Research Article

Wound healing and coagulant activity of crude extract metabolites from fungal endophytes

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Abstract: Bleeding from a wound as a result of physical injury is a life-threatening condition. In pursuing new drug structures, the effect of crude fungal extracts fungal isolated from *Jatropha multifida* on wound healing and coagulation of mouse whole blood was investigated. *Jatropha multifida* leaves were sterilized, cut into small segments, and then incubated in Potato Dextrose Agar for seven days. Four isolates were purified and their morphologies were characterized. Identification of isolates was confirmed by a molecular protocol. Two crude extracts from *Phlebiopsis gigantea* (OK021602) and *Phyllosticta* sp (OK021603), which exhibited higher phytochemicals composition, were selected and evaluated using wound excision and coagulation of mouse whole blood, by administering 30 µg/mL, 50 µg/mL and 70 µg/mL crude extracts respectively. The percentage of wound healing in mice was higher (p<0.05) for the crude extracts of *Phlebiopsis gigantea* (OK021602) as compared to that of *Phyllosticta* sp (OK021603). The highest percentages of wound contraction were 99% at 70 µg/mL, and 53% at 70 µg/mL for *Phlebiopsis gigantea* (OK021602) and *Phyllosticta* sp (OK021603), respectively as compared to the control group which had 42% wound contraction at day 15 post-treatment. The results of the present study clearly indicate that *Jatropha multifida* leaves harbor endophytic fungi that produce pharmacologically important bioactive secondary metabolites with wound and hemostatic effects; therefore, further exploration is inevitable, particularly for the purification and identification of specific chemical structures of bioactive compounds.

1. INTRODUCTION

Bleeding from a wound as a result of physical injury and hemorrhage is a life-threatening condition. For example, in the United States of America only, it was estimated that about 60,000 reported deaths every year are attributed by bleeding due to hemorrhage. Notably, it has been extrapolated that about 1.9 million deaths attributed by bleeding are reported every year worldwide (Cannon, 2018). The problem is even more alarming in sub-Saharan (SSA). For example, among 480,000 maternal deaths were attributed to bleeding worldwide between 2003 and 2004, 41.6% were reported from SSA (Tochie *et al*., 2019). Likewise, Tanzania is not exceptional, the problem of bleeding is very significant, astonishingly, about 21% of deaths

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among women of reproductive age were associated with bleeding related to postpartum hemorrhage (Bishanga *et al*., 2018).

Although bleeding often stops on its own in minor injuries, in many situations, the use of mechanical barriers, thermal and hemostatic drugs is essential for the rapid prevention of life-threatening bleeding (Spotnitz, 2007). Pharmacological drugs such as tranexamic acid (TXA) have been used for the prevention of excessive blood loss from major trauma, postpartum bleeding, surgery, and heavy menstruation (Chauncey &vWieters, 2020). However, similar to other synthetic pharmacological drugs, most hemostatic agents have side effects. For instance, an anti-fibrinolytic agent (aprotinin) has nonspecific serine protease inhibitory effects, which may cause serious allergic reactions (Ebrahimi *et al*., 2020). Furthermore, there is a paucity of discovery of novel drug structures with coagulation and wound-healing effects. Existing challenges have triggered the search for new drug molecules, for example by exploring bioactive compounds generated by endophytic fungi isolated from medicinal plants with wound healing and coagulation activity.

