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Research Article

Ultrasound and microwave extraction from *Moringa oleifera* Lam.: Characterization and antiproliferative effect

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Abstract: *Moringa oleifera* has been a focus of interest because of the different properties (anticancer, antioxidant, etc.) that have been attributed to this plant. However, the most used methodology is soxhlet, which requires long periods of reaction (18 hours), generating greater energy expenditure. Recently, green extraction technologies have been developed like ultrasound and microwaves, reducing reaction time by up to 97%. The objectives of this study were to extract and identify the polyphenolic compounds present in aqueous and hydro-alcoholic extracts from *Moringa oleifera* dried leaves using ultrasound and microwave, as well as to evaluate their in vitro cytotoxic effect using cancer and non-cancer cells. A combination of ultrasound and microwave was utilized to extract polyphenolic compounds from *Moringa* dried leaves. HPLC–MS analysis was conducted to qualitatively identify the polyphenols in the samples. The cytotoxic effect was evaluated by MTT and comet assays using non-cancer (3T3, Hek293, and Vero) and cancer (HepG2) cells lines. Results: 30 polyphenolic compounds from 9 different families were identified by HPLC. Data suggested that hydro-alcoholic extracts from *Moringa* leaves have potent cytotoxic activities in a depend-doses response. Also, compounds from aqueous extracts did not cause cell death, while polyphenol extract from hydro-alcoholic extracts decreased populations in both cancer and non-cancer cell lines measurement by MTT. HepG2 cells showed DNA damage by comet assay. The extraction using ultrasound and microwaves at 30 minutes of reaction has an antiproliferative effect through apoptosis in cancer cells, in addition ethanolic extracts have higher cytotoxicity compared to aqueous extracts.

1. INTRODUCTION

Moringa oleifera (MO) has been widely used as food or food supplement (human and animal) because of its high energy value. It is consumed as fresh, powders or aqueous extracts (Nayak *et al*., 2016) but the current worldwide boom is due to its biological effects such as antimicrobial

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activity and treatment of cardiovascular, gastrointestinal, hematological, and hepatorenal diseases (Jayawardana *et al*., 2015); urinary diseases (Nayak *et al*., 2016); activity against malaria, and even to combat the symptoms of HIV/AIDS by stimulating the immune system (Ramabulana *et al*., 2016); to treat nervous disorders (González-Trujano *et al*., 2018); hypertension, catarrh, gastric ulcers, and skin diseases (Chen *et al*., 2017), hypoglycemic (Stohs & Hartman, 2015), impotence, hemorrhoids, headaches, and gum pain (Bakre, Aderibigbe & Ademowo, 2013). In another study, it has been proven *Moringa* effects as a palliative against the pain caused by arthritis (Mahdi *et al*., 2018). The main *Moringa oleifera* phytochemicals are quercetin, alkaloids, tannins, flavonoids, and glycosides which are present in all parts of the plant. Phytochemicals generally have fewer side effects than synthetic substances (Jarriyawattanachaikul, Chaveerach & Chokesajjawatee, 2016).

It is important to note that the quantity and quality of bioactive molecules, extracted from vegetal sources depend on the optimal selection of the extraction method and solvents (Fu *et al*., 2016). There is a wide variety of extraction methods and solvents (Vongsak *et al*., 2013), from conventional techniques (soxhlet, maceration, and distillation) to the most innovative and considered green (ultrasound, microwaves, high pressure, among others) technologies (Guerrouj *et al*., 2016), which have recently increased their popularity over traditional methods because they are environmental friendly (Yang *et al*., 2011), offer savings in time and energy, applicability to smaller samples and are performed at lower temperatures, which guarantee a recovery real and effective of phytocompounds (Azmir *et al*., 2013). The most commonly used solvents are ethanol, methanol, dichloromethane, acetone, and water (Tapia-Torres *et al*., 2015). Microwave and Ultrasound Extractions have previously been done with this technology (Kayanan & Sagum, 2021) however it is the first time that its activity has been tested in both cancerous and non-cancerous cells and to know if the effect previously reported in conventional extractions is still maintained (Khor *et al*.,2018; Bhadresha *et al*., 2022).

Therefore, the objectives of this study were to identify the bioactive compounds obtained from aqueous and hydroalcoholic extracts of the MO leaves obtained using a combination of ultrasound and microwave and to evaluate their cytotoxic effect on cancer and non-cancer cells lines, besides evaluating the genotoxic behaviour of those bioactive compounds.

