SUPPLEMENTARY MATERIAL

Enhancement of Phenolic Content, Antioxidant and Cytotoxic Activities of *Moringa oleifera* Leaf and Seed by Suspension Culture

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Abstract

Moringa oleifera Lam. family *Moringaceae* is well known for a wide range of biological activities and a complex phytochemical composition. The current study investigates tissue culture protocols for *Moringa oleifera* leaves and seeds. For static culture initiation, Murashige and skooge (MS) as a basal medium with hormonal supply of (0-10 µM) of 2,4-Dicholorophenoxy acetic acid and 6-benzyl aminopurine for *Moringa oleifera* seeds and leaves was employed. Suspension cultures with the optimum hormonal combination was initiated for both seeds and leaves calli. Liquid chromatography/mass spectroscopy (LC/MS) analysis performed, for the first time, on the methanolic extracts of plant parts and the produced calli revealed varying concentrations of nine major components (six flavonoids and three phenolic acids). Antioxidant and cytotoxic activities, against three cell lines, were evaluated for the obtained methanolic extracts. In general, superior biological activities were identified for the produced calli when compared to plant parts.

Experimental

Plant material

M. oleifera seeds were obtained from Faculty of Agriculture Tanta University, Egypt. *M. oleifera* leaves were cultivated in the garden in the faculty of Pharmacy Damnhour University. The species was identified by Dr Rasha Abdel Rhaman, Tanta University, Egypt. Reference specimens were given a code MO32018 and kept at the herbarium of the Faculty of Pharmacy, Damnhour University, Egypt.

Initiation of static and suspension cultures of M. oleifera seeds and leaves

A hormonal combination of 2,4-D and BA was used with a concentration range between (0-10) µM. The best results and calli from leaves and seeds of *M. oleifera* were initiated on MS (Caission Laboratory, USA) solid media supplemented with 8.88 µM of benzyladenine (BA; Sigma Chemical Company, St Louis, Mo63178) and 9.05 µM of 2, 4-dicholorophenoxy-acetic acid (2, 4-D; Eastman Kodak Chemical Company, Rochester, NY14650), 1% agar (Bact agar agar type800, B&V, Parma, Italy) , 30 gl^{-1} sucrose, pH

adjusted at 5.6, 16 h photoperiod at 22°C. Also, calli from *M. oleifera* seeds were initiated on MS basal medium without adding any growth regulators. Cell suspensions for all were established by transferring 1-2 g fresh weight callus into 100 ml MS liquid medium supplemented with of 8.88 µM BA and 9.05 µM 2, 4-D and subcultured every 2 weeks for 16 weeks.

Methanolic extract preparation

For the leaves of *M. oleifera*, the dried powdered leaves (2g) were extracted in 70% methanol for 96 hr at room temperature, concentrated to a dark green residue using rotary evaporator at 50-60° C under reduced pressure, dissolved in 1% HCL and the solution was extracted with 70% Methanol and stored in air tight containers at 4° C to be used in analysis. For *M. oleifera* seeds, the peeled dried powdered seeds (2g) were defatted in petroleum ether for 72 hr at room temperature, then the residue was treated as previously mentioned for the leaves to obtain the methanolic extract. For calli harvested from all suspension cultures of *M. oleifera* seeds and leaves (16 weeks of suspension culture time), freeze dried and powdered calli (2g for each) were extracted and concentrated as previously illustrated.

Identification of compounds by LC/MS analysis

For LC/MS, the separation was conducted on Infinity Lab Poroshell 120EC-C18, (4.6 x100 mm, 2.7µm, Agilent Company, USA). (A) Methanol with 0.1% formic acid and 1 mM Ammonium formate (B) Water with 0.1% formic acid and 1 mM ammonium formate. Separation of compounds was carried out with gradient elution profile: 0 min, A: B 10:90; 36 min, A: B 100:0; 50 min, A: B 100:0. Post run time 16 min. Chromatography was performed at 30 °C with a flow-rate of 0.3 ml/min. UV spectra Diode array detector (DAD) were recorded between 190 and 900 nm. Finally, the eluents were auto injected into the electrospray ionization interface of the mass spectrometer. The ionization was on negative ion mode with Turbo Spray source, Scan type Q1 MS 50-1200 m/z, Scan rate 2000 Da/s, CUR gas 25, Temperature 450°C, Gas 1 50, Gas 2 40, Ion spray voltage 4500 V. Standards used for identification of tested extracts were Rutin, Isorhamentin, Kaempferol, Apigenin, Querecetin, Luteolin, p- coumaric acid, Gallic acid and Chlorogenic acid (Sigma Chemical Co. St. Louis, Mo, USA). Identification of the compounds was done through matching the detected molecules with pure standard references in-house library (Department of chemical theory of Drugs, Faculty of Pharmacy, Comenius university Bratislava, Slovakia) using the molecular weight (MW), retention time (t_R), fragmentation pattern and the specific Ultraviolet light (UV) spectrum.

Experimental design for comparing antioxidant activity

Different chemical assays for judging the effect of our tissue culture protocol on the antioxidant activity of the studied plant were performed by comparing the same serial dilutions of the methanolic extracts for both the produced calli and the *in-vivo* originated plant.

a) The total antioxidant capacity (TAC) assay (Saxena and Patel 2010)
b) Determination of radical scavenging activity using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Molyneux 2004), Hydroxyl(OH) (Smirnoff and Q.J. Cumbes 1989), Superoxide anion (O_2) (McCord and Fridovich 1969) , and Nitric oxide (NO) (Marcocci et al. 1994) radicals.
c) Investigation of the reducing power of the tested extracts (Oyaizu 1986).

d) Investigation of the anti-lipid peroxidation activity of the tested extracts (Wang et al. 2009).

Experimental design for comparing cytotoxic activity

Cytotoxicity assay was performed using both normal PBMCs as control and three different proliferating cancerous cell lines; Hepatic cancer cell line [HepG-2 (ATCC® Number: HTB-8065™)], Breast cancer cell line [MCF-7 (ATCC® Number: HTB-22™)] and Colorectal cancer cell line [HCT-116 (ATCC® Number: HTB-37™)]. The selected concentrations of the sample extracts were added and cell viability was measured at 24 h intervals for maximum of 72 h using neutral red uptake assay. IC50 was calculated using Prism statistics software program (Sebaugh 2011).

