



EVALUATION OF PHYTOCHEMICAL COMPOUNDS IN *Moringa oleifera* Lam. EXTRACTS ON CYTOTOXICITY OF MCF-7 CELL LINE

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Marwa S. Abd El Alem¹, Ali S.H.², Sarwat M.I.² and Hussein S.H.¹

1. Medicinal and Aromatic Plants Dept., Horticulture Research Institute, Agriculture Research Center, Giza, Egypt

2. Agric. Biochemistry Dept., Fac. of Agric., Ain Shams Univ., P.O. Box 68 Hadyek Shoubra 11241, Cairo, Egypt

*Corresponding author: marwa.saad54@yahoo.com

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ABSTRACT

Moringa oleifera Lam. which called "Miracle tree" was an enormous usage of medical, nutritional and economic benefits so its leaves and roots extracts were vitro examined for phytochemicals contents, antioxidant, and cytotoxicity against MCF-7 cell line. Leaves and roots both extracts were estimate using 2,2-di phenyl-1-picryl hydrazyl (DPPH) radical scavenging assay and high performance liquid chromatography (HPLC) to determine phenolic and flavonoids compounds. The leaves aqueous and ethyl acetate extracts contained a significantly ($p < 0.05$) more phenolic compounds than root extracts, while roots ethyl acetate extracts were significantly more active in DPPH assay comparing with aqueous extracts. However, the leaves aqueous extract and roots ethyl extracts were contained high values flavonoids compound but in case of phenolic compounds the aqueous roots extract recorded the greatest highest number followed by the leaves ethyl acetate extract. The ethyl acetate extractions for leaves and roots have a high inhibition percentage against breast adenocarcinoma MCF-7 cell line comparing with aqueous extracts.

Keywords: Antioxidant, MCF-7 cell line, HPLC, *Moringa oleifera* leaves and roots, ethyl acetate extract

INTRODUCTION

The few decades have seen side effect of chemical drugs on human health and increasing ecological pollutant. Also, depending of peoples on fast foods and bad behaviors in nutrition all of

this leads to appearance several diseases due to formation of free radical in bio-systems. So, approximately 80% of the world population turns to using medicinal plants which consider one of curative and against harmful diseases. *Moringa oleifera* Lam. is the most vastly cultivated species of monogeneric family, moringaceae which is local to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan, **Fahey (2005)**. This rapidly-growing tree was also utilized by the ancient Romans, Greeks and Egyptians against diseases. It is now widely cultivated and has become naturalized in many locations in the tropics. All *Moringa oleifera* parts are edible and have long been utilized by human beings **Fuglie (1999)**. *Moringa oleifera* leaves are well-known to have numerous good biological activities, as reported by **Iqbal and Bhanger (2006)**, **Chumark et al (2008)**. **Gupta et al (1999)** mentioned that *Moringa oleifera* Lam. Methanol extraction of root showed central nervous system depressant action.

Many studies have investigated that some naturally occurring phytochemical agents, such as phenolic compounds (e.g. alkaloids and flavonoids), specifically those ingested in the human beings diet, trigger cancer-cell death machineries and can be used as chemo preventive candidates against certain cancerous cell types **Goodman (2000)**, **Gao et al (2002)**, **Ahmed et al (2015)** and **Abd-Rabou (2016)**.

Chumark et al (2008) and **Singh et al (2009)**. stated that *Moringa oleifera* Lam. is a rich source of antioxidant, as, its aqueous extracts of in some plant parts performance as an antioxidant. **Lalas and Tsaknis (2002)** and **Siddhuraju & Becker (2003)** concluded that *Moringa oleifera* Lam freeze

dried leaves. has different high amount of extraction, such as methanol and ethanol with 65.1 and 66.8%, respectively. *Moringa oleifera* Lam. has a more strong anticancer plant beside several bioactive compounds (e.g., niazimicin and thiocarbamate) with discovered significant antitumor bounce, **Guevaraa et al (1999)**.

