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# Phyto-metabolomic investigation and anti-migratory potential of *Moringa oleifera* Lam. and *Moringa peregrina* (Forssk.) Fiori against hepatic carcinoma cell line

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**ABSTRACT**

The present study aims to characterize the active ingredients of *Moringa oleifera* (MO) and *Moringa peregrina* (MP) leaf extracts and evaluate their cytotoxic and anti-migration efficacies against liver cancer cells (HepG2). The chemical profiling of the aqueous and methanolic extracts of both *Moringa* species was carried out using LC-ESI-MS led to identifying of 37 compounds classified as flavonoids, phenolic acids, glucosinolate, and phenyl ethanoid. The MTT assay was used to assess the cytotoxic effect, which revealed to, the increasing of the MO and MP extracts' concentration adversely decreased the treated cells' viability. Treating of HepG2 cells by MO and MP for different time intervals effectively inhibited wound closure, while the cells migratory ability in the treated cells was decreased. This study reports that MO and MP extracts exhibited promising cytotoxic effects and inhibited migration of highly metastatic HepG2 cell lines, which may be due to the presence of polyphenolic compounds.

**Keywords:** *Moringa*, anti-migration, HepG2, LC-ESI-MS, phenolic compounds

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## INTRODUCTION

Cancer is one of the leading causes of death globally, and the incidence of this group of diseases will continue to grow because of the increasing ageing of the population[1]. Hepatocellular carcinoma (HCC) is a frequent primary liver malignancy that primarily affects individuals, and it is the third leading cause of death worldwide[2]. The incidence of HCC has increased globally over the past 20 years, and in some countries, including the USA, it is anticipated to continue to rise through the year 2030[1]. The standard treatment for HCC mainly involves liver transplantation, surgical resection and chemotherapy[3]. Unfortunately, surgical resections are not suitable for HCC patients with advanced stages, especially those with liver cancer metastasis[4]. In addition, the currently available chemotherapeutic drugs are not effective to treat advanced HCC because of acquired resistance to the treatment[5]. In this regard, it is necessary to find more effective compounds, which may provide a novel therapy for HCC treatment, especially in the advanced stage.

Although there have been significant advancements in cancer therapy, metastasis still accounts for more than 90% of cancer-related deaths[6]. However, to treat cancer more effectively, we should further focus on preventing the formation and growth of metastatic carcinoma cells. We should consider that inhibition of migration, associated with the process of metastasis, might be as important as inhibition of cell proliferation.

At present, there is no effective therapy that can be used for patients diagnosed with HCC. There is a need for chemopreventive products targeting middle-aged patients. This is because treatment results are not sustainable for the health system, which does not cover the population[7]. Developing such a product from traditional medicinal plants would facilitate better compliance among a lot of patients, as the use of traditional plants and herbs is culturally embedded into the local lifestyle[8].

In recent years, the use of plants in primary health care and phytotherapeutic research has increased owing to the identification of bioactive molecules in medicinal plants and growing interest in alternative medicines. The World Health Organization (WHO) estimates that 80% of people receive their main medical care from plant-based traditional medicine. Also, in the clinical studies, more than 50% of the anticancer medications used came from naturally occurring plant sources[9].

The *Moringa* genus (Moringaceae family) contains 13 species found in Asia and Africa. *Moringa* species possess many biological properties, including an-

ti-inflammatory, antioxidant, anticancer, and antihyperglycemic due to their high alkaloids, flavonoids, glucosides, terpenes, and glucosinolate contents[10]. *Moringa oleifer*a Lam. (MO) is the most widely known and utilized due to its broad spectrum of health benefits[11]. While *Moringa peregrina* (Forssk.) Fiori (MP) is a potential crop candidate and is renowned for its ability to withstand drought and for its rich nutritional and therapeutic benefits[12].

The present study aims to identify the active constituents of MO and MP extracts using LC-ESI-MS as well as evaluate their *in vitro* cytotoxic and anti-migratory effects on liver cancer cells.

## METHODOLOGY

### Collection of plant material

The leaves of MO were obtained from the Egyptian Scientific Association of *Moringa*, National Research Center. While MP leaves were obtained from the Orman Garden, Giza, Egypt. The samples of the two plants were kindly identified by Mrs. Therese Labib, a consultant in plant taxonomy at the Orman Garden. Voucher specimen numbers 27318 (MO) and 27319 (MP) were kept at the Department of Medicinal Chemistry, Theodor Bilharz Research Institute, Giza, Egypt. The leaves of both *Moringa* species were dried in the shade at room temperature, then crushed to a fine powder by the electric mill and kept for the extraction process.

### Preparation of the extracts

One hundred grams of both *Moringa* species' dried leaf powder were extracted with 500 mL of pure methanol (MeOH). The filtrate was evaporated using a rotary evaporator (Buchi, Switzerland) under vacuum till dryness, this step was repeated three times to obtain semi solid crude MeOH ext. (17 g). Furthermore, 10 g of the dried MeOH ext. were fractionated by *n*-butanol (*n*-BuOH). The soluble constituents in *n*-BuOH were filtered and concentrated using a rotatory evaporator to obtain 5.24 g of a dry *n*-BuOH fraction (fr.). On the other hand, another amount of leaf powder (100 g) of each *Moringa* species was extracted using boiling $H_2O$ (500 mL) for 6 hours (h) with stirring until all the soluble constituents dissolved in the solvent, followed by filtration. The aqueous filtrate was concentrated using a rotary evaporator and this step was repeated three times to obtain $H_2O$ ext. (8.50 g). All extracts were stored in brown glass vials until further use.

### LC-ESI-MS phytochemical analysis of MO and MP extracts

The aqueous ($H_2O$) and MeOH extracts of both MO and MP species were chemically investigated via liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS). The experiments were carried out according to El-Wakil et al.[13] with little modification using the LC system (Waters Alliance 2695, Waters, USA) and reversed-phase analytical column C18, 250 mm, and 5 μm particle size (Phenomenex, USA). The mobile phase consists of two eluents; A ($H_2O$ + 0.1% formic acid) and B ($CH_3CN$: MeOH [1:1] +0.1% formic acid). The injection volume was 20 μL of 5 mg/mL of each extract, and the elution flow was 400 μL/min. The LC time program was as follows: 0.0–2.0 min (5% B), 2.0–10 min (5.0%–10% B), 10–70 min (10%–50% B), 70–80 min (50%–70% B), 80–95 min (70%–5% B) and 95–100 min (5% B). The ESI-MS spectra were calculated by scanning in the 50–1000 m/z range with the following parameters: the source temperature was set at 150 °C, the capillary voltage was at 3 kV, the cone voltage was at 70 eV, the desolvation gas flow was at 600 L/h, the desolvation temperature was at 350°C, and the cone gas flow was at 50 L/h. The compounds were assigned by retention time, and mass spectroscopic results were compared to literature data.

### Cell culture maintenance

The HepG2 cells were acquired from Nawah Scientific Inc., (Mokattam, Cairo, Egypt). The cells were seeded in Dulbecco's Modified Eagle Medium (DMEM) additionally with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. Then, incubated in a humidified 5% $CO_2$ atmosphere at 37°C. Cell passage was between 12 and 15, at 75-85% confluence used for seeding and treatment throughout the experiment.

### 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay

This assay is a sensitive, quantitative, and reliable colorimetric method that measures the viability of cells. The assay is based on the ability of mitochondrial lactate dehydrogenase enzymes (LDH) in viable cells to convert the water-soluble substrate of MTT into dark blue formazan crystals, which are water insoluble.