Traditional medicines have been used for centuries worldwide for the treatment and prevention of diseases such as malaria, cholera, tuberculosis, and asthma *(Rakotoarivelo et al*., 2015). Traditional medicine in Eastern and Southern Asia has been used for at least 3000 years (Park *et al*., 2012). For example, the roots of *Alkanna tinctoria* have been used for antiviral treatment in India (Shaheen *et al*., 2020). Some medicinal plants have been reported to have wound healing and hemostatic effect (Kumar *et al*., 2007). A notable medicinal plant is *Jatropha multifida*, a member of the family Euphorbiaceae, which has been reported to have bioactive compounds with pharmaceutical effects (Anani *et al*., 2016; Kumar and Sharma, 2008). Various studies have been conducted to evaluate the effectiveness of *Jatropha multifida* leaf latex and crude leaf extracts on wound healing and coagulation of whole rat blood (Anani *et al*., 2016; Dougnon *et al*., 2012; Victorien *et al*., 2012). Information from these studies suggests that endophytes residing in *Jatropha multifida* may produce bioactive secondary metabolites with similar pharmacological activities to the wound and hemostatic effects.

Endophytic fungi belong to a class of plant symbionts that reside within plant tissues (Guo *et al*., 2008). Fungal endophytes are fungi that under normal circumstances live within living plant tissues without causing any noticeable symptoms (Tadych and White, 2009). Endophytes produce bioactive compounds that enable plants to adapt to abiotic and biotic stressors (Rana *et al*., 2019). They are known to produce a multitude of bioactive compounds with medicinal values (Dhayanithy *et al*., 2019). However, information on the wound healing and the coagulant effects of crude extract of endophytic fungi isolated from *Jatropha multifida* was lacking; therefore, this study aimed to evaluate the wound healing and coagulant activity of crude extracts of endophytic fungi isolated from the leaves of *Jatropha multifida*.

2. MATERIAL and METHODS

2.1. Plant Materials and Authentication

Plant materials were collected between January and April 2021 in the Ilala and Kisarawe districts of the Dar es Salaam and Coastal (Pwani) regions Tanzania, respectively. Initially, representative leaf samples were taken to the Department of Botany at the University of Dar es Salaam (UDSM) for identification and authentication (voucher number: FMM 4184). Following authentication, the samples were collected and packed in sterile well-labeled plastic bags placed in a container containing silica gel, and immediately transported to the Microbiology laboratory of the Department of Molecular Biology and Biotechnology (UDSM) for further processing.

2.2. Description of Experimental Animals

Six-weeks-old Swiss albino mice were obtained from the Tanzania Veterinary Laboratory Agency (TVLA) and taken to the Zoology Department of the University of Dar es Salaam,

where they were further housed for two weeks before experimentation. The mice were fed a layer mash (FUGA mills, Dar es Salaam, Tanzania) diet at *ad libitum*.

2.3. Isolation and Characterization of Endophytic Fungi

Leaf samples were sterilized as previously described (Kjer *et al.*, 2010) with some modifications. Briefly, leaf samples were washed in running water for 10 min to remove soil particles and stick debris followed by washing in 95% ethanol for 1 min, 2% sodium hypochlorite for 10 seconds, and in 95% ethanol for 1 min. Finally, samples were washed with sterile distilled water for 2 min. To test the efficiency of sterilization a non-sterilized leaf segment (control) and a segment of sterilized leaf were inoculated into growth media. The absence of epiphytic fungi and bacteria on the sterilized leaf segments indicated a high efficiency of surface sterilization. Sterilized leaves were cut into small segments of approximately 2 to 3 mm^2 and then inoculated onto Petri dishes with potato dextrose agar (PDA) and incubated at room temperature for seven days. Pure colonies were obtained after sub-culturing on PDA. The colonies were selected based on their color, texture, elevation, and morphology.

2.3.1. Molecular characterization of endophytes isolated

Fungal genomic DNA was extracted using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, USA), according to the manufacturer's instructions. Initially, genomic DNA was quantified by using Nanodrop Spectrophotometer 2000 (Thermo Fisher Scientific), which was then followed by quality evaluation by running the genomic DNA on 0.8% agarose gel electrophoresis and observed under UV light.

The nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) of the fungal was amplified using the forward primer, ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and the reverse primer, ITS-4 (5′- TCCTCCGCTTATTGATATGC-3′) (Hamzah *et al*., 2018). The PCR thermal protocol was as follows: initial denaturation was at 95 ^oC for 2 min, denaturation at 95 ^oC for 30 seconds, annealing at 55 ^oC for 30 seconds, extension at 72 ^oC for 30 seconds, and the final extension at 72 ^oC for 10 min (Manter and Vivanco, 2007). The expected amplicon size for the ITS region was about 600 bp.

2.3.2. Sequencing of amplicons and Bioinformatics analysis

Sanger sequencing of PCR amplicons was done at Inqaba Biotec™ (South Africa). The raw sequences were then transferred electronically for Bioinformatics analysis. First, consensus DNA sequences were reconstructed by using Geneious Prime (Biomatters, New Zealand). Then, the phylogenetic tree was constructed by using the neighbor-joining tree algorithm to establish species relatedness (Araújo *et al*., 2018).

2.4. Fungal Mass Cultivation and Extraction of Crude Extracts

Mass cultivation of the fungal endophytes was performed as previously described (Mbilu *et al*., 2018). Briefly, agar blocks of actively growing pure isolates were inoculated in a 500 mL conical flask containing 300 mL of sterile potato dextrose broth (PDB) followed by incubation at room temperature in a shaker at 90 rpm for 14 days. After the incubation, the fungal mycelia were filtered using Whatman No. 1 filter paper. The filtrate was mixed with an equal volume of ethyl acetate and shaken for 10 minutes. Ethyl acetate was evaporated to dryness using a vacuum rotary evaporator at 35 ^oC to obtain a crude extract. The crude extracts obtained were stored at −4 ^oC for further analysis (Mbilu et al., 2018). The selection of crude extracts of fungal secondary metabolites for wound healing and coagulant activity testing was based on the presence and abundance of the tested functional groups.

2.5. Phytochemical Analysis of Fungal Crude Extract

Fungal crude extracts were assessed for the presence of natural compounds (bioactive secondary metabolites) qualitatively by using method previously described by Fitokimia *et al.*,

(2016). The presence of alkaloids, flavonoids, phenolics, saponins and tannis was assessed in the present study. To test the presence of alkaloids, flavonoids and phenolics, for each, 1 mL of fungal crude extracts were placed in test tube, which was then followed by addition of few drops of Wagner's reagent (iodine, potassium iodide, and distilled water solution), 10% ferric chloride ($FeCl_3$) and 5% ferric chloride, respectively. The crude extract colour change to brown, dark green and dark green was an indication of the presence of alkaloids, flavonoids and phenolics in the fungal crude extracts, respectively. Likewise, to test tannins, 2 mL of 5% $FeCl_3$ solution added in test tube containing 2 mL of fungal crude extract, and the extract colour change to greenish-black precipitate indicated the presence of tannins. On the other hand, the presence of saponins was determined using frothing test. The fungal crude extract was vigorously shaken with distilled water and allowed to stand for 10 minutes. The formation of fairly emulsion indicated the presence of saponins.

2.5. Wound Healing Activity of the Fungal Crude Extract

Two fungal crude extracts (FUCE) named FUCE 1 and FUCE 2, which exhibited the highest phytochemical content, were selected for the evaluation of wound healing and whole mice blood coagulation effectiveness. Forty mice were divided into eight groups: control, FUCE 1 (30 µg/mL) FUCE 1 (50 µg/mL) FUCE 1 (70 µg/mL), FUCE 2 (30 µg/mL), FUCE 2 (50 µg/mL), FUCE 2 (70 µg/mL) and the vehicle group. Each group consisted of five mice (n=5). Mice in the control group were not treated, whereas mice in the FUCE groups were treated with 0.1mL of 30 µg/mL, 50 µg/mL and 70 µg/mL of the respective extract. Mice in the vehicle group were treated with 10% DMSO, solvent used to reconstitute extracts.

