IJPSR (2024), Volume 15, Issue 3 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 24 July 2023; received in revised form, 20 October 2023; accepted, 30 December 2023; published 01 March 2024

PHYTOCHEMICAL ANALYSIS, ELEMENTAL CONTENT, AND FIBROBLAST PROLIFERATION ACTIVITY OF THE HYDRO-ETHANOLIC EXTRACT OF *MORINGA OLEIFERA* LAM LEAVES

E. Oppong Bekoe [*1], E. Orman [2], N. A. Offei [3, 4], K. Donkor [5] and A. R. Aikins [3, 4]

Department of Pharmacognosy & Herbal Medicine [1], School of Pharmacy, University of Ghana, Ghana.
Department of Pharmaceutical Chemistry [2], University of Health and Allied Sciences, Ghana.
Department of Biochemistry [3], Cell and Molecular Biology, University of Ghana.
West African Centre of Infectious Pathogens (WACCBIP) [4], University of Ghana, Ghana.
Centre for Plant Medicine Research Mampong-Akuapem [5], Ghana.

Keywords:

Moringa oleifera, Phytochemical analysis, Elemental analysis, Fibroblast proliferation, Wound healing

Correspondence to Author:
Emelia Oppong Bekoe

Department of Pharmacognosy & Herbal Medicine, School of Pharmacy, University of Ghana, Ghana.

E-mail: eoppongbekoe@ug.edu.gh

ABSTRACT: *Moringa oleifera* is one plant that is multi-purposefully used for the prevention and treatment of numerous diseases. This study analyzed the phytochemical and elemental contents of *M. oleifera* leaf extract, and evaluated its effect on fibroblast proliferation, which is crucial for wound healing. *M. oleifera* leaf samples were collected, identified, and extracted using solvents of different polarities. High-performance thin-layer chromatography (HPTLC) and ultra-high-performance liquid chromatography (UPLC) were employed to analyze the phytochemical composition, while inductively coupled plasma-optical emission spectrometry (ICP-OES) was used to determine the elemental content. The effect of the hydroethanolic extract was investigated on proliferation of Hs27 fibroblast skin cells by the MTT assay. Water extraction yielded the highest extract of 13.0±3% w/w, while petroleum ether extraction yielded the least of 3.03±0.8% w/w. HPTLC fingerprinting of the 50% hydroethanolic extract identified bioactive compounds including flavonoids, phenol carboxylic acids fatty acids, and sterols. UPLC analysis confirmed the presence of rutin and kaempferol in the extract. Elemental analysis showed a high concentration of Ca, K, Br, Mg, and P and the presence of high concentrations of heavy metals. Furthermore, the extract exhibited significant fibroblast proliferation activity at concentration of 0.1 - 1 µg/mL. The presence of bioactive compounds and essential elements in *M. oleifera* along with the evaluation of Hs27 fibroblast proliferation, highlights its possible role in enhancing wound healing and possible therapeutic applications. The documented fingerprints are also essential for quality control.

INTRODUCTION: *Moringa oleifera* Lam, commonly known as drumstick or moringa, is a medicinal plant belonging to the Moringaceae family, and is distributed in many countries of the tropics and subtropics. It is the best known of all the native *Moringa genus*. For centuries, it has been used in Ayurvedic and Unani medicine [1, 2]. *M. oleifera* is a versatile plant, with almost all its parts having beneficial effects in both nutrition and medicine [3].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.15(3).813-21

This article can be accessed online on www.ijpsr.com

DOI link: https://doi.org/10.13040/IJPSR.0975-8232.15(3).813-21

It has widespread uses in nutritional supplementation, folkloric medicine, culinary uses, water purification, cosmetics and skincare, agricultural purposes among others [4-9]. Moringa is available on the market as Moringa powder (pulverized dried leaves), moringa oil (oil extracted from seeds), moringa teas, moringa capsules or