So, this study was conducted to evaluate effect of different extracts of *Moringa oleifera* Lam. against cancer disease.

MATERIALS AND METHODS

Plant material

Moringa oleifera dry powder: Both dry powder of leaves and roots were kindly obtained from National Center for Research (NCR), Giza, Egypt.

Preparation of *Moringa oleifera* extracts

Organic extraction of *Moringa oleifera* Lam. powder of leaves and roots was carried out by ethyl acetate according to the method described by **Harbone (1984)**.

The residue was weighted and the yield percentage was calculated, then the residue was stored at 4°C to be ready for using.

Aqueous *Moringa olifera* Lam. extract was prepared according the method of **Shah et al (2015)**.

Determination of phenolic compound

Total phenolic compounds were determined with Folin-Ciocalteu reagent using Gallic acid as a standard according to the method described by **Singleton and Rossi (1965)**.

Determination of antioxidant activity

DPPH radical scavenging assay

The scavenging effect of crude extracts of *Moringa oleifera* Lam. leaves and roots on DPPH radical was estimated according to **Jain et al (2008)**.

The radical scavenging activity was determined based on percentage inhibition of absorbance, which was calculated using the following equations:

$$(\%) \text{ DPPH radical scavenging activity} = [(A_{\text{control}} - A_{\text{sample}}) / (A_{\text{control}}) \times 100]$$

Separation and quantification of phenolic compounds and flavonoids.

High pressure liquid chromatography (HPLC) analysis carried out at the Food Technology Institute, Agriculture Research Center (NRC), Giza, Egypt. According to the method of **Goupy et al (1999)**.

In-vitro studies (Cell culture, maintenance and sub-culture)

The *in-vitro* cytotoxicity assay was assessed in the cell culture lab, Faculty of Agriculture, Cairo University, According to the method of **Repetto et al (2008)**.

A- Cultivation, propagation and adaptation

Breast cancer MCF-7 cell lines and cultured using Dulbecco's modified Eagle's Medium (DMEM), and Complete media: DPBS supplemented with 10 % Fetal bovine serum (Sera Lab)+1% antibiotic, i.e. penicillin G potassium+ streptomycin (sigma). Cells were cultured in 5% CO₂ at 37°C then treated with 0.25% (w/v) trypsin/EDTA to affect cell release from the culture flask. After washing the cells with phosphate buffered saline (PBS), to get rid of media.

B- Sub-culturing, counting and plating

After formation of a complete healthy cell sheet, the cells were harvested by trypsinization using 1 to 2 ml warm (37°C), 0.5% trypsin-EDTA in order to lysis between cells but EDTA playing important role in removing Mg⁺², Ca⁺² ions which help in adhering cells with-ve ions in flask surface. So, cells fell apart surface. The trypsin reaction was stopped after 2 min by addition of 10 ml complete media containing 10% FBS. The detached cells were harvested by centrifugation at 2000 rpm for 10 min using falcon tubes. The cell pellet was re-suspended in 1 ml of fresh complete media containing 10% FBS. The number of viable cells was counted using short-term viability assay and 0.4% trypan blue (1:1) was used to stain dead cells and examined under microscope using hemocytomete. The number of viable cells was not less than 80%.

C- Testing of the cells for cytotoxicity

First day: a cell suspension containing 25.000 cells was delivered to each well of a 96 well micro-liter tissue culture plate and incubate at 37 °C in a

humidified carbon dioxide chamber (5%) for 24 h in order to allow the cells to attach to the plate surface and to grow normally. Then, examinations of cells were performing to detect contamination and apoptosis on cell replication.