Figures

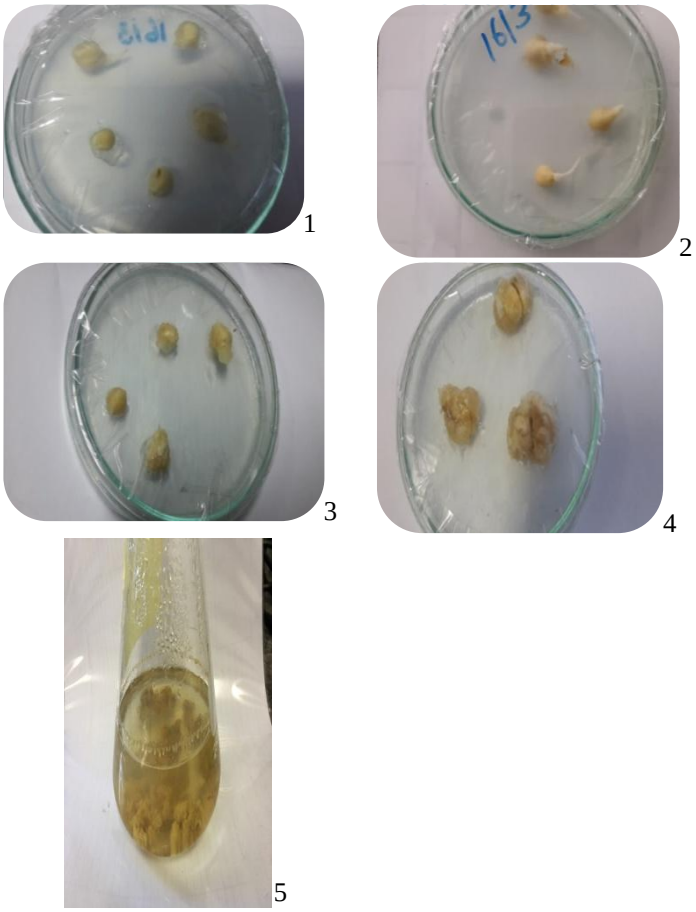
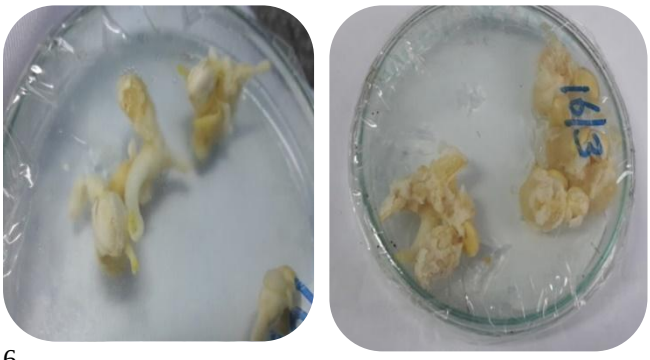


Figure S (1-5): Formation of *M. oleifera* seeds callus in MS basal static culture.

1. Seeds of *M. Oleifera* cultured on MS basal static medium with little shooting after 13 days .
2. Yellowish white small callus of *M. oleifera* begins to form after 20 days of the culture time.
3. Incomplete Calli of *M. oleifera* seed after about 25 days of the culture time.
4. Complete calli of *M. oleifera* seed after 40 days of the culture time.
5. Initiation of suspension culture of *M. oleifera* seed calli containing MS (BA , 8.88 µM) and (2,4-D, 9.05 µM) liquid medium.



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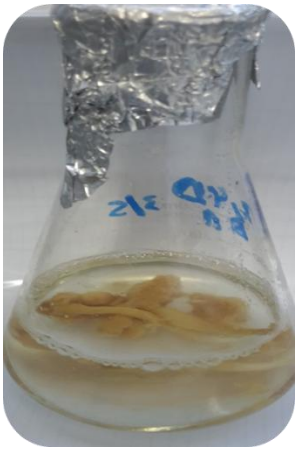

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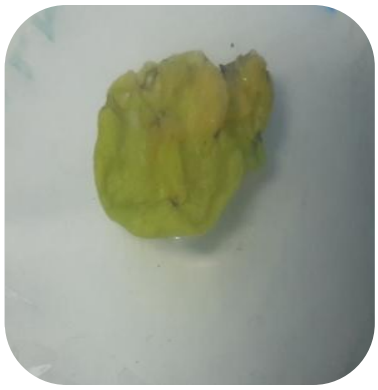
Figure S (6-8): Formation of *M. oleifera* seeds calli produced on MS static culture with a hormonal combination of (2, 4- D, 9.05 µM) and (BA, 8.88 µM).

6. *M. oleifera* seeds calli cultured on MS (2,4- D, 9.05 µM) and (BA, 8.88 µM) static medium with a conspicuous shooting system.
7. Yellowish white callus of *M. oleifera* seeds calli after 30 days of the culture time formed from shoots mainly.
8. Initiation of suspension culture for *M. oleifera* seeds calli containing MS (2,4- D, 9.05 µM) and (BA, 8.88 µM) liquid medium



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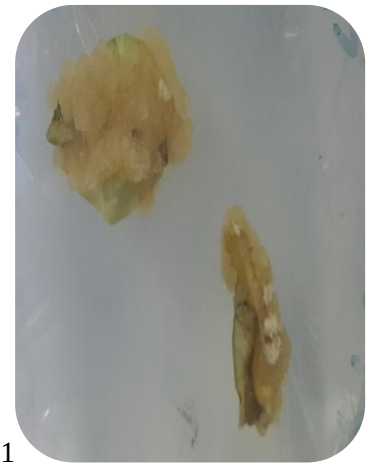

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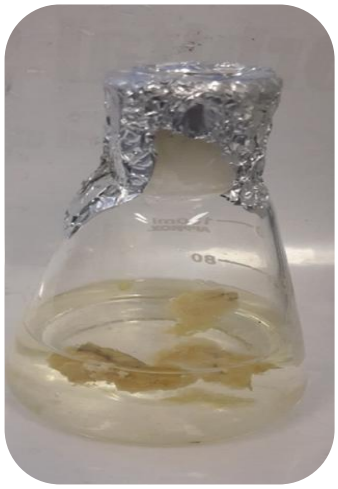

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Figure S (9-12): Formation of *M. oleifera* leaf calli produced on MS static culture with a hormonal combination of (2, 4- D, 9.05 µM) and (BA, 8.88 µM) .

