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Bioactive Potential of *Macadamia integrifolia* and *In vitro* Elicitation of Flavonoid Production in its Callus Cultures

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Abstract: *Macadamia integrifolia* is a highly valued nut tree crop. The application of tissue culture from macadamia in secondary metabolites production is of value to ensure permanent source of desired substances. The present study aims to investigate the anti-aging activity of extracts of three different parts viz. kernel, leaf and pericarp of *M. integrifolia* and to maximize *in vitro* production of its flavonoids. Data revealed that kernel extract exhibited the strongest anti-aging activity in which it showed the highest reversion of D-galactose induced memory deficit, highest promotion of motor function. Additionally, kernel extract possessed the highest antioxidant activity via reduction of malonaldehyde MDA concurrent with an increase of glutathione GSH serum levels in comparison with D-galactose treated group. The maximal hepato-renal protective and antihyperlipidemic activities were recorded for kernel extract compared to leaf and pericarp extracts. Of 25 treatments MS medium fortified with BAP 1.0 mg/L plus NAA 1.0 mg/L plus Caisen hydrolysate 1g/L was the suitable medium for callus initiation from kernel. Flavonoids production was enhanced in callus culture supplemented with phenylalanine at 100 mg/L and exposed to UV radiation at 15 W for 1 h. In contrast, callus grown on medium augmented with silver nitrate 200 mg/L and exposed to 15W UV produced the highest total phenolics and flavonoids (1196.59 and 239.32 mg/100 g d.w), respectively.

Key words: *Macadamia integrifolia* · Callus · Flavonoids · Bioactive

INTRODUCTION

Macadamia integrifolia (Maiden & Betche) nut, known as Queensland nut, Australian nut, bauple nut is a dark green spreading semi-hard tree [1]. The genus *Macadamia* consists of nine species, but only the smooth-shelled *M. integrifolia*, and the rough shelled *M. tetraphylla* (L.A.S. Johnson) that are cultivated for their edible nuts [2].

Oxidative stress is believed to be a primary factor in neurodegenerative diseases, as well as in the normal process of aging. Oxygen-derived free radicals exert detrimental effects including peroxidation of membrane lipids, enzyme inactivation, DNA fragmentation and activation of apoptosis [3].

D-galactose (D-gal) is a reducing sugar that can be metabolized at normal concentration. However, at high levels, D-gal can be converted into aldose and hydroperoxide under the catalysis of galactose oxidase, resulting in the generation of a superoxide anion and oxygen-derived free radicals [4]. D-galactose also reacts readily with free amine groups in proteins and peptides both *in vivo* and *in vitro* to form advanced glycation end products. Evidence shows that D-gal can remarkably lead to the generation of reactive oxygen species, especially superoxide radicals and hydrogen peroxide [5].

The capacity for plant cell, tissue and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been well-recognized almost since the inception of *in vitro* biotechnology. The deliberate stimulation of defined chemical products within carefully regulated *in in-vitro* cultures provides an excellent forum for in-depth investigation of biochemical and metabolic pathways, under highly controlled micro-environment conditions [6]. There are several advantages to producing a valuable

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secondary product in plant cell culture, rather than in the whole crop plant [7] to include i.e., ensuring consistency in production, avoiding climatic changes or soil conditions, microbial free, easier to extract being free of chlorophyll. The present study aimed to investigate the medicinal activity of extracts of different parts of *M. integrifolia* as well as enhancing the flavonoid production in its callus cultures.

MATERIALS AND METHODS

Plant Material: Samples of *M. integrifolia* used in this study were collected in March 2015 from the unique cultivated tree in the experimental farm of Horticulture Research Institute, Agriculture Research Center, Giza, Egypt. Plant identity was kindly authenticated by Dr. Reem Sameer Hamdi assistant professor of Plant Taxonomy, Department of Botany, Faculty of Sciences, Cairo University.

Leaves, kernels and pericarps of *M. integrifolia* were air-dried, coarsely powdered and kept in tightly closed amber-colored glass containers at room temperature.