Second day: the aqueous and ethyl acetate extracts of (leaves and roots) *Moringa Oleifera* with concentration 100, 200 and 400 µg/well were applied in triplicates were adding with 1% antibiotic without using BSA to known effect of extract only. But, in negative control complete media (10% Fetal bovine serum+1% antibiotic) and positive control using (Doxorubicin HCl 3 µg/ml) as chemotherapy and incubate at 37°C in a humidified carbon dioxide chamber (5%) for 24 h in order to examined toxicity test.

Third day; Toxicity test: the culture media was removed 0.2 ml of Dulbecco 's phosphate buffered saline solution (DPBS) containing 0.01% neutral red dye was added to each well to penetrate viable cells and binding with lysosome, and the cells were incubated for an additional 2-4 h. De-stained step; the dye was removed and the cells were washed once with DPBS. Then, 0.2 ml of 50% ethanol in 1% acetic acid was added to each well. After, gentle shaking for 30 minutes at room temperature, the absorbance at 570nm was measured by Ellissa instrument.

High concentration: indicate to cell viability.

Negative control: instead red colour.

Toxicity case: red colour diluted.

Calculation: percentage of viability cell= $(A_{\text{sample}}/A_{\text{control}}) \times 100$

RESULTS AND DISCUSSIONS

The results listed in **Table (1)**. Showed that total phenols yield in aqua-leaves (43.02±3.77 µg/ml) was higher than aqua-roots (26.05±1.98 µg/ml) extracts. While, ethyl-roots extracts (20.78±0.92 µg/ml) showed the lowest total phenols content comparing with ethyl-leaves and aqua-extracts. It can be noticed that total phenols of the two different extracts of *Moringa oleifera* Lam. under study were significantly ($p \leq 0.05$) differed due to depending on type of solvent. This may be due to the differences in the polarity of the solvents used; such observation was in agreement with the finding of **Unuigbe et al (2015)**

The results listed in **Table (1)** present DPPH free radical scavenging activity of aqueous and ethyl acetate extracts of *Moringa oleifera* Lam.

Compared to vitamin C as a reference antioxidant. The highest percentage of antioxidant activity

comparing with vitamin C followed by ethyl-roots extract and aqua-extract. These obtained results showed that the ethyl-roots extract has higher scavenging activity (82.75±4.37%) than ethyl-leaves extract (79.61±7.2%). While, aqua-leaves extract showed the lowest scavenging activity (66.65±1.45%) compared with aqua-roots and ethyl acetate extract.

Generally, it could be concluded that ethyl acetate extract possessed the highest antioxidant activity comparing with aqueous extract of *Moringa oleifera* Lam. higher antioxidant of ethyl acetate and aqueous extracts may be due to the presence of high content of antioxidant phenol in phenolic sample extract as mentioned before, these results were agreement with **Charoensin (2014)**.

Table 1. Yield and activity of antioxidant phenolic compounds of *Moringa oleifera* Lam. leaves and root on dry weight basis.

<i>Moringa oleifera</i> Lam.Extracts				
	Aqueous Extracts 10 g/100 ml		Ethyl acetate Ex- tracts 400 mg/Kg	
	Leaves	Roots	Leaves	Roots
Phenolic compounds (µg/ml)	43.02± 3.77 ^a	26.05± 1.98 ^{bc}	29.09± 0.91 ^b	20.78± 0.92 ^c
DPPH (%A.A)	66.65± 1.45 ^b	75.33± 7.32 ^a	79.61± 7.20 ^a	82.75± 4.34 ^a

Each value represents the average of three replicates ± stander errors.

The small letter compared in rows and the same letters means non-significant effect at $p < 0.05$ significant level.

Therefore, it's more beneficial as a medicinal plant for alternative anticancer drugs and nutraceutical products, **Igbo et al (2015)**. **Leelavinathan (2007)** and **Abas et al (2015)** conducted that *M. oleifera* have antioxidant indicates, so the plant could be promising agent in scavenging free radicals.

Cytotoxic effect of *Moringa oleifera* Lam. aqueous and ethyl acetate extracts of leaves and roots.