tablets, Moringa leaf extract, moringa seed powder, moringa soap *etc*. [4, 6]. This plant has a wide range of nutritional and bioactive compounds, including proteins, essential amino acids, carbohydrates, lipids, fibre, vitamins, minerals, phenolic compounds, saponins, isothiocyanates, phytosterols and others [1, 2]. The leaves, roots, seed, bark, fruit, flowers, and immature pods of this plant have been demonstrated experimentally to be beneficial in several chronic conditions, including high blood pressure, diabetes, hypercholesterolemia, cancer, non-alcoholic liver disease, cardioprotective, antimicrobial, hepatoprotective, and general inflammation. It also has antipyretic, antiepileptic, antispasmodic, diuretic, and antioxidant effects. This plant is also known to possess wound healing activity [10, 11]. For such a plant with numerous formulations, quality control is necessary to ensure safety, consistency, and in effect efficacy. This study therefore sought to investigate the phytochemical and elemental contents, of the extract of *Moringa oleifera* leaves through HPTLC and UPLC analysis and also to evaluate its effect on fibroblast proliferation which could be critical to wound healing. **Fig. 1** displays a picture of *M. oleifera* leaves and flowers.

FIG. 1: PICTURE OF PARTS OF *M. OLEIFERA* A. LEAVES B. SEEDS C. FLOWERS D. FRUITS

MATERIALS AND METHODS:

Plant Sample Collection and Identification: *M. oleifera* samples leaves were harvested in March 2020 from Kwabenya in the Ga East Municipality and the University of Ghana, both in the Greater Accra Region of Ghana. Plant materials were identified by a Botanist at the Department of Pharmacognosy and Herbal Medicine, University of Ghana, where herbarium specimens have been deposited with voucher numbers MO03/2020/1-5. The herbal materials were thoroughly washed with distilled water and air dried in a dust-free environment for 3 weeks.

Extraction: Extracts were prepared from pulverized plant material of 2 g each with 20 mL each of water, ethanol 50% v/v and petroleum ether separately at room temperature. Extractions were made by ultra-sonication for 30 minutes in each solvent, and subsequently centrifuged at 8000 × g for 5 min. The supernatants were concentrated *in-vacuo* at 40 ^{o}C and lyophilized to obtain crude extracts.

High-Performance Thin Layer Chromatographic (HPTLC) Analysis:

Chemicals and Reagents: All chemicals were purchased in the highest quality available and used as received from VWR (Darmstadt, Germany). Highly purified deionized water was freshly obtained from Millipore® Simplicity (Billerica, U.S.A.).

Sample Preparation and Application: Ten microliters (10 µL) of 5 mg/mL extracts dissolved in methanol were then applied to HPTLC plates of 20 × 10 cm dimensions of silica gel 60 F254 (Merck). The extract solutions were applied as an 8 mm band with minimum of 11.4 mm distance between bands and 8 mm from lower edge of the plate, and the bands were dried.

Development: A 20 × 10 cm Twin Trough Chamber (CAMAG, Muttenz Switzerland), saturated for 15 minutes with about 10 mL mobile phase in each trough was used.

For the 50% ethanol extract, ethyl acetate-water-formic acid 90:5:5 (v/v) was used as the mobile phase, and for the petroleum ether extract, toluene-ethyl acetate 90:10 (v/v) was the mobile phase. The developing distance was about 70 mm from the lower edge of the plate. The plates were then allowed to air dry and documented before and after derivatization.

Reagent Preparation: Reagents were prepared according to protocols described by Wagner and Bladt [12]. Anisaldehyde sulphuric acid and Natural Product Reagent (naturstoff) reagents were used to detect the presence fatty acids and sterols, and flavonoids respectively. Anisaldehyde reagent was prepared by adding 20 mL of acetic acid, 10 mL of sulfuric acid, and 1 mL of anisaldehyde to 170 mL of ice-cooled methanol and well mixed. Naturstoff reagent was prepared by dissolving diphenyl boryloxyethylamine in methanol to produce 1 % w/v solution.

Detection and Documentation: The HPTLC analysis was performed on CAMAG TLC Visualizer 2 equipped with vision CATS software (version: 3.0) (Muttenz, Switzerland). The plates were examined both when underivatized and derivatized under white light, short UV λ 254 nm and long UV λ366 nm. For the petroleum ether extracts, the plates were derivatized with anisaldehyde reagent and then heated at 100 °C for five min. For the 50% ethanol extracts, the plates were derivatized with Naturstoff reagent and examination was performed under 366 nm for the presence of flavonoids.

