Menoufia J. Agric. Biotechnology, Vol. 2 June (2017): 15 - 24

ESTIMATION OF TOTAL PHENOLIC, FLAVONOID CONTENTS AND *IN VITRO* ANTIOXIDANT ACTIVITY OF *MORINGA OLEIFERA* VARIETY GROWN IN EGYPT

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Received: Jan. 29, 2017 Accepted: Mar. 19 , 2017

ABSTRACT: *The present research determined the chemical composition and the antioxidant activity of leaves extracts (aqueous – acetone) of Moringa oleifera variety grown in Egypt. It was found that moringa leaves contain total ash 8.03%, crude lipids 3.14%, crude protein 26.31%, total carbohydrates 44.89%, and crude fiber 17.63%. Total phenolics in leaves aqueous extract were 243.8 mg/g and total flavonoids were 145.22 mg/g, while in acetone extract total phenolics were 142.51 mg/g and total flavonoids were 124.64 mg/g. HPLC results showed that moringa leaves contained 23 phenolic compounds and 22 flavonoid contents and the main constituents of both were (e-vanillic, Pyrogallol, Salycilic, Catechin, P-hydroxybenzoic, Ellagic, Alpha-coumaric, 3,4,5-trimethoxy-cinnamic, Protocatechuic and Chlorogenic) and (Luteo-6-arabinose-8-gloucose, Hesperidin and Rosmarinic) respectively. In comparison with the acetone extract, the aqueous extract showed the highest activity in reducing power assay. Egyptian moringa leaves extracts were found to have antioxidant activity due to their high amount of total phenolics and falvonoids.*

***Key words*:** *Moringa – Phenolic compounds – Flavonoids – antioxidant.*

INTRODUCTION

Herbal medicine is still the most abundant, affordable, reliable, trusted and well understood form of health care in virtually all African villages (Abalaka *et al.,* 2009), and 80% of the population within developing countries uses herbal and other traditional medicines to treat their common ailments (WHO, 2002; Willcox and Bodeker, 2004).

Moringa oleifera Lam. *(M. oleifera),* commonly known as horse-radish or drumstick tree in English, belongs to family Moringaceae. It is a small sized tree, which is native to South Asia, Africa and Arabia and is used as traditional medicine in many tropical and subtropical countries (Moyo *et al.,* 2011). It is a deciduous tree growing rapidly even in poor soils, well adapted to droughts and able to reach up to 15 m in height. It is one of the 14 species of genus moringa, which is native to India, Africa, Arabia, Southeast Asia, the Pacific and Caribbean islands, and South America (Muhl *et al.,* 2011). The flowers and the fruits appear twice each year, and seeds or cuttings are used to propagate the tree. Almost all the parts of *M. oleifera* are used for various ailments in the indigenous medicine of South Asia, including the treatment of diabetes، hypertension, inflammation and infectious diseases (Verma *et al.,* 2009). Its leaves, pods and flowers are generally consumed for nourishment.

A variety of natural products, including crude extracts and isolated compounds from plants, can induce body weight reduction and prevent diet-induced obesity. Different parts of this plant are used in the indigenous systems of human medicine for the treatment of a variety of human ailments.

The leaf extracts of *M. oleifera* have been reported to exhibit antioxidant activity both *in vitro* and *in vivo* due to the high amount of polyphenols such as, phenolics and

flavonoids components (Atawodi *et al.,* 2010). The leaves were found to be used for the treatment of fever, catarrh, gonorrhea, headache, blood pressure and anxiety. The root, bark and steam are used to treat epilepsy, nervous debility, hysteria, kidney pains, asthma and snake bite. While seeds and seeds oil are used against fever, back pain, rheumatism and bladder troubles (Morton 1991 and Fuglie 2001).

Pharmacological studies have demonstrated that *M. oleifera* known to possess hypoglycemic, hypotensive، anti-microbial, hepatoprotective, immunomodulatory، antioxidant and antitumor activities (Anwar *et al.,* 2007; Sudha *et al.,* 2010; Mahajan and Mehta 2010). These biological activities could be attributed to the presence of secondary plant metabolites present in *M. oleifera* such as phenolics, flavonoids, carotenoids، alkaloids, glycosides, sterols, amino acids, vitamins, and minerals (Auwal *et al.,* 2013). Therefore, the present study was conducted to estimate the major classes of phytochemicals present in the *M. oleifera* variety grown in Egypt, as well as total phenolic and flavonoid contents in *M. oleifera* extracts.

