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ORIGINAL ARTICLE

Chemical Composition of *Moringa oleifera* Ethyl Acetate Fraction and Its Biological Activity in Diabetic Human Dermal Fibroblasts

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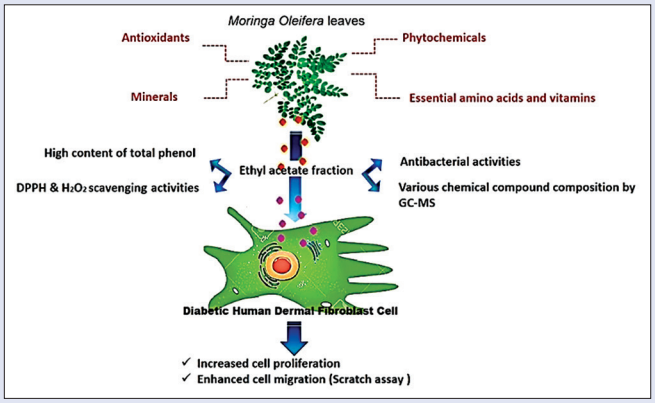
ABSTRACT

Background: *Moringa oleifera* (MO), commonly known as the drumstick tree, is used in folklore medicine for the treatment of skin disease. **Objective:** The objective of this study is to evaluate the ethyl acetate (EtOAc) fraction of MO leaves for *in vitro* antibacterial, antioxidant, and wound healing activities and conduct gas chromatography-mass spectrometry (GC-MS) analysis. **Materials and Methods:** Antibacterial activity was evaluated against six Gram-positive bacteria and 10 Gram-negative bacteria by disc diffusion method. Free radical scavenging activity was assessed by 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical hydrogen peroxide scavenging and total phenolic content (TPC). Wound healing efficiency was studied using cell viability, proliferation, and scratch assays in diabetic human dermal fibroblast (HDF-D) cells. **Results:** The EtOAc fraction showed moderate activity against all bacterial strains tested, and the maximum inhibition zone was observed against *Streptococcus pyogenes* (30 mm in diameter). The fraction showed higher sensitivity to Gram-positive strains than Gram-negative strains. In the quantitative analysis of antioxidant content, the EtOAc fraction was found to have a TPC of 65.81 ± 0.01. The DPPH scavenging activity and the hydrogen peroxide assay were correlated with the TPC value, with IC_{50} values of 18.21 ± 0.06 and 59.22 ± 0.04, respectively. The wound healing experiment revealed a significant enhancement of cell proliferation and migration of HDF-D cells. GC-MS analysis confirmed the presence of 17 bioactive constituents that may be the principal factors in the significant antibacterial, antioxidant, and wound healing activity. **Conclusion:** The EtOAc fraction of MO leaves possesses remarkable wound healing properties, which can be attributed to the antibacterial and antioxidant activities of the fraction.

Key words: Diabetic wound healing, gas chromatography-mass spectrometry, migration rate, phenolic content, scratch assay, skin pathogen

SUMMARY

- *Moringa oleifera* (MO) leaf ethyl acetate (EtOAc) fraction possesses antibacterial activities toward Gram-positive bacteria such as *Streptococcus pyogenes, Streptococcus faecalis, Bacillus subtilis, Bacillus cereus* and *Staphylococcus aureus,* and Gram-negative bacteria such as *Proteus mirabilis* and *Salmonella typhimurium*
- MO leaf EtOAc fraction contained the phenolic content of 65.81 ± 0.01 and flavonoid content of 37.1 ± 0.03, respectively. In addition, the fraction contained 17 bioactive constituents associated with the antibacterial, antioxidant, and wound healing properties that were identified using gas chromatography-mass spectrometry analysis
- MO leaf EtOAc fraction supports wound closure rate about 80% for treatments when compared with control group.



Abbreviations used: MO: *Moringa oleifera*; EtOAc: Ethyl acetate; GC-MS: Gas Chromatography-Mass Spectrometry; HDF-D: Diabetic Human Dermal Fibroblast cells.

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INTRODUCTION

The rising epidemic of diabetes is resulting in a calamitous physical, emotional, and financial toll on our country, Malaysia as well as worldwide. The World Health Organization anticipates that by 2030, more than 347 million people worldwide will suffer from diabetes; this is 10 times greater than the number of people affected by HIV/AIDS.[1] Patients who develop diabetes will be subject to debilitating complications and higher health-care costs. More than 298 million cases are expected in developing countries, where most patients will not have access to adequate healthcare.[2] Diabetic foot ulcers (DFUs), a leading cause of amputations, affect 15% of people with diabetes. Due to the lack of healing in diabetic patients, the ulcers represent a substantial morbid event.[3] DFUs increase the risk of infections and other associated complications. Five-year mortality rates after new-onset diabetic

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ulceration have been reported to be between 43% and 55%, and rise to 74% for patients receiving lower-extremity amputation.[4] The magnitude of the challenge faced by DFU patients is reflected in the high cost of treatment.

DFUs often fail to heal because of a persistently high concentration of pro-inflammatory cytokines in the wound site. Pro-inflammatory cytokines are strongly upregulated in hyperglycemic conditions. A high glucose concentration substantially disturbs cell-dependent responses and induces high concentrations of proteases, which degrades multiple growth factors, receptors, and matrix proteins that are essential for wound healing.[5] In addition, the diabetic wound does not follow the orderly cascade of events that characterize normal wound healing. Instead, the inflammatory reaction in diabetic wounds is prolonged. Thus, the end results are ongoing tissue destruction rather than repair.[6] In addition, a longstanding open wound generates vulnerability to opportunistic infections, which aggravate the healing process. Open wounds are susceptible to infection, especially by bacteria, and also offer an entry point for systemic infections.[7]

One of the most important considerations in caring for patients with DFU is the avoidance of serious complications by expeditious and complete wound healing. Regardless of the multitude of wound healing drugs and technologies that have been reported in recent years, the management of chronic diabetic ulcers remains a challenge among biomedical researchers. The emerging scientific perspective of wound physiology has introduced natural therapeutic approaches targeting specific wound healing abnormalities. Antibacterial and healing compounds present in a traditional remedy can support this approach and may be valuable in the treatment of wounds.[8] Virtually all diabetes involves free radicals and although the free radicals are secondary to the disease; in some cases, the free radicals are causal and lead to oxidative stress. Cellular antioxidants augment wound healing by a reduction of the damage caused by oxygen radicals. The antioxidant complement in diabetic patients is massively depleted by free radicals. Thus, a delicate balance between free radicals and antioxidants is essential to ensure healthy wound healing in diabetic patients.[9] Studies have confirmed that medicinal plants with high antioxidant polyphenol content are cost-effective, efficacious wound healing agents in the management of diabetes, with few side effects.[10]

