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Full Length Research Paper

Effect of UV-C irradiation on antioxidant activities, total phenolic and flavonoid contents and quantitative determination of bioactive components of *Moringa oleifera* Lam. shoot culture

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Effect of UV-C irradiation on the antioxidant activities of shoot cultures of *Moringa oleifeira* Lam. was investigated. Total phenolic and flavonoid contents and the antioxidant bioactive components were determined. The shoots of *M. oleifeira* were cultured for 6 weeks on Murashige and Skoog (MS) mediums containing 0.5 mg/L 6- benzyladenine (BA) for multiple shoot formation. Multiple shoots were treated with UV-C irradiation for 0 min (for the control group) 5, 10 and 15 min (for the experimental groups). After 4 weeks of culture, the shoots were extracted with methanol and analyzed for antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging and ferric reducing power (FRP) assay and the total phenolic and flavonoid contents were determined. Quantitative analysis of active compounds was accomplished by high performance liquid chromatography (HPLC). The results indicate that the shoots treated with UV-C irradiation for 10 min exhibited the highest antioxidant activities at IC_{50} of 31.43 mg/mL using DPPH scavenging assay, 58.98 mg TEAC/100 g fresh weight (FW) using ABTS scavenging assay and 33.78 mM $FeSO_4$ /100 g FW using FRP assay. The total phenolic and flavonoid contents were 112.56 mg GAE/ 100 g FW and 65.31 mg QE/100 g FW, respectively. Crypto-chlorogenic acid, isoquercetin and astragalin were the highest antioxidant bioactive components with values of 30.10, 61.21 and 12.67 ng/mL, respectively. UV-C irradiation can stimulate the antioxidant capacities of *M. oleifera* shoot cultures. Our study will provide useful knowledge and can be utilized for improving the quality of *M. oleifera* raw materials in herbal supplementary food and medical uses.

Key words: *Moringa oleifeira* Lam., antioxidant activities, UV-C irradition, plant tissue culture.

INTRODUCTION

Moringa oleifera Lam. (Horse radish tree or Drumstick tree) is in the Moringaceae family. It is a plant that originated in Asia, Asia minor and Africa (Mughul et al., 1999). The medicinal properties of it are anti-inflam-

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Figure 1. A 6 week old shoot of *M. oleifera* that had been cultured on MS medium containing 0.5 mg/L BA.

matory, bactericide, anti-diuretic, anti-cancer, reducing blood pressure, relieving joint pain, reducing cardiovascular disease and reducing rheumatism (Anwar et al., 2007; Chumark et al., 2008; Anjula et al., 2011). Besides these it contains some phytochemicals such as group glucosinolates effects against the formation of cancer cells and increasing glutathione. It contains important antioxidants which are phenolic compounds and flavonoids such as rhamnetin, gallic acid, cryptochlorogenic acid, isoquercetin and kaemferol which are high in antioxidants (Bennett et al., 2003; Brahma et al., 2009; Vongsak et al., 2012). The IC_{50} of *M. oleifeira* leaves were extracted by methanol using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay was 246.06 μg / mL (Maksab and Wichairam, 2009). At present leaves of *M. oleifera* are used as traditional medicines in capsule form. A shortage of young leaves occurred due to insect pests which resulted in the reduction of these traditional medicines.

Some secondary metabolites in plant tissue culture can be stimulated; for example, increasing the amount of isoflavonoid, anthraquinone and anthocyanin production in callus culture (Fedoreyer et al., 2000; Mischenko et al., 1999). The kinds and amount of growth regulators could enhance antioxidant features and total phenolics in plants. The Murashige and Skoog (1962) medium containing 6-benzyladenin (BA) could produce secondary metabolites which have the same chemical composition as the natural plants (Suriyaphan and Matchachip, 2009; Polsak, 2003). The condition of a culture media could be adjusted to incorporate biotic elicitors such as chitosan, chitin and enzymes as well as abiotic elicitors such as oxidative stress as ultraviolet and plant wounded (Benhamou, 1996).