MATERIALS AND METHODS

1. Plant material

Fresh leaves of *M oleifera* were collected from Elmedoran, Elmesharak Keblly, Senoris, Faiom, Egypt in February 2014. The leaves were identified in Horticulture department, Faculty of Agriculture, Menoufia University. The leaves were washed and air-dried for 24 hours, then dried at 50 °C. The dried samples were grinded into fine powder and kept in refrigerator for analysis.

2. Determination of chemical composition

Ash content was determined by ignition of dried sample at 550 °C until a constant weight according to the Association of Official Analytical Chemists, (AOAC, 2000). Crude fiber was determined according to AOAC, (2000). Total nitrogen was determined (dry basis) according to the modified micro-Kjeldahl method as described by AOAC., (2000). The crude protein contents were calculated using the conversion factor 6.25. Crude lipid was determined according to AOAC., (2000). The total carbohydrate determined by difference = 100 – (% protein + % fat + % ash).

3. Preparation of plant extracts

Of total (1 Kg) dried powdered leaves, (½ Kg) was extracted by distilled water and (½ Kg) was extracted by acetone at room temperature for 3 days. The resulting extracts were filtered using Whatman no. 1 filter paper and the residues were re-extracted by the same process until plant materials were exhausted. The collected filtrates were pooled and evaporated to dryness under reduced pressure to give a semisolid residue, which was then lyophilized to get powder and were stored at - 20 °C until used. The yields were 99.58 and 74.5 g per 500 g of dried powdered leaves of aqueous and acetone extracts respectively.

4. Determination of total phenolic compounds

The amount of total phenolics in extracts was determined with the Folin- Ciocalteu reagent. Gallic acid was used as a standard and the total phenolics were expressed as mg gallic acid equivalents (GAE)/g dry weigh. 10 ml of samples were extracted in methanol. 0.5 ml of each sample and standard were introduced into test tubes and mixed with 2.5 ml of a 10 fold dilute Folin-Ciocalteu reagent and 2 mL of 7.5% sodium carbonate. The tubes were covered tightly and allowed to stand for 30 minutes at room temperature. The absorbance was measured spectrometrically at wavelength 760 nm (Kim *et al.,* 2003).

5. Determination of total flavonoids compounds

The total flavonoids were determined

using the method reported by Dewanto *et al*., (2002). Briefly, an aliquot of 250 µl of each extract or a standard solution was mixed with 1.25 ml of deionized water followed by 75 µl of a 5% $NaNO_2$ solution. After 6 min, 150 µl of 10% $AlCl_3.6H_2O$ solution was added to each mixture. After 5 min, 0.5 ml of 1 M NaOH was added, and the total volume was adjusted to 3.0 ml with deionized water. Catechin was used as a standard and absorbance was measured at 510 nm, which was corrected using a blank, the results were expressed as mg of catechin equivalents (CE)/ g dry weight.

6. Fractionation and quantitative determination of phenolic compounds by HPLC

Phenolic compounds were fractionated and determined by HPLC according to the method of Goupy *et al.,* (1999) as follow : 5 g of samples were extracted by methanol and centrifuged at 10000 rpm for 10 min and supernatant was filtered through a 0.2 µm Millipore membrane filter then 1-3 ml was collected in a vial, using 200 µl for injection in HPLC Hewilet Packared (series 1050) equipped with autosamplling injection, solvent degasser, ultraviolet (UV) detector set at 280 nm and quaternary HP pump (series 1050). Hewlett Packard using a column Alltima C18, 5mm (150mm x 4.6mm Alltech). The column temperature was maintained at 35 ^{o}C. Gradient separation was carried out with methanol and acetonitrile as a mobile phase at flow rate of 1 ml/min. Phenolic acid standards from sigma Co. were dissolved in a mobile phase and injected into HPLC. Retention time and peak area were used to calculate phenolic compounds concentration by the data of Hewllet Packared software. The data were reported with convergence limit in triplicate.