The growing body of evidence of the effectiveness of *Moringa oleifera* (MO) as a wound healing agent has led to a significant increase in its uses. Different parts of this plant act as antihyperthyroidism agents,[11] natural coagulants,[12] and possess antitumor,[13] antiinflammatory,[14] antiulcer,[15] antispasmodic,[16] antihypertensive,[17] cholesterol-lowering,[18,19] antidiabetic, hepatoprotective, antibacterial, and antifungal activities.[20] This versatility encouraged us to explore the effect of the ethyl acetate (EtOAc) fraction of MO leaves on the different parameters of wound healing, including antibacterial activity, antioxidant activity, cell proliferation, and migration rate in an *in vitro* wound healing model. In addition, we identified the constituent chemical compounds of the fraction by gas chromatography-mass spectrometry (GC-MS) analysis.

MATERIALS AND METHODS

Plant collection and extract preparation

Fresh and mature MO leaves were acquired from Garden No. 2 at Universiti Putra Malaysia (UPM), Malaysia, with the voucher specimen SK 1561/08 and stored in the IBS Herbarium unit.[21] The leaves were further extracted with 90% ethanol and then filtered through Whatman filter paper. The filtrates were evaporated on a rotary evaporator, and the extracts were concentrated under reduced pressure and lyophilized to obtain a powder (Virtis Bench Top K, United States). The ethanol extract was extracted successively with different organic solvents such as hexane, chloroform, EtOAc, and butanol to obtain the hexane, EtOAc, chloroform, and butanol fractions, respectively, in addition to the residual methanol fraction. All crude extracts were filtered separately through Whatman No. 41 filter paper to remove particles and were evaporated to dryness in a rotary evaporator. The residue left in the separating funnel was reextracted twice following the same procedure and then filtered. The combined extracts were concentrated and dried in a rotary evaporator under reduced pressure. Only the MO EtOAc fraction was used in further experiments, and this was stored at − 20°C.

Antibacterial studies

Agar disc diffusion method

The antibacterial effects of the MO leaf EtOAc fraction were evaluated using agar disc diffusion method. A total of 10^5 CFU/mL of overnight bacteria culture was spread on agar. Numbers of sterilized discs were dipped into the solvents (negative controls) and fraction solutions (100 mg/mL) and placed on the plates. After incubation at 37°C for 24 h, the antibacterial activity was assessed by the measurement of the diameter of the inhibition zone formed around the disc. A comparison test of the antibiotic control, streptomycin, was made using commercial discs. The antibacterial experiments were conducted in triplicate and inhibition zone diameters were recorded from each independent experiment.

Antioxidant studies

Total phenolic content

The total phenolic content (TPC) in the MO leaf EtOAc fraction was estimated using Folin-Ciocalteu reagent (Singleton *et al.*, 1999). In a series of test tubes, 0.5 mL of the fraction in methanol was mixed with 2.5 mL of 10% Folin-Ciocalteau reagent and 2.5 mL of 7.5% sodium carbonate. After shaking, the mixture was left to stand for 2 h and then the absorbance was measured at 765 nm. A standard curve was prepared using gallic acid. From the standard curve, the TPC was calculated and expressed as gallic acid equivalent in mg/g of fraction.

Total flavonoid content

The total flavonoid content was measured by a colorimetric assay.[22] 1 mL of extract was added to 1 mL of distilled water, then 0.15 mL of 2% aluminum chloride solution was added, and the sample was thoroughly mixed. The absorbance at 415 nm was measured relative to a blank sample. Rutin was used as the standard for the calibration curve. The total flavonoid content of the extract was expressed as rutin equivalent in mg/g of sample.

1-diphenyl-2-picryl hydrazyl free radical scavenging assay

The free radical scavenging activity of the fractions was measured *in vitro* by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay.[23] DPPH solution (0.1 mM in methanol) was prepared, and 150 µL of this solution was mixed with 150 µL of fraction or ascorbic acid (standard) in methanol at various concentrations. The reaction mixture was vortexed thoroughly, left in the dark at room temperature for 30 min, and the absorbance was measured at 517 nm. The percentage inhibition of scavenging was determined by the following equation:

$$\text{DPPH radical scavenging activity}\,(\%) = \frac{A0 - A1}{A0} \times 100\%$$

Where $A0$ is the absorbance of DPPH radicals and methanol, and $A1$ is the absorbance of DPPH radicals and sample extract or standard.

The IC_{50} values of the fraction and standard were determined. All analyses were performed in triplicate, and the average was reported.

Hydrogen peroxide scavenging assay

The hydrogen peroxide radical scavenging activity of the MO leaf EtOAc fraction was estimated using the method of[24] with a rational modification. Hydrogen peroxide solution (4 mM) was prepared in a standard phosphate buffer (pH 7.4). Briefly, 174 µL of various concentrations of fraction solutions and the standard (ascorbic acid) were mixed with 26 µL H_2O_2 solution (2 mM) and incubated for 10 min. After incubation, the absorbance was measured at 230 nm relative to a blank solution containing phosphate buffer without hydrogen peroxide. The percentage inhibition of hydrogen peroxide radicals scavenged by the fraction was calculated using this formula:

$$\text{Hydroxyl radical scavenging activity} = \frac{A0 - A1}{Ao} \times 100\%$$

Where *A*0 is the absorbance of the control, and *A*1 is the absorbance in the presence of the MO leaf EtOAc fraction.