UV-C irradiation (200-280 nm) could increase the activity of defense enzymes and could increase antioxidant activity such as ascorbic acid, anthocyanin synthesis, and total phenolic. It could also help delay senescence in strawberrys (Erkan et al., 2008). There were several reports that UV-C irradiation was used to stimulate the production of antioxidant capacities in the broccoli and Ceylon spinach (Costa et al., 2006; Pumchaosuan and Wongroung, 2008). UV-C irradiation could also stimulate enzyme-associated antioxidant activities including superoxide dismutase, catalase and peroxidase (Erkan et al., 2008). UV-C irradiation at 3.6 KJ/m^2 could inhibit the declining of vitamin C and carotenoids (Burana and Srilaong, 2009). Therefore, this research aimed to stimulate the oxidative stress in shoots of *M. oleifera* by UV-C irradiation in order to promote antioxidant activities, total phenolic and flavonoid contents and antioxidant bioactive components.

MATERIALS AND METHODS

Chemicals

Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium was prepared in-house. 6- benzyladenine (BA), folin-ciocalteaut reagent and phosphate buffered saline pH 7.4 (PBS) were purchased from Sigma-Aldrich, USA. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), trolox, gallic acid, aluminum chloride, trichloroacetic acid, potassium dihydrogen phosphate and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). Potassium ferricyanide and ferric chloride were obtained from Fluka Biochemika (Steinheim, Germany).

Sodium bicarbonate, and ferrous sulfate were purchased from Ajax Finechem (NSW, Australia). Isoquercetin, crypto-chlorogenic acid and astragalin were purchased from Biopurify China with purity more than 95%. Methanol (HPLC grade) was purchased from RCI Labscan (Thailand). Deionized water was purified from the Ultra clear series TWF (Siemens, Germany). All chemicals and solvents were of analytical grade if not stated otherwise.

Plant materials

M. oleifera plants in this study were collected from a natural source in Uttaradit Province, Thailand. The plant was identified by the author. A voucher specimen was kept at the Science and Technology Center, Uttaradit Rajabhat University, Uttaradit, Thailand.

Plant culture

Shoots of *M. oleifera* were rinsed in sterilized water. Then explants were sterilized by soaking in 15% clorox solution for 7 min and in 10% clorox solution for 7 min. Then they were washed three times with sterile distilled water, cut into pieces of 0.5-1.0 cm in length, and put on the MS medium containing 0.5 mg/L BA (Petchang, 2011) to induce multiple shoot formation (Figure 1). The culture conditions were set at a temperature of 25±3°C and 16 h photoperiod (40 μmol/m^2/s) by mercury fluorescent lamps for 6 weeks.

UV-C illumination

The UV-C illumination method was modified from Erkan et al.