2. MATERIAL and METHODS

2.1. Metabolite extraction

2.1.1. *Vegetal material collection*

The vegetal material was collected from July to August 2017, from an experimental planting, at the Zaragoza Experimental Station, Zaragoza Coahuila Mexico, which belongs to the Universidad Autonoma Agraria Antonio Narro (UAAAN) and is located at -100° 55' west longitude and 28° 33' north latitude. Leaves of MO were removed from petioles and were left at room temperature for 5 days, for drying. Dried leaves were subsequently pulverized using an Oster brand industrial blender and stored under dark conditions.

2.1.2. *Extraction*

The obtained MO powder was homogenized and used to obtain five combinations of extraction variables: mass/volume ratio (g/mL) of 1:8, 1:12, and 1:16 in a mixture of EtOH / H_2O at 0, 30 and 70% at a volume of 500 mL (Table 1). Samples were sonicated at room temperature (25 ^{0}C) for 20 minutes using an ultrasound bath (Branson 5510), then subjected to a microwave treatment with a CEM Mars model 230/60, with a 5 min heating ramp until reaching 70 ° C, (power of 800 watts) that was maintained for 5 minutes. The extraction was carried out in triplicate. The obtained extracts were filtered, measuring the volume recovered, and stored at 4°C in amber glass bottles until further analysis.

Table 1. Design of extraction variables combination.

Mass/Volume ratio (g/mL)	Dissolvent EtOH-water (%)	Final ratio	Name
1:8	0/100	Mor - 1g/8 mL - 0% EtOH	Mor-8-0%
1:8	70/30	Mor- 1g/ 8mL - 70% EtOH	Mor-8-70%
1:12	30/70	Mor - 1g/12mL- 30% EtOH	Mor-12-30%
1:16	70/30	Mor - 1g/16 mL - 70% EtOH	Mor-16-70%
1:16	0/100	Mor - 1g/16 mL - 0% EtOH	Mor-16-0%

2.2. Identification of Compounds

2.2.1. *Chromatographic separation of phenolic compounds*

The separation of the polyphenolic fraction was carried out with the methodology expressed by Ascacio-Valdés *et al*., 2010. A column of Amberlite® XAD 16N resin was previously activated (10 minutes) in absolute methanol and packed as a stationary phase. Water was used as the first eluent to remove water-soluble compounds and subsequently eluted with ethanol to recover the polyphenolic fraction. Then, this fraction was placed on a glass Petri dish and dried at room temperature, without exposing it to light for 3 days, after that, the powder was collected in amber bottles and stored for later analysis.

2.2.2. *High efficiency liquid chromatography -mass spectrometry (HPLC-MS) analysis*

The compounds obtained after Amberlite XAD-16 chromatography were analyzed by HPLC (Varian Prostar), model 330 with a UV-visible diode array detector and coupled to a Varian brand mass detector, model 500-MS. A reversed-phase C18 column was used with a flow of 0.2 mL/min with a mass detection limit of 100 to 2000 m/z. For sample injection, these were prepared by weighing 10 mg of each extract and dissolving them in 1 mL of methanol. Samples were sonicated for 5 min at room temperature and finally filtered through 0.45 µm membranes.

2.3. Cytotoxicity

2.3.1. *Cell lines*

The lines used were: Hek 293 (293 [HEK-293] (ATCC® CRL-1573™)) (human cells of embryonic kidney tissue), Vero (ATCC® CCL-81™) (monkey kidney cell), NIH/3T3(ATCC® CRL-1658™) (fibroblast cell line) and Hep-G2 [HEPG2] (ATCC® HB-8065™) (transformed line, hepatocellular carcinoma). All cell lines were obtained from ATCC and were grown on Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 1% buffer HEPES, 1% non-essential amino acids, 1% penicillin/streptomycin and 1% pyruvate. The cultures with 90% confluence were treated with trypsin (5 minutes, 37°C), obtaining a cell suspension that was counted in the Neubauer chamber by the trypan blue exclusion method (Schwarzlin *et al*., 2016).

2.3.2. *Dilutions of phenolic compounds*

The tested concentrations of each phenolic compounds sample were 10, 50 and 100 µg/mL (1 mg of phenolic powder was weighed and proceeded to obtain dilutions). The phenolic powder was resuspended in 1mL of DMEM (culture medium) with 5 µL of DMSO (Houdkova et al., 2017).