In vitro diabetic wound healing study

Cell culture and cell viability assay

Diabetic human dermal fibroblast cells (HDF-D, ZenBio Inc.) were cultured in Dulbecco's Modified Eagle Medium containing 5% fetal bovine serum, 100 µg/mL streptomycin, and 100 U/mL penicillin at 37°C in an atmosphere of 5% CO_2. The effect of the EtOAc fraction on cell cytotoxicity was investigated by 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in HDF-D. Briefly, cultured cells were seeded into a 96-well plate at a density of 1×10^5 and left overnight. Subsequently, the cells were treated with serial dilutions of MO leaf EtOAc fraction of 15–500 µg/mL. After a 24-h incubation period, 10 µL MTT reagent (5 mg/mL) was then added to each of the wells and incubated for 4 h. The resulting formazan crystals were dissolved by the addition of 100 µL dimethyl sulfoxide to each well, well mixed, and left to stand in the dark at room temperature for 30 min. Absorbance was recorded at 570 nm using an ELISA microplate reader, and the cell viability was calculated according by comparing with the control group without treatment.

Cell proliferation assay

Briefly, HDF-D cells were seeded on 96-well plates at a density of 1×10^5 cell/well and incubated at 37°C overnight. The cells were treated with various concentrations (15–500 µg/mL) of the MO leaf EtOAc fraction as described in the cell viability section and incubated for 24 h. The plates were then incubated with 10 µL of Cell Counting Kit-8 (CCK-8) solution for 4 h. The absorbance of samples was measured by microplate reader at 450 nm with a reference at 630 nm. To determine the cell proliferation rate, the graph of absorbance against the number of cells was plotted according to the manufacturer's instructions. All the experiments were performed in triplicate.

Scratch assay

The scratch assay was conducted according to a previously established method in our research group.[25] Briefly, HDF-D cells at a cell density of 2×10^5 cells/well were seeded into a 24-well plate and incubated at 37°C in an atmosphere of 5% CO_2. After incubation, cells were scraped with a P200 pipette tip, and the media was removed from each well. MO leaf EtOAc fractions (12.5, 25, and 50 µg/mL), and the positive control drug, allantoin, were added to each well and photographed by phase contrast microscopy at ×4 magnification at 0 h. After an incubation period of 24 h, the second set of images was recorded. To determine the percentage of cell migration rate, the images were analyzed by ImageJ software (NIH, Bethesda, MD, USA).

Gas chromatography-mass spectrometry analysis

The GC-MS analysis of the MO leaf EtOAc fraction was performed using an Agilent 6890N series II GC interfaced with an Agilent 5973 series quadrupole mass spectrometer (Palo Alto, CA, USA) and equipped with an Agilent 76,73A autosampler. Helium gas (99.999%) was used as the carrier gas with a constant flow rate of approximately 1 mL/min. Mass transfer line and injector temperatures were set at 220°C and 290°C, respectively. The oven temperature was set to increase from 50°C to 150°C at 3°C/min, held isothermally for 10 min, and finally raised to 250°C at 10°C/min.

The MO leaf EtOAc fractions were diluted to 10 mg/mL in methanol. The diluted samples were injected in the split mode with a split ratio of 120:1. The delay time was 2 min, and the total running time was 120 min. The relative amounts of the chemical ingredients in the MO leaf EtOAc fractions were expressed as a percentage by peak area normalization. The relative percentage of each component was determined by comparing its average peak area to the total area. The software used to handle mass spectra and chromatograms was a GC-MS solution (Version 2.53).

Statistical analysis

Data are presented as the mean ± standard deviation. The statistical significance between groups was analyzed by one-way ANOVA using SPSS software version 21.0 (SPSS, Chicago, Illinois, USA). The results obtained at the end of experiment were compared with those of the control and diabetic groups using a Student's *t*-test. Values of $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant.

RESULTS

Antibacterial activity of *Moringa oleifera* ethyl acetate fraction

The antibacterial activity of the MO leaf EtOAc fraction was tested against 16 pathogenic bacterial strains: six Gram-positive (*Staphylococcus aureus*, *Streptococcus faecalis*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus thuringiensis*) and 10 Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Salmonella typhimurium*, *Pseudomonas solanacearum*, *Enterobacter aerogenes*, *Acinetobacter anitratus*, *Klebsiella pneumonia*, *Serratia marcescens*, *Staphylococcus epidermidis*, and *Salmonella choleraesuis*). These bacterial strains were selected on the basis of their skin infection-causing behavior in diabetic patients. The results of the antibacterial activities are presented in Table 1. The MO leaf EtOAc fraction produced inhibition zone diameters ranging from 7 to 31 mm, and the most noteworthy results were exhibited by Gram-positive bacteria. The diameters were calculated including the 6-mm filter paper disc. Streptomycin was used as the standard antibiotic and showed significant antibacterial activity against all the test organisms.

Antioxidant activity of *Moringa oleifera* ethyl acetate fraction

Total phenolic content and total flavonoid content

Table 2 shows the total phenolic and total flavonoid contents of the MO leaf EtOAc fraction. The TPC was calculated from the calibration curve ($y = 0.0015x + 0.0475$) with a regression value of 0.9931, and the MO leaf EtOAc fraction had a TPC value of 65.81 ± 0.01 mg/g. This value indicated that 1 mg of plant extract contained an amount of phenol equivalent to 65.81 mg of pure gallic acid. TPC in the EtOAc fraction is predicted to be higher than other solvent fractions.[26] In short, the results suggest the MO leaf EtOAc fraction is a rich source of polyphenolic compounds. The total flavonoid content of the MO leaf EtOAc fraction is

Table 1: Antibacterial activity of *Moringa oleifera* leaf ethyl acetate fractions by agar disc diffusion method

Inhibition zone (mm) MO EtOAc fraction (100 mg/mL)			
Test microbes	**EtOAc fraction**	**Gram-positive (%)**	**Standard**
Streptococcus pyogenes	30.33±0.6	72.8	41.67±2.5
Streptococcus faecalis	13.33±2.1	57.95	23±2
Bacillus subtilis	13±10.1	43.33	30±0.1
Bacillus cereus	11.67±0.6	35.72	32.67±1.2
Staphylococcus aureus	7.67±1.2	20.54	37.33±2.1
Bacillus thuringiensis	-		37±2.6
Test microbes	**EtOAc fraction**	**Gram-negative (%)**	**Standard**
Proteus mirabilis	20±0	48.78	41±1
Salmonella typhimurium	10±1	27.78	36±4.6
Pseudomonas solanacearum	-		32.67±3.7
Enterobacter aerogenes	-		33±2.61
Pseudomonas aeruginosa	-		30±2
Acinetobacter anitratus	-		33±2
Klebsiella pneumoniae	-		24.67±4.51
Serratia marcescens	-		27±0
Staphylococcus epidermidis	-		25±0.58
Salmonella choleraesuis	-		31±1