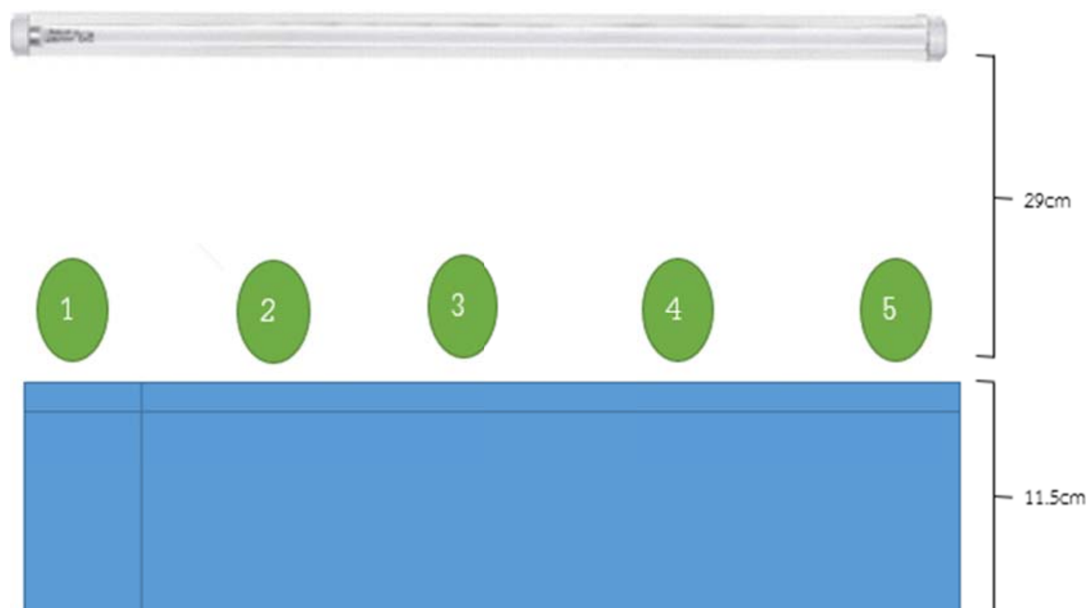

Figure 2. Diagram showing the UV-C treatment of this study.

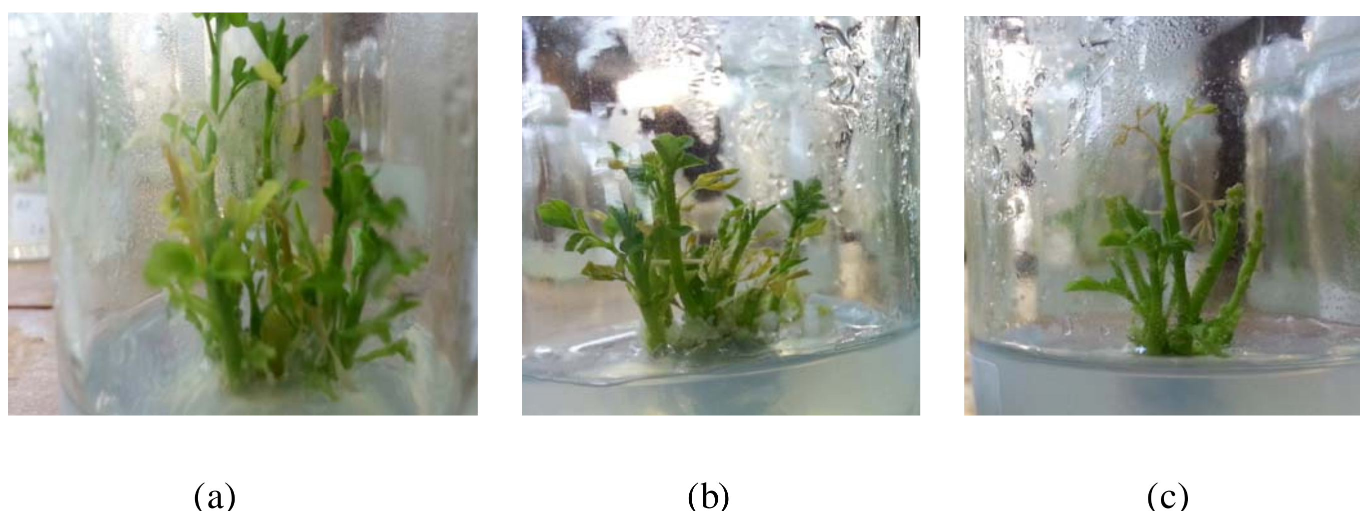

Figure 3. One week old multiple shoots were treated with UV-C irradiation from the UV lamp for (a) 5 min, (b) 10 min and (c) 15 min.

(2008). The UV-C illumination device consisted of an unfiltered germicidal UV lamp (EI series UV-C lamp, UVP model UVS-28, Holland) located 29 cm above the radiation vessel. The UV-C intensities at the irradiation area were determined by using a UV-C light meter (Model: UV-C-254SD, Lutron Electronic, Germany). Peak radiation region was at approximately 254 nm. The schematic diagram is shown in Figure 2. The different UV-C illumination doses were obtained by altering the duration of the exposure at a fixed distance, that is 5, 10 and 15 min. Prior to use, the UV lamps were allowed to stabilize by turning them on for 15 min. A non-illuminated sample was considered as the control treatment. After illumination, samples were cultured to maintain the temperature at 25±3°C with 16 h photoperiod (40 µmol/m^2/s) with mercury fluorescent lamps for four weeks (Figure 3).