HepG2 cells were cultured in a 24-well plate to get a cell count of 500,000/well and grown for 24 h at 37°C to reach 85% confluency. Once the cells had adhered, they were treated with serial dilutions of MO and MP extracts (MeOH ext., $H_2O$ ext. and *n*-BuOH fr.) with concentrations of 3.25, 6.25, 12.5, 25, 50, and 100 μg/mL. Untreated cells were used as the control and the plate was

incubated for 24 h at 37°C, then 10 µL of 5 mg/ml MTT reagent were added to each well and incubated again for 4 h in the dark. The supernatant was removed and 200 µL of dimethyl sulfoxide (DMSO) was added to each well, including the control. Followed by shaking and then the plate was kept in a dark place for about 10 min at room temperature. The absorbance of each well was recorded at 570 nm using a microplate reader (Molecular Devices Co., CA, USA). The viability percentage was calculated as follows:

The cell viability % = (OD of treated cells/OD of untreated cells) × 100

The drug concentrations needed to inhibit 50% of cell growth relative to untreated control cells were calculated as the half maximal inhibitory concentration ($IC_{50}$) values.

### *In-vitro* wound healing migration assay

The migration of HepG2 was examined using the scratch assay method. Cells were plated at a density of $3x10^5$/well onto a coated 24-well plate for scratch wound assay and cultured overnight in 5% FBS-DMEM at 37°C and 5% $CO_2$. The cells were allowed to adhere. On the next day, a sterilized micro-pipette tip was used to scratch across the cell layer and horizontal scratches were introduced into the confluent monolayer. After removing the supernatant, the cells were treated with different extracts of both MO and MP at a concentration of 10 µg/mL. Images were taken using an inverted microscope at 0, 24, 48, 72, and 96 h. The plate was incubated in 5% $CO_2$ at 37°C in-between time points. To determine the migration rate, images were analyzed by MII Image View software version 3.7.

### Statistical analysis

The data were calculated using the statistical package for social science, IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., USA) and Microsoft Excel 2016. Continuous normally distributed variables were represented as Mean ± SD with a 95% confidence interval and using the frequencies and percentage for categorical variables; a p value<0.05 was considered statistically significant. The student's t-test was used to evaluate the means of normally distributed variables between groups. The Pearson correlation coefficient (r) was used to show the correlation between different parameters in this study. The migration rate was calculated by dividing the time spent in migration according to the formula: Rm = Wi–Wf / t; where Rm is the rate of cell migration, Wi is the average initial wound width, Wf is the average final wound width, and t is the duration of migration (in hours).

## RESULTS and DISCUSSION

### LC-ESI-MS phytochemical characterization of MO and MP extracts

The use of plant extracts, containing hundreds of chemicals as pharmaceutical agents, is no longer a black box or the primary obstacle to understanding their mechanisms of action and the contained active compounds[14]. Over the past three decades, there has been a significant rise in the use of herbal medicines and supplements, with at least 80% of people using them for some aspect of primary healthcare[15]. Multiple molecules of medicinal plant origin are currently used as drugs to combat cancer[16]. *Moringa* species contain numerous phytoconstituents, including alkaloids, flavonoids, phenolic acids, tannins, steroids, glucosinolates, saponins, and terpenes. Its diverse pharmacological benefits are a result of the variety of these phytochemicals in this genus[10].

In the present study, the phytochemical analysis of 70% MeOH and aqueous extracts of both *Moringa* leaf extracts was carried out by LC-ESI-MS. The identification of compounds was confirmed by mass fragmentation analysis, as shown in Figure 1. Figure 2 shows the total ion chromatograms of these extracts. A total of 37 polyphenolic compounds, including 20 flavonoids, 11 phenolic acids, 5 glucosinolates, and 1 phenyl ethanoid were identified, as shown in Table 1. These compounds were detailed tentatively identified and classified as follows:

**Table 1.** Tentative assignment of the chemical composition of MO and MP extracts

| Comp. No. | $t_R$ (Min) | MW | [ $[M-H]^-$ m/z | MS fragments | Tentative assignment | MO | | MP | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | MeOH ext. | $H_2O$ ext. | MeOH ext. | $H_2O$ ext. |
| 1 | 2.92 | 342 | 341 | 179, 135 | Caffeoyl-glucoside | - | - | + | + |
| 2 | 3.00 | 192 | 191 | 173, 93, 85 | Quinic acid | + | - | - | - |
| 3 | 4.09 | 196 | 195 | 97, 80 | Hydroxytyrosol acetate | + | + | + | + |
| 4 | 7.26 | 361 | 360 | 259, 97 | Glucosinolate derivatives | + | - | - | - |
| 5 | 12.27 | 571 | 570 | 328, 275, 97 | Glucomoringin | + | + | + | - |
| 6 | 13.94 | 516 | 515 | 353, 341, 179 (100%), 135 | 3-Caffeoylquinic glycoside | - | - | - | + |
| 7 | 17.03 | 330 | 329 | 191, 167 (100%), 135 | Vanillic acid-4-*O*-β-D-glucopyranoside | - | + | - | - |
| 8 | 17.53 | 354 | 353 | 191 (100%), 179, 150, 135 | 3-Caffeoylquinic acid | + | + | + | + |
| 9 | 21.63 | 613 | 612 | 259, 97 | 4-(2′-*O*-Acetyl-α-L-rhamnopyranosyloxy) benzyl glucosinolate | - | - | + | - |
| 10 | 22.21 | 338 | 337 | 191, 163 (100%), 119 | 3-Coumaroylquinic acid | + | + | + | + |
| 11 | 23.63 | 338 | 337 | 191, 163, 119 | 3-Coumaroylquinic acid isomer | + | + | - | + |
| 12 | 24.63 | 613 | 612 | 259, 97 | 4-(3′-*O*-Acetyl-α-L-rhamnopyranosyloxy) benzyl glucosinolate | - | - | + | - |
| 13 | 25.97 | 354 | 353 | 191, 179 (100%), 173, 135 | 3-Caffeoylquinic acid isomer | + | + | + | + |
| 14 | 27.47 | 626 | 625 | 463, 301 | Quercetin-di-*O*-glycoside | - | - | + | + |
| 15 | 31.15 | 338 | 337 | 191, 173 (100%), 163, 119 | 4-Coumaroylquinic acid | - | + | - | + |
| 16 | 32.06 | 594 | 593 | 503, 473, 383, 353 | Apigenin-6,8-di-C-glycopyranoside (Vicenin-2) | + | - | + | + |
| 17 | 32.48 | 338 | 337 | 191, 173 (100%), 163, 119 | 4-Coumaroylquinic acid isomer | - | + | - | - |
| 18 | 32.73 | 613 | 612 | 259, 97 | 4-(4′-*O*-Acetyl-α-L-rhamnopyranosyloxy) benzyl glucosinolate | - | - | + | - |
| 19 | 33.07 | 594 | 593 | 503, 473, 383, 353 | Apigenin-6,8-di-C-glycopyranoside (Vicenin-2) isomer | - | + | - | - |
| 20 | 33.23 | 368 | 367 | 193, 173 (100%), 135 | 4-Feruloylquinic acid | - | + | - | + |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 21 | 36.32 | 626 | 625 | 609, 463, 301 | Quercetin-di-*O*-glycoside isomer | - | - | + | + |
| 22 | 39.99 | 432 | 431 | 341, 311 (100%), 283 | Vitexin or isovitexin | - | - | + | - |
| 23 | 41.50 | 432 | 431 | 341, 311 (100%), 283 | Vitexin or isovitexin | - | - | + | - |
| 24 | 42.50 | 610 | 609 | 463, 301, 179 | Quercetin-3-*O*-rutinoside | + | + | + | + |
| 25 | 43.42 | 464 | 463 | 301 (100%), 271, 179 | Quercetin-3-*O*-glycoside | + | + | + | + |
| 26 | 46.51 | 608 | 607 | 505, 463, 301 (100%), 179 | Quercetin-hydroxymethylglutaroyl glycoside | - | - | + | - |
| 27 | 47.01 | 506 | 505 | 463, 301 (100%), 271, 179 | Quercetin-3-*O*-acetyl-glycoside | - | - | + | + |
| 28 | 48.18 | 448 | 447 | 285 (100%), 255 | Kaempferol-3-*O*-glycoside | - | - | + | + |
| 29 | 48.51 | 506 | 505 | 301 (100%), 271, 179 | Quercetin-3-*O*-acetyl-glycoside isomer | - | - | + | + |
| 30 | 48.68 | 594 | 593 | 447, 285, 151 | Kaempferol-3-*O*-rutinoside | + | + | - | - |
| 31 | 49.43 | 624 | 623 | 447, 285, 151 | Kaempferol-3-*O*-glycoside-glucoronoid | + | + | - | - |
| 32 | 50.18 | 478 | 477 | 315, 151 | Isorhamnetin 3-*O*-glycoside | + | + | - | - |
| 33 | 50.35 | 302 | 301 | 271 (100%), 165 | Quercetin | - | - | + | - |
| 34 | 52.60 | 286 | 285 | 255, 179 | Kaempferol | - | - | + | + |
| 35 | 54.61 | 490 | 489 | 285 (100%), 255 | Kaempferol-*O*-acetyl-glycoside | - | - | + | - |
| 36 | 55.44 | 520 | 519 | 421, 315 (100%), 285 | Isorhamnetin-*O*-acyl-glycoside | - | - | + | - |
| 37 | 72.14 | 574 | 573 | 463, 301 (100%), 179 | Quercetin derivative | - | - | + | - |