Ultra High-Performance Liquid Chromatographic (UPLC) Analysis: The UPLC analysis of the 50% ethanolic extracts of *M. oleifera* was performed to develop the fingerprint chromatogram for the extract for its quality control with analytical markers established to be rutin and kaempferol. Instrumentation: Acquity™ UPLC, PDA eλ Detector, Quaternary Solvent Manager, Acquity UPLC H-Class Manager, Sample Manager FTN Acquity UPLC, Milford, U.S.A. Stationary Phase: Acquity UPLC HSS T3, 1.8 µm, C_{18} 2.1 × 100 mm (Waters, Milford, U.S.A.). Mobile phase: A: HCOOH (0.1%) in water, B: HCOOH (0.1%) in Acetonitrile; Gradient: t_{0min} A 98%, t_{1min} A 90%, t_{5min} A 90%, $t_{9\ min}$ A75%, t_{11min} A 75%, t_{14min} A 50%, t_{15min} A 50%, t_{20min} A 100%; flow 0.5 mL/min; injection volume 2µL; column temperature 40°C; detection λ 210–400 nm. Reference standards (97% HPLC) for external calibration: rutin (Sigma-Aldrich, Taufkirchen, Germany), and kaempferol (Roth, Karlsruhe, Germany) were dissolved in methanol at concentrations of 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL.

Elemental Content Analysis by Inductively Coupled Plasma-Optical Emission Spectrometric (ICP-OES)

Standard Solutions: The volumetric flasks for ICP-OES measurements were pre-treated with 2% v/v supra pure HNO_3 and purified water to minimize adsorption effects. The standard solutions of the metals were diluted between 1 and 5,000 µg/L with 2% *v/v* HNO_3 added from 1 g/L stock solutions of single-element standards.

Sample Preparation: A microwave digestion system (Multiwave Go, Anton Parr GmbH, Austria) was used to digest the plant samples. The samples (approximately 0.1 g each) were placed in Teflon vessels and 4 mL of HNO_3: HCl (3:1) was added to each. The vessels were heated at a temperature programme as follows: 10 minutes heating to 100 °C and holding for 5 minutes, then heating from 100 °C to 150 °C for the next 10 minutes and holding for additional 5 minutes. Complete digestion was confirmed by decolorization of the sample solutions. The digests were cooled down to room temperature, transferred to 50 mL volumetric flasks, and made to volume with deionized water. A blank of HNO_3: HCl (3:1) was used for the analysis.

Elemental Analysis by ICP-OES: The elemental analysis was carried out using ArCos MV II (Spectro Ametek, Kleve, Germany) ICP-OES with axial plasma viewing. Gas flows were controlled by internal mass-flow controllers. A standard D-torch was employed.

For sample introduction, the peristaltic pump of the system combined with a crossflow spray chamber was used under the following plasma conditions: 1200 W (RF-power), 13 L/min cooling gas, 0.8 L/min, and 0.8 L/min nebulizer gas.

Cell Culture: Human skin fibroblast line Hs27 was purchased from American Type Culture Collection. The cells were maintained in high glucose Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) (Gibco-Life Technologies, Carlsbad, CA) and 1% penicillin-streptomycin in 5 % CO_2 humidified atmosphere at 37 ^{o}C. Hs27 cells used were between passages of 20 to 28. The medium was replaced every 2 to 3 days. When the adherent cells reached 80% confluence, they were detached using 0.05% trypsin- EDTA, collected at 1000 rpm for 3 minutes, and seeded into fresh flasks.

Cell Proliferation Assay (MTT): Cytotoxicity and cell proliferation were assessed using the MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay previously described in literature [13]. A total of 6×10^3 Hs 27 cells in 90 µL of serum-free media were seeded into each well of 96 well tissue culture plate. The cells were then treated with 100, 10, 1, 0.1 µg/mL of the ethanol 50% extract for 48 h. TNF- α (0.1 ng/mL) (Sigma-Aldrich, St Louis, MO, USA) was used as a positive control. The cells were treated for 48 h with extract alone and TNF- α as a positive control. Afterward, 20 µL of 2.5 mg/ml MTT (Sigma-Aldrich, St Louis, MO, USA) was added to the cells and incubated for 4 hours at 37 °C. Acidified isopropanol (100 µL) was added to each well and kept in the dark for 30 minutes to stop the conversion of MTT to formazan and absorbance was measured at 590 nm using a microplate reader (Varioskan LUX, Thermo Fisher Scientific, USA).