7. Fractionation and quantitative determination of flavonoids by HPLC

HPLC analyses were performed with Dionex Ultimate 3000 liquid chromatograph (Germany) with four solvents delivery system quaternary pump (LPG 3400 SD) including a diode array detector (DAD 3000) with 5 cm flow cell, a manual sample injection valve equipped with a 20 µl loop and Chromeleon 6.8 system manager as data processor. The separation was achieved by a reversed-phase acclaim TM 120 C18 column (5 µm particle size, 4.6 x 250 mm). A modified method of Zuo *et al*., (2002) was used where the mobile phase contains 1% aq. acetic acid solution (Solvent A) and acetonitrile (Solvent B), the flow rate was adjusted to 0.7 ml/min, the column was thermostatically controlled at 28 ^{O}C and the injection volume was kept at 20 µl. A gradient elution was performed by varying the proportion of solvent B to solvent A. The gradient elution was changed from 10 % to 40% B in a linear fashion for duration of 28 min, from 40 to 60 % B in 39 min and from 60 to 90 % B in 50 min. The mobile phase composition comes back to initial condition (solvent B: solvent A: 10: 90) in 55 min and allowed to run for another 10 min, before the injection of another sample. Total analysis time per sample was 65 min. HPLC chromatograms were detected using a photo diode array UV detector at three different wavelengths (272, 280 and 310 nm) according to absorption maxima of analysed compounds. Each compound was identified by its retention time and by spiking with standards under the same conditions. The quantification of the sample was done by the measurement of the integrated peak area and the content was calculated using the calibration curve by plotting peak area against concentration of the respective standard sample. The data were reported with convergence limit in triplicate.

8. *In vitro* antioxidant activity reducing power assay

A spectrophotometric method (Oyaizu 1986) was used for the measurement of reducing power. For this determination 2.5 ml of each extracts were mixed with 2.5 ml

of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After adding 2.5 ml of 10% trichloroacetic acid (w/v), the mixture was centrifuged at650 rpm for 10 min. The upper layer (5 ml) was mixed with5 ml deionized water and 1 ml of 0.1% of ferric chloride, and the absorbance was then measured at 700 nm. Higher absorbance indicates higher reducing power. Vit.C was used as standard.

RESULTS AND DISCUSSION.

1. Chemical composition of *moringa oliefera* leaves

The obtained results in table (1) indicate that *M oliefera* leaves contain total ash 8.03 %, crude lipid 3.14 %, total protein 26.31 %, crude fiber 17.63 %, and total carbohydrates 44.89 %. The results are in accordance with those of Richter, *et al.,* (2003), Oduro, *et al.,* (2008), Moyo, *et al.,* (2011), Simonsohn, (2012) and Borges, *et al.,* (2014) who refer that moringa leaves chemical composition is complex, total ash 7.1 %, total lipids 4.7 %, crude protein 25.0 %, crude fiber 19.9 %, and total carbohydrates 43.9 %.

2. Total phenolic compounds and total flavonoids of *moringa oliefera* leaves extract.

Plant polyphenols are a major group of compounds acting as primary antioxidants or free radical scavengers. Therefore, it was reasonable to determine the total phenolic and flavonoid contents in the plant extracts. The results obtained are shown in table (2) which indicate that the total phenolics in leaves aqueous extract were 243.8 mg/g GAE equivalent, while total flavonoids were 145.22 mg/g catechin equivalent, on the other hand total phenolics and total flavonoids were 142.51 and 124.6 mg/g for acetone extract respectively. These data agree with those of Siddhuraju and Becker (2003), Iqbal and Bhanger (2006), who found that, the total phenolics results were 83 – 135 mg/g and 23 – 132.3 mg/g. the total phenolics results were 59 – 140 mg/g and 69.3 – 125 mg/g.

Table (1): The chemical composition of moringa leaves (W/W %)

Components	Percentage (w/w %)
Ash	8.03
crude lipid	3.14
Total Protein	26.31
Crude Fiber	17.63
Total carbohydrate	62.52

Table (2): Total phenolic and total flavonoid contents of moringa leaves extracts

Extracts	Total phenolics (mg/g)	Total flavonoids (mg/g)
aqueous extract	243.8	145.22
Acetone extract	142.51	124.64

3. Quantitative analysis of phenolic and flavonoids compounds in moringa leaves by HPLC

Phenolic compounds in moringa leaves were analyzed by High Performance Liquid Chromatography (HPLC), and the concentrations of all tested phenolic compounds were given in table (3).

HPLC analysis for the phenolic compounds showed the presence of 23 compounds which were varied in their amounts. It was observed that e-vanillic, Pyrogallol, Salycilic, Catechin, *P*-OH-benzoic, Ellagic, Alpha-coumaric, 3,4,5-methoxy-cinnamic, Protocatechuic and Chlorogenic were found in the high level, their amounts were 1366.17, 724.73, 582.68, 438.94, 236.55, 203.37, 189.68, 179.45, 167.33 and 166.26 mg/100g dry weight respectively. While Caffeine (127.42), Catechol (117.68), Caffeic (115.9), Ferulic (115.67) and Epicatechin (107.82) mg/100g dry weight, were found in moderate amounts.

Coumarin, Phenol, Iso-ferulic, Vanillic, Gallic, Cinnamic, 4-aminobenzoic and *P*-coumaric were found in low amounts in the leaves of (*M.O*) methanolic extract.