## Flavonoids

Flavonoids are the most abundant group of polyphenolic compounds detected in MO and MP extracts in which quercetin, kaempferol, apigenin and isorhamnetin derivatives are the major flavonoids. Compounds 14 and 21 ($t_R$=27.47 and 36.32 min, respectively) had $[M-H]^-$ at *m/z* 625 which afforded main fragments at *m/z* 463 $[M-H-162]^-$ and 301 $[M-H-(162\times2)]^-$ which means the presence of quercetin aglycon attached with two glycoside moieties. Thus, these compounds were assigned as quercetin-di-*O*-glycoside[17]. Compound

24 ($t_R$=42.50 min) represented a precursor ion at *m/z* 609 along with other fragment peaks at *m/z* 463, 301 and 179 which characteristics for quercetin-3-*O*-rutinoside. Compound 25 ($t_R$=43.42 min) exhibited an *m/z* 463 $[M-H]^-$ which was assigned as quercetin-3-*O*-glycoside. Compound 26 ($t_R$=46.51 min) showed a deprotonated molecule at *m/z* 607 was assigned as quercetin-hydroxymethylglutaroyl glycoside based on hydroxymethylglutaroyl moiety produced the ion *m*/z 463 $[M-H-144]^-$ and ion at *m/z* 301 $[M-H-144-162]^-$ of quercetin aglycone. Compounds 27 and 29 ($t_R$=47.01 and 48.51 min) afforded a $[M-H]^-$ at *m/z* 505 and product ions at 463 and 301 (quercetin) which reflect the elimination of acetyl and glycoside moieties, respectively. Thus, they were identified as quercetin-3-*O*-acetyl-glycoside. Compound 33 ($t_R$=50.35 min) was characterized as quercetin with m/z 301 along with product ions at *m/z* 271, 165. In addition, compound 37 ($t_R$=72.14 min) was annotated as a quercetin derivative with *m/z* 573 $[M-H]^-$ and product ions at m/z 463, 301, and 179.

Compound 34 ($t_R$=52.60 min) was characterized as kaempferol with *m/z* 285 $[M-H]^-$ and product ions at *m/z* 255 and 179. Compound 28 ($t_R$=48.18 min) was assigned as kaempferol-3-*O*-glycoside due to the existence of $[M-H]^-$ at *m/z* 447 and 285 $[M-H-162]^-$ due to the loss of the glycoside moiety. Compound 30 ($t_R$=48.68 min) was assigned as kaempferol-3-O-rutinoside with $[M-H]^-$ at *m/z* 593 and product ions at m/z 447 [M-H-146]- and 285 [M-H-146-162]- due the elimination of the rutinoside moiety. Compound 31 ($t_R$=49.43 min) was characterized as kaempferol-3-*O*-glycoside-glucuronide which had *m/z* 623 $[M-H]^-$ and product ions at *m/z* 447 $[M-H-176]^-$ and 285 $[M-H-176-162]^-$ due to the loss of the glucuronide and glycoside units, respectively. Moreover, Compound 35 ($t_R$=54.61 min) was identified as kaempferol-*O*-acetyl-glycoside due to the appearance of *m/z* 489 $[M-H]^-$ and main signal at *m/z* 285 $[M-H-204]^-$ corresponding to the elimination of the acetyl-glycoside unit. Compounds 32 and 36 ($t_R$=50.18 and 55.44 min) were assigned as isorhamnetin 3-*O*-glycoside and isorhamnetin-*O*-acyl-glycoside which had precursor ion peaks at *m/z* 477 and 519, respectively. Most of these compounds were identified before in MO leaf extracts. Most of these compounds were detected and identified in *M. oleifera* leaves which growing in different localities in the world as reported by Amaglo et al., Oldoni et al., Ralepelea et al.,and Abdel Shakour et al.[17-20].

Compounds 16 and 19 ($t_R$=32.06 and 33.07 min) exhibited a pseudomolecular ion peak at *m/z* 593 which fragments into 503, 473, 473, 383, and ions. These peaks represented characteristic fragment ions of an apigenin-6,8-di-C-glycopyranoside (vicenin-2). Meanwhile, compounds 22 and 23 ($t_R$=39.99 and 41.50 min, respectively) showed *m/z* 431 is consistent with the presence of the

apigenin 8-C-glucoside (vitexin) or and the apigenin-6-C glucoside (isovitexin) with a typical fragmentation pattern (*m/z* 341, 311 (100%) and 283), this compound was reported in *M. oleifera* leaf extract[17].