The values are the mean±SD of three parallel measurements. >21 mm: Large zone of inhibition; 10-20 mm: Moderate zone of inhibition; 1-9 mm: weak zone of inhibition; -: No inhibition zone. The results are expressed as a percentage of inhibition (%) compared with the standard antibiotic streptomycin. EtOAc: Ethyl acetate; SD: Standard deviation; MO: *Moringa oleifera*

Table 2: Total phenolic and total flavonoid content in the *Moringa oleifera* leaf ethyl acetate fraction

Total phenolic content	Total flavonoid content
65.81±0.01	37.1±0.03

The values given are the mean of triplicate measurements±SD. Phenolic content is expressed as mg GAE/g of plant extract. Flavonoid content is expressed as mg Ru/g of plant material. GAE: Gallic acid equivalents; RU: Rutin equivalents; SD: Standard deviation

presented in Table 2. The total flavonoid content was calculated from the calibration curve (y = 0.001x + 0.0635) with a regression value 0.9964. From the rutin standard curve of various concentrations, the MO leaf EtOAc fraction had a total flavonoid content of 37.1 ± 0.03 mg/g.

1-diphenyl-2-picryl hydrazyl free radical scavenging activity

The scavenging effects of the MO leaf EtOAc fraction on the DPPH radical are illustrated in Figure 1 and show that the fraction significantly reduced DPPH radicals. The scavenging activity increased in a concentration-dependent manner due to the scavenging capacity of the fraction and was comparable to ascorbic acid. The IC_{50} value signifies the concentration required to scavenge 50% of the initial DPPH radicals. The IC_{50} value of the MO leaf EtOAc fraction was 18.21 ± 0.06 µg/mL; in comparison, the standard (ascorbic acid) had an IC_{50} of 13.78 ± 0.02 µg/mL.

Hydrogen peroxide scavenging activity

Figure 2 shows the scavenging activity of the MO leaf EtOAc fraction on hydrogen peroxide. The fraction scavenged hydrogen peroxide in a concentration-dependent manner. Evaluation of the IC_{50} shown in Figure 2 demonstrated that the fraction exhibited moderate antioxidant activity with an IC_{50} value of 59.22 ± 0.04 µg/mL. This result was lower than the reference standard, ascorbic acid (IC_{50}: 47.27 ± 0.09 µg/mL).

Effect of Moringa oleifera ethyl acetate fraction on cell viability

The cytotoxic effect of isolated MO leaf EtOAc fraction was evaluated by MTT assay on HDF-D cells exposed to various concentrations (15.62, 31.25, 62.5, 125, 250, and 500 µg/mL) of fraction. Cell survival analyses [Figure 3] showed concentration-dependent inhibition of

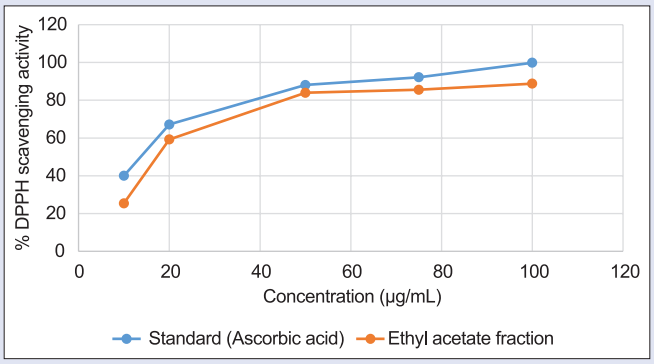

Figure 1: 1-diphenyl-2-picryl hydrazyl scavenging activity of the ethyl acetate fraction of *Moringa oleifera* leaves at various concentrations. Values are mean ± standard deviation (n = 3). The 1-diphenyl-2-picryl hydrazyl scavenging activity of ethyl acetate fraction increased in a concentration-dependent manner comparable to ascorbic acid

the fraction on HDF-D cell growth. Higher concentrations (125, 250, and 500 µg/mL) of the fraction increased cytotoxicity by about 40% in HDF-D cells; hence, we selected a nontoxic concentration of the fraction to investigate its wound healing properties.

Effect of Moringa oleifera ethyl acetate fraction on cell proliferation

The cell proliferative properties of MO leaf EtOAc fraction were evaluated in HDF-D cells using a CCK-8 assay kit according to the manufacturer's instructions. Cells were treated with a serial dilution of the EtOAc fraction for 24 h as described in the cell viability section. As shown in Figure 4, treatment with this fraction caused an irregular fluctuation in the percentage of cells, which indicated that the fraction did not exert a concentration-dependent effect on the growth of HDF-D cells although lower concentrations of the fraction slightly increased cell proliferation.

Effect of Moringa oleifera on wound healing activity

In vitro migration assays were performed to investigate the wound healing potential of the MO leaf EtOAc fraction in HDF-D cells

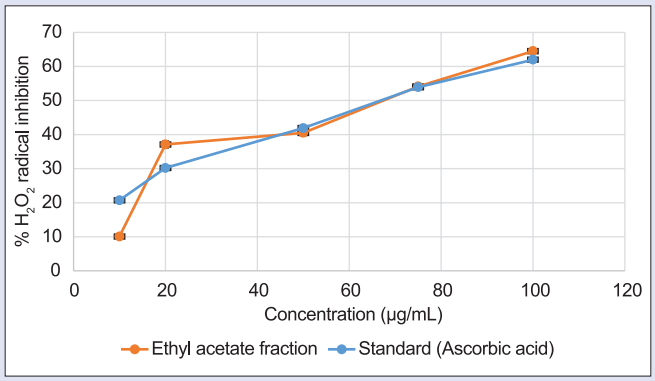

Figure 2: Hydrogen peroxide scavenging activity of *Moringa oleifera* leaf ethyl acetate fraction at various concentrations. Values are mean ± SD (n = 3). The H_2O_2 radical inhibition activity of ethyl acetate fraction increased in a concentration-dependent manner comparable to ascorbic acid