The percent value of viability cell concentration (MCF-7) cell line of applied moringa extracts listed in **Table (2)** and illustrated in **Figs. (1a-n)**.

This data refers to inhibition % of ethyl acetate extract of *M. oleifera* roots and leaves 93-69% inhibition respectively, showing higher Inhibition percent than aqueous one at concentration 400 µg/ml. In contrast to the trends noted for %viability the ethyl acetate extract for roots and leaves denoted

decrease in viability percent of the cells. We can reason that, due to contain compounds that have selective proliferative activity in different cancer cell lines specially MCF-7 under study. The anticancer activity of ethyl acetate roots and leaf extract of *M. oleifera* may in part be attributed to the presence of phenolic compounds in the plant as reported by (Khalafalla et al 2010).

In addition to its anticancer properties, *M. oleifera* is also, a potent antioxidant Das sujoy et al (2012) and Verma et al (2012) and portrays a wide spectrum antibiotic activity Peixoto et al (2011). Pathogenic slides from (3a) to (30) were in accordance with the results illustrated in Table (2).

Table 2. Cytotoxic effect of *Moringa oleifera* Lam. aqueous and ethyl acetate extractS of Leaves and Roots.

Treatments Con. µg/ml	Viability %				Inhibition %			
	Ethyl. leaves	Ethyl. Roots	Aqua leaves	Aqua roots	Ethyl. leaves	Ethyl. Roots	Aqua leaves	Aqua roots
100	55	65	76	75	45	35	24	25
200	46	50	75	74	54	50	25	26
400	31	6.0	69	71	69	94	31	26

Each value represents the average of three replicates.

Ethyl acetate sample was dissolved first in DMSO.

Aqua: Aqueous extract. Ethyl: Ethyl acetate extract.

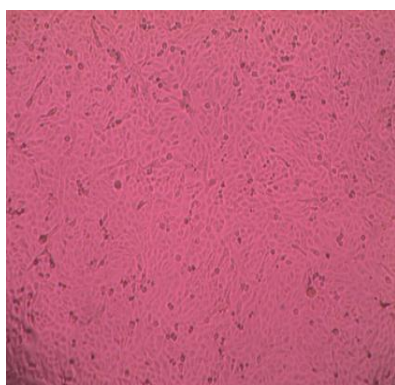


Fig. 1a. Negative control.

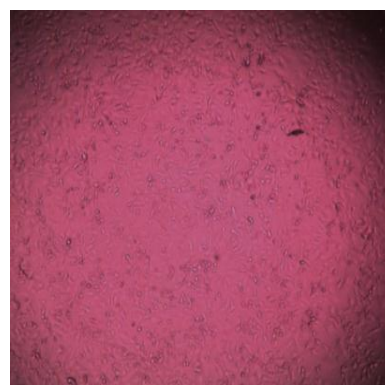


Fig. 1b. Positive control MCF-7 cells.

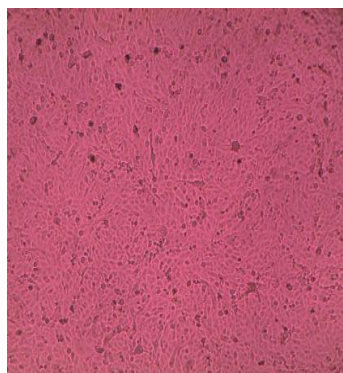


Fig. 1c. Aqua-Leaves (100 µg/ml)

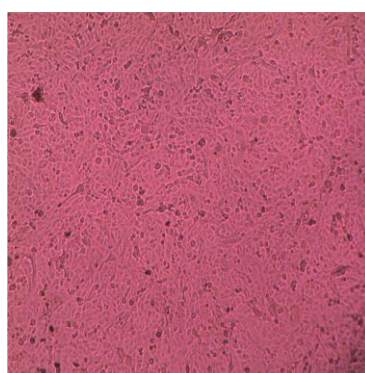


Fig. 1d. Aqua-Leaves (200 µg/ml).