Plant Extracts: 70 g of the air dried powder kernels, 1350 g of leaves and 100 g of pericarp were separately macerated in successive portions of ethyl alcohol 95% till exhaustion. The alcoholic extracts were pooled and evaporated under vacuum to obtain 27.83 g, 277.66 g and 18.43 g of seeds, leaves and pericarp extracts, respectively. Extracts were stored for further biological and phytochemical studies.

Experimental Animals: Swiss male mice of 20-30 g body weight were used for the determination of acute toxicity. Wister albino male rats, weighing from 200-225 g were used for D-galactose induced toxicity experiment. The animals were obtained from the animal house colony of the National Research Centre, Giza, Egypt. The animals were housed in standard metal cages in an air conditioned room at 22±3°C, 55± 5% humidity and provided with standard laboratory diet and water ad libitum. Group of 8 rats or 8 mice were used for each experiment. All experimental procedures were conducted in accordance with the guide for care and use of laboratory animals and in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health guide for care and use of laboratory animals.

***In vivo* Modulatory Activity Against D-Galactose Induced Aging in Rats**

Experimental Protocol: Animals were divided into five groups (each containing 8 rats). One served as normal control receiving saline water and the rest were treated with D-galactose (Sigma Aldrich Co.) at a dose of 150 mg/kg intrapretonial (i.p) [8] once a day for 60 days using saline water as a vehicle [9]. The tested ethanol extract of *M. integrifolia* leaf, kernel and pericarp were administered intragastrically at a dose of 200 mg/kg/day in distilled water for 30 days. At the end, rats were tested for Memory assessment using Morris water maze [10] and motor coordination assessment [11]. Decrease in fall off time is suggestive of central nervous system depression [12]. Motor coordination was assessed on the 83rd days before probe trial in Morris water maze.

Biochemical Analysis: Animals were mildly anaesthetized at the end of the experimental period with diethyl ether and the blood samples were collected by retroorbital sinus puncture into micro-centrifuge tubes. Blood was allowed to clot for 30 min then centrifuged at 10000 rpm for 10 min to obtain serum. Samples were divided into aliquots and stored at-20°C until further analysis which included. Serum was analysed for these biomarkers to evaluate these effects: a) *serum oxidative stress biomarkers i.e.* glutathione (GSH) and malonaldehyde, *b) hepato-protective activity i.e.*, AST, ALT and total bilirubin, c) *nephro-protective activity i.e.*, urea, creatinine and total protein and d) *anti-hyperlipidemic activity* i.e. triglycerides, total cholesterol, LDL and HDL.

All parameters were measured in samples by end point method as per the manufacturer's instructions of the suitable diagnostic kit.

For determination of total phenolic and flavonoid contents, plant materials were extracted according to the method of Ivanova *et al*. [13].

Determination of Total Phenolics and Flavonoids Content: The total phenolic content was determined by the Folin-Ciocalteu method [14], whereas the total flavonoids was evaluated according to the colorimetric assay with aluminium chloride reagent [15].

Determination of Phenolics and Flavonoids via HPLC: HPLC Hewlett Packard (Series 1050) equipped with solvent degasser, ultraviolet (UV) detector set at (280 nm for phenolics and flavonoids determination) and quaternary pump was used for analysis. The column used for separation was Phenomenex C18 (250mm length)

isocratic separation was carried out using of methanol: acetic acid: water (36: 0.9: 63.1) (v/v) as a mobile phase at flow rate of 1 ml/min. Authentic phenolics and flavonoids were dissolved in mobile phase and injected into HPLC. The retention time and peak area were used to calculate the phenolic and flavonoids concentrations by the data analysis of Hewllet packared software [16, 17]. Quantification was based on peak area computation (external standard method) and concentrations were determined according to the following formula:

$$\textbf{Conc.}_{\text{unknown}} = (\textbf{Area}_{\text{unknown}}/\textbf{Area}_{\text{known}})\ \textbf{Conc.}_{\text{known}}$$

Identification of individual components was performed by comparison of their retention times with those of available authentic samples similarly analyzed.