The mice were anesthetized with chloroform (Ezike et al., 2010). The dorsal thoracic central region of the animals was shaved, and the entire thickness of the skin was excised using a circular biopsy punch of with a diameter of 1 cm to obtain a wound of approximately 80 mm^2 (Masson-Meyers et al., 2020). The mice were then treated daily from day zero to the day after wound healing. The wound area was measured at three-day intervals. The following formula was used to calculate the wound contraction percentage (%):

$$\text{wound contraction (\%)} = \frac{W_{AO} - W_{AT}}{W_{AO}} \times 100\ \%$$

where W_{AO} = wound area at day zero, and W_{AT} = wound area on a specific day (Ezike et al., 2010).

2.6. Coagulation Time of Whole Blood

Hemostasis is a physiological process that aids in preventing blood loss from damaged blood vessels (Blanco & Blanco, 2017). Thirty-five mice were randomly divided into seven groups. The control group which was not treated, FUCE 1 (30 µg/mL) group, FUCE 1 (50 µg/mL) group, FUCE 1 (70 µg/mL) group and FUCE 2 (30 µg/mL) group, FUCE 2 (50 µg/mL) group, FUCE 2 (70 µg/mL) group. Each animal was anesthetized with chloroform and the thoracic cavity was open to expose the aorta (Ezike *et al.,* 2010). The aorta was cut and 1 mL of blood was quickly withdrawn using a plastic disposable syringe, and then transferred into paraffin-coated plastic tubes containing small amount of 30 µg/mL, 50 µg/mL and 70 µg/mL fungal crude extracts. The plastic tube was swirled every 15 s to check the fluidity of the contents. The interval between the introduction of the blood and the time of clot formation was taken as the coagulation time in seconds (Ezike *et al.,* 2010).

2.7. Statistical Analysis

Descriptive and inferential statistical analyses were performed using the origin pro software (Seifert, 2014). One-way analysis of variance (ANOVA) was conducted to compare means among wound contraction groups. Results were expressed as mean ± standard error of means

(SEM). Pairwise comparison of means was performed by Tukey's HSD, and a significant difference was taken at $p < 0.05$.

3. RESULTS

3.1. Phenotypic and Molecular Characteristics of Endophytic Fungi

In this study, four endophytic fungi were isolated and characterized. Phenotypic characteristics based on color, texture, shape, edges, and elevation of mycelia on agar surface were variable (Figure 1).