9. Initiation *of M. oleifera* leaf greenish- yellow calli on the periphery exposed to the media of MS (2,4- D, 9.05 µM) and (BA, 8.88 µM) static culture after 13 days
10. *M . oleifera* leaf calli on MS (2, 4- D, 9.05 µM) and (BA, 8.88 µM) static culture after 20 days.
11. *M . oleifera* leaf calli on MS (2, 4- D, 9.05 µM) and (BA, 8.88 µM) static culture after 42days.
12. Initiation of suspension culture of *M . oleifera* leaf calli containing MS (2, 4- D, 9.05 µM) and (BA, 8.88 µM) liquid medium.

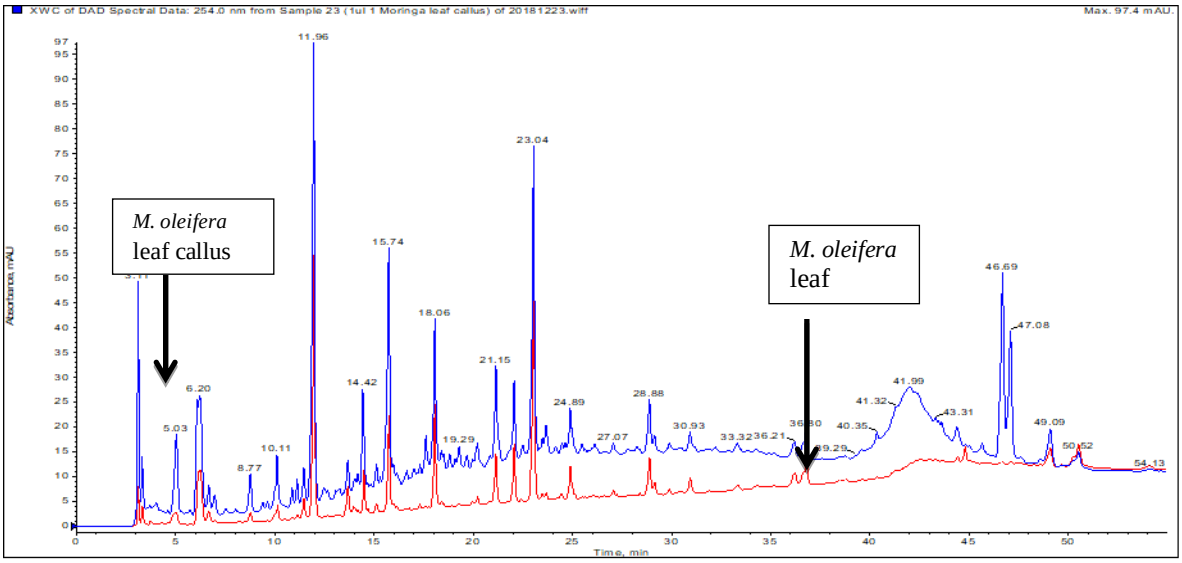


Figure S13: HPLC/DAD chromatogram of *M. oleifera* leaf callus extract and *M. oleifera* leaf extract measured at 254 nm.

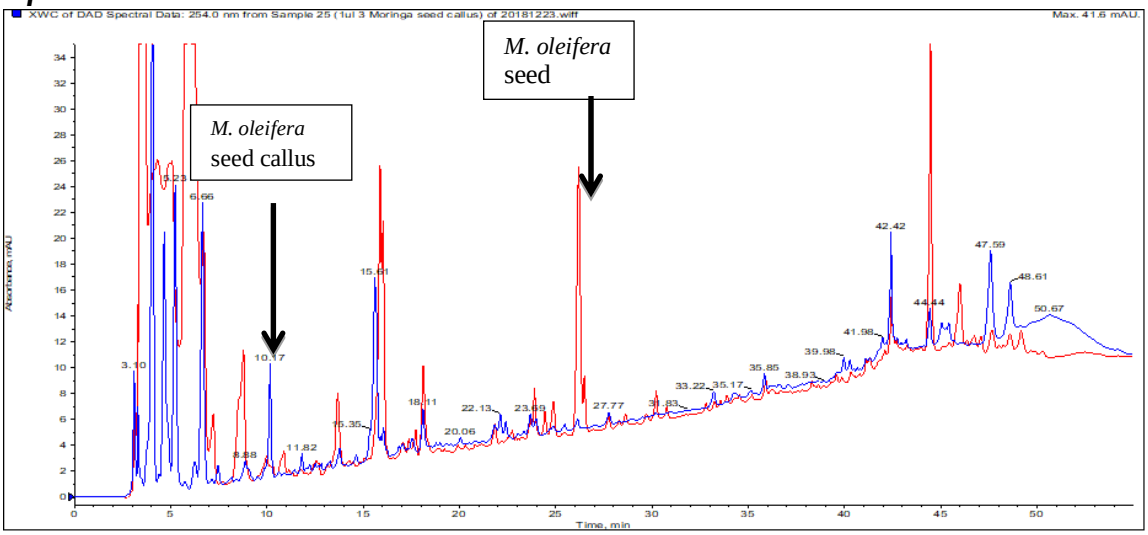


Figure S14: HPLC/DAD chromatogram of *M. oleifera* seed callus extract and *M. oleifera* seed extract measured at 254 nm.