2.3.3. *In vitro Cell Stimulation with Extracts and MTT Assay*

A cell density of 8.500 cells per well was plated in 96-well flat bottom plates in a volume of 100 µL medium (DMEM) per well. The cell densities were inoculated in triplicate for each cell line. Subsequently, plates were incubated for 24 h at 37°C with 5% CO_2 to allow cell adhesion.

After 24 h (incubation), the medium was replaced by 200 µL of culture medium with different concentrations of extract, then incubated for 24 h at 37°C with 5% CO_2.

After the last step, the culture medium was eliminated and 100 µL of new culture medium was added. Subsequently, 20 µL of MTT (5 mg / mL Phosphate Bufferes Saline (PBS)) were added. The plates were incubated for 4 h (37°C with 5% CO_2). Then, the supernatant was carefully removed and 100 µL of DMSO were added. The culture plates were read using a microplate reader at 570 nm (Thermo Scientific Multiskan FC). Cell viability was expressed as a percentage. The resulting values were taken as an indirect measure of viable cell mass, compared with untreated control cells. The percentage of viable cells was determined according to the following equation:

cell viability (%) = (absorbance of treated cell/absorbance of control cell) * 100.

2.3.4. *Genotoxicity*

DNA damage was determined by the comet assays (Cell Biolabs). Cells were seeded in 6-well plates (200,000 cells/well), and then incubated at 37º C and 5% CO2 for 24 h, to promote adhesion to the plate. Then, the medium was removed to place the dilutions (10, 50, and 100 µg/mL)of the extract prepared in 1 mL (final volume) of culture medium for 24 h. After that, the supernatant was removed and cells were washed twice with 1X saline solution. Subsequently, cells were removed with a scrapper, resuspended in PBS, and centrifuged, then, cells were resuspended in a final volume at a concentration of 1x10 5 cells/mL. After, cells were mixed with agarose (1:10) and 20 µL of the mixture were placed on the slide, which was incubated at 4 ºC for 15 min under dark conditions, and then submerged in the alkaline solution buffer for 1 h. Subsequently, the slides were immersed in the lysis buffer at 4 ºC for 1 h, the buffer was removed and replaced by an alkaline solution, then, the slide was left at 4ºC for 30 min. The alkaline solution was replaced twice by Tris-Borate-EDTA (TBE), and the slide was left for 5 min. Afterward, the slide was carefully transferred to the electrophoresis chamber, and covered with Tris Buffered Saline (TBS) solution, then 35 volts were applied for 15 min, at the end, the slide was kept in a horizontal position, and washed twice with water for 5 min. The slide was placed in 70% ethanol for 5 min, and green Vista Dye was added. DNA damage was assessed by fluorescence microscopy (Axioscope 5 LED, Carl Zeiss, Germany) with the specific filter for FITC. The images were analyzed with Comet Score 2.0 software to measure DNA fragmentation. Four replicates for each concentration were made.

2.4 Statistical Analysis

The means and standard errors of three replications of cell viability are shown in graphs. Differences between tested and control groups were determined by ANOVA using a statistical significance at $p < 0.05$, when it was needed, treatment mean differences were accessed using a Tukey´s multiple comparison test. Statistical analyses were performed using the SAS Software. Graph Pad Prism 6.0. was utilized to create the graphics.

3. RESULTS

3.1 Extraction and Identification of Polyphenolic Compounds

The color of the aqueous fractions varied from green to straw yellow, while the ethanolic fractions showed transparent colors with intense color saturation to transparent yellowish-green. In this study, 30 different compounds were identified with different combinations of mass/volume, ethanol relations, and a combination of ultrasound and microwaves, these compounds belong to 9 different families. Table 2 shows the distribution of the compounds from each m/v combination, as well as the retention time and compound family. It was observed that two compounds are present in all five extracts, quercetin 3-O-galactoside, and peonidin 3-O- (6 "-acetyl-galactoside), from flavanols and anthocyanidins groups, respectively.

Table 2. Polyphenolic compounds extracted from *Moringa oleifera* leaves using different combinations of mass/volume, ethanol relations and a combination of ultrasound and microwaves.