***In vitro* Initiation and Growth of Callus**

Surface Sterilization of Explants: The entire creamy white seed kernels (after removing the hard fruit testa) were sterilized by 0.1% mercuric chloride for 5 minutes and washed by sterile distilled water thrice and used for culture.

Culture Medium: MS medium [18] supplemented with two different plant growth regulators (Auxins and cytokinins) was used in 25 treatments and 4 jars per treatment for callus initiation as follows: a) MS free medium as control, b) 2,4-D (1,2-dichlorophenoxy acetic acid) at 1, 2 or 4 mg/L, c) NAA (naphthalene acetic acid) at 1, at 1, 2 or 4 mg/L, d)2,4,5-Trichloro-phenoxy acetic acid 1, at 1, 2 or 4 mg/L, e) Parachloro-phenoxy acetic acid 1, at 1, 2 or 4 mg/L and f) Effect of adding BAP (benzyl amino purine) at 1.0 mg/L plus Caisen hydrolysate 1.0 g/L to all the previous auxin types and concentrations.

Physical and Chemical Elicitation of Callus for Secondary Metabolites Production: Chemical and physical elicitors were assed for their effect on flavonoids production to include phenylalanine at 100 or 200 mg/L, silver nitrate at 5 or 10 mg/L in presence or absence of ultraviolet irradiation exposure for 60 min at 15 or 30 Watts. The source of ultraviolet rays was Philps TuV, 15W, 54 V, 0.34 A. Model G15T8 ultraviolet UV-C lamp (45 cm long and 2.8 cm diameter and contains mercury (Hg) 2.0 mg, water air disinfection, 253.7 nm-254 nm UV-C were used to evaluate their effects on callus fresh weight, dry weight and secondary metabolites production in means of total phenolics, total flavonoids and determination of individual phenolics and flavonoids concentrations by HPLC.

Statistical Analysis: Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Data were expressed as mean±standard error and the values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Acute Toxicity: Results obtained from acute toxicity study revealed that ethanolic extracts of kernel, pericarp and leaves of *M. integrifolia* are consedered safe upto dose reaching 5 g/kg of body weight.

Morris Water Maze: Rats treated with D-galactose for 90 days showed significant impairment in memory as indicated by Morris water maze test during the retention trial conducted Table (1). D-galactose (150 mg/kg i.p.) treatment significantly ($P < 0.05$) raised escape latency time (ELT) and delay northwest latency (NW). During the probe trial on day 90, D-galactose treated animals were found to spend significantly less time in the target quadrant (NW) than the control group. The memory deficit caused by D-galactose was significantly ($P < 0.05$) reversed by all tested extracts, where kernel extract showed the highest reversion of D-galactose induced memory deficit, followed by pericarp and leaves extracts.

Motor Coordination Assessment: The effect of D-galactose on balance and coordination was tested on an accelerating Rota-Rod. All animals in D-galactose treated group showed decreased latency to fall than normal group. Administration of tested extracts showed a slight advantage in promoting motor function than normal group with more significant advantage than D-galactose treated group, where kernel extract showed the highest promotion of motor function followed by pericarp extract while leaves extract showed the lowest effect.

Effect on Serum Oxidative Stress Biomarkers: Administration of D-galactose for 90 days was associated with marked elevation in serum levels of MDA by about 2.2 fold and a decrease in serum GSH by about 52% ($P< 0.05$) of the normal control value Table (2). Administration of all three extracts at 200 mg/kg daily to rats treated with D-galactose reduced the serum levels of MDA and increased serum GSH in comparison with D-galactose treated group. The highest activity was obtained from kernel extract followed by pericarp extract and leaves extract which showed the lowest antioxidant activity.