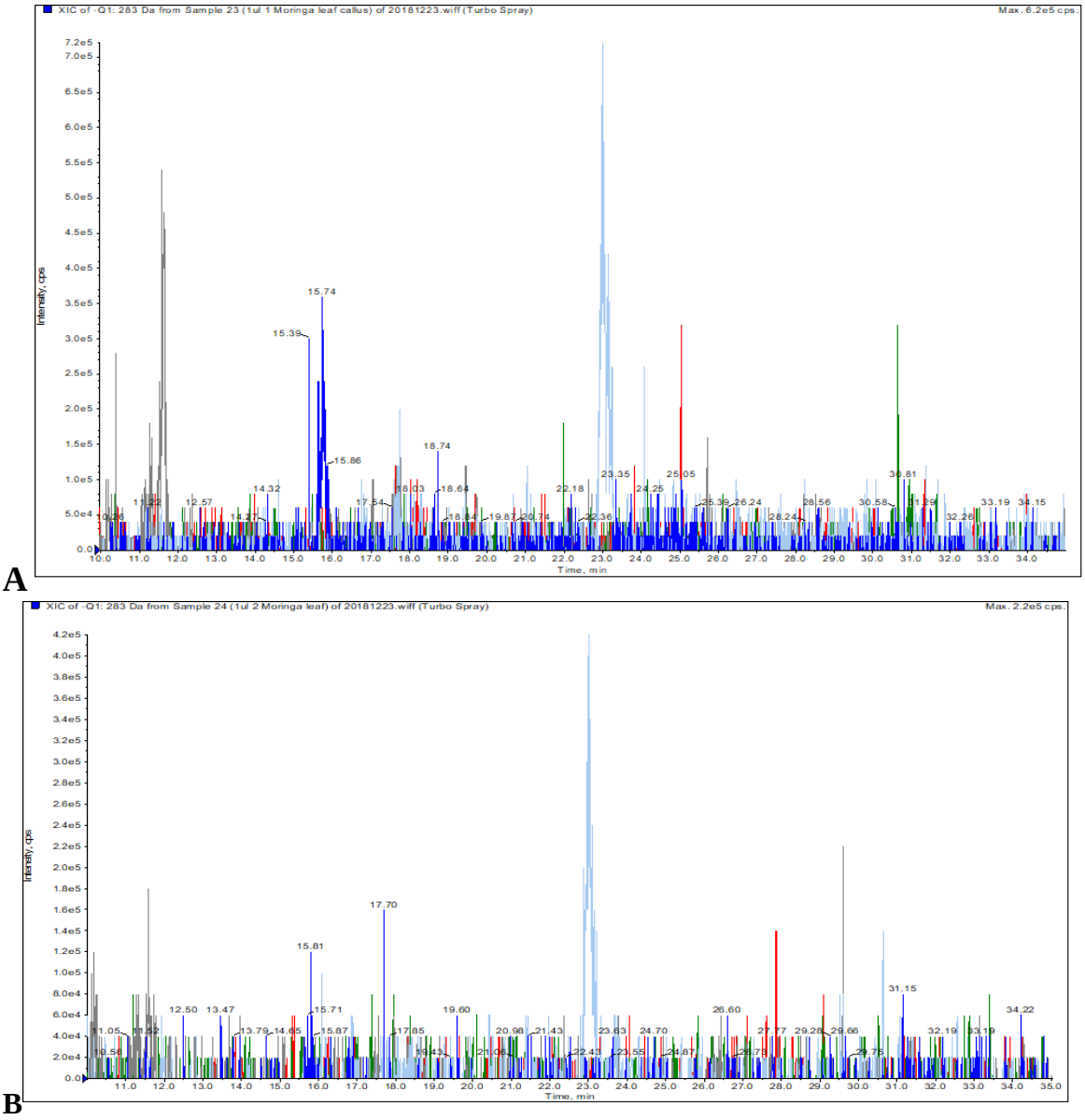
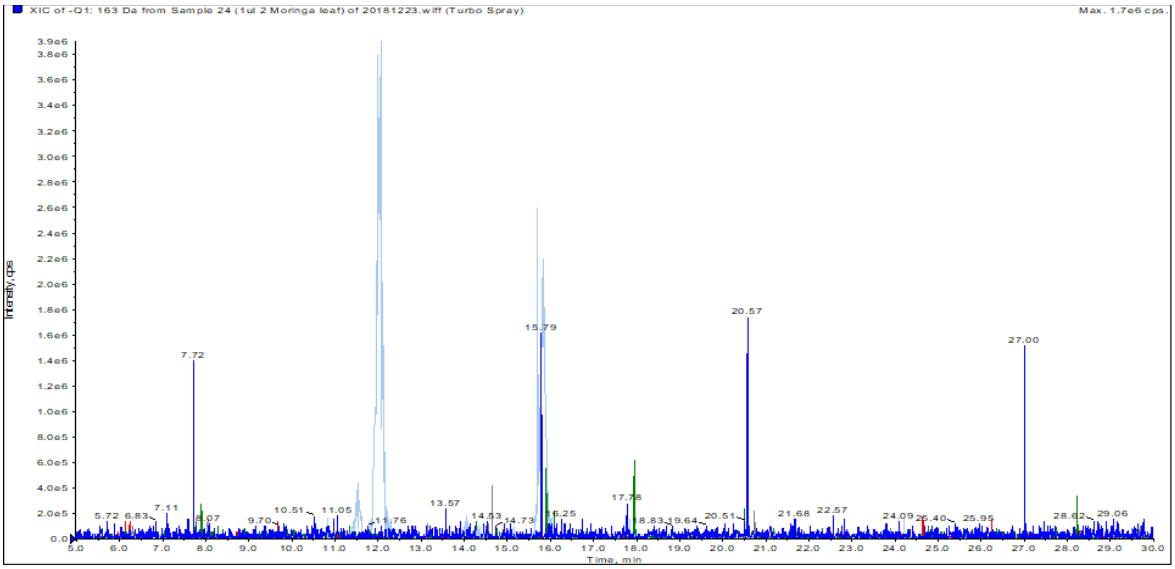