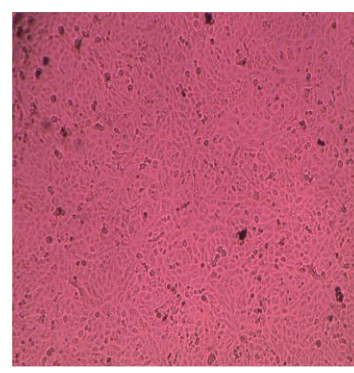


Fig. 1e. Aqua-Leaves (400 µg/ml).

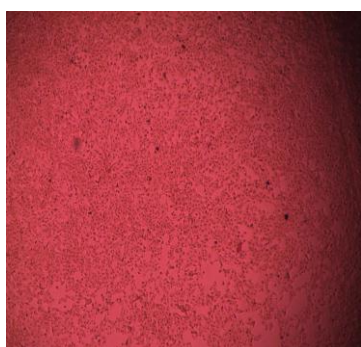


Fig. 1f. Ethyl-Leaves (100 µg/ml)

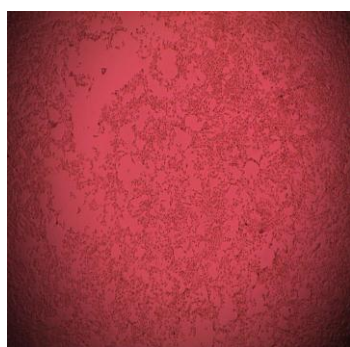


Fig. 1g. Ethyl-Leaves (200 µg/ml)



Fig. 1h. Ethyl-Leaves (400 µg/ml)

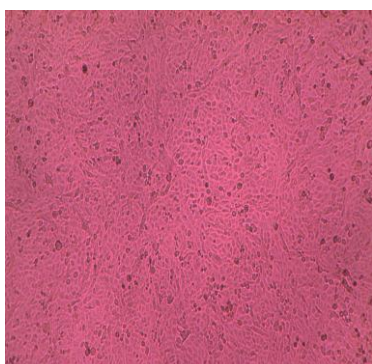


Fig. 1i. Aqua Roots (100 µg/ml)

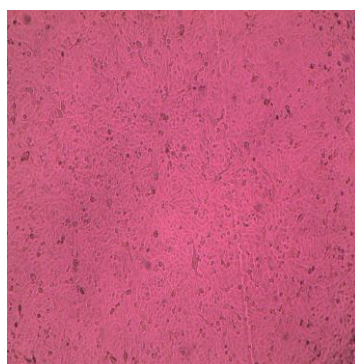


Fig. 1j. Aqua Roots (200 µg/ml)

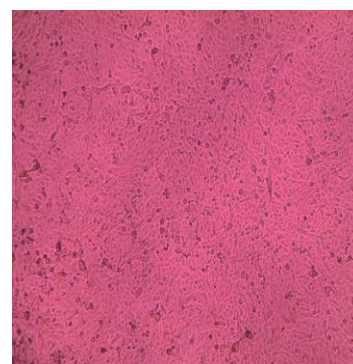


Fig. 1k. Aqua Roots (400 µg/ml)



Fig.1l. Ethyl - Roots(100 µg/ml)

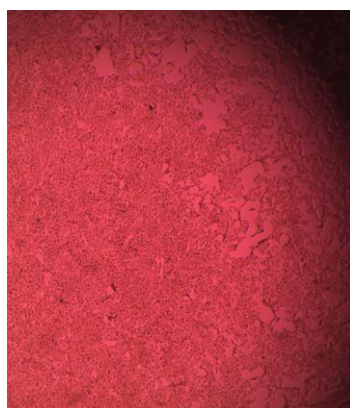


Fig.1m. Ethyl – Roots (200 µg/ml)