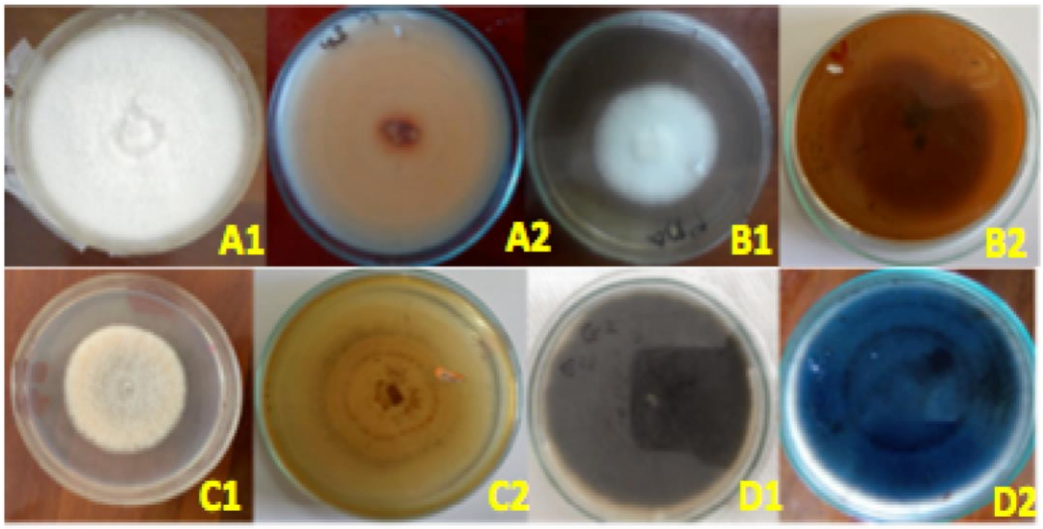

Figure 1. Morphological appearance of endophytic fungi isolated from *Jatropha multifida. Phlebiopsis gigantea (*OK021602) front (A1) and back view (A2); *Phyllosticta* sp (OK021603) front (B1) and back (B2) view*; Colletotrichum* sp (OK021604) front (C1) and (C2) view; *Phyllosticta elongata (*OK021605) front (D1) and back (D2) view.

The phenotypic characterization was further confirmed by genotypic characterization by sequencing the ITS region of the isolates by using ITS1 and ITS4 primers. The Accession numbers and suggested scientific names are presented in Table 1. In addition, DNA sequences were used to generate a phylogenetic tree to establish genetic relatedness between endophytic fungi that were isolated in the present study with the ones reported in GenBank (Figure 2).

Table 1. Name and accession numbers of endophytic fungi that were isolated from *Jatropha multifida* and successfully characterized in the present study.

Isolate ID	Accession number	Scientific name
FC 1	OK021602	*Phlebiopsis gigantea*
FC 2	OK021603	*Phyllosticta sp*
FC 4	OK021604	*Colletotrichum sp*
FC 6	OK021605	*Phyllosticta elongata*

Note: FC stands for fungal culture, so FC1 indicates fungal culture 1 for pure isolate 1.