Figure S15: Mass chromatograms with selected ions 269 (apigenin), 285 (luteolin and kaempferol), 301 (quercetin), 315 (isorhamnetin) and 609 (rutin).
A) Mass chromatogram of *M. oleifera* leaf callus methanolic extract
B)Mass chromatogram of *M. oleifera* leaf methanolic extract



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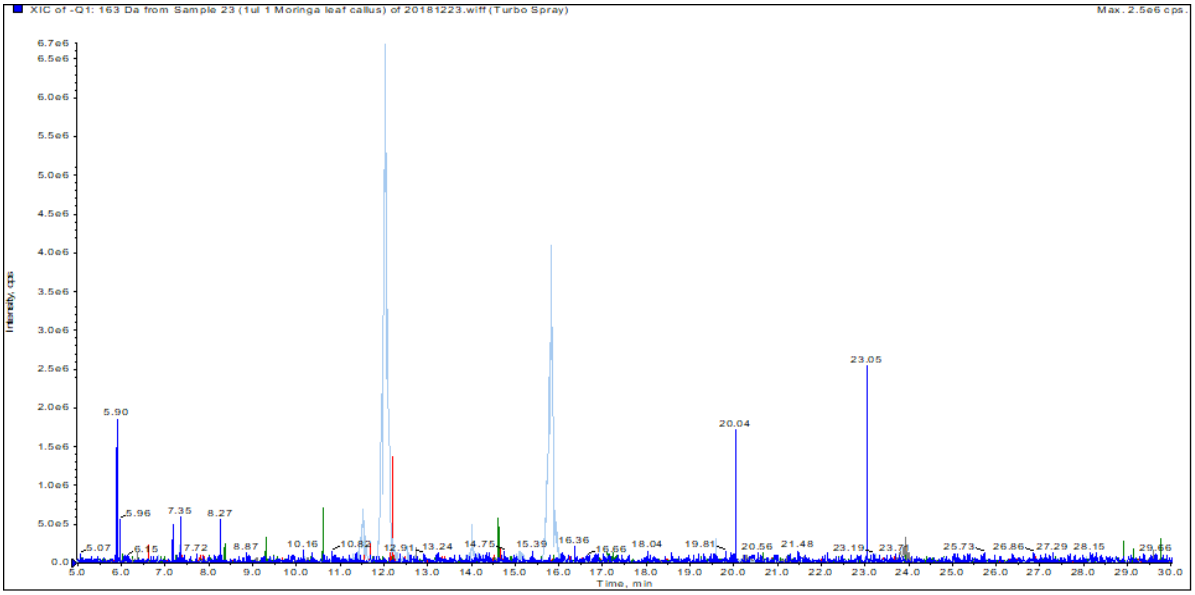

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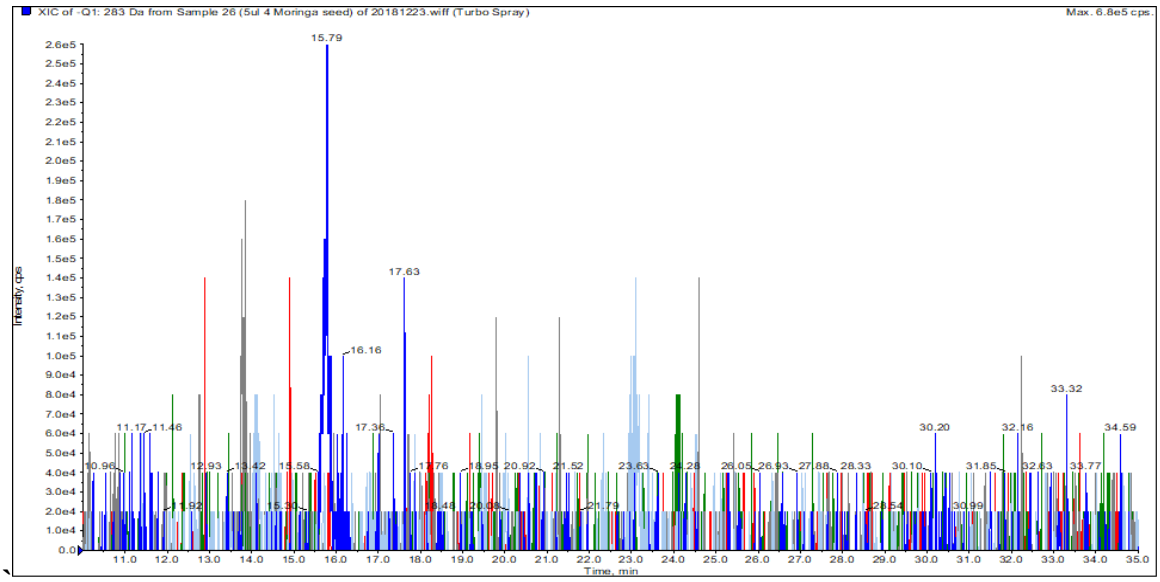
Figure S16: Mass chromatograms with selected ions 163 (p-coumaric acid), 285 (gallic acid), 179 (caffeic acid), 193 (ferulic acid) and 353 (chlorogenic acid).

A) Mass chromatogram of *M. oleifera* leaf callus methanolic extract
B) Mass chromatogram of *M. oleifera* leaf methanolic extract