Fresh multiple shoot extraction

The fresh multiple shoots were minced into small pieces, weighed, and macerated in methanol for 72 h at room temperature (37±2°C) with occasional shaking. The extracts were centrifuged at 650 rpm for 6 min. The supernatants were stored at -20ºC until analysis.

Antioxidant activities determination

DPPH scavenging assay

The antioxidant activities were determined by DPPH scavenging assay using the procedure adapted from Vongsak et al. (2013). The anti-

oxidant activities of the extracts and trolox (standard solutions) were investigated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA). A total of 500 µL of the extract or of the standard was added to 500 µL of DPPH in methanol solution (152 µM). After incubation at room temperature for 20 min, the absorbance of each solution was determined at 517 nm using a UV-VIS spectrophotometer (Perkin Elmer, USA). The corresponding blank readings were also taken and the inhibition percentage was then calculated as follows:

$$\% \text{ Inhibition} = \frac{(A_1 - A_2)}{A_1} \times 100$$

Where, A_1 was the absorbance of the control reaction (containing all reagents except the test compound) A_2 was the absorbance of the test compound.

The concentration of sample required for 50% scavenging of the DPPH free radical value (IC_{50}) was determined from the curve of scavenging percentage plotted against the concentration of test compound or standard.

ABTS scavenging assay

The antioxidant activities were determined by ABTS scavenging assay using the procedure adapted method from Arnao et al. (2001). The stock solutions contained 7.4 mM $ABTS^{\bullet+}$(2,2'-azino-bis (3-ethylbenzothiazoline- 6 -sulfonic acid) diammonium salt radical solution) and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing 7.4 mM $ABTS^{\bullet+}$ solution and 2.6 mM potassium persulfate solution (1:1) and allowing them to react for 12 h at room temperature in the dark.

The solution was then diluted by mixing 1 mL $ABTS^{\bullet+}$ solution with 24 mL methanol to obtain an absorbance of 1.1±0.02 units at 734 nm using the UV-VIS spectrophotometer. Fresh $ABTS^{\bullet+}$ solution was prepared for each assay. Extracted samples (150 µL) were allowed to react with 2,850 mL of the $ABTS^{\bullet+}$ solution for 2 h in a dark condition.

Then the absorbance was taken at 734 nm using the UV-VIS spectrophotometer. The standard curve was linear between 25 and 600 mM trolox. Results were expressed in milligrams of trolox equivalents antioxidant capacity (TEAC)/100 g. fresh weight (FW). Additional dilution was needed if the ABTS value measured was over the linear range of the standard curve.

Ferric reducing power (FRP) method

The FRP method was adapted from Ferreira et al. (2007). The 250 µL extracted samples were mixed with 250 µL of 0.2 M sodium phosphate buffer and 250 µL of 1% (w/v) potassium ferric cyanide solution and then incubated at 50°C for 20 min. The mixtures were added with 1 ml of 10% (w/v) trichloro acetic acid and centrifuged at 650 rpm for 10 min.

The 250 µL supernatant was drawn and mixed with 250 µL of deionized water and 50 µL of 0.1% (w/v) ferric chloride solution. The absorbance of the mixtures was measured at 700 nm using the UV-VIS spectrophotometer. The content of Fe^{2+} was evaluated and expressed as mM $FeSO_4$ /100 g FW.