Percentage viability was calculated for the concentrations using the absorbances of the formula:

% Cell viability = (Absorbance of treated wells-Absorbance of color control) / (Absorbance of untreated wells-Absorbance of blank) × 100%

Results were analyzed using One Way ANOVA, followed by Dunnett's multiple comparisons test.

RESULTS AND DISCUSSION:

Extract Yields: After extraction with the various solvents, the highest yield was observed in water, and the least in petroleum ether and methanol **Table 1.**

TABLE 1: EXTRACT YIELDS *M. OLEIFERA* LEAVES

Solvent	Water	Methanol 70%	Ethanol 50 %	Petroleum ether
Extract yield [%w/w]	13.0±3	4.6±1	10.4±1.8	3.03±0.8

HPTLC Fingerprinting: Analysis of the 50% hydroethanolic extract of *M. oleifera* showed possible presence of flavonoids as demonstrated by the presence of blue and orange fluorescent bands detected with the natural product reagent **Fig. 2**. Phenol carboxylic acids could be present due to the presence of blue bands. Anisaldehyde-sulphuric acid reagent showed the presence of phenols, terpenes, sugars, and steroids by the formation of violet, blue, red, grey or green bands at day light **Fig. 3** while typical blue, green or violet fluorescent zones were formed for saponins at λ 366 nm [12]. The TLC profiles under different detection conditions showed some degrees of differences and thus, demonstrates variations in the phytochemical compositions of the two extracts. Considering the hydroethanolic extract, the profile developed at 254 nm showed spots with R_fs similar to that observed in the profiles developed at 366 nm. However, there were some differences; for instance, after spraying with the Natural product reagent, two spots at R_fs ~0.58 and ~0.73 were conspicuous and distinguished its profile from the underivatized one. This was also confirmed from the videodensitograms of the profiles, where the red channel fingerprint for the derivatized profile **Fig. 3B** showed peaks for these two spots at the stated R_fs but were absent in the red channel extract of the underivatized profile **Fig. 2B**. Similarly, the profiles of petroleum ether extract developed at 366 nm also showed differences: one blue fluorescent spot at R_f~0.39 in the underivatized profile, and two blue fluorescent spots at R_fs ~0.25 and ~0.39, and one white fluorescent spot at R_f~0.15 in the derivatized profile. This was also confirmed in the videodensitograms, where the blue, green, and grayscale channels showed differences in peaks at the stated R_fs **Fig. 2-3.** The presence of flavonoids, phenol carboxylic acids, terpenes, sugars, steroids and saponins is confirmed by literature [1, 2]. As observed, the number of spots in the hydroethanolic extract were more than the number of spots in the

petroleum ether extract and this could be due to the different polarities of the extraction solvents. HPTLC analysis is a rapid chromatographic method for the detection of quality in raw plant materials and botanicals [14]. The HPTLC and UPLC fingerprints developed in this study can be used as a basis to establish the identification of moringa leaf samples, and for quantification of flavonoids and other bioactive components of the plant the fingerprint analysis forms the basis for further analysis, characterization, and standardization. By comparing the fingerprints of other leaves extract with the reference provided in this study, it becomes possible to identify some variations or possibly, falsification and adulteration in moringa-containing products. This information is crucial for ensuring consumer safety and confidence in nutraceuticals.