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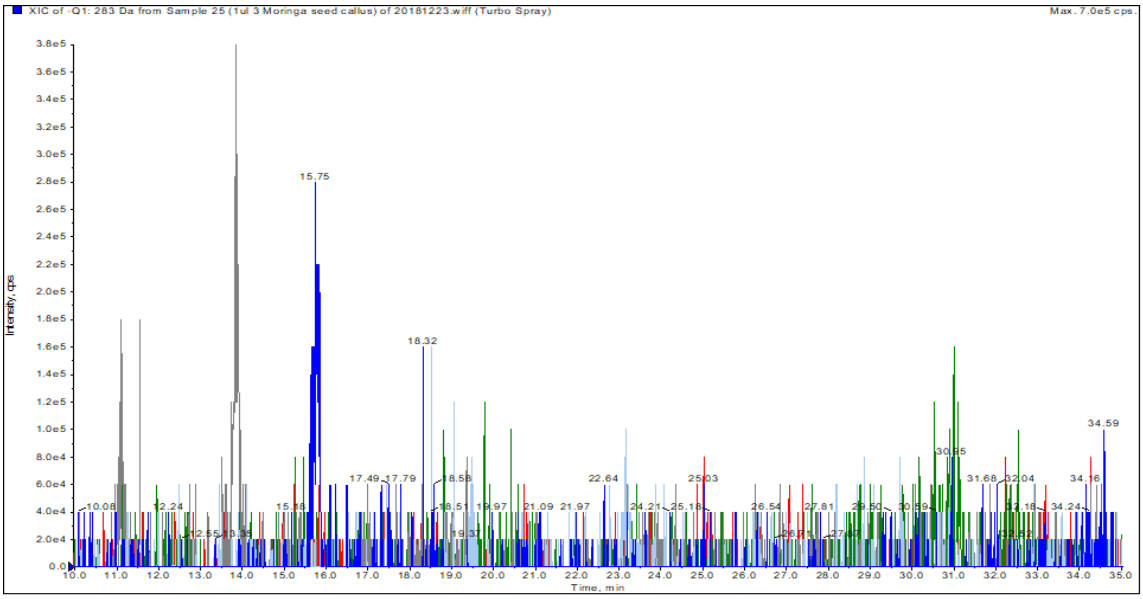

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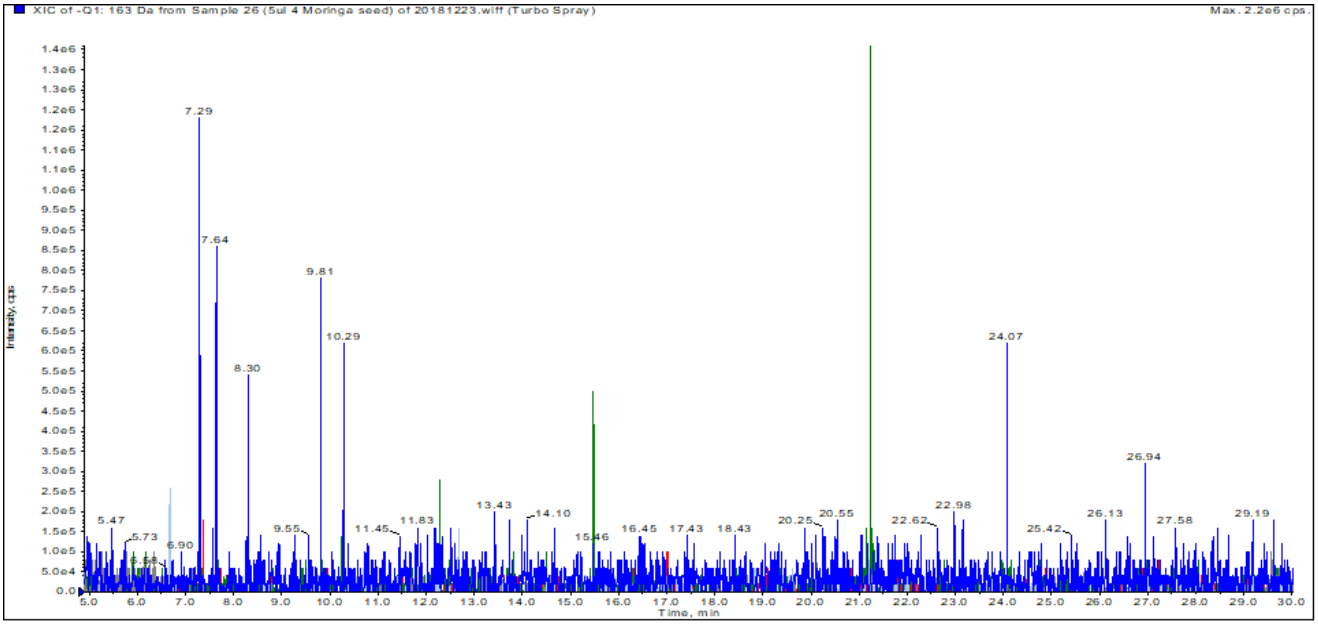
Figure S17: Mass chromatograms with selected ions 269 (apigenin), 285 (luteolin and kaempferol), 301 (quercetin), 315 (isorhamnetin) and 609 (rutin).

A) Mass chromatogram of *M. oleifera* seed callus methanolic extract.
B) Mass chromatogram of *M. oleifera* seed methanolic extract.



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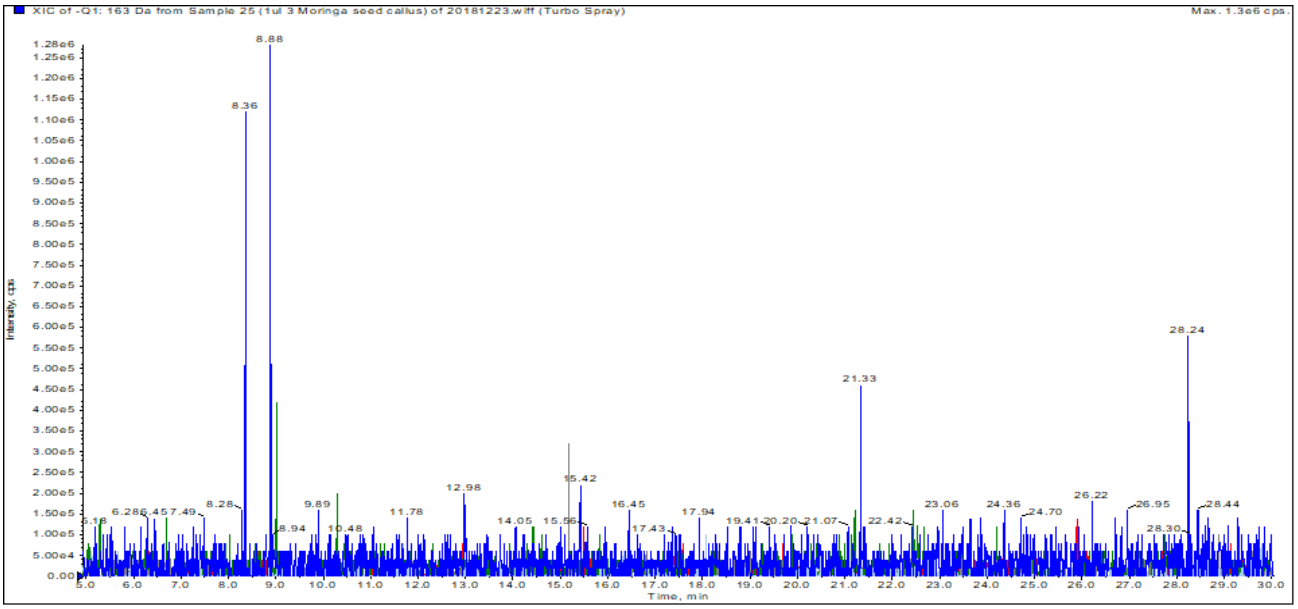