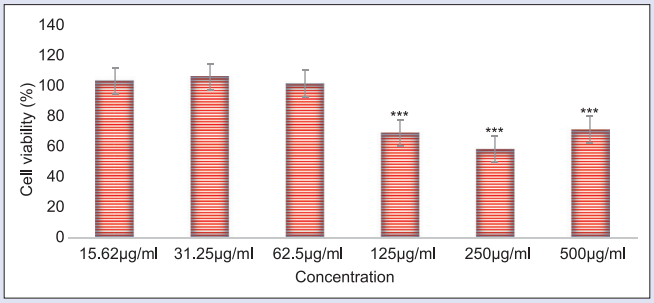

Figure 3: The cytotoxicity of *Moringa oleifera* leaf ethyl acetate fraction treatment in diabetic human dermal fibroblast cells was assessed. Cells were treated with various concentrations of isolated fractions (15.62, 31.25, 62.5, 125, 250, and 500 µg/mL) for 24 h. After the incubation period, the viability of fraction-treated cells was evaluated by 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide assay. Values are presented as the mean percentage ± standard deviation of three replicates. ***P < 0.001 versus control group

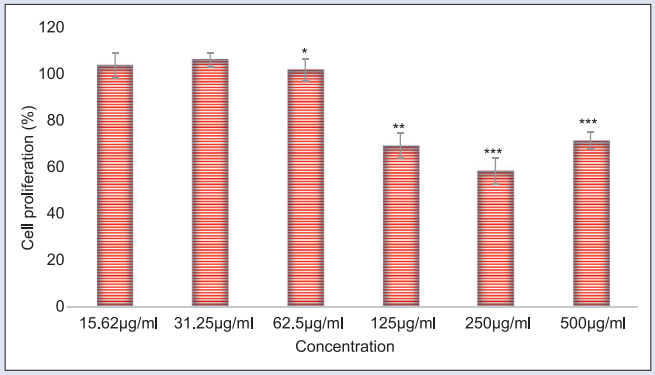

Figure 4: The proliferative effect of the *Moringa oleifera* leaf ethyl acetate fraction on diabetic human dermal fibroblast cells. Cells were seeded in a 96-well plate, different concentrations of *Moringa oleifera* ethyl acetate fraction were added, and the cells were left to stand for 24 h. The proliferative effect was measured by Cell Counting Kit-8 assay kit and calculated by a comparison of the values from the ethyl acetate fraction treatment group with the control group. Data are expressed as mean ± standard deviation from three individual experiments. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group

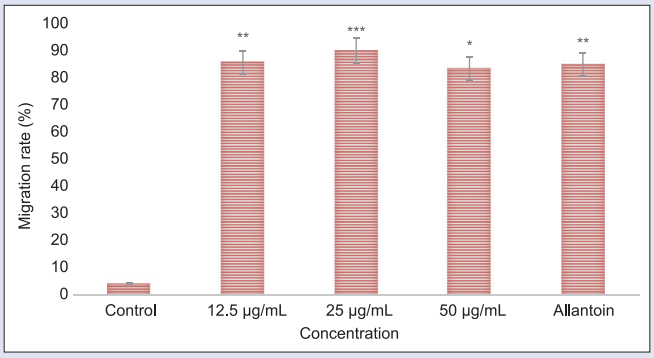

Figure 5: The percentage of cell migration rate of *Moringa oleifera* leaf ethyl acetate fraction treated wound in diabetic human dermal fibroblast. Cells were seeded into 6-well plates with the addition of different concentrations of *Moringa oleifera* ethyl acetate leaves fraction for 24 h. The migration rate of *Moringa oleifera* leaf ethyl acetate fraction-treated wounds in diabetic human dermal fibroblast cells was evaluated at 0 and 24 h after treatment and calculated by Image-J software. Results are expressed as mean ± standard deviation from each individual experiment. *P < 0.05, **P < 0.01***P < 0.001 versus control group

[Figures 5 and 6]. Different concentrations of the MO EtOAc leaves fraction (12.5, 25, and 50 µg/mL) were applied for 24 h after wound scratching. The wound coverage was significantly increased for HDF-D cells at all concentrations compared with the control group. Although the control group cells slightly closed the scratched area, MO leaf EtOAc fraction-treated HDF-D cells were found to migrate faster following a 24-h incubation period. Rapid cell migration and wound closure rate of the fraction-treated HDF-D cells was observed, and these effects were comparable with the positive control drug allantoin, especially at a treatment concentration of 25 µg/mL [Figure 6]. In comparison with the control group, wound closure rates were enhanced by 82% and 87% for treatment.

Gas chromatography-mass spectrometry analysis

Interpretation of the GC-MS spectra of the MO leaf EtOAc fraction was conducted using the National Institute Standard and Technique (NIST) database. The spectrum of the unidentified components was compared with the spectra of known components stored in the NIST library. Qualitative analyses of the fraction using GC-MS showed the presence of 17 prominent peaks in the chromatogram out of a total of 30 [Figure 7]. The peak area concentration (%), retention time, and peak identities of the compounds are presented in Table 3.

DISCUSSION

MO is known as a valuable food source because of its high nutritional content and physiological properties.[33] The principal constituents of MO leaves are water, protein, sugar, mineral salts, and fatty acids.[34,35] Several of these sugar substances are pharmacologically active, including L-arabinose, D-mannose, D-galactose, L-rhamnose, and D-xylose and have been shown to enhance wound healing in multiple extensive studies. Furthermore, many researchers have demonstrated that natural sugars, such as D-mannose and D-glucose, have wide variety of antimicrobial functionalities.[36,37] In addition, MO leaves contain many fatty acids including lauric acid, myristic acid, palmitic acid, arachidonic acid, and oleic acid[38,39] that have a similar molecular structure to 10-HDA. 10-HDA is a bioactive compound found in royal honey that has been shown to enhance wound healing in various extensive studies. This type