**Phenolic acids**

The identified phenolic acids in both *Moringa* species were 10 hydroxycinnamic acids and 1 benzoic acid derivative. hydroxycinnamic acids such as compound 1 ($t_R$=2.92 min) showed the $[M-H]^-$ ion at *m/z* value 341 corresponding to caffeoyl-*O*-glucoside[17]. Compound 6 ($t_R$=13.94 min) had a deprotonated molecular ion peak at *m/z* 515 with a typical fragmentation pattern of 3-caffeoylquinic glycoside[21]. Compound 7 ($t_R$=17.03 min) was characterized as vanillic acid-4-*O*-β-D-glucopyranoside with characteristic *m/z* ion at 329[22]. Compounds 8 and 13 ($t_R$=17.53 and 25.97 min, respectively) were afforded ion peaks at *m/z* 353 corresponding to 3-caffeoylquinic acid and its isomer. Compounds 10 and 11 ($t_R$=22.21 and 23.63 min, respectively) showed molecular ion peaks with *m/z* values of 337 along with characteristic fragments at *m/z* 191, 163 (100%) and 119 of 3-coumaroylquinic acid and its isomer. Compounds 15 and 17 ($t_R$=31.15 and 32.48 min, respectively) showed the same molecular ion peaks with *m/z* 337 with fragment ions at *m/z* 191, 173 (100%) and 119 characteristics for 4-coumaroylquinic acid and its isomer. Moreover, compound 20 ($t_R$=33.23 min) was identified as feruloylquinic acid, which had a characteristic *m/z* value of 367. While only one benzoic acid derivative was detected, such as compound 2 ($t_R$=3.00 min) which was assigned as quinic acid with an *m/z* value of 191[23]. Most of these phenolic acids were detected in *M. oleifera* and *M. ovalifolia* leaf extracts[17,21-23].

**Glucosinolates**

Glucosinolates are a class of alkaloids whose structures are characterized by a β-D-glucoside unit and an *O*-sulfated anomeric (Z)-thiohydroximate function linked to a variable side chain based on the plant species' metabolism of amino acids. Previous studies reported that, *M. oleifera* had several uncommon glucosinolates compounds with a second saccharide residue in the aglycon side chain, which possess a promising antioxidant and anticancer activities[24].

Compound 5 ($t_R$=12.27 min) exhibited a deprotonated molecule at *m/z* 570 which displayed fragment ions at *m/z* 424, 328, 275 and 97 corresponding to the deprotonated ion of glucomoringin. Compounds 9, 12, and 18 ($t_R$=21.63, 24.63 and 32.73 min, respectively) afforded a precursor ion peak at *m/z* 612 with the same fragmentation pattern (*m/z* 259, 97) of three glucosinolates isomers and their structures similar to glucomoringin, except for the presence of an acetyl group at C-2′, C-3′, and C-4′ on the α-L-rhamnopyranosyl

unit. They identified as 4-(-2′-*O*-acetyl-α-L-rhamnopyranosyloxy) benzyl glucosinolate, 4-(-3′-*O*-acetyl-α-L-rhamnopyranosyloxy) benzyl glucosinolate, 4-(-4′-*O*-acetyl-α-L-rhamnopyranosyloxy) benzyl glucosinolate. In addition, compound 4 ($t_R$=7.26 min) was assigned as a glucosinolate derivatives. These compounds possess promising antioxidant and anticancer activities as well as were reported in *M. oleifera* leaves but not reported in *M. peregrina*[20,24].

**Phenylethanoids**

Just one compound (compound **3**) was detected in both MO and MP extracts, with a precursor ion peak at *m/z* 195 and fragment ions at *m/z* 97 and 80. This compound was annotated as hydroxytyrosol acetate[25].

In present results (Table 1 and Figure 1), it appears that the $H_2O$ ext. of both *Moringa* species (MO and MP) are rich in phenolic acids and the MP extracts are famous for quercetin and apigenin derivatives. In addition, the peak intensity of quercetin-3-*O*-rutinoside is much higher in MO extracts than in MP extracts while the intensity of quercetin-3-*O*-glycoside peak is much higher in MP extracts than in MO extracts. Moreover, MP MeOH ext. is rich in glucosinolate. Therefore, the presence of these phytochemicals in both species may be responsible for the plant bioactivities.

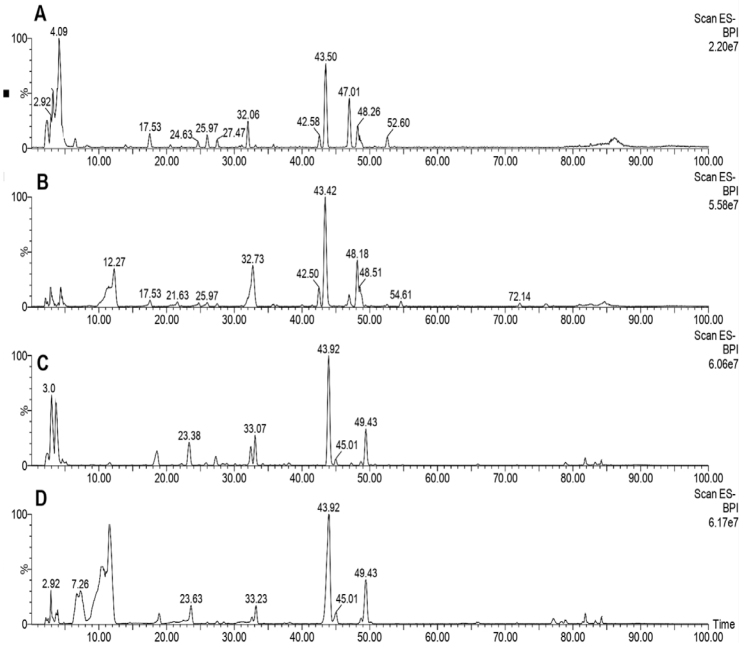



**Figure 1.** Total ion chromatograms (TIC) of (A) MP-$H_2O$, (B) MP-MeOH, (C) MO-$H_2O$ and (D) MO-MeOH extracts

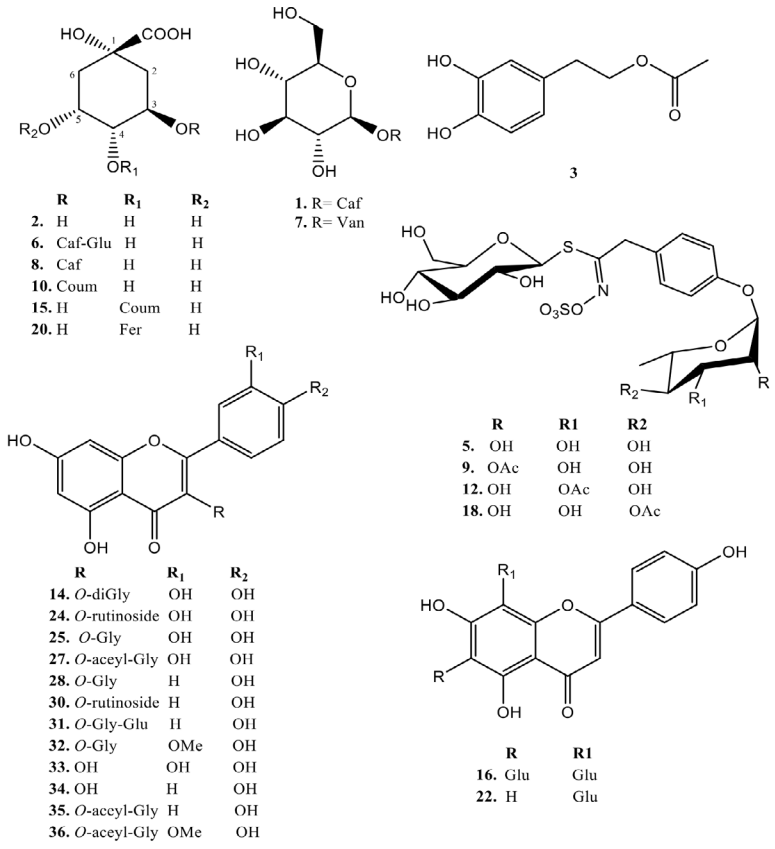



**Figure 2.** Structures of some identified compounds in $H_2O$ and MeOH extracts of both *Moringa* species (MO and MP)

### Effect of MO and MP extracts on cell viability

The development of new drugs from herbal plant ingredients has been the basic agenda in the R&D of the drug industry for many decades. Despite recent therapeutic advances in cancer treatment, metastasis remains the principal cause of cancer death. Recent work has uncovered the unique biology of metastasis-initiating cells that results in tumor growth in distant organs[6].