B

Figure S 18 : Mass chromatograms with selected ions 163 (p-coumaric acid), 285 (gallic acid), 179 (caffeic acid), 193 (ferulic acid) and 353 (chlorogenic acid).

A) Mass chromatogram of *M. oleifera* seed callus methanolic extract
B) Mass chromatogram of *M. oleifera* seed methanolic extract

Figure S19: Antioxidant activity of *M. oleifera* leaf and *M. oleifera* leaf callus methanolic extracts

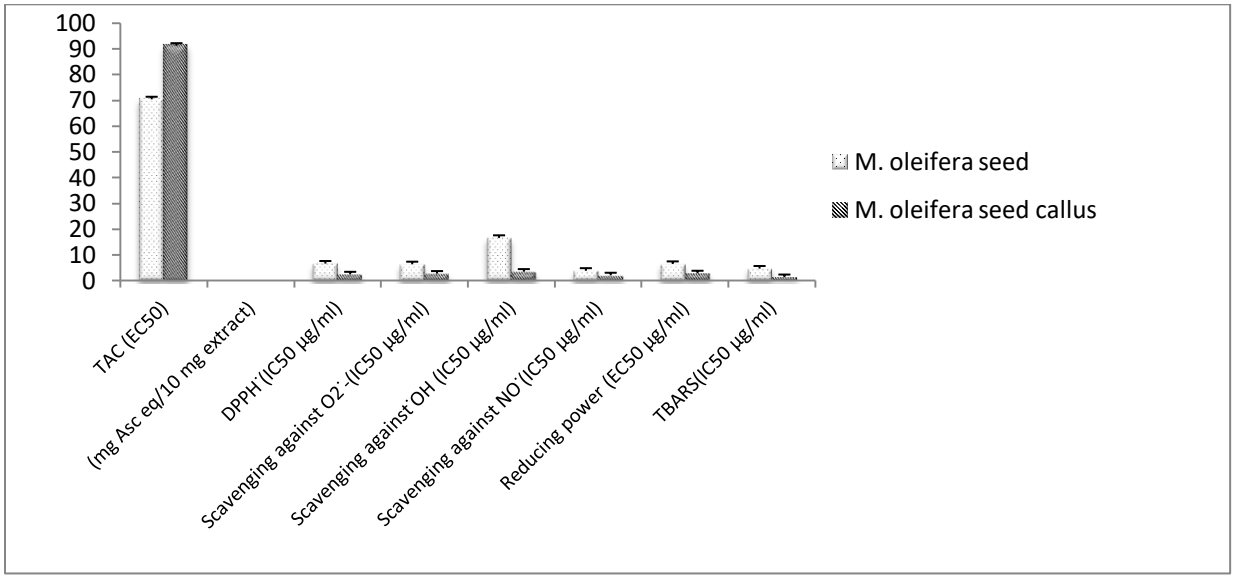
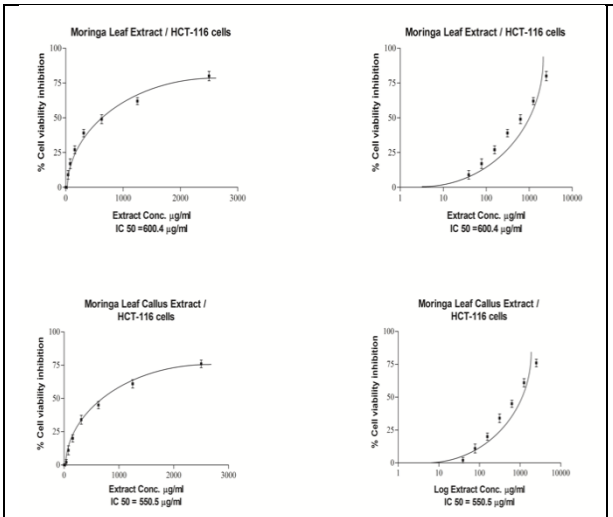


Figure S20: Antioxidant activity of *M. oleifera* seed and *M. oleifera* seed callus methanolic extracts.



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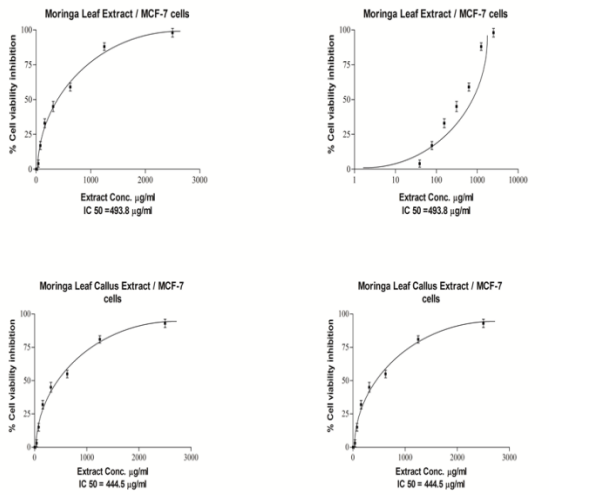