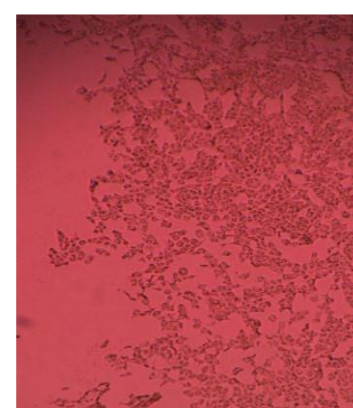


Fig.1n. Ethyl - Roots(100 µg/ml)

Identification and quantification of flavonoids compounds of *Moringa oliefera* Lam. (leaves and roots) aqueous and ethyl acetate extracts by HPLC.

Moringa oliefera Lam. extracts were subjected to HPLC analysis to investigate the types of flavonoid compounds responsible for their antioxidant, anti lipidemic and anti-carcinogenic activities. In this investigation, 18±4 flavonoid compounds were identified and quantified in *Moringa oliefera* Lam. and its extracts by comparing the HPLC chromatograms

of them with the HPLC chromatograms of standard compounds based on the retention time. The obtained data were listed in **Table (3)**. Flavonoids which identified were naringin, hesperidin, naringin, kampferol, quercetin and other flavonoids.

Remarkable data analysis of both extracts revealed higher content of most flavonoid compounds in *Moringa* in two extracts, In case, of flavonoid compounds in aqua-leaves extract showed the highest values of flavonoid compounds (17) containing naringin (1678.33ppm) and rutin

(1557.81ppm) with high concentration comparing with ethyl-leaves extract naringin(82.03 ppm) and rutin (173.63ppm), also, high level of apig. 6-arabinose-8-galactose (832.44ppm), luteo.7-glucose (619.02 ppm), apigenin-7-glucose (409.74 ppm) and acacetin neo-rutinoside (520.57 ppm) found in aqua-leaves extract comparing with ethyl-leaves were recorded apig. 6-arabinose-8-galactose (94.25 ppm), apigenin-7-glucose (36.93 ppm) and no detection were found in acacetin neo-rutinoside and luteo.7-glucose flavone. On the other hand, flavones were found in aqua- leaves extract but in low concentration such as quercetrin (281.16 ppm), kamp. 3,7-dirhamoside (258.50 ppm), apig.6-rhamnose-8-glucose (254.54 ppm), hespirtin (163 ppm), quercetin (119.41 ppm), rhamentin (133.26 ppm), naringenin (62.75 ppm), finally kampferol (15.49 ppm) and apegnin (25.22 ppm) comparing with ethyl-leaves extract quercetrin (87.7ppm), kamp.3,7-dirhamoside (97.41ppm), apig.6-rhamnose-8-glucose (95.31ppm), hespirtin (72.51 ppm), quercetin (101.1ppm), finally kampferol (28.20 ppm)and apegnin (15.73 ppm) but rhamentin, naringenin flavon not detected in this extract.

Table 3. Value of flavonoids compounds in *Moringa oleifera* Lam. leaves and roots determined by HPLC analysis.

HPLC analysis Test results of flavonoids (ppm)				
Flavonoids	Leaves		Roots	
	Aqueous	Ethyl acetate	Aqueous	Ethyl acetate
Apig. 6-arabinose-8-Galactose	832.44	94.25	169.42	969.11
Apig. 6-rhamnose-8-Glucose	254.54	95.31	78	213.79
Naringin	1678.33	82.03	330.18	596.78
Luteo.7-glucose	619.02	ND	584.50	203.04
Hesperidin	ND	1145.97	1689.75	3123.15
Rutin	1557.81	173.63	144.66	423.18
Apig.7-o-neo hespirtin	547.88	46.76	102.32	164.85
Kamp.3,7-dirhamoside	258.50	97.41	54.37	83.14
Quercetin	281.16	87.69	92.29	479.07
Apigenin-7-glucose	409.74	36.93	18.19	57.64
Acacetin neo.hespirtin	102.22	53.71	116.41	382.27
Acacetin neo. rutinoside	520.57	ND	ND	ND
Quercetin	119.41	101.11	48	155.63
Naringenin	62.75	ND	37.64	74.94
Hespirtin	163.01	72.51	68.45	498.97
Kampferol	15.49	28.20	12.75	77.83
Rhamentin	133.26	ND	ND	142.93
Apegnin	25.22	15.73	15.16	28.19