No. Compound	Family	Compound	8-0% (RT)	8-70% (RT)	12-30% (RT)	16-70% (RT)	16-0% (RT)	Reference
1	Catechins	(+)- Gallo catechin	2.26				8.89	
2		(+)- Catechin		2.22			2.49	Cuellar-Nuñez *et al*., 2018
3	Hydroxycinnamic acid	1-caffeoylquinic acid	17.37		18.87	18.67		Nouman *et al*., 2016
4		3-caffeoylquinic acid	18.07			19.22		
5		4-caffeoylquinic acid	24.90					Bing *et al*., 2015
6		Tyrosine p coumar oil	45.07					
7		Aspartate coffee oil					18.75	
8		3-p-coumaroylquinic acid					33.34	Nouman *et al*., 2016
9		Tartaricpcoumaroil acid					2.28	
10	Flavanols	Quercetin-3-O galactoside	32.93	34.88	34.58	25.48	37.32	Makita, 2017
11		Quercetin	40.77	42.27	41.99			Cuellar-Nuñez *et al*., 2018
12		Quercetin 3-O-glucoside				32.82	37.38	Nouman *et al*., 2016
13		Quercetin 3-O-acetyl-rhamnoside		37.46				
14		Quercetin 4'-O glucoside				32.82		
15		Kaempferol 3-O-galactoside-7-O- rhamnoside		20.27	23.63			
16		Kaempferol 3-O-rutinoside		29.47	25.84			Makita, 2017
17		Luteolin 7-O-rutoniside			28.57			
18		Kaempferol 3-O-rutinoside			33.50			
19	Antocianinas	Peonidin 3-O- (6 "-acetyl-galactose)	34.15	35.70	35.41	35.29	36.79	Nkechinyere Onyekwere & Felix I., 2014

20		Peonidin 3-O- (6 "-acetyl glucoside)	35.08	36.95	36.95	36.72	36.50	
21		Delphinidin 3-O galactoside				28.43		
22	Phenolic terpenes	Rosmadial					3.67	
23	Methoxy cinnamic acid dimers	5-5'-dehydro ferulic acid					29.18	
24	Metoxi flavones	Sinensetin		33.22			35.39	
25	Methoxy cinnamic acid	3-feruloylquinic acid					36.50	Nouman *et al*., 2016
26	Hydroxybenzoic acids	Gallic acid 3-O-gallate					58.66	
27	Dihydrochalcones	Phloretin			2.24			
28	Flavones	Apigenin 6-C-glucoside		33.89				Makita, 2017
29	Curcuminoids	Bisdemethoxyrcurcumin		51.56	51.46	51.35		Nurcholis *et al*. 2016
30	Tyrosols	p-HPEA-EA (possible)		20.27				

3.2. Cytotoxicity of *Moringa oleifera* Extract

To corroborate the cytotoxic effect, extracts were placed in contact with non-cancer cells (3T3, Hek293, and Vero) for 24 h and later the MTT assay was performed. The optical microscopy on 3T3 (before MTT assays) showed that control cells showed smooth and regular surfaces with normal morphology, whereas cells treated with the Mor-8-70%, Mor-12-30%, and Mor-16-70% extracts at 50 and 100 µg/mL showed changes in cell morphology due to cell death (data no shown). In Figure 1 is observed that in 50 and 100 µg/mL extracts concentration with any ethanol gradient (Mor-8-70%, Mor-12-30%, and Mor-16-70%), cell viability of three normal cells lines was affected compared to DMEM effects (assay medium with 5 microliters of DMSO) used as negative control. Extracts behaviour on Hek 293 and Vero cell lines were very similar, in this context, it is possible to observe also, that polyphenols from aqueous extracts (Mor-8-0% and Mor16-0%), which are in low or high concentrations (10-100 µg/mL) did not reduce cell populations, for the conversely, the percentage of cells viability incremented in the case of Mor-16-0%.