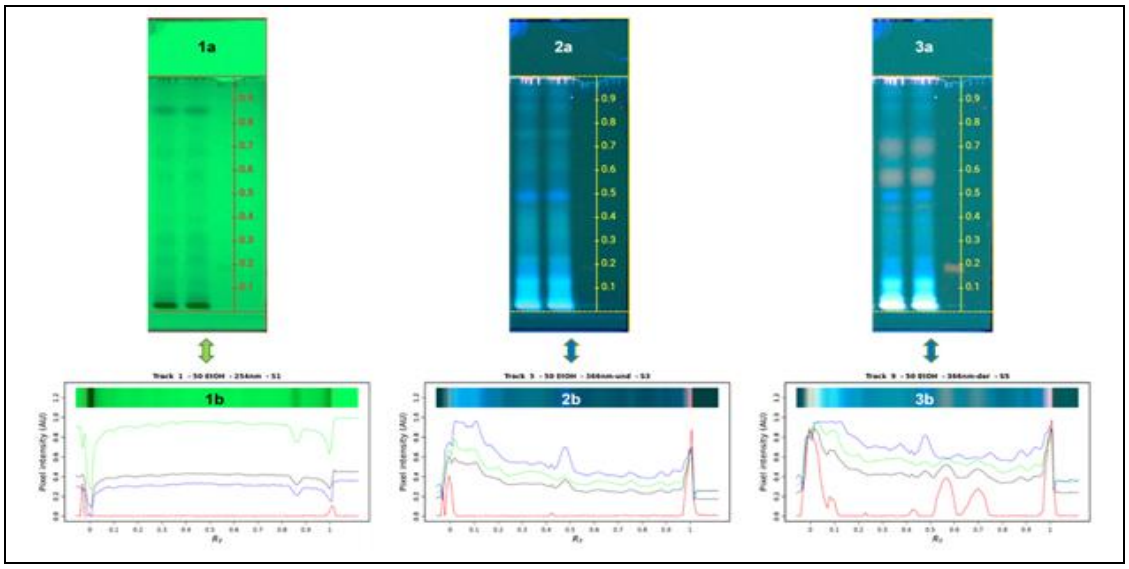

FIG. 2: TLC PROFILES AND VIDEO-DENSITOGRAMS OF HYDROETHANOLIC EXTRACT (50%) OF *MORINGA OLEIFERA* UNDER DIFFERENT DETECTION CONDITIONS. [1A, 1B] DETECTION WAS CARRIED OUT AT 254 NM ON AN UNDERIVATIZED PLATE. [2A, 2B] DETECTION WAS CARRIED OUT AT 366 NM ON AN UNDERIVATIZED PLATE. [3A, 3B] DETECTION WAS CARRIED OUT AT 366 NM ON A PLATE DERIVATIZED WITH NATURAL PRODUCT REAGENT