All values were the mean of two injections

In case of roots extracts data recorded that, ethyl-roots extract show highest concentration of

flavonoids such as hesperidin (3123.15 ppm) comparing with aqua-roots (1689.75 ppm). Also, shows high concentration in this extract as follow apig.6-arabinose-8-galactose (969.11 ppm), naringin (596.7 ppm), hespirtin (498.97ppm), quercetin (479 ppm), rutin (423ppm), acacetin neo. hesperside (382.27 ppm), quercetin (155.63 ppm), follow by low concentration of kampferol (77.83 ppm), and naringenin (74.94 ppm) and apegnin (28.19 ppm) comparing with aqua-roots extract were shows apig.6-arabinose-8-galactose(169.4 ppm), naringin (330 ppm), hespirtin (68.45 ppm), quercetrin (92.29 ppm), rutin(144 ppm), acacetin neo. hesperside (116.4 ppm), quercetin(48 ppm), follow by low concentration of kampferol (12.75 ppm),and naringenin (37.64 ppm) and apegnin (15.16 ppm) finally, there is no detection for acacetin neo. rutinoside in both aqua-roots and ethyl-roots extracts.

In dried leaves, myricetin is found with low concentration, while kaempferol and quercetin are found with medium concentrations, whereas, higher amounts are presented in freeze-dried leaves (Siddhuraju & Becker, 2003 and Amaglo et al 2010). Yang et al (2008) reported that flavonoids, for example apigenin, genistein and luteolin are not found in *M. oleifera* leaves. Modi et al (2010) illustrated that all parts of *M. oleifera* Lam. has combination between flavonoids and phenolic compounds, which treatment of numerous diseases for human.

Table 4. Values of Phenolic compounds in *Moringa oleifera* Lam. Leaves and roots determined by HPLC analysis.

HPLC analysis Test results of phenolic compounds (ppm)				
Phenolic compounds	Leaves		Roots	
	Aqueous	Ethyl acetate	Aqueous	Ethyl acetate
Gallic Acid	176.75	3.02	97.11	446.78
Pyrogallol	2799.80	232.51	3288.70	46913.55
4-Amino-benzoic acid	ND	3.17	19.63	93.86
Protocatechin	ND	42.87	539.86	710.97
Catechin	4349.36	172.38	652.88	2169.34
Chlorogenic acid	169.75	66.48	161.34	458.24
Catechol	2157.05	121.64	11622.83	1321.27
Caffeine	240.30	81.45	157.97	928.37
p-OH-benzoic acid	951.20	229.94	269.32	1096.26
Caffeic acid	80.99	18.14	116.25	ND
Vanillic acid	295.84	ND	119.19	889.57
p-Coumaric acid	90.23	76.05	23.80	164.21
Ferulic acid	234.09	99.66	65.70	360.19
Iso-Ferulic acid	54.26	16.79	15.71	131.39
Alpha-coumarin	114.08	14.79	35.81	ND
Ellagic acid	ND	77.15	ND	1338.60
Benzoic acid	825.34	460.67	1152.47	1778.92
Coumarin	282.46	41.46	136.12	463.27
Coumarin-3,4,5-tri.methoxy	121.89	ND	44.55	ND
Salicylic acid	280.86	192.42	974.70	1163.07
Cinnamic acid	64.21	9.54	15.0	51.94

All values were the mean of two injections.