The cell culture wound closure assay is a useful method to examine cell migration, in which a scratch is generated on a confluent cell monolayer. The speed of wound closure and cell migration can be quantified by taking snapshot pictures with a regular inverted microscope at several time intervals[26].

Herein, the cytotoxic effect of MO and MP extracts on HCC, HepG2 cell lines was studied using various gradient concentrations of the two species of *Moringa* extracts using the MTT assay. The different fractions have been treated with increasing concentrations (1.563 -100 µg/mL) for 24 h. The obtained results showed clearly that increasing the concentration of the MO and MP extracts adversely decreased the viability of the treated HepG2 cells. Moreover, the highest concentration applied (100 µg/mL) showed the highest decrease in cell viability in all fractions. The data were analyzed by the Pearson correlation coefficient (r), which is a measure of linear correlation between two sets of data.

For MO, the mean of the viability of $H_2O$ ext., MeOH ext. and *n*-BuOH fr. was 52.1 ± 15.7, 62.5 ± 7.7 and 52.0 ± 11.1, respectively, with correlation coefficient (r) -0.771, -0.976, and -0.66 with a significant effect of p value=0.042 and 0.107 for $H_2O$ ext. and *n*-BuOH fr., respectively. A highly significant effect was observed for MeOH fr. with p<0.001 (Table 2). In addition, the lowest viability percentage of cells was observed for the $H_2O$ ext. at concentrations of 100 and 50 µg/mL (Figure 3).

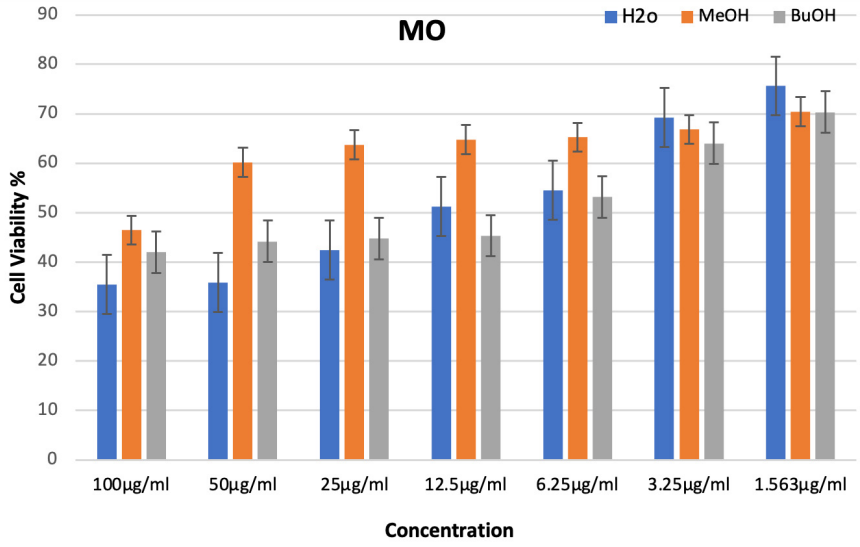



**Figure 3.** Effect of MO extracts on viability of HepG2 cells

While for MP, the mean of viability for $H_2O$ ext., MeOH ext., and *n*-BuOH fr. was 51.6 ± 9.6, 44.8 ± 8.5 and 56.1 ± 5.0, respectively, with a correlation coefficient (r) -0.959, -0.792, and -0.997, respectively. The $H_2O$ ext. and *n*-BuOH fr. showed a highly significant effect with p values=0.002 and <0.001, respectively. The non-significant effect was observed with MeOH ext. (p=0.06) as shown

in Table 2. Hence, the results exhibited that the lowest viability percentage of cells was observed for $H_2O$ and MeOH extracts at a concentration of 100 µg/mL (Figure 4). These results showed that treatment with MO and MP extracts decreased the viability of HepG2 cells in a dose-dependent manner.

**Table 2.** The viability % of MP and MO extracts

| Conc. (µg/mL) | Viability % | | | | | |
|---|---|---|---|---|---|---|
| | MP extracts | | | MP extracts | | |
| | $H_2O$ ext. | MeOH ext. | n-BuOH fr. | $H_2O$ ext. | MeOH ext. | n-BuOH fr. |
| 100 | 35.18 | 35.73 | 46.69 | 35.51 | 46.43 | 41.98 |
| 50 | 48.69 | 38.65 | 54.28 | 35.84 | 60.17 | 44.18 |
| 25 | 49.68 | 39.19 | 57.29 | 42.46 | 63.74 | 44.77 |
| 12.5 | 55.88 | 47.26 | 59.09 | 51.27 | 64.8 | 45.34 |
| 6.25 | 57 | 49.67 | 59.23 | 54.53 | 65.22 | 53.15 |
| 3.25 | 63.22 | 58.21 | 59.86 | 69.27 | 66.79 | 64 |
| 1.563 | 70.23 | 63.65 | 65.21 | 75.61 | 70.45 | 70.33 |
| Mean of Viability | 51.6 ± 9.6 | 44.8 ± 8.5 | 56.1 ± 5.0 | 52.1 ± 15.7 | 62.5 ± 7.7 | 52.0 ± 11.1 |
| The correlation between the concentration and viability % | r=-0.959**, p=0.002 | r=-0.792, p=0.06 | r=-0.997**, p=<0.001 | r=-0.771*, p=0.042 | r=-0.976**, p= <0.001 | r=-0.66*, p= 0.107 |
| $IC_{50}$ (µg/ml) | 35.56 | 67.91 | 77.75 | 34.47 | 88.75 | 37.98 |

Viability is represented as Mean ± SD, while the correlation coefficient (r); the data were analyzed by Person correlation p=p. value.
*p. value<0.05 is significant, **p. value<0.01 is highly significant.

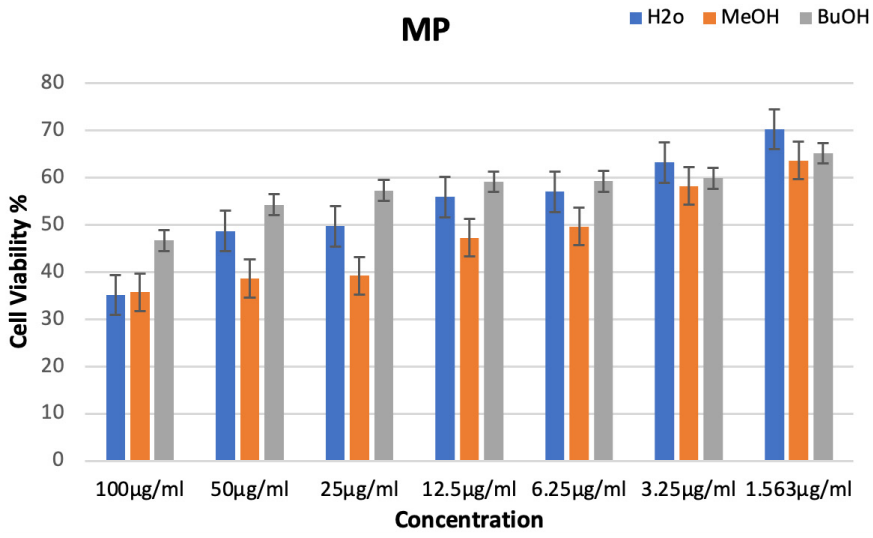



**Figure 4.** Effect of MP extracts on viability of HepG2 cells

The concentration of $H_2O$ ext., MeOH ext., and an *n*-BuOH fr. of both MO and MP required to inhibit 50% of the cells ($IC_{50}$) was calculated. The results showed that the $IC_{50}$ values of MO extracts on HepG2 cells were 34.473, 88.75, and 37.98 µg/mL for $H_2O$ ext., MeOH ext. and *n*-BuOH fr., respectively. While the values recorded for MP extracts were 35.56, 67.91, and 77.75 µg/mL for $H_2O$ ext., MeOH ext. and *n*-BuOH fr., respectively.