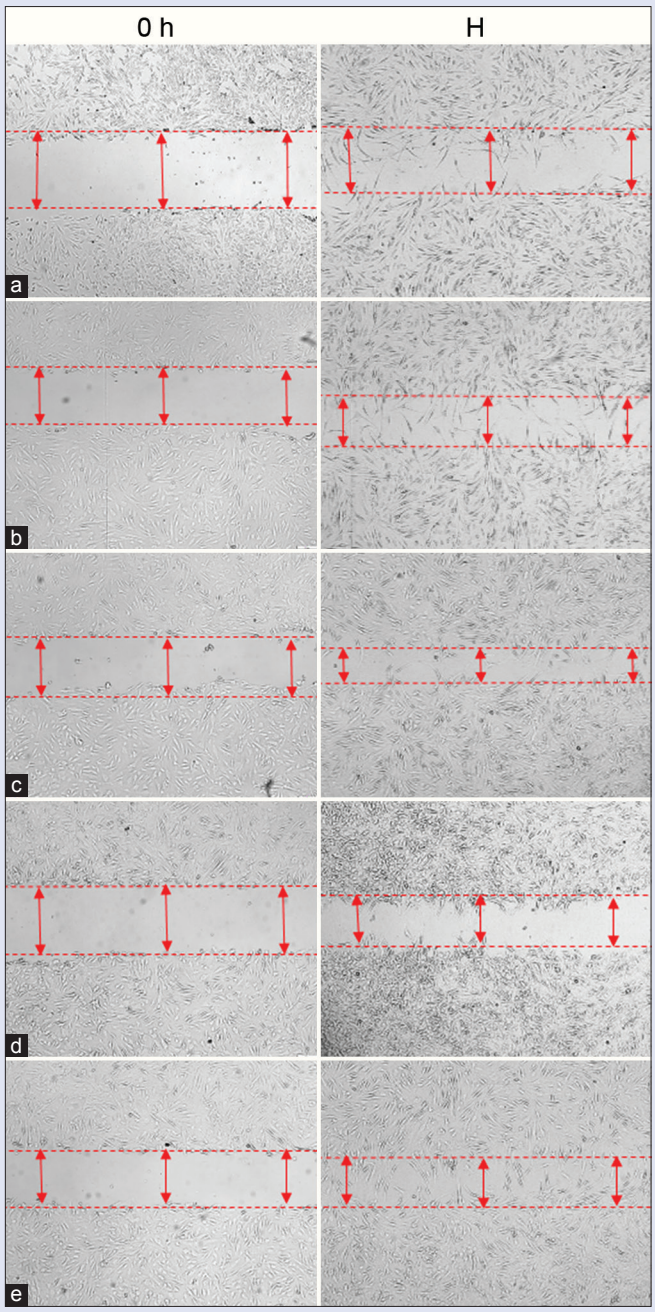

Figure 6: The migration rate of the fraction on diabetic human dermal fibroblast cells. The various concentrations (12.5, 25, and 50 µg/mL) of *Moringa oleifera* leaf ethyl acetate fraction were treated on a wound created in diabetic human dermal fibroblast observed by scratch assay. Human dermal fibroblast-diabetic cells were scratched with p200 pipette tips, and photos were captured at 0 h. After the treatment with various concentrations of *Moringa oleifera* leaf ethyl acetate fraction, the cells were photographed after 24 h incubation (a) Control, (b) *Moringa oleifera* leaf ethyl acetate fraction 12.5 µg/mL, (c) *Moringa oleifera* leaf ethyl acetate fraction 25 µg/mL, (d) *Moringa oleifera* leaf ethyl acetate fraction 50 µg/mL, (e) allantoin 50 µg/mL

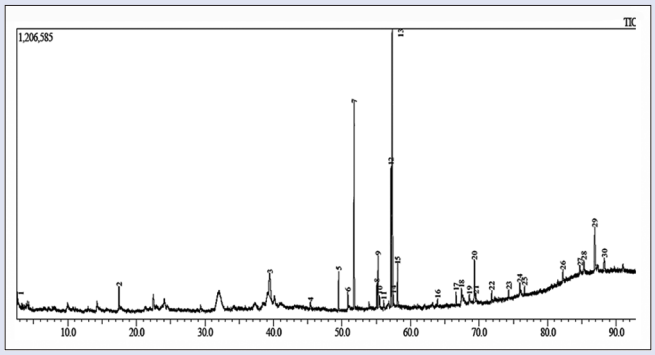

Figure 7: Gas chromatogram obtained for ethyl acetate fraction of *Moringa oleifera* leaf extract

of fatty acids triggers fibroblasts to induce various growth factors in the wound, in particular transforming growth factor-β1 (TGF-β1) and vascular endothelial growth factor (VEGF).[40]

In vitro antibacterial analysis of MO leaf EtOAc fraction showed a potential inhibition of Gram-positive bacteria but only moderate activity against Gram-negative bacteria. The difference in antibacterial efficiency may be attributed to the different bacterial structures: the cell wall in Gram-positive bacteria consists of a single layer while the Gram-negative cell wall is a multilayered structure confined by an outer cell membrane.[41] Our findings indicated that the MO leaf EtOAc fraction may be a novel candidate for dermal wound healing because of the effectiveness of its antibacterial properties against various pathogens, especially skin infection-causing pathogens.

Phenols are major phytoconstituents in plants; flavonoids are primarily derivatives of phenolic compounds. Phenolics act mainly as free radical scavengers, conferring oxidative stress tolerance on plants.[42] In the present study, phenolic levels in MO leaf EtOAc MO fraction were found to be relatively high and the estimation of antioxidant activity of showed a positive correlation between antioxidant activity and phenolic content. However, the flavonoid content the fraction was much lower than the phenol content. A similar trend of antioxidant content in MO leaves was reported by Iqbal and Bhanger.[43] Antioxidants present in plants enhance the healing of wounds by quenching the free radicals and the prevention of the cellular damage caused by free radicals. The redox properties of antioxidants could delay or prevent the onset of degenerative diseases. This property allows them to act as hydrogen donors or reducing agents, which improves regeneration and organization of the new tissue in wound healing.[44] Thus, the enhanced wound healing may be due to the free radical scavenging action of the fraction.