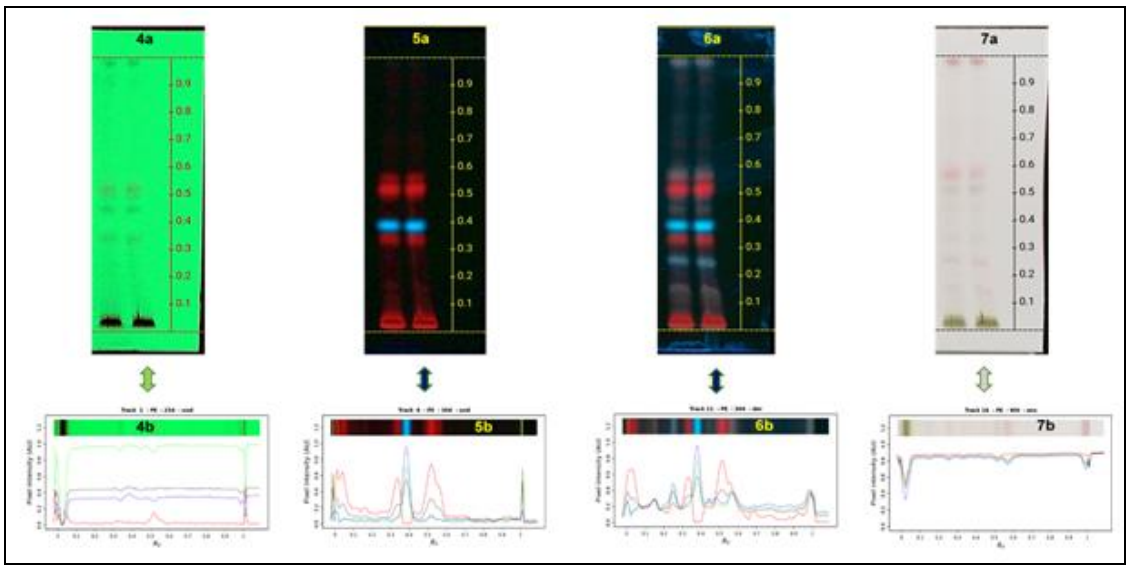

FIG. 3: TLC PROFILES AND VIDEO-DENSITOGRAMS OF PETROLEUM ETHER EXTRACT OF *M. OLEIFERA* UNDER DIFFERENT DETECTION CONDITIONS. [4A, 4B] DETECTION WAS CARRIED OUT AT 254 NM ON AN UNDERIVATIZED PLATE. [5A, 5B] DETECTION WAS CARRIED OUT AT 366 NM ON AN UNDERIVATIZED PLATE. [6A, 6B] DETECTION WAS CARRIED OUT AT 366 NM ON A PLATE DERIVATIZED WITH NATURAL PRODUCT REAGENT. [7A, 7B] DETECTION CARRIED OUT IN WHITE LIGHT ON A PLATE DERIVATIZED WITH ANISALDEHYDE-SULPHURIC ACID REAGENT

UPLC Fingerprinting: UPLC fingerprint analysis of the 50% ethanol extract of *M. oleifera* leaves is as provided below **Fig. 4**, with 6 distinct peaks. Peaks 4 and 7 at 8.01 and 12.76 minutes were identified as rutin and kaempferol respectively.

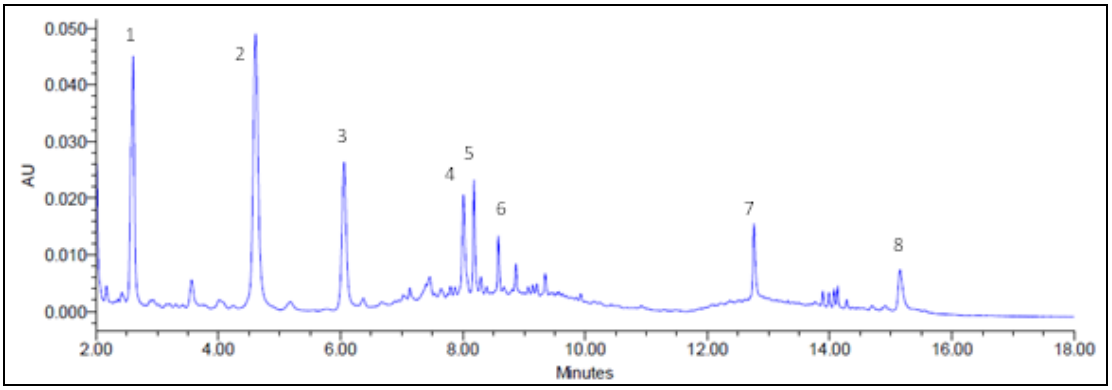

FIG. 4: TYPICAL UPLC CHROMATOGRAM OF 50% ETHANOL EXTRACT OF *M. OLEIFERA* Λ 260 NM. The retention times for the peaks were as follows: Peak 1 = 2.03±0.02 min; Peak 2 = 4.61±0.02 min; Peak 3 = 6.06±0.01 min; Peak 4 = 8.01±0.01 min; Peak 5 = 8.18±0.01 min; Peak 6 = 8.86±0.01 min, Peak 7= 12.76±0.01 min, Peak 8= 15.15±0.01 min