However, our results revealed that all the extracts have significant cytotoxic effects, and increasing the concentration of MO and MP extracts adversely decreased the viability of the treated HepG2 cells.

The $IC_{50}$ curves determine the drug concentration that can inhibit the growth of tumor cells by half when compared to the cells grown with no exposure to the drug, low $IC_{50}$ value means that the drug is effective at low concentrations, and thus will show lower systemic toxicity when administered to the patient. Interestingly, the three extracts mentioned above showed similar $IC_{50}$ values close to this concentration and exhibited cytotoxic activity against HepG2 cells.

Our results are in agreement with those of Jung et al.[27] who reported that water extracts of *M. oleifera* leaves have been shown to inhibit the growth of liver cancer and also consistent with Mansour et al.[28] who mentioned that *M. peregrina* and *M. oleifera* leaves ethyl acetate extracts exhibited the highest inhibition activity against HCC HepG2 cell line (78% and 80.7% inhibition, respectively). Furthermore, the results of the present study are close to Emami et al.[29]. results who proved that HepG2 viability methanolic ext. was decreased

with increasing of extract concentration. Also, he mentioned that the $IC_{50}$ for the MeOH ext. of a leaf of MO was 12.89 μg/mL which differs from our result as it indicated that the $IC_{50}$ of MO-methanolic ext. was 88.75 μg/ml and this difference in the $IC_{50}$ values may be attributed to the type of solvents and methods of extraction used. A different fraction that was not included in our study is isothiocyanate from MO (MIC-1) was evaluated for anticancer activity against 30 cancer cell lines including HepG2, which were treated with MIC-1 (0 or 10 μM) for 48 h. The research, conducted by Xie et al.[30] demonstrated that MIC-1 considerably reduced the HepG2 cell line's ability to proliferate and produced strong growth inhibitory effects on the cells with a high growth inhibitory rate.

It is clear from the current research why MO and MP extracts have potent anticancer properties against HepG2 cells. This may be because the plant's leaves contain promising polyphenolic compounds. As a result, it is evident that the polar solvents employed in the extraction procedure are capable of dissolving the active components from the source to the MP extracts, which demonstrated their anti-cancer effects in comparison to other extracts.

**The anti-migration capacity of MO and MP extracts against HepG2 cells**

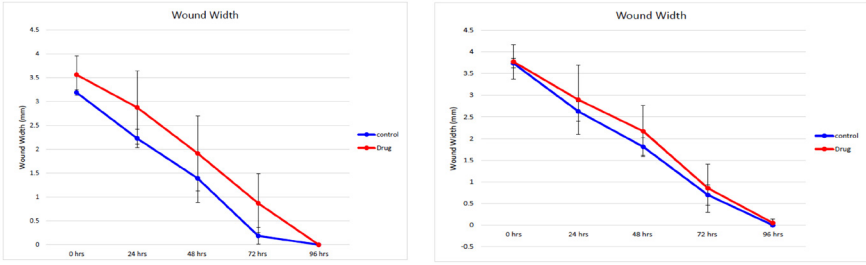
Migration is a primary step in cancer metastasis. We studied the anti-migration potential of MO and MP extracts against the HepG2 cell line, wound healing assay was performed in the presence of various types of extracts. The 85% confluent HepG2 cell was wounded using a micro-pipette tip. After 24, 48, 72, and 96 h of incubation, the wound width was observed and imaged under an inverted microscope. The wound width was calculated as the average distance between the edges of the scratches.

Paired t-test was used to analyze the data, This test showed that the treatment with $H_2O$ extract of MO for 72 h demonstrated a significant dependent migration inhibition of HepG2 cells with p=0.049, while for $H_2O$ ext. of MP, the migration of HepG2 cells was inhibited and cells migrated slower following a 96 h incubation period with a significant p=0.016 and a decrease in wound width was observed as cell migration was induced, as shown in Table 3 and Figure 5. On the contrary, MeOH ext. of MO showed no inhibition for HepG2 cell migration as the cells had migrated into the scratched area and the untreated cells showed a less extensive wound with a significant p=0.047 after 72 h incubation whereas the treated cells migrated significantly more, while no significant anti-migration effect was observed for MeOH ext. of MP at all-time intervals (Table 4). A significant anti-migration effect of *n*-BuOH fr. of MO was observed with p=0.019, while *n*-BuOH fr. of MP showed no significant migration inhibition for HepG2 cells (Table 5).

**Table 3.** Comparison between $H_2O$ extracts of MO and MP species on HepG2 regarding the anti-migration effect

| Time (h) | MO ($H_2O$ ext.) | | | MP ($H_2O$ ext.) | | |
|---|---|---|---|---|---|---|
| | Control | Drug | p. value | Control | Drug | p. value |
| 0 | 3.1 9 ± 0.06 | 3.56 ± 0.40 | 0.158 | 3.74 ± 0.11 | 3.77 ± 0.40 | 0.216 |
| 24 | 2.23 ± 0.19 | 2.87 ± 0.77 | 0.218 | 2.63 ± 0.22 | 2.90 ± 0.80 | 0.15 |
| 48 | 1.39 ± 0.51 | 1.91 ± 0.78 | 0.335 | 1.81 ± 0.21 | 2.17 ± 0.59 | 0.256 |
| 72 | 0.19 ± 0.18 | 0.87 ± 0.62 | 0.049* | 0.70 ± 0.23 | 0.85 ± 0.56 | 0.16 |
| 96 | 0.00 ± 0.00 | 0.00 ± 0.00 | - | 0.00 ± 0.00 | 0.05 ± 0.09 | 0.016* |

The wound widths are represented as Mean ± SD; the data were analyzed by paired t test. *p. value<0.05 is significant, **p. value<0.01 is highly significant.




A) MO B) MP

**Figure 5.** Effect of $H_2O$ extracts of MO and MP on HepG2 migration

**Table 4.** Comparison between MeOH extracts of MO and MP on HepG2 regarding anti-migration effect

| Time (h) | MO (MeOH ext.) | | | MP (MeOH ext.) | | |
|---|---|---|---|---|---|---|
| | Control | Drug | p. value | Control | Drug | p. value |
| 0 | 4.02 ± 0.12 | 3.95 ± 0.13 | 0.97 | 3.90 ± 0.28 | 4.04 ± 0.73 | 0.128 |
| 24 | 3.28 ± 0.53 | 3.09 ± 0.11 | 0.115 | 2.13 ± 0.51 | 2.18 ± 0.99 | 0.216 |
| 48 | 2.30 ± 0.11 | 2.06 ± 0.13 | 0.795 | 0.44 ± 0.36 | 0.52 ± 0.89 | 0.092 |
| 72 | 1.05 ± 0.24 | 0.94 ± 0.05 | 0.047* | 0.00 ± 0.00 | 0.00 ± 0.00 | - |
| 96 | 0.00 ± 0.00 | 0.00 ± 0.00 | - | 0.00 ± 0.00 | 0.00 ± 0.00 | - |

The wound widths are represented as Mean ± SD; the data were analyzed by paired t test. *p. value<0.05 is significant, **p. value<0.01 is highly significant.