According to Guo and Dipietro,[45] healing impairment in diabetic ulcers has a number of physiological causes including diminished fibroblast proliferation and angiogenesis. The wound healing effect on HDF-D after treatment with the fraction supports the wound healing activity by promotion of the proliferation of fibroblast cells. As the crucial migration step of the wound healing process is impaired in diabetes, the fraction demonstrated a potent promotion of migration as determined by the increased response in migration scratch assay. Proliferation and migration are the manifestations for the development of new blood vessels from the preexisting vascular bed in angiogenesis.[46]

The growth factors TGF-β and VEGF are involved in fibroblast migration and angiogenesis, respectively.[47] The increased migration rate observed in HDF-D cells may be the influence of growth factors which are involved in the wound healing process. The wound healing properties may be attributed to the phytocompounds presents in the EtOAc fraction. These statements were justified by the GC-MS analysis of the fraction which identified various medicinal compounds with wound healing effects including antioxidant, antibacterial, anti-inflammatory, and antidiabetic activity. Therefore, the MO leaf EtOAc fraction is a therapeutically beneficial agent with vital bioactive compounds that can improve diabetic wound healing by the stimulation of fibroblast

Table 3: Total ion chromatogram of *Moringa oleifera* leaf ethyl acetate fraction with retention time, peak area, and reported biological activities

Peak	RT	Area	Compound	Reported biological activities
1	49.504	743,026	Hexadecanoate<methyl->	Antioxidant, anti-inflammatory, antibacterial[27]
2	51.766	3,802,309	n-hexadecanoic acid	Antioxidant, hypocholesterolemic, anti-inflammatory, antibacterial[28]
3	51.766	3,802,309	Hexadecanoic acid, ethyl ester	Antioxidant, hypocholesterolemic nematicide, pesticide, lubricant, antiandrogenic, flavor, hemolytic 5-alpha reductase inhibitor
4	55.070	496,083	9,12-octadecadienoic acid (Z, Z)-, methyl ester	Anti-inflammatory, hypocholesterolemic, antibacterial, hepatoprotective, antihistaminic, antiarthritic, anticoronary[28]
5	55.272	1,169,479	9-octadecenoic acid (Z)-, methyl ester	
7	55.491	175,211	Octadecanoate<methyl->	
8	56.072	2,732,882	Z-6,17-octadecadien-1-ol acetate	
9	57.139	747,625	6-octadecenoic acid, (Z)-	
10	58.110	763,360	Octadecanoic acid, ethyl ester	
11	55.491	31,479	Phytol	Antimicrobial, anticancer, cancer preventive, diuretic, anti-inflammatory[28]
12	63.937	172,373	Eicosanoate<ethyl->	Antioxidant, pesticide, flavor, 5-alpha-reductasein-hibitor, antifibrinolytic, hemolytic[29]
13	66.649	763,360	Pentacosane<n->	Antibacterial[30]
14	71.818	265,511	Heptacosane<n->	Antibacterial[30]
15	74.331	203,748	Tetracosanoate<ethyl->	Antibacterial[31]
16	76.626	184,272	Nonacosane<n->	Antibacterial[30]
17	82.217	292,776	Vitamin E	Antiaging, analgesic, antidiabetic, anti-inflammatory, antioxidant, antidermatitic, antileukemic, antitumor, anticancer, hepatoprotective, hypocholesterolemic, antiulcerogenic, vasodilator, antispasmodic, antibronchitic, anticoronary[32]

RT: Retention time

growth, promotion of angiogenesis, and acceleration of healing rate with antibacterial properties.

CONCLUSION

Our results demonstrated that the MO leaf EtOAc fraction has significant wound healing activity. These conclusions validate the use of this plant in folkloric medicine for the treatment of wounds and provide valuable scientific evidence that the EtOAc fraction was a promising complementary supplement for diabetic patients with wound healing defects. Further *in vivo* experiments on the detailed molecular mechanisms responsible for the enhancement the wound healing treatments in diabetes need to be conducted.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. World Health Organization. Global Report on Diabetes. Geneva: World Health Organization; 2016.
2. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: Estimates for the year 2000 and projections for 2030. Diabetes Care 2004;27:1047-53.
3. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: Prevalence, numerical estimates, and projections. Diabetes Care 1998;21:1414-31.
4. Singh N, Armstrong DG, Lipsky BA. Preventing foot ulcers in patients with diabetes. JAMA 2005;293:217-28.
5. Younes NA, Ahmad AT. Diabetic foot disease. Endocr Pract 2006;12:583-92.
6. Menke NB, Ward KR, Witten TM, Bonchev DG, Diegelmann RF. Impaired wound healing. Clin Dermatol 2007;25:19-25.
7. Edwards R, Harding KG. Bacteria and wound healing. Curr Opin Infect Dis 2004;17:91-6.
8. Singhal A, Gupta H, Bhati V. Wound healing activity of *Argyreia nervosa* leaves extract. Int J Appl Basic Med Res 2011;1:36-9.
9. Gbedema SY, Emelia K, Francis A, Kofi A, Eric W. Wound healing properties and kill kinetics of *Clerodendron splendens* G. Don, a Ghanaian wound healing plant. Pharmacognosy Res 2010;2:63-8.
10. Balachandar R, Saran PL, Ashok KK, Ragavi A, Gurumoorthy P. Antioxidant activity and wound healing potential of selected medicinal plants. J Chem Pharm Sci 2014;2:100-3.
11. Pal SK, Mukherjee PK, Saha K, Pal M, Saha BP. Antimicrobial action of the leaf extract of *Moringa oleifera* lam. Anc Sci Life 1995;14:197-9.
12. Kalogo Y, Rosillon F, Hammes F, Verstraete W. Effect of a water extract of *Moringa oleifera* seeds on the hydrolytic microbial species diversity of a UASB reactor treating domestic wastewater. Lett Appl Microbiol 2000;31:259-64.
13. Fernandes EE, Pulwale AV, Patil GA, Moghe AS. Probing regenerative potential of *Moringa oleifera* aqueous extracts using *in vitro* cellular assays. Pharmacognosy Res 2016;8:231-7.
14. Fard MT, Arulselvan P, Karthivashan G, Adam SK, Fakurazi S. Bioactive extract from *Moringa oleifera* inhibits the pro-inflammatory mediators in lipopolysaccharide stimulated macrophages. Pharmacogn Mag 2015;11 Suppl 4:S556-63.
15. Choudhary MK, Bodakhe SH, Gupta SK. Assessment of the antiulcer potential of *Moringa oleifera* root-bark extract in rats. J Acupunct Meridian Stud 2013;6:214-20.
16. Vyas S, Kachhwaha S, Kothari SL. Comparative analysis of phenolic contents and total antioxidant capacity of *Moringa oleifera* lam. Pharmacogn Res 2015;7:44-51.
17. Ryo K, Sayaka N, Tadahiko S, Kenya S, Masako I, Koji S, *et al.* Antihypertensive effect of water extracts from leaves of *Moringa oleifera* lam. on spontaneously hypertensive rats. Jpn Soc Nutr Food Sci 2008;55:183-5.
18. Mehta K, Balaraman R, Amin AH, Bafna PA, Gulati OD. Effect of fruits of *Moringa oleifera* on the lipid profile of normal and hypercholesterolaemic rabbits. J Ethnopharmacol 2003;86:191-5.
19. Jain PJ, Patil SD, Haswani NG, Girase MV, Surana SJ. Hypolipidemic activity of *Moringa Oleifera* Lam, Moringaceae, on high fat diet-induced hyperlipidemia in albino rats. Braz J Pharmacogn 2010;20:969-73.
20. Kalappurayil TM, Joseph BP. A review of pharmacognostical studies on *Moringa oleifera* lam. flowers. Pharmacogn J 2016;9:1-7.
21. Fakurazi S, Sharifudin SA, Arulselvan P. *Moringa oleifera* hydroethanolic extracts effectively alleviate acetaminophen-induced hepatotoxicity in experimental rats through their antioxidant nature. Molecules 2012;17:8334-50.
22. Quettier-Deleu C, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx M, *et al.* Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. J Ethnopharmacol 2000;72:35-42.