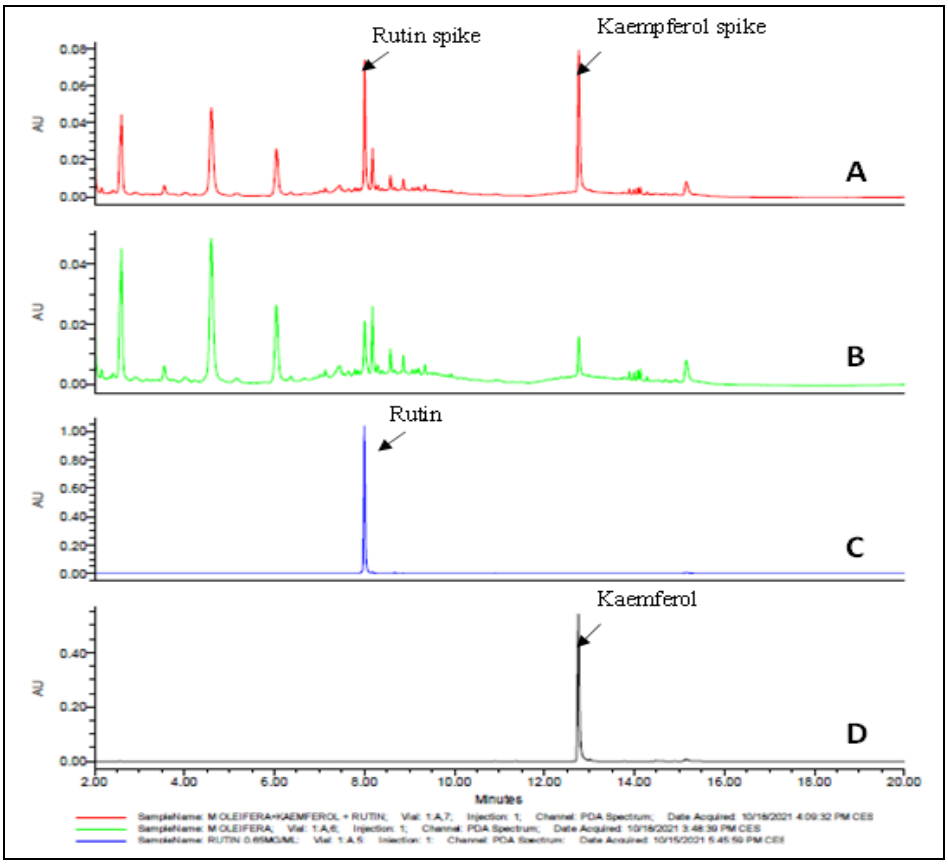

FIG. 5: UPLC CHROMATOGRAMS OF 50 % ETHANOL EXTRACT OF *M. OLEIFERA* AT Λ 260 NM; A. 50 % ETHANOL EXTRACT SPIKED WITH RUTIN AND KAEMPFEROL; B. 50 % ETHANOL EXTRACT; C. RUTIN 0.0625 MG/ML; D. KAEMPFEROL 0.0625 MG/ML

Elemental Content: Table 1 provides the details of the elemental content below. The highest concentrations of elements were observed with Ca, K, Br, Mg and P. On another hand As, Cd, Cr, Hg and Pb exceeded the recommended limits. The presence of Ca, K, Mg, P, S, Mn, Fe, Zn, and Cu in *M. oleifera* is well known [15]. However, calcium exhibited a relatively high concentration in comparison to many of the elements in moringa. Calcium also plays a crucial role in cell signalling which stimulate wound healing through the enhancement of angiogenesis, collagen synthesis, extracellular matrix protein synthesis and tissue granulation [16].

TABLE 2: ELEMENTAL CONTENT OF *M. OLEIFERA* LEAF SAMPLES

Element	Median (IQR) amount in *M. oleifera* (mg/kg)	Limit (mg/kg)
Al	61.94 (55.52–68.36)	
As	13.99 (7.00–20.97)	5
Ba	15.00 (11.26–15.27)	
Br	4244	
Ca	19340 (13639–19340)	
Cd	45.84	0.3
Cr	13.25 (12.67–17.19)	2
Cu	< LOD	150
Fe	330.4 (304.8–355.9)	
Hg	2.38	0.5
Ir	< LOD	
K	13687 (12528–14847)	
Mg	4020 (3982–4267)	
Mn	20.97 (20.32–20.98)	
Ni	39.79	
P	3257 (2919–3610)	
Pb	26.52	10
S	9271 (8835 – 10702)	
Sn	< LOD	
Sr	54.08	
Ti	< LOD	
Zn	13.98	

IQR – Interquartile range showing the lower quartile (Q1) and upper quartile (Q3) where applicable; LOD – Limit of detection.