**Table 5.** Comparison between *n*-BuOH fractions of MO and MP on HepG2 regarding the anti-migration effect

| Time (h) | MO (*n*-BuOH fr.) | | | MP (*n*-BuOH fr.) | | |
|---|---|---|---|---|---|---|
| | Control | Drug | p. value | Control | Drug | p. value |
| 0 | 3.47 ± 0.02 | 3.57 ± 0.35 | 0.019* | 4.76 ± 0.16 | 4.86 ± 0.20 | 0.393 |
| 24 | 2.32 ± 0.10 | 2.68 ± 0.27 | 0.09 | 2.76 ± 0.63 | 2.89 ± 0.21 | 0.099 |
| 48 | 1.76 ± 0.27 | 1.88 ± 0.10 | 0.112 | 1.16 ± 0.55 | 1.17 ± 0.16 | 0.147 |
| 72 | 0.47 ± 0.19 | 0.72 ± 0.31 | 0.337 | 0.00 ± 0.00 | 0.00 ± 0.00 | - |
| 96 | 0.00 ± 0.00 | 0.00 ± 0.00 | - | 0.00 ± 0.00 | 0.00 ± 0.00 | - |

The wound widths are represented as Mean ± SD; the data were analyzed by paired t test. *p. value<0.05 is significant, **p. value<0.01 is highly significant.

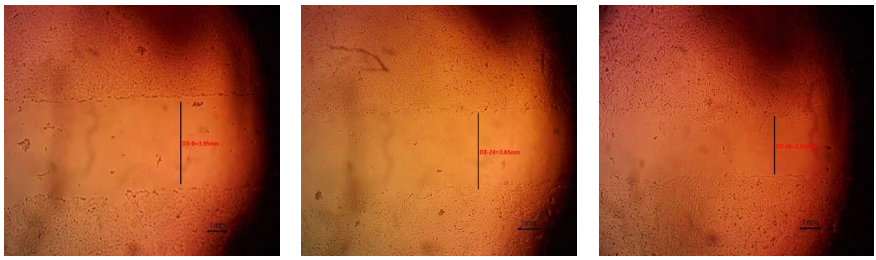
## Comparison between MO and MP fractions regarding their anti-migration effect

Student t-test was used to analyze data, it was observed that MP-$H_2O$ ext. gives a better anti-migration effect of HepG2 cells than MO-$H_2O$ ext. following a 96 h incubation period with significant p=0.016 (Table 6, Figures 6 and 7). Also, a better effect was observed for MP-MeOH than MO at 0 hour with significant p=0.05, while at 24, 48, and 72 h, MO-MeOH showed a significantly better anti-migration effect for HepG2 cells than MP-MeOH with p=0.041, 0.028, and 0.041, respectively (Table 7). It was found that the MO-*n*-BuOH fr. Showed a better significant anti-migration effect than MP after 72 h incubation with p=0.033 (Table 8).

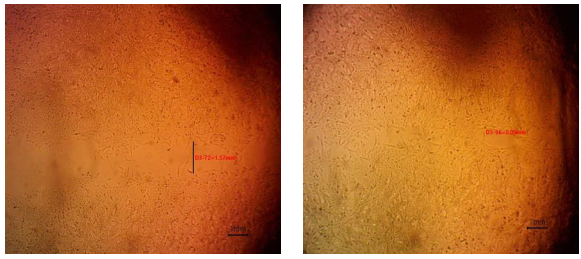
**Table 6.** Comparison between MO-H2O and MP-$H_2O$ extracts regarding their anti-migration effect

| Time (h) | MO-$H_2O$ ext. | MP-$H_2O$ ext. | p. value |
|---|---|---|---|
| 0 | 3.56 ± 0.40 | 3.77 ± 0.40 | 0.991 |
| 24 | 2.87 ± 0.77 | 2.90 ± 0.80 | 0.882 |
| 48 | 1.91 ± 0.78 | 2.17 ± 0.59 | 0.461 |
| 72 | 0.87 ± 0.62 | 0.85 ± 0.56 | 0.797 |
| 96 | 0.00 ± 0.00 | 0.05 ± 0.09 | 0.016* |

The wound widths are represented as Mean ± SD; the data were analyzed by student t test. *p. value<0.05 is significant, **p. value<0.01 is highly significant.

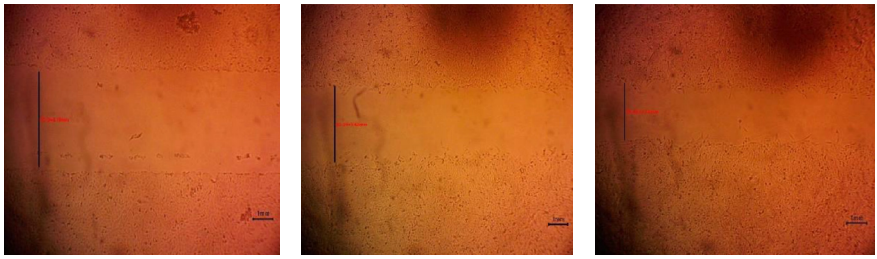


0 h 24 h 48 h

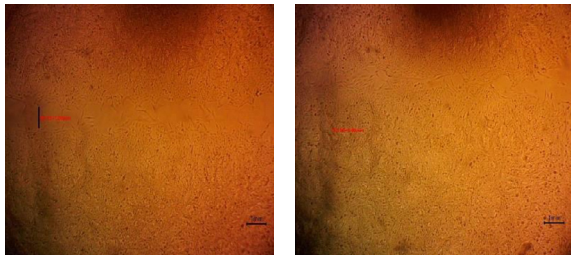


72 h 96 h

**Figure 6.** Effect of MO-$H_2O$ ext. on cellular migration in HepG2 cells determined by wound healing assay (Images were taken at time zero, 24, 48, 72, and 96 h)



0 h 24 h 48 h



72 h 96 h

**Figure 7.** Effect of MP-$H_2O$ ext. on cellular migration in HepG2 cells determined by wound healing assay. Images were taken at time zero, 24, 48, 72 and 96 hr.

**Table 7.** Comparison between MO-MeOH and MP-MeOH extracts regarding their anti-migration effect

| Time (h) | MO- MeOH ext. | MP- MeOH ext. | p. value |
|---|---|---|---|
| 0 | 3.95 ± 0.13 | 4.04 ± 0.73 | 0.05* |
| 24 | 3.09 ± 0.11 | 2.18 ± 0.99 | 0.041* |
| 48 | 2.06 ± 0.13 | 0.52 ± 0.89 | 0.028* |
| 72 | 0.94 ± 0.05 | 0.00 ± 0.00 | 0.041* |
| 96 | 0.00 ± 0.00 | 0.00 ± 0.00 | - |

The wound widths are represented as Mean ± SD; the data were analyzed by student t test. *p. value<0.05 is significant, **p. value<0.01 is highly significant.