Table 1: The effect of tested extracts on cognitive impairment and unbalance caused by D-galactose administration

Groups	ELT (S)	NW latency rats crossing the target quadrant	% of time spent in target quadrant	Latency to fall (s)
Normal	18.5±0.23	51±1.3	85	285±3
D- galactose	57.7±1.2[α]	27±1.2[α]	45	187±12[α]
Leaves	24.6±1.7*	45±1.7*	75	240±7*
Pericarp	22.5±0.9*	47±1.9*	78	255±9*
Kernel	21.3±5*	49±2.5*	81.5	271±5*

Data are mean±SD (n = 8). ELT: Escape latency time; NW: Northern west

[α] Significant versus control (P < 0.05). * Significant versus D-galactose (P < 0.05).

Table 2: The effect of tested extracts on GSH and MDA in rats treated with D-galactose

Groups	GSH (uM/ml)	MDA (mmol/ml)
Normal	7.3±0.91	8.4±0.39
D- galactose	3.53±0.41[α]	18.5±1.23[α]
Leaves	6.5±0.44[*]	10.6±0.84*
Pericarp	6.7±0.35[*]	9.9±0.57*
Kernel	7.1±0.28[*]	9.2±0.47*

Data are mean±SD (n = 8). GSH: Glutathione, MDA: Malonaldehyde

[α] Significant versus control (P < 0.05). * Significant versus D-galactose (P < 0.05).

Table 3: Effect of tested extracts on elevated liver and renal functions caused by D-galactose administration

	Liver function			Renal function			Lipid profile			
Animal groups	AST (U/ml)	ALT (U/ml)	Total Bilirubin (mg/dl)	Creatinine (mg/dL)	Urea (mg/dL)	Total protein (g/dl)	Cholesterol (mmol/L)	Triglyceride (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
Normal	36.4±1.39	25.8±1.37	0.92±0.03	0.54±0.08	84.38±2. 2	7.8±0.68	98±5.8	82±7.2	45±3.8	65±5.7
D- galactose	112±8.42	88.6±5.8*	1.3±0.12	0.98±0.02*	108.6±7.8*	5.1±0.41*	105±4.8	102±8.8	59±5.4	77±5.3
Leaves	50±1.36	46.7±3.6[*a]	1.08±0.07	0.79±0.06[*a]	104.7±4.6	6.9±0.56[a]	101±8.6	95±3.6	49±4.6	72±6.4
Pericarp	47±2.72	42.4±4.2[*a]	1.05±0.05	0.77±0.07[*a]	97.4±3.2	7±0.38[a]	101±7.4	91.4±4.2	48±3.8	70±4.2
Kernel	41±1.94	32.5±2.2[a]	0.99±0.03	0.61±0.04[a]	92.5±3.5[a]	7.2±0.24[a]	99±6.8	88±4.5	47±4.4	69±3.8

Data are mean±SD (n = 8) ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, HDL: High density lipoprotein, LDL: Low density lipoprotein

[a] Significant versus control (P < 0.05).

* Significant versus D-galactose (P < 0.05).

The ethanolic extract of kernel of *M. integrifolia* showed the highest improvement in cognition impairment and motor coordination in D-galactose induced aging rat-model followed by pericarp ethanolic extract while leaves extract showed the least improvement.

Ethanolic extracts of kernel, pericarp and leaves showed hepatoprotective, antihyperlipidemic, renal protection and antioxidative stress activities in D-galactose treated rats. Kernel extract was the most effective in all these aspects followed by pericarp extract and leaves extract came third. Administering rutin, or flavonoid extracted from ethyl acetate extract of *Saussurea involucrata* (30mg/kg/d and 30mg/kg/d) for 6 weeks, concomitant with D-gal injection, significantly increased superoxide dismutase and glutathione peroxidase activities and decreased the MDA level in plasma [3]. Furthermore, the result showed that the percentages of cleaved caspase and PARP in the D-gal-treated mice were much higher than those in the control. Furthermore, our results also showed that oral administration of rutin to these mice significantly improved behavioral performance in a step-through passive avoidance task and these results suggest that flavonoid or rutin exerts potent antiaging effects on D-gal in mice via antioxidative mechanisms.