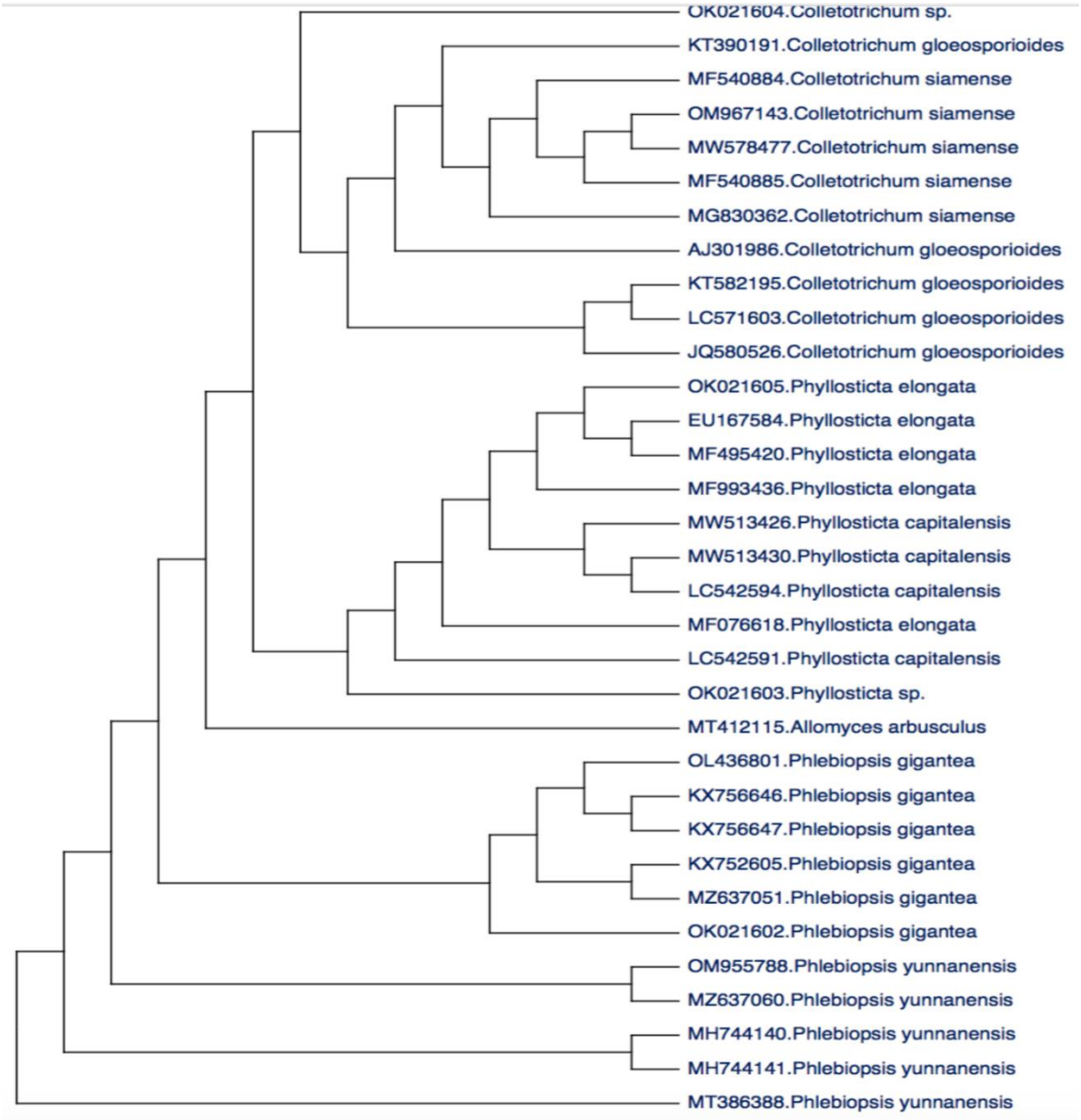

Figure 2. A neighbor-joining phylogenetic tree depicting the relationship of *Phlebiopsis gigantea* (OK021602), *Phyllosticta sp* (OK021603), *Colletotrichum sp* (OK021604), *Phyllosticta elongata* (OK021605) with other related fungi obtained from GenBank. The *Allomyces arbusculus (*MT412115) was used as an out-group.

3.2. Qualitative Phytochemical Analysis

Initial screening for the performance of crude extracts on wound healing and hemostatic activity was done by evaluation of the presence of essential classes of bioactive compounds. Therefore, phytochemical analysis was conducted, and the results revealed the presence of tested classes of bioactive compounds in all four fungal crude extracts (FUCE) as summarized in Table 2.

Table 2. Qualitative phytochemical analysis of fungal crude extracts

Compound test	alkaloid	flavonoid	saponin	tannin	phenol
FUCE 1	++	++	++	++	++
FUCE 2	+	+	+	+	+
FUCE 3	–	–	–	+	+
FUCE 4	+	–	–	–	–

FUCE stands for fungal crude extract, + means present, – means absent, and the number of + indicates the abundance of a particular phytochemical

3.3. Wound Healing Activity of Crude Extracts

Animals treated with crude fungal extract (FUCE 1) had the highest percentage of wound contraction in the fifteenth day post-treatment. Animals treated with FUCE 1 showed a significant reduction in wound diameter (p<0.05) compared to animals in the control group as shown in Table 3.

Table 3. effect of fungal crude extracts on wound contraction in mice wounds.

Treatment group	Concentration µg/mL	Wound contraction (%)				
		The day of observation post treatment				
		Day 3	Day 6	Day 9	Day 12	Day 15
Control	-	2^a ±2.00	4^a ±1.87	12^a ±2.55	24^a ±1.87	42^a ±1.22
FUCE 1	30	5^a ±2.2	9^a ±4.00	25^a ±3.53	35^a ±2.73	53^a ±2.54
	50	19^a ±1.00	39^a ±1.00	58^a ±1.22	79^a ±1.00	98^a ±2.00
	70	27^b ±2.54	47^b ±2.00	63^b ±2.73	83^b ±3.39	99^b ±3.39
FUCE 2	30	1^a ±1.00	4^a ±1.87	13^a ±2.54	26^a ±2.91	41^a ±2.91
	50	3^a±2.00	10^a±2.74	21^a±2.45	36^a±2.92	53^a±3.39
	70	2^a ±1.22	11^a ±2.91	25^a ±3.16	42^a ±3.00	58^a ±2.54
Vehicle	-	1^a±1.00	4^a±2.00	10^a±1.87	23^a±1.22	40^a±2.45

[a] no significance difference; [b] there is a significance difference (p<0.05); FUCE stands for fungal crude extract.