23. Kumarasamy Y, Byres M, Cox PJ, Jaspars M, Nahar L, Sarker SD. Screening seeds of some Scottish plants for free radical scavenging activity. Phytother Res 2007;21:615-21.
24. Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis 1989;10:1003-8.
25. Muhammad AA, Pauzi NA, Arulselvan P, Abas F, Fakurazi S. *In vitro* wound healing potential and identification of bioactive compounds from *Moringa oleifera* Lam. Biomed Res Int 2013;2013:974580.
26. Mooza A, Nora A, Shah AK. GC-MS analysis, determination of total phenolics, flavonoid content and free radical scavenging activities of various crude extracts of *Moringa peregrina* (Forssk.) Fiori leaves. Asian Pac J Trop Biomed 2014;4:964-70.
27. Wang LJ, Muh L, Liu HJ, Bhandari B, Saito M, Li LT. Volatile components in three commercial douchies: A Chinese traditional salt-fermented soybean food. Int J Food Properties 2010;13:1117-33.
28. Sermakkani M, Thangapandian V. GC-MS analysis of *Cassia italica* leaf methanol extract. Asian J Pharm Clin Res 2012;5:110-4.
29. Priya D, Patil A, Niranjana S, Chavan A. Potential testing of fatty acids from mangrove *Aegiceras corniculatum* (L.) blanco. Int J Pharm Sci 2012;3:569-71.
30. Mihailović V, Vukovic N, Niciforovic N, Solujic S, Mladenovic M, Masko-Vic P, *et al*. Studies on the antimicrobial activity and chemical of the essential oils and alcoholic extracts of *Gentiana asclepiadeal*. J Med Plant Res 2011;5:1164-74.
31. Mellou F, Loutrari H, Stamatis H, Roussos C, Kolisis FN. Enzymatic esterification of flavonoids with unsaturated fatty acids: Effect of the novel esters on vascular endothelial growth factor release from K562 cells. Process Biochem 2006;41:2029-34.
32. Rajeswari V, Vijayalakshmi S, Gajalakshmi S. Phytochemical and pharmacological properties of *Annona muricata*. Int J Pharm Sci 2012;4:3-6.
33. Bhomika RG, Babita BA, Ramesh KG, Anita AM. Phyto-pharmacology of *Moringa oleifera*. Nat Prod Radiance 2007;6:347-53.
34. Faizi S, Siddiqui BS, Saleem R, Siddiqui S, Aftab K, Gilani AH. Isolation and structure elucidation of new nitrile and mustard oil glycosides from *Moringa oleifera* and their effect on blood pressure. J Nat Prod 1994;57:1256-61.
35. Makkar HP, Becker K. Nutritional value and antinutritional components of whole and ethanol extracted *Moringa oleifera* leaves. Anim Feed Sci Technol 1996;63:211-28.
36. Abdul KM, Kabir Pijush D, Anwar MN. Antibacterial and antifungal evaluation of some derivatives of methyl α-D-Mannopyranoside. Int J Agric Biol 2005;7:754-6.
37. Kabir AK, Matin MM, Hossain A, Sattar MA. Synthesis and antimicrobial activities of some rhamnopyranoside derivatives. J Bangladesh Chem Soc 2003;16:85-93.
38. Farooq A, Sajid L, Muhammad A, Anwarul HG. *Moringa oleifera*: A food plant with multiple medicinal uses. Phytother Res 2006;21:17-25.
39. Chinwe CI, Maria JS, Carmita JJ, Fausto D. Phytochemical and nutritional properties of dried leaf powder of *Moringa oleifera*. Asian J Plant Sci Res 2015;5:8-16.
40. Izuta H, Chikaraishi Y, Shimazawa M, Mishima S, Hara H. 10-Hydroxy-2-decenoic acid, a major fatty acid from royal jelly, inhibits VEGF-induced angiogenesis in human umbilical vein endothelial cells. Evid Based Complement Alternat Med 2009;6:489-94.
41. Mahomoodally MF, Gurib-Fakim A, Subratty AH. Screening for alternative antibiotics: An investigation into the antimicrobial activities of medicinal food plants of Mauritius. J Food Sci 2010;75:M173-7.
42. Rashed AN, Afifi FU, Disi AM. Simple evaluation of the wound healing activity of a crude extract of *Portulaca oleracea* L. (growing in Jordan) in *Mus musculus* JVI-1. J Ethnopharmacol 2003;88:131-6.
43. Iqbal S, Bhanger MI. Effect of season and production location on antioxidant activity of *Moringa oleifera* leaves grown in Pakistan. J Food Compos Anal 2006;19:544-51.
44. Leite SN, Palhano G, Almeida S, Biavattii MW. Wound healing activity and systemic effects of *Vernonia scorpioides* gel in guinea pig. Fitoterapia 2002;73:496-500.
45. Guo S, Dipietro LA. Factors affecting wound healing. J Dent Res 2010;89:219-29.
46. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000;407:249-57.
47. Ucuzian AA, Gassman AA, East AT, Greisler HP. Molecular mediators of angiogenesis. J Burn Care Res 2010;31:158-75.