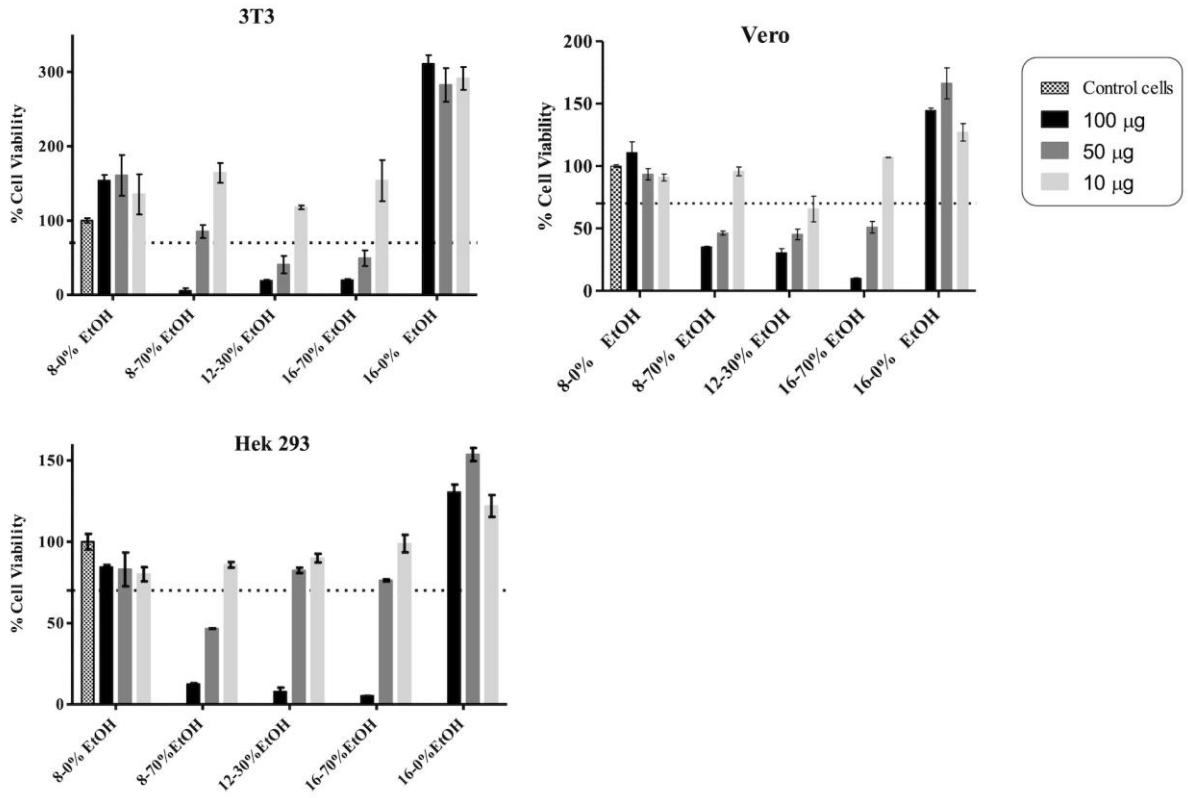

Figure 1. Cytotoxic effect of several dose (100, 50 y 10 µg) aqueous and hydroalcoholic extracts from *Moringa oleifera* on normal cells after 24 h of exposure. a) Effect on fibroblast cell (3T3), b) Effect on cells of embryonic kidney tissue (HEK-293) and c) Effect on monkey kidney cell (Vero).

Figure 2 shows the behaviour of HepG2 after 24 h of stimulation with each extract. Regarding the cells without treatment, the hydroalcoholic extracts show evident cytotoxicity at concentrations of 50 and 100 µg/mL without showing a significant difference between both doses ($p< 0.05$), while aqueous extractions do not show cytotoxicity according to the ISO standard 10993 maintaining cellular viability above 70% indicated as the permissible limit (Miller *et al*, 2017). On the contrary, the viability of the cells is increased particularly when the extraction volume is increased (16-0%).

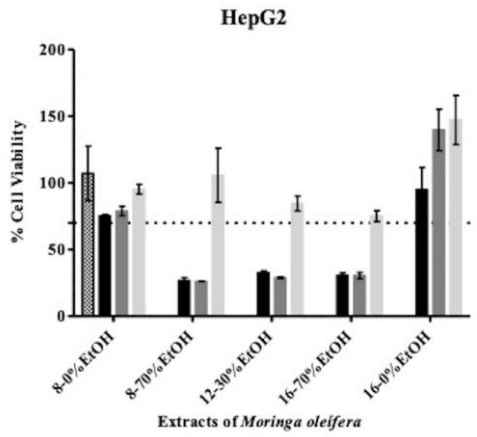

Figure 2. Effect of several doses (100, 50 and 10 µg) of aqueous and hydro-alcoholic of *Moringa oleifera* extract on Hep-G2 (transformed line, hepatocellular carcinoma) cytotoxicity after 24 h of exposure.