Fibroblast Proliferation Activity: At a concentration of 1 µg/mL, *M. oleifera* extract gave a significant two-fold increase in the number of fibroblast cells. The increase in proliferation was significantly higher than that of the TNF-α positive control for all the extract concentration except for 10 µg/mL **Fig. 6.**

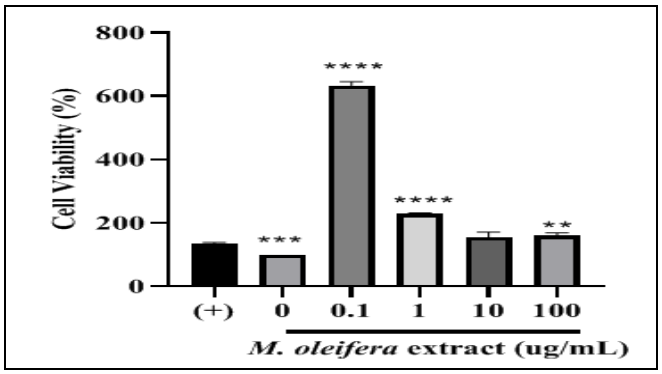

FIG. 6: EFFECT OF EXTRACTS OF *M. OLEIFERA* EXTRACT ON CELLULAR PROLIFERATION OF DERMAL HS27 FIBROBLAST **P<0.01, ***P<0.001, ****P<0.0001

Wounds are defined as injuries that disrupt the normal anatomical structure and function of living tissues. This damage compromises the epithelial layer of the skin and spreads into the subcutaneous tissue, disrupting other structures such as tendons, muscles, and nerves [17, 18]. Wound healing progresses through a series of events that include inflammation, proliferation, and migration of various cell types such as fibroblasts. The proliferation stage is a critical stage of the wound healing process that involves the activity of many cells, including fibroblasts, keratinocytes, and endothelial cells. In injured tissues, fibroblasts proliferate and differentiate into myofibroblasts, which contract and aid in healing by shrinking the wound and secreting extracellular matrix (ECM) proteins to pull the wound edges [19, 20].

Fibroblasts play an important role in the tissue repair process by secreting growth factors, cytokines, collagen, and ECM components from the late inflammatory phase until the full final epithelization of the injured tissue [21]. Products derived from various herbs and plants are relatively safe to consume because they contain multifunctional curing agents and bioactive compounds. Of notable importance are products from the Moringaceae family, especially *M. oleifera*. The various parts of this plant have been used for various ailments, including the treatment of inflammation and infectious diseases, as well as cardiovascular, gastrointestinal, hematological, hepato-renal disorders, diabetes mellitus, CNS depressant, antifertility effects, and wound healing effects [7, 10, 11, 22]. The significant *in-vitro* effect on the proliferation of fibroblast substantiates its

usefulness in wound healing. At 1 µg/mL, *M. oleifera* significantly stimulated proliferation of fibroblast cells, significantly higher than TNF-α positive control. Studies have revealed that extracts of *M. oleifera* have the potential to stimulate cell proliferation and protect against oxidative stress [23, 24]. In conclusion, the HPTLC and UPLC fingerprints provide a basis for the authentification of *M. oleifera* drug materials for identity and quality. The ethanol extract also showed that *M. oleifera* had a high potential for wound healing. Elemental content also showed some heavy metals being above permissible limits, and hence could be of concern. High concentrations of heavy metals for example could inhibit fibroblast proliferation. This inhibition could occur due to the metals' interference with protein folding and their ability to enhance aggregation, ultimately disrupting normal physiological activity [25]. The evaluation of fibroblast proliferation highlights its possible role in wound healing and the presence of bioactive compounds and essential elements could be attributable to its various therapeutic applications. Further research is needed to explore the specific mechanisms underlying the observed effects and to develop standardized formulations for the effective utilization of *M. oleifera* in healthcare.

ACKNOWLEDGEMENTS: Authors will like to acknowledge Dr. Cindy Kitcher for authenticating the plant materials.

Funding: This study is part of a larger project funded by Deutsch Forschungs Gemeinschaft (DFG), new insights into the molecular mechanisms of anthelmintic proanthocyanidins. Grant number HE 1642/12-1 (AOBJ: 661072) - work package 3 on the quality assurance of herbal medicines.

CONFLICT OF INTERESTS: The authors declare that there exist no conflict of interests regarding the publication of this manuscript.

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How to cite this article:
Bekoe EO, Orman E, Offei NA, Donkor K and Aikins AR: Phytochemical analysis, elemental content, and fibroblast proliferation activity of the hydro-ethanolic extract of *Moringa oleifera* lam leaves. Int J Pharm Sci & Res 2024; 15(3): 813-21. doi: 10.13040/IJPSR.0975-8232.15(3).813-21.