Effect on Serum Liver Biomarkers: Administration of D-galactose for 90 days was associated with marked elevation in serum levels of liver biomarkers including alanine transaminase (ALT) and aspartate transaminase (AST) which were estimated using kits provided by Quimica Clinica Aplicada (Spain) and serum total bilirubin level which was measured by using a commercial kit supplied by Bio-diagnostic (Egypt). Biochemical changes due to D-galactose administration manifested as an increase in the serum levels of ALT by about 3.1 fold, were AST by about 3.6 fold and total bilirubin by about 1.4 fold of the normal values (Table 3).

Administration of all tested extracts 200 mg/kg daily to rats treated with D- galactose reduced the serum levels of AST, ALT and total bilirubin in comparison with D- galactose treated group, with kernel extract showing the highest reducing activity on elevated AST, ALT and total bilirubin, followed by pericarp extract and leaves

extract showed the lowest reduction on elevated ALT, AST and total bilirubin. The term flavonoids refers to thousands of plant compounds with a common basic structure, phenylchromane, which allows the generation of multiple flavonoid subclasses including flavonols, flavones, catechins, anthocyanidins, isoflavones, dihydroflavonols and chalcones [19]. Flavonoids have numerous pharmacological activities, exert antioxidant effects which enabled them to possess different therapeutic activities like, anti hyperlipidemic, hepatoprotective and kidney (nephro) protective [20]. Flavonoids having wide range of nephro protective activities in different conditions like glomerulonephritis in diabetic nephropathy and other chemical induced kidney failure like gentamycin, ccl4 and cyclosporine.

Effect on Renal Biomarkers: Administration of D-galactose for 90 days w as associated with marked elevation in serum levels of renal biomarkers. These changes were manifested as an increase in the serum levels of creatinine by about 1.8 fold and Urea by about 1.3 fold of the normal values. Furthermore, D-galactose caused a decrease in serum protein by about 35% ($P< 0.05$) of the normal control value (Table 3).

Administration of all three extracts 200 mg/kg daily to rats treated with D- galactose reduced the serum levels of creatinine and urea while increased serum protein in comparison to D- galactose treated group. Kernel ethanolic extract showed the highest renal protection in all of the tested parameters, followed by pericarp extract and leaves extract which showed the lowest activity on elevated kidney function. Lien *et al*. [21] demonstrated that flavonoids can protect kidney through different mechanisms including xanthine oxidase pathway which has been implicated as an important route in the oxidative injury to renal tissues, especially after ischemia-reperfusion. Xanthine oxidase is a source of oxygen free radicals. In the reperfusion phase (i.e, reoxygenation), xanthine oxidase reacts with molecular oxygen, thereby releasing superoxide free radicals. quercetin and silibin, inhibit xanthine oxidase activity, thereby resulting in decreased oxidative injury.

Effect on Lipid Profile: Administration of D-galactose for 90 days was associated with slight elevation in serum levels of lipid (cholesterol, triglyceride, LDL and HDL) Table (3). Administration of all three extracts at 200 mg/kg dose daily to rats treated with D- galactose caused insignificant variation in the serum levels of cholesterol, triglyceride, HDL and serum LDL in comparison with D-galactose treated group and normal control group with the highest antihyperlipidemic activity obtained from kernel extract. Leaves extract showed the lowest activity.

***In vitro* Callus Culture:** Over the last few years, the scope of plant species employed in tissue culture has been broadened particularly due to perceived or actual needs to extend micro-propagation to plants which are of commercial value or are rare or threatened by extinction. Due to a lack of proper cultivation practices, destruction of plant habitats and the illegal and indiscriminate collection of plants from these habitats, many medicinal plants are severely threatened [22].