Table (3): HPLC analysis of polyphenolics in moringa leaves extract

No	Phenolic compounds	mg / 100g D.W
1	Pyrogallol	724.73
2	Gallic	44.58
3	4 – Amino-benzoic	12.88
4	Protocatechuic	167.33
5	Catechin	438.94
6	Catechol	117.68
7	Chlorogenic	166.26
8	Epicatechin	107.82
9	*P*-OH-benzoic	236.55
10	Caffeine	127.42
11	Caffeic	115.9
12	Vanillic	57.81
13	P-coumaric	11.19
14	Ferulic	115.67
15	Iso-ferulic	63.45
16	e-vanillic	1366.17
17	Ellagic	203.37
18	Alpha-coumaric	189.68
19	Benzoic	77.67
20	Coumarin	81.34
21	3,4,5-methoxy-cinnamic	179.45
22	Salycilic	582.68
23	cinnamic	17.57

Data in table (4) presented the HPLC analysis of flavonoids. It can be seen that, there were 22 Components, Luteo-6-arabinose 8-gloucose, Hesperidin and Rosmarinic flavonoids were found in high levels, their amounts were 209.16, 171.05 and 129.58 mg/100g D.W respectively. While Apig-6-rhamnose-8-glucose, Kaemp-3, (2-*p*-comaroyl) glucose and Luteolin were found in moderate amounts. their amounts were 69.24, 54.69 and 52.05 mg/100g D.W respectively. The other compounds i.e. Quercetrin, Apig-6- glucose -8- rhamnose, Hesperetin, Quercetrin-3-O-glucoside, Naringin, Apig.7-O-neohespiroside and Acacetin were found in low levels, their amounts were 31.85, 30.12, 29.52, 28.16, 25.53, 21.74 and 17.24 mg/100 gm D.W respectively

Table (4): HPLC analysis of flavonoids in moringa leaves extract

No	flavonoids	Mg / 100g D.W
1	Luteo-6-arabinose- 8-glucose	209.16
2	Luteo-6-glocose- 8-arabinose	3.36
3	Apig- 6-rhamnose- 8-glucose	69.24
4	Apig-6-glucose- 8- rhamnose	30.12
5	Naringin	25.53
6	Luteolin	52.05
7	Hesperidin	171.05
8	Rutin	4.17
9	Quercetrin-3-O-glucoside	28.16
10	Rosmarinic	129.58
11	Apig-7-O-neohespiroside	5.03
12	Kamp-3,7-dirhamoside	13.98
13	Apig-7-glucose	21.74
14	Quercetrin	31.85
15	Quercetin	2.19
16	Kaemp-3,(2-p-comaroyl) glucose	54.69
17	Naringenin	5.67
18	Hesperetin	29.52
19	Kampferol	7.06
20	Rhamnetin	4.68
21	Apigenin	3.64
22	Acacetin	17.24

Eight flavonoids, were detected in very minor amounts including Kamp-3,7-dirhamoside, Kampferol, Naringenin, Apig-7-O-neohespiroside, Rhamnetin, Rutin, Apigenin and Luteo-6-glocose-8-arabinose at amounts (13.98, 7.06, 5.67, 5.03, 4.68, 4.17, 3.64 and 3.36 mg/100 gm D.W respectively), which agree with those of Vongsak *et al.,* (2013).

4. Reducing power activity for moringa leaves extracts

The leaf extracts of *M. oleifera* have been reported to exhibit antioxidant activity both *in vitro* and *in vivo* in numerous studies due to their high amount of polyphenols such as, phenolics and flavonoids contents (Verma *et al.,* 2009; Atawodi *et al.,* 2010; Hossain *et al.,* 2012 and Dolly *et al.,* 2013).

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism in phenolic antioxidant action (Nabavi *et al.,* 2009). In this assay, the presence of reductants (antioxidants) in the samples would result in the reduction of Fe^{+3} to Fe^{+2} by donating an electron.

Data in Fig (1) showed the reducing power of moringa leaves aqueous extracts (2.5% and 5% concentration), which were (35.3 and 197.88 mMol Ascorbic Eq), while the reducing power for 2.5% and 5% acetone extracts were (22.55 and 147.82 mMol Ascorbic Eq). These results are in accordance with those of Hossain, *et al.,* (2012) who found that reducing power was (53.925 ± 5.25 mMol Ascorbic Eq) for moringa leaves ethanol extract and it was (50.675 ± 3.699 mMol Ascorbic Eq) for moringa leaves methanol extract.