**Table 8.** Comparison between MO-*n*-BuOH and MP-*n*-BuOH fractions regarding their anti-migration effect

| Time (h) | MO- *n*-BuOH fr. | MP- *n*-BuOH fr. | p. value |
|---|---|---|---|
| 0 | 3.57 ± 0.35 | 4.86 ± 0.20 | 0.231 |
| 24 | 2.68 ± 0.27 | 2.89 ± 0.21 | 0.522 |
| 48 | 1.88 ± 0.10 | 1.17 ± 0.16 | 0.221 |
| 72 | 0.72 ± 0.31 | 0.00 ± 0.00 | 0.033* |
| 96 | 0.00 ± 0.00 | 0.00 ± 0.00 | - |

The wound widths are represented as Mean ± SD; the data were analyzed by student t test. *p. value<0.05 is significant, **p. value<0.01 is highly significant.

In the present study, we demonstrated for the first time that the aqueous extracts of MO and MP significantly inhibit HepG2 migration, as MO-$H_2O$ ext. significantly inhibits the cell migration following 72 h incubation, whilst the significant anti-migration effect of MP-$H_2O$ was observed after 96 h incubation, these results showed that MO and MP treatment inhibits the migration of HepG2 cells in a time-dependent manner.

Student t test proved a significant anti-migration effect of the MeOH ext. at all-time intervals and a non-significant suppression of cell migration for the *n*-BuOH fr. except after 72 h incubation; this means that the migration capacity of HepG2 cells decreased at specific time intervals which confirm the time-dependent manner of anti-migration.

Cell migration plays an important role in many physiological and pathological processes, such as tissue repair and tumor cell metastasis. Our study used a wound healing assay to determine the migration velocity of the HepG2 cell line. Statistical analysis showed that the migration rates of HepG2 cells treated with MO-MeOH and the MP-$H_2O$ extracts were slower and significantly suppressed.

### Migration rate

The wound healing assay is used to investigate cell migration. Cell migration is the movement of individual cells, cell sheets, and clusters from one location to another. Migration is considered the rate-limiting process during healing, and therefore migration process is a key part of investigating wound healing.

In the present comparative study between MO and MP, the Student t-test was used to calculate the migration rates of HepG2 cells for all extracts, it was found that the MeOH ext. of MO has a slower significant migration rate with p=0.01 but for the other extracts, they showed faster significant migration rate than the untreated cells with p=0.01. While for MP, the HepG2 cells treated with water ext. showed a slower significant migration rate with p=0.01 but for the cells treated with methanol and butanol extracts of MP, the migration rate of HepG2 cells was significantly faster than the untreated control with p=0.01 (Table 9 and Figure 8).

**Table 9.** The migration rate of both *Moringa* species extracts

| Extract | MO | | | MP | | |
|---|---|---|---|---|---|---|
| | Control | Drug | p. value | Control | Drug | p. value |
| $H_2O$ ext. | 3.18 | 3.55 | 0.01* | 3.73 | 2.90 | 0.01* |
| MeOH ext. | 4.00 | 3.93 | 0.01* | 3.89 | 4.03 | 0.01* |
| *n*-BuOH fr. | 3.46 | 3.56 | 0.01* | 4.74 | 4.84 | 0.01* |

Migration rate was calculated by dividing the time spent in migration according to the formula: Rm = Wi–Wf / t; where Rm is the rate of cell migration, Wi is the average initial wound width, Wf is the average final wound width, and t is duration of migration (in hours). The data were analyzed by student t test.
*p. value<0.05 is significant, **p. value<0.01 is highly significant.

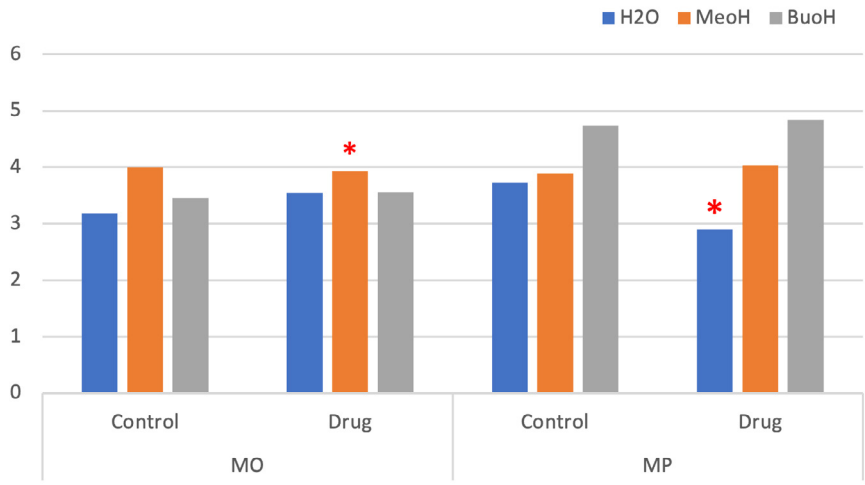



**Figure 8.** Migration rate within control comparison of MO and MP

The anti-migration effects of MO and MP have not been demonstrated before on liver cancer cell lines and accordingly, there is no available published research concerning this subject, so we discussed the migration effect of this medicinal plant on other cell lines. The study of Xie et al.[31] who examined the migratory inhibition of PC3 human prostate cancer cells by *M. oleifera* alkaloids (MOA) *in vitro*, they reported that MOA significantly suppress PC3 cell migration in a wound healing assay by inhibiting the expression of cyclooxygenase 2 (COX-2), and inhibiting the production of prostaglandin E2 (PGE2), these results agreed with Xie et al.[30] who assessed the effects of Isothiocyanate from *Moringa oleifera* (MIC-1) on the migration of renal cell carcinoma cell line (786-O and 769-P), the results showed that the exposure to MIC-1 (0, 1, and 2 μM) substantially reduced the migration ability of 786-O and 769-P cells and they mentioned that the cell migration rate of 786-O and 769-P cells decreased in the wound healing assay. The above results support our findings regarding the anti-migration effect of different fractions of *Moringa*. Additionally, to the best of our knowledge, this research is the first to demonstrate a substantial inhibition of HepG2 migration by the aqueous extracts of MO and MP, a treatment that may prevent the metastasis of HCC.

It can be concluded that the LC-ESI-MS analysis of $H_2O$ and MeOH extracts of MO and MP leaves in negative ion mode led to characterize of major chemical constituents as flavonoid (*O*-and C-glycosides), phenolic acids, glucosinolates and phenyl ethanoid. The flavonoids consist mainly of the quercetin and kaempferol, isorhamnetin and apigenin derivatives including glycosides

and acetyl glycosides. The cytotoxic activity of MO and MP extracts on HepG2 cell lines showed $IC_{50}$ values ranging from 34.473 to 88.75 μg/mL, in which the $H_2O$ extracts of both *Moringa* species had the highest activities as well as exhibited significant antimigratory potential.

Hence, the promising cytotoxic and anti-migratory effects of MO and MP extracts could contribute to the development of a new anticancer drug for HCC from natural sources.

## STATEMENT OF ETHICS

This study does not involve experiments on animals or human subjects.

## CONFLICT OF INTEREST STATEMENT

All authors declare that there are no conflicts of interests.

## AUTHOR CONTRIBUTIONS

Ezzat E.A. Osman: Study Design, Performing the Experiments, Data analysis, Writing, Preparing, and Editing the manuscript. Sayed S. Abdel-Hameed: Study Design, Data Interpretation, Writing, and Reviewing the manuscript. Mohamed A. Shemis: Study Design, Reviewing, and Editing the Manuscript. Samah Mamdouh: Performing the Experiments, Data Analysis, Writing and Preparing the Manuscript.

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