3.3. Hydro-Ethanolic Extracts Cause DNA Damage of HepG2 Cell Line

After learning that hydroalcoholic extracts affected cell viability, DNA damage was determined (Figure 3). Cells were exposed to 10, 50, and 100 µg/mL concentrations, with different m/v extraction ratios (8-70, 12-30, and 16-70). In all cases, greater DNA fragmentation was observed in relation to concentration, conferring a dose-response phenomenon. Regarding the % of DNA in the tail, it is observed that as the concentration increased, the percentage of DNA in the tail also increased, and this agrees with the results of the graphs of both, the length of the tail and the moment, which are parameters that are associated with the DNA fragmentation. In the 3 parameters (Tail DNA%, Length, and Moment) model, all treatments show significant differences in comparison to the control. The results obtained indicated a significant damage in the DNA of HepG2 cells after incubating with the ethanol extracts obtained from leaves.

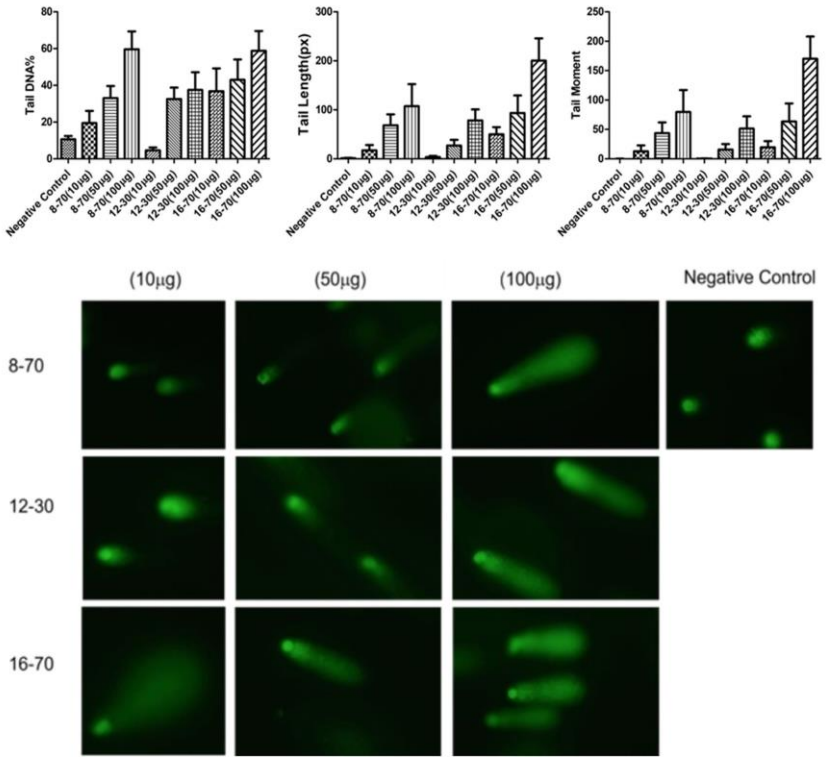

Figure 3. DNA Damage of HepG2 cell in response to the three m/v extract concentrations from *Moringa oleifera* extract.

4. DISCUSSION and CONCLUSION

The highest number and different polyphenols were extracted using a higher mass/volume ratio and low levels of ethanol (0 or 30%). The optimization of time in an extraction process is an important parameter to consider (Azmir *et al*., 2013), results showed that different polyphenols can be obtained for 30 min extraction time, compared to the literature that ranges up to 24 hours (Makkar & Becker, 1996). In this study, polyphenols were obtained under environmentally friendly extraction conditions such as water and ethanol and ultrasound and microwave technologies, contrasted with other authors who use other types of solvents and conventional technologies (Cuellar-Nuñez *et al*., 2018).

The flavonoids are the group of phytochemicals with the highest presence in the five extracts, however, they were more frequently found in the extracts with ethanol, Mor-8-70%, Mor-12-30%, and Mor-16-70% than in aqueous extracts Mor-8-0% and Mor-16-0%. In overview, the present study revealed a higher number of polyphenolic compounds in Mor-8-70% and Mor-16-0% extracts. The observed result could be due to different degrees of the polarity of the solvents used for the extraction of polyphenolic compounds and thus could contribute significantly to the cytotoxicity and pharmacology activity (Moyo*et al.*, 2012).