3.4. Coagulation Effect of Crude Fungal Extract

The reference range of mice's whole blood clotting time was about 100 seconds. FUCE 1 demonstrated higher blood clotting activity as compared to FUCE 2. Surprisingly, FUCE 2 demonstrated anticoagulant activity (Table 4). Interestingly, the pattern of coagulation and anticoagulation activity of FUCE 1 and FUCE 2, respectively was correspondingly to the increase in extract concentration (Table 4), demonstrating dose-response relationship.

Table 4. Effect of fungal crude extracts in mice whole blood coagulation.

Treatment	Dose (µg/mL)	Coagulation time (s)
Control	-	98.6
FUCE 1	30	42.6
	50	32.33
	70	14.6
	30	986
FUCE 2	50	$2.67x10^3$
	70	$3.04x10^3$

FUCE stands for fungal crude extract, for FUCE1 is fungal crude extract of isolate 1 under the present study

4. DISCUSSION and CONCLUSION

A multitude of reports have demonstrated wound healing and the hemostatic effect of *Jatropha multifida* (Anani *et al.*, 2016; Ezike *et al.*, 2010). Although not supported by empirical evidence, *Jatropha multifida* is used in most of Tanzania communities for the prevention of bleeding and treatment of wounds. Notably, there is a plethora of evidence, which suggests the relationship between endophytes and their host plant in terms of bioactive secondary metabolites. Based on this reality, it was hypothesized that endophytic fungi isolated from *Jatropha multifida* generate bioactive secondary metabolites with wound healing and coagulant activity. Results of the present study corroborated wound healing activity of crude extracts of fungal endophytes from *Jatropha multifida*; however, the coagulation activity of the fungal endophytes is still debatable based on the present findings (Tables 3 and Table 4).

In this study *Jatropha multifida* leaves were collected from six different plants that were used for the isolation of endophytic fungi. Based on the molecular identification, the genus

Phyllosticta was observed to be the dominant genus on *Jatropha multifida* leaves, compared to other genera isolated. The *Phebiopsis* genus was the only Basidiomycota isolate identified in this study whereas other genera belong to the class Dothideomycetes and Sordariomycetes in phylum Ascomycota. All genera isolated are pathogens to other plant hosts but there are no reports of the pathogenesis of these species in *Jatropha multifida*. In addition, all endophytes isolated in this study have previously been isolated in other host plants (Rampadarath et al., 2018; Wikee et al., 2013). The abundance, diversity, and species composition of endophytes vary according to host species, tissue types, site characteristics, local microclimates, and anthropogenic factors (Torres *et al.*, 2011). It is strongly believed that the isolation of a relatively small number of endophytes in this study could have been due to the aforementioned reasons. Interestingly, this is the first study report on the isolation and identification of endophytic fungi isolated from *Jatropha multifida*.

Phytochemical analysis results revealed that *Phlebiopsis gigantea* extract had a high level of bioactive compounds compared to other extracts. The similarity between bioactive compounds present in this study and the ones that have been previously reported from the crude extract of *Jatropha multifida* leaves justifies the hypothesis that fungal endophytes and their host plants might have been producing similar bioactive compounds. Results from this study are in accordance with the previous report (Devi *et al*., 2012) wherein endophytes have shown the presence of different bioactive compounds like flavonoids, saponin, alkaloids, and phenol. Also, it has been reported that alkaloids, phenol, saponin, and flavonoids all have wound healing and hemostatic activity (Fetse *et al*., 2014; Sharma *et al*., 2021). In addition, the similarity between bioactive compounds present in this study and the ones that have been previously reported from leaves of *Jatropha multifida* (Chioma *et al*., 2021; Rampadarath *et al*., 2014) justifies the hypothesis that fungal endophytes and their host plants might have been producing similar bioactive compounds.