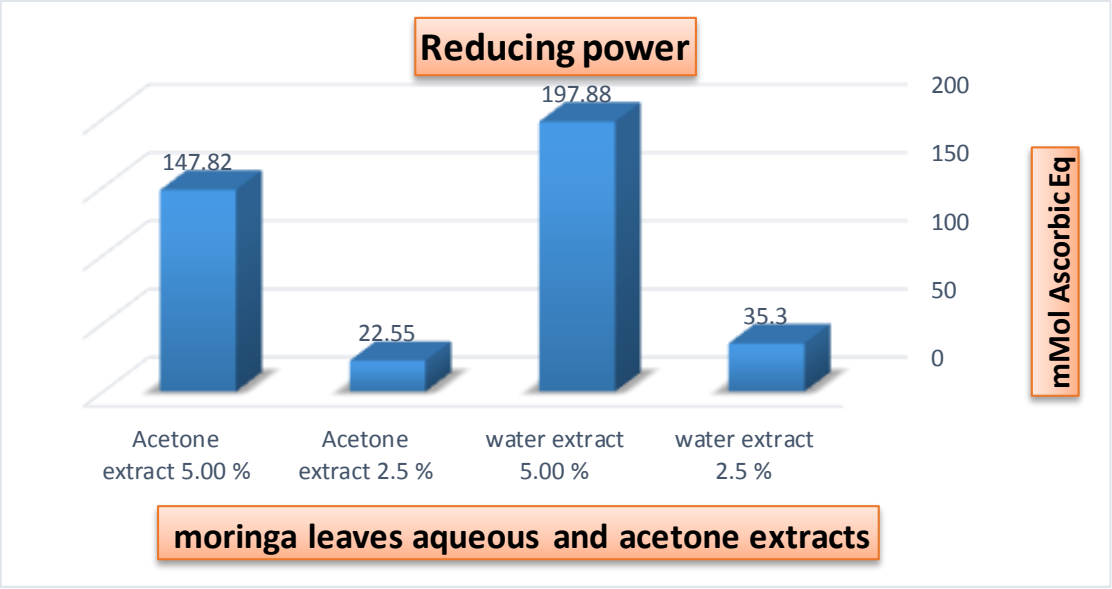

Fig (1): Reducing power activity for moringa leaf extracts.

CONCLUSION

The results of our study suggest that leaves extract of *M. oleifera* variety grown in Egypt are rich in phenolic and flavonoid compounds, which means that theses leaves can be used as a natural source of antioxidants to prevent the progression of many diseases. *M. oleifera* leaves extracts also produced marked *in-vitro* antioxidant activity that justifies its use in traditional system of medicine in Egypt.

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تقدير الفينولات الكلية ومحتوى الفلافونات والنشاط المضاد للأكسدة معمليا فى المورينجا أوليفيرا التى تم زراعتها في مصر

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الملخص العربى

وقد حدد البحث الحالي التركيب الكيميائي والنشاط المضاد للأكسدة لمستخلصات الأوراق (مائي – أسيتون) فى نبات *المورينجا أوليفيرا* الذي يزرع في مصر. ووجد أن أوراق المورينجا تحتوي على الرماد الكلي 8.03٪ والدهون الخام 3.14٪ والبروتين الخام 26.31٪ والكربوهيدرات الكلية 44.89٪ والألياف الخام 17.63٪. وبلغ مجموع الفينولات في الأوراق المستخلص المائي 243.8 ملجرام / جرام وكان مجموع الفلافونويدات 145.22 ملجرام / جرام ، بينما بلغ مجموع الفينوليات 142.51 ملجرام / جرام وكان مجموع الفلافونويد 124.64 ملجرام / جرام. وأظهرت نتائج HPLC أن أوراق المورينجا تحتوي على 23 مركب فينولى و 22 مركب فلافونى وكانت المكونات الرئيسية لكل من (الفاليين، بيروجالول، ساليسيليك، كاتشين، P– هيدروكسي بنزويك، إلاجيك، ألفا–كوماريك، 3،4،5–ثلاثي ميثوكسي– سيناميك، بروتاكاتشويك و كلوروجينيك) و (لوتيو–6–أرابينوس–8–جلوكوس، هسبريدين و روزمارينيك) على التوالي. وبالمقارنة مع مستخلص الأسيتون، أظهر المستخلص المائي أعلى نشاط في تقليل مقايسة الطاقة. تم إثبات أن أوراق المورينجا المصرية ذات نشاط مضاد للأكسدة نظرا لوجود كمية عالية من الفينولات الكلية والفالفونويدات.