Identification and quantification of phenolic compounds of *Moringa oleifera* Lam. (leaves and roots) aqueous and ethyl acetate extracts by HPLC.

Moringa oleifera Lam. extracts were subjected to HPLC analysis to investigate the types of phenolic compounds responsible for their antioxidant and anti-carcinogenic activities. In this investigation, 21±4 phenolic compounds were identified and quantified in *Moringa oleifera* Lam. and its extracts by comparing the HPLC chromatograms of them with the HPLC chromatograms of standard compounds based on the retention time. The obtained data were listed in **Table (4)**. Phenolic compounds which identified were gallic, pyrogallol, caechin and chlorogenic and other phenols.

Remarkable data analysis of both extracts revealed higher content of most phenolic and flavonoids compounds in *Moringa* in two extracts, In case, of phenolic compounds Pyrogallol compound showed the highest level of phenolic compounds in both extracts of roots and leaves. On the other hand, aqueous extract of root showed higher contents of phenolic compound except Ellagic acid which detected in ethyl acetate extract for both leaves and roots.

Coumarin 3, 4, 5 tri-methoxy not detected in ethyl acetate extracts. Chlorogenic, caffeine, caffeic acid, vanillic and coumarine contents were found in moderate concentrations but, content of p-coumaric, ferulic acid, iso-ferulic and alfa-coumarine were found in low concentration, as well as cinnamic acid were detected in lower concentration in both extracts.

Oboh et al (2015) memorized that Gallic and chlorogenic acid, kaempferol, and quercetin are compounds of phenolic found in the leaves extract.

Prakash et al (2007) and Singh et al (2009) reported that dried leaves containing, gallic acid, chlorogenic and caffeic with low concentration but, ratable concentrations were found for ellagic and ferulic acids. Also, **Bajpai et al (2005)** noted the poorly detectable amounts from this compounds .

In this case, the maximum studies difference for flavonoids and phenolic compounds probably due to variation of environmental conditions extraction method and sensitivity of the analytical methods.

Generally it can be noticed that flavonoids and phenolic compounds are believed to be the major phytochemicals responsible for antioxidant activity resulting to their scavenging ability due to their

hydroxyl groups and also, acted the following pharmaceutical roles:

1. Kaempferol has been reported to possess antioxidant and anti-inflammatory activity (**karthivashan et al 2013**).
2. chlorogenic acid has many biological properties, including antibacterial, antioxidant, particularly anticarcinogenic activities (**Bassoli et al 2008 and Santos et al 2006**).

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تقيم المركبات الفينولية في مستخلصات نبات المورينجا على التسهم الخلوى للخلايا MCF-7 [2]

مروة سعد عبدالعليم¹ - صفوت حسن علي² - مصطفى إبراهيم ثروث² - سيد حسن حسين¹

1. قسم النباتات الطبية والعطرية- معهد بحوث البساتين- مركز البحوث الزراعية- جيزة- مصر
2. قسم الكيمياء الحيوية الزراعية- كلية الزراعة- جامعة عين شمس- ص.ب 68- حدائق شبرا 11241- القاهرة- مصر

*Corresponding author: marwa.saad54@yahoo.com

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وكان مستخلص خلايا الأثيل للجذور والأوراق أعلى
في نسبة التنشيط مقارنة بالمستخلص المائي للجذور
والأوراق.

الكلمات الدالة: المورينجا أوليفيرا، المستخلصات المائية
والعضوية، خط الخلايا MCF-7

الموجز

يهدف هذا البحث الى دراسة تأثير المستخلصات
المائية والعضوية لخلايا الأثيل على جذور وأوراق
نبات المورينجا أوليفيرا وذلك لمعرفة تأثير كل من هذه
المستخلصات على خلايا MCF-7 وأظهرت النتائج
أن المستخلصات المائية والعضوية لخلايا الأثيل قد

تحكيم: ا.د. نجاح الشحات

ا.د. محمود عبدالرازق دهيم