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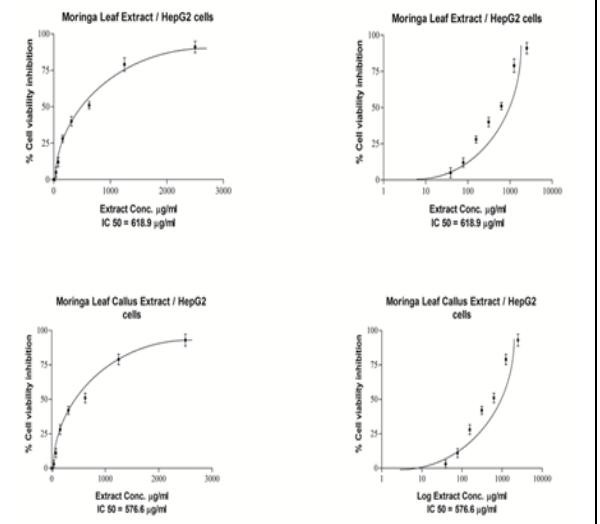
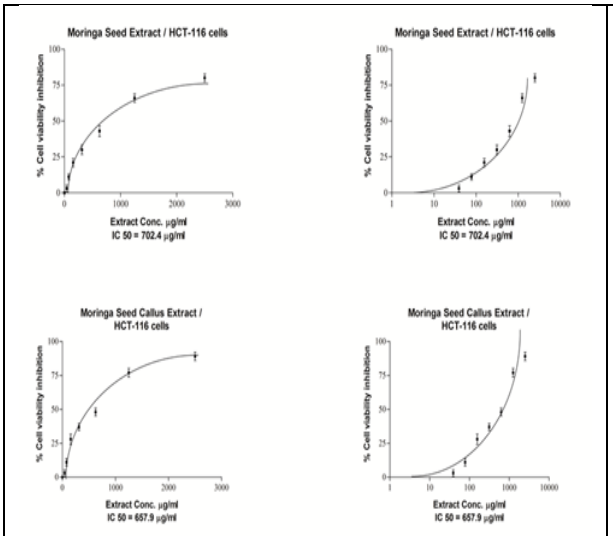


C

Figure S21: cytotoxic activity of serial concentrations of *M. oleifera* leaf and *M. oleifera* leaf callus extracts on
A)HCT-116 cell lines
B) MCF-7 cell lines.
C) HepG-2 cell lines.



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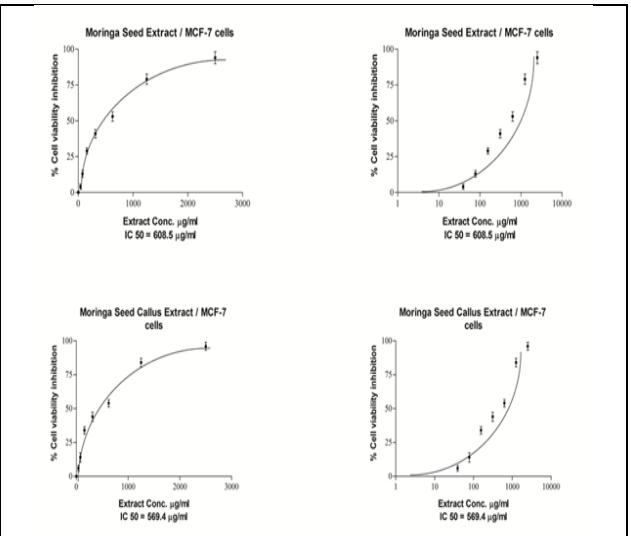

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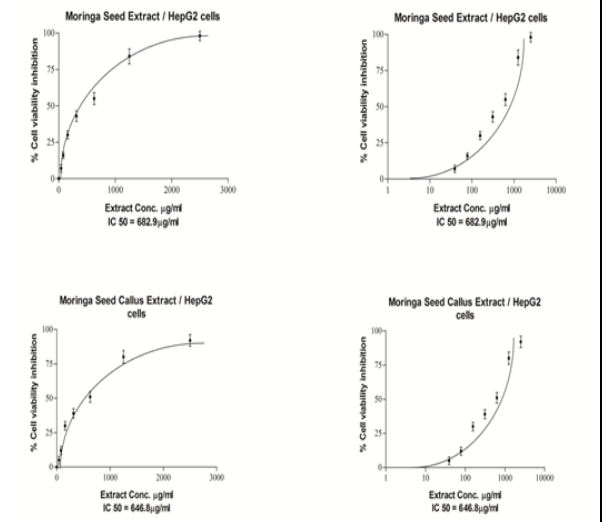

C

Figure S22: cytotoxic activity of serial concentrations of *M. oleifera* seed and *M. oleifera* seed callus extracts on
A)HCT-116 cell lines.
B) MCF-7 cell lines
C) HepG-2 cell lines.

Tables

Table S 1: Comparison of flavonoidal and phenolic acid content (µg/g dry weight) in *M. oleifera* leaf, seed and their calli methanolic extracts.

Flavonoids/ Phenolic acids	*M. oleifera* leaf	*M. oleifera* leaf callus	*M. oleifera* seed	*M. oleifera* seed callus
Rutin	603.6 ± 5.79	1500 ± 6.0 9	788.18 ± 5.38	528 ± 9.17
Querecetin	46.54 ± 3.07	ND	83.42 ± 6.0 3	49.51 ± 2.27

Luteolin	44.56 ± 2.03	110.42 ± 6.09	ND	46.07 ± 3.39
Kaempferol	ND	115.74 ± 7.13	80.5 6 ± 6.8 3	49.95 ± 3.09
Apigenin	24.41 ± 2.16	56.51 ± 4.19	46.73 ± 3.17	40.11 ± 5.02
Isorhamentin	223 ± 1.95	1270.54 ± 14.07	228 ± 1.93	ND
Gallic acid	28.54 ± 2.34	ND	ND	42.32 ± 2.34
***p*- coumaric acid**	52.61 ± 4.29	29.83 ± 5.0 9	48.31 ± 3.35	52.31 ± 5.22

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