Total phenolics content determination

The total phenolic content was determined by the method adapted from Vongsak et al. (2013) using the folin-ciocalteu reagent. Each 100 µL of the 0.2 g/mL (w/v) samples was mixed with the 250 µL folin-ciocalteu reagent (diluted 1:10) with deionized water and 400 µL of 7.5% (w/v) sodium bicarbonate solution. The mixture was allowed to stand for 30 min at room temperature with intermittent shaking. The absorbance was measured at 765 nm using a UV-VIS spectrophotometer. The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) / 100 g FW.

Total flavonoids content determination

The total flavonoid content was determined by the method adapted from Vongsak et al. (2013) using aluminum chloride. Each 400 µL of 0.04 g/mL (w/v) samples was mixed with 400 µL of 2% aluminum chloride solution. The mixture was allowed to stand for 10 min at room temperature with intermittent shaking. The absorbance of the mixture was measured at 415 nm against a blank sample without aluminum chloride using the UV-VIS spectrophotometer. The total flavonoids content was expressed as milligrams quercetin equivalent (QE)/100 g FW.

Quantitative analysis of major active compounds by HPLC

HPLC was performed on an Agilent 1260 series equipped with a quaternary pump 1260 Quat Pump VL, auto-sampler 1260 ALS, column thermostat 1260 TCC, and diode array detector 1260 DAD VL. The separation was carried out on a Hypersil BDS C-18 column (4.6 x 100 mm i.d., 3 µm) with a C-18 guard column. The mobile phase was 0.5% acetic acid (A) and methanol (B). The gradient elution was performed from 10% to 70% B in A for 20 min, and 100% B for 10 min. The column was equilibrated with 10% B in A for 10 min prior to each analysis. The flow rate was 1.0 mL/min at 25°C. The DAD detector was monitored at a wavelength of 334 nm for crypto-chlorogenic acid and 360 nm for isoquercetin and astagalin detection. The injection volumes for all samples including the standards were 20 µL.

Data collection and analysis

Each treatment was three replicates and the completely

Table 1. Antioxidant activities, total phenolic and flavonoid contents of shoot extraction of *M. oleifera* in the natural group, control group and experimental groups.

Treatment	DPPH assay (IC_{50}) (mg/mL)	ABTS assay mg TEAC/100 g FW)	FRP assay (mM $FeSO_4$ /100g FW)	Total phenolics (mg.GAE/ 100g FW)	Total flavonoids (mg.QE /100 g. FW)
Natural	45.69±0.62[d]	33.62±1.83[c]	18.10±0.80[c]	46.62±2.26[c]	37.83±0.98[c]
Control	44.29±0.56[d]	36.78±0.88[c]	20.70±0.38[c]	70.67±1.61[b]	41.52±0.85[c]
UV-C 5	40.89±0.56[c]	37.19±0.59[c]	22.52±0.74[bc]	73.48±1.64[b]	41.99±1.50[c]
UV-C 10	31.43±0.78[a]	58.98±2.09[a]	33.78±2.75[a]	112.56±2.94[a]	65.31±2.07[a]
UV-15	38.28±0.99[b]	50.22±0.63[b]	25.33±0.60[b]	81.34±1.14[b]	49.29±0.44[b]

[a,b,c,d] Dissimilar letters in the same column indicate a significant different at $p < 0.05$ using one-way ANOVA.

randomized design (CRD) was carried out. The data was analyzed using analysis of variance (ANOVA) followed by Duncan's multiple range test for the mean comparison.

RESULTS AND DISCUSSION

The antioxidant activities of *M. oleifera* in the natural group, the control group (UV-C for 0 min) and the experimental groups (UV-C for 5, 10 and 15 min) determined by the DPPH scavenging, ABTS scavenging and FRP assay showed that UV-C irradiation for 10 min showed the highest value with IC_{50} of 31.43 mg / mL, 58.98 mg TEAC/100 g FW ($y = 1.485x + 7.104$, $R^2 = 0.994$, where y is percentage inhibition and x is concentration of trolox in µg/mL) and 33.78 mM $FeSO_4$ equivalents /100 g FW ($y = 0.000x - 0.016$, $R^2 = 0.998$, where y is the absorbance unit of Fe^{2+} and x is the concentration of Fe^{2+}), respectively (Table 1). The antioxidant activities of *M. oleifera* detected by these three assays were statistically significantly different among each treatment ($p < 0.05$).