Quercetin 3-o-galactoside was found in all extracts. In a parallel comparison to other authors reported that this compound extracted from another plant source, and under various extraction stages, using solvents such as water, ether, and ethyl acetate provided a protective effect on PC12 cells against the cytotoxicity induced by hydrogen peroxide, and tert-butyl hydroperoxide (Liu *et al.*, 2005). Kaempferol 3-O-rutinoside was found in Mor-8-70% and Mor-12-30% extracts and these results are homologous to those reported in the literature from two *M. oleifera* species (Makita *et al.*, 2017). The extracts were obtained with ultrasound-assisted extraction (30 minutes) using 80% methanol as solvent. Several trials demonstrated the pharmacological properties of glycosides of quercetin and kaempferol, as well as the positive relationship between the consumption of vegetables with flavonoid content and a positive effect on health (Makita *et al.*, 2017). The identification of flavanols (Quercetin 3-O galactoside) in all extraction is consistent with previous reports in the literature, which is one of the most reported phytochemicals extracted from *M. oleifera* (França *et al*., 2017). Also, all hydroalcoholic or aqueous extracts contained anthocyanidins (Peonidin 3-O- (6 "-acetyl-galactoside) (Nkechinyere Onyekwere & Felix I, 2014). The compounds from the catechins group found were: gallocatechin, present in the extracts of Mor-8-0% and Mor-16-0%, and the catechin in the extracts Mor-8-70% and Mor-16-0%.

Cytotoxicity results are in concordance with the data (Mekonnen, Houghton & Timbrell, 2005), who evaluated the cytotoxicity of ethanol (80%) and aqueous extracts of *Moringa stenopetala* (Baker f.) Cufod. on HepG2 cells using the lactate dehydrogenase (LDH) test to measure cytotoxicity.

The results obtained indicated a significant decrease in HepG2 cells viability after incubating with the ethanol extracts obtained from leaves, while the aqueous extract of the leaves did not alter the levels of cellular viability, which suggests that ethanol extracts may be toxic (Mekonnen, Houghton & Timbrell, 2005). According to *in vitro* result, the aqueous extract does not cause cytotoxic damage; on the contrary, they induce cell proliferation, which may be associated with the presence of (+)-Gallocatechin, an exclusive molecule in this aqueous fraction, which has recently been reported to induce the synthesis of growth factors and collagen, promoting the regeneration of wounds (Vendidandala *et al*., 2021). These results are in agreement with literature where *Moringa* polyphenols obtained with water protect Hek293 cells against the damage generated by cadmium (Souid *et al*., 2020).

On the other hand, the presence of Bisdemethoxyrcurcumin exclusively in the ethanolic fraction suggests the participation of this molecule causing the death of cells in the 3t3 line, this agrees with the literature, since it induces apoptosis in fibroblasts (Jin *et al*., 2019), as well as Bisdemethoxyrcurcumin has been shown to induce apoptosis, and inhibit the proliferation of cancer cells (Liu *et al*., 2011; Li *et al.*, 2013).There are few reports in the literature of *in vitro* genotoxicity caused by *Moringa oleifera* extracts (Alkan *et al*., 2022), according to these results, *Moringa* extracts can cause DNA damage causing fragmentation, which is related to the mechanism of apoptosis already reported. In this study, it was found that ethanolic extracts cause DNA fragmentation, and this damage is related to the concentration of the extract.

Ultrasound and microwave technology at reaction times of 30 min demonstrated the obtaining of polyphenols from *Moringa oleifera* leaves identified by HPLC. In this study, it was observed that polyphenols obtained from aqueous extracts preserve a percentage of cellular viability higher than 70% established as the limit allowed by the Norma ISO standard 10993-5: 2009-10 test to evaluate the *in vitro* cytotoxicity of medical devices. Also, it was found that Mor-8-70%, Mor-12-30% and Mor-16-70% (hydroalcoholic extracts) had a high cytotoxic effect on all tested cell line, decreasing cell viability in a dose-dependent manner and that in HepG2 (cancer cell line) they induce apoptosis.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Rosario Estrada contributes to cell experiments, **Diana Salazar** contribute extraction of polyphenols, **Aide Saenz** and **Juan Ascasio** contributed to the characterization of the compounds, **Raul Rodriguez**, **Carolina Flores** and **Cecilia Esparza** contributed to the experimental analyzes and to the writing of the article.

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