Wound contraction is the healing response that functions to reduce the size of the tissue defect and eventually decrease the size of damaged tissue that needs repair (Lesperance *et al*., 2006). Wound contraction involves three distinct phases i.e. inflammation, proliferation (fibroblastic), and tissue remodeling (maturation) (Fetse *et al*., 2014). In this study wound healing and coagulation test results revealed that *Phlebiopsis gigantea* (OK021602) extract had a high level of bioactivity compared to *Phyllosticta sp* (OK021603) extract, which had slight bioactivity. Animals in the FUCE 1 group treated with a crude fungal extract of 30 µg/mL had slightly higher bioactivity with 53% wound contraction compared to animals in the control group which had 42% wound contraction. This indicates that the presence of bioactive compounds in the crude fungal extract promoted wound healing even in a small concentration. On the other hand, FUCE 1 group treated with 50 µg/mL and 70 µg/mL had even higher bioactivity with 98% and 99% wound contractions respectively compared to the control group. Results indicated that as the concentration of bioactive compounds increases the wound contraction percentage increases as well hence, promote fast wound healing in mice. Animals in FUCE 2 groups treated with crude fungal extracts with 30 µg/mL, 50 µg/mL and 70 µg/mL respectively, had less wound contraction percentages compared to animals treated with FUCE 1 extracts and had almost similar wound contraction percentage of animals in the control group.

In the evaluation of mice whole blood coagulation, the *Phlebiopsis gigantea* (OK021602) extract had a high level of bioactivity compared to that of *Phyllosticta sp* (OK021603) extract. The blood treated with FUCE 1 of 30 µg/mL coagulated in 42.6 s, two times faster than blood in the control group. This result indicates that bioactive compounds with coagulant activity in FUCE 1 extract speed up the blood coagulation process. On the other hand, FUCE 1 extracts with 50 µg/mL and 70 µg/mL coagulated whole blood in 32.33 s, three times faster than the blood coagulation in the control group and 14.6 s which is seven times faster than blood coagulation in the control group, respectively. These results indicate that the presence of bioactive compounds in high concentrations promotes high bioactivities. In this case, high

bioactive compounds with coagulant activity promoted or sped up mice's whole blood coagulation process. Surprisingly, FUCE 2 extracts with 30 µg/mL, 50 µg/mL, and 70 µg/mL coagulated mice while blood in 986 s, 2.67×10^3 s, and 3.04×10^3 s, which is 10, 27, and 30 times slower than blood coagulation in the control group respectively. These results could be due to the presence of blood thinner compounds (anticoagulants) that prevent blood from clotting, increasing the mice's whole blood coagulation time.

Results from the present study clearly indicate that *Jatropha multifida* leaves harbor endophytic fungi that produce pharmacologically important bioactive metabolites that can be used as an alternative in the treatment of wounds and prevention of bleeding (hemorrhage). Further studies on functional analysis of individual compounds are highly recommended.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s). The experiment was conducted in compliance with the Guidelines on the Humane Treatment of Laboratory Animals as stipulated in the Tanzania Animal Welfare Act, 2008.

Availability of Data

The data in the study is available to other research upon request.

Authorship Contribution Statement

Fulgence Ntangere Mpenda: Envision of study, data analysis, supervision, revision, original draft, proof reading; **George Madaha:** Envision of study, data collection, data analysis; **Fortunatus Jacob:** Envision of the study, original draft, revision of manuscript

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