Total phenolic and flavonoid contents determination showed that UV-C irradiation for 10 min had the highest value at 112.56 mg GAE /100 g FW ($y = 0.036x + 0.143$-, $R^2 = 0.996$, where y is the absorbance unit of gallic acid and x is the concentration of gallic acid in µg/mL) and 65.31 mg QE/100 g FW ($y = 0.033x - 0.022$; $R^2 = 0.998$, where y is absorbance unit of quercetin and x is concentration of quercetin in µg/mL)., respectively (Table 1). The total phenolic and flavonoid contents of *M. oleifera* detected were statistically significantly different among each treatment ($p<0.05$).

The higher antioxidant properties of the control group than that of the natural group might be explained by the plant growth regulator in the MS medium containing 0.5 mg/L BA enhancing the antioxidant activities as well as total phenolic and flavonoid contents. Our finding is consistent with the previous work of Suriyapan and Machachip (2009), Fedoreyer et al. (2000) and Mischenko et al. (1999) and Polsak (2003). As described, UV-C irradiation as the elicitor to cause oxidative stress (Benhamou, 1996) that could increase the biosynthesis of an important secondary metabolite such as antioxidative components, and stimulate the activities of the superoxide dismutase, catalase and peroxidase enzyme (Dornenberg and Knorr, 1995; Benhamou, 1996; Erkan et al., 2008). They could prevent and reduce cell and DNA damage from oxidative stress (Pongprasert et al., 2011) and UV-C irradiation at 3.6 KJ/m^2 could inhibit the declining of vitamin C and carotenoids of *Brassica alboglabra* var. *alboglabra* (Burana and Srilaong, 2009). UV-C irradiation was used to stimulate the production of antioxidants in the broccoli (Costa et al., 2006) and increase anthocyanin product in the callus culture of Ceylon spinach (*Basella rubra* Linn.) (Pumchaosuan and Wongroung, 2008).

Our findings show that UV-C irradiation for 10 min was the optimum duration to increase antioxidant activities which resulted in higher growth of *M. oleifera.* Applying UV-C irradiation for 5 min was too short to stimulate the antioxidant activities of *M. oleifera* while applying for 15 min was so long that it damaged cells and caused harm to *M. oleifera*.

The antioxidant bioactive components including crypto-chlorogenic acid, isoquercetin and astragalin was analyzed by HPLC using the modified method from Vongsak et al. (2012). The results showed that UV-C irradiation for 10 min had the highest value at 30.10 ng / mL (as determined by calibration curve: $y=0.026x+1.264$; $R^2=0.999$, where y is peak area and x is the concentration in ng/mL), 61.21 ng/mL (as determined by calibration curve: $y = 0.052x - 2.557$; $R^2=0.999$, where y is peak area and x is the concentration in ng/mL) and 12.67 ng / mL (as determined by calibration curve: $y=0.046x - 2.704$; $R^2=0.999$, where y is peak area and x is the concentration in ng/mL), respectively (Table 2 and Figure 4). The crypto-chlorogenic acid, isoquercetin and astragalin determined were statistically significant different ($p<0.05$) among each treatment. UV-C irradiation for 10 min caused the total phenolic and flavonoid contents to be higher than those of the control group and the natural group as well as the antioxidative components crypto-chlorogenic acid, isoquercetin and

Table 2. Contents of crypto-chlorogenic acid (Cryp), isoquercetin (Iso) and astragalin (Astra) by HPLC (ng/mL).

Treatment	Cryp	Iso	Astra
Natural	4.49±1.30[c]	15.53±1.36[c]	4.54±0.72[c]
Control	4.53±1.11[c]	31.99±3.72[b]	5.38±0.94[c]
UV-C 5	14.40±4.09[b]	36.58±2.60[b]	8.90±3.07[b]
UV-C 10	30.10±3.88[a]	61.21±4.70[a]	12.67±5.40[a]
UV-C 15	15.61±2.72[b]	37.82±4.87[b]	9.13±2.02[b]

[a,b,c,d]Dissimilar letters in the same column indicate a significant difference at $p < 0.05$ using one-way ANOVA.

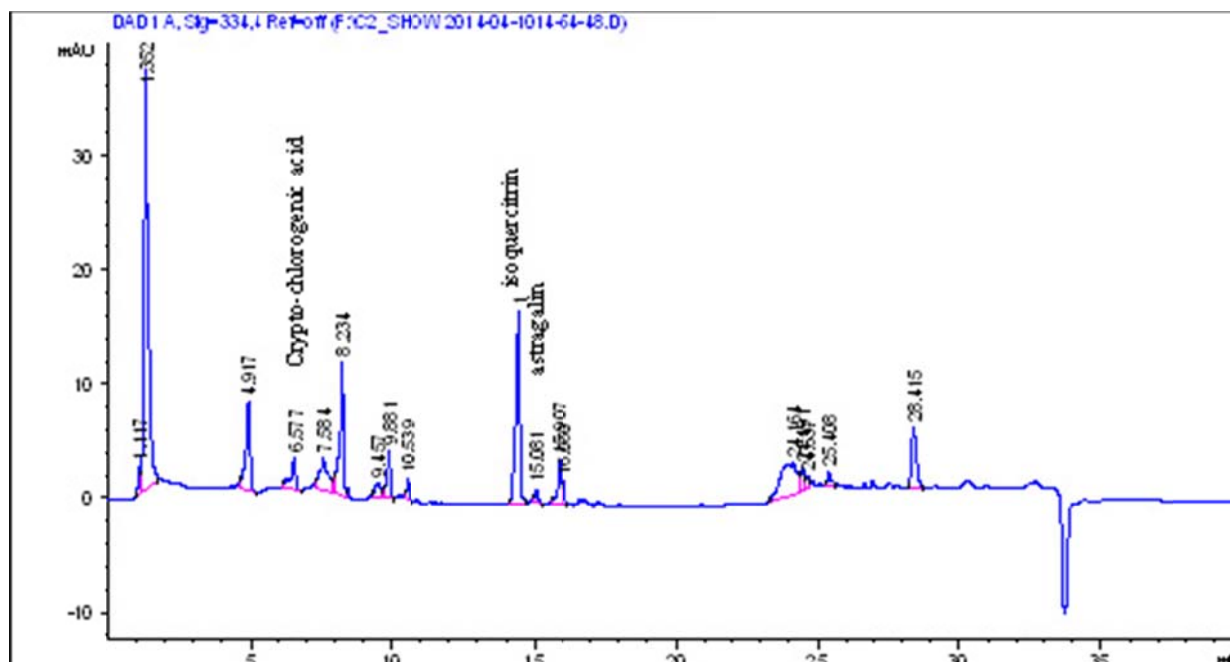

Figure 4. HPLC chromatogram showing the *Moringa oleifera* shoot culture profile.

astragalin. It can be concluded that UV-C irradiation could stimulate the activities of the enzyme in the biosynthesis of the antioxidative compounds resulting in increasing antioxidant capacities of *M. oleifera*.

Conclusion

UV-C irradiation for 10 min was the optimal duration for stimulating antioxidant capacities and antioxidant compounds in shoot cultures of *M. oleifera,* resulting in higher antioxidant activities, total phenolic and flavonoid contents as well as the amount of antioxidative components crypto-chlorogenic acid, isoquercetin and astargalin than those of the control group and the natural group. UV-C irradiation may also stimulate the activities of the superoxide dismutase, catalase and peroxidase enzyme that are involved in the biosynthesis of antioxidative compounds. Our study could provide useful knowledge that can be utilized for improving the quality of *M. oleifera* raw materials in herbal supplementary food and medical uses.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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