***In vitro* Initiation and Growth of Callus:** Application of 0.1% mercuric chloride for 5 minutes was sufficient to get contamination free kernel culture and this may be due to that the kernel is protected far away inside fruits. Explants survival was 90% From all of the 25 treatments prepared for callus initiation, only one produced callus which was the MS media fortified with BAP 1ml/L + NAA 1ml/L + Caisen hydrolysate 1g/L, while all of the other treatments were not suitable for callus initiation from *M. integrifolia* kernel explants.

The kernel showed greening in color in the first week (Fig 1) and with time, brown mass started to form that continued to grow and getting brighter in color. Finally yellow, friable, non-differentiated callus was formed.

Callus cultures of kernel explants grown on MS medium supplemented with BAP 1.0 mg/L + NAA 1.0 mg/L + caisen hydrolysate 1.0 g/L were harvested after an incubation period of 6 weeks, at which approximately ultimate growth occurred.

Callus fresh weight was measured after three successive subcultures, each lasting for 6 weeks and the resulted weights are illustrated in Figure (2)**.** It is assumed that differentiated tissue cells in plant tissues are capable of de-differentiating and regenerating wounded tissue or even the entire plant; it is also assumed that they can form totipotent callus cells [23]. A more recent concept claims that plant cells do not re-differentiate, but that callus is rather formed from pre-existing stem cells [24].

Exogenous application of auxin and cytokinin induces callus in various plant species. Generally speaking, an intermediate ratio of auxin and cytokinin promotes callus induction, while a high ratio of auxin-to-cytokinin or cytokinin-to-auxin induces root and shoot regeneration, respectively [25]. Auxin signaling is transduced via ARF transcription factors, especially

Fig. 1: Kernel greening and Callus formation

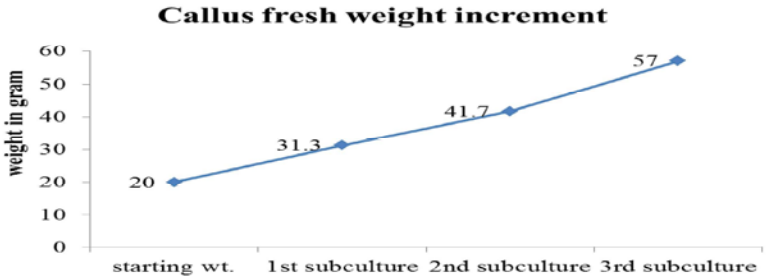

Fig. 2: *M. integrifolia* callus fresh weight increment in 3 successive subcultures

Table 4: Effect of pheylalanine, silver nitrate and UV irradiation on *M. integrifolia* callus fresh and dry weights (g) and moisture content (%)

Treatment	Fresh weight(g) Means±SD	Dry weight (g) Means±SD	Moisture content
Control	1.2975±0.0467	0.1699±0.0061	86.9056%
Phenylalanine 100mg/L	1.2418±0.2407	0.1705±0.0331	86.2699%
Phenylalanine 200mg/L	1.1098±0.1528	0.1558±0.0214	85.9614%
Silver nitrate 100mg/L	1.474±0.401	0.2170±0.059	85.2782%
Silver nitrate 200mg/L	1.4145±0.1955	0.207±0.0286	85.3659%
Phenylalanine 100mg/L + 15W UV for 60 min	1.3528±0.4214	0.2365±0.0737	82.5177%
Phenylalanine 100mg/L + 30W UV for 60 min	1.009±0.0109	0.121±0.0011	88.0079%
Phenylalanine 200mg/L + 15W UV for 60 min	1.7228±0.7793	0.2343±0.1060	86.4000%
Phenylalanine 200mg/L + 30W UV for 60 min	1.4315±0.4072	0.2075±0.0857	85.5047%
Silver nitrate 100mg/L + 15W UV for 60 min	1.4058±0.2484	0.166±0.0294	88.1918%
Silver nitrate 100mg/L + 30W UV for 60 min	1.5278±0.3839	0.1963±0.0493	87.1515%
Silver nitrate 200mg/L + 15W UV for 60 min	1.4565±0.4155	0.153±0.0437	89.4954%
Silver nitrate 200mg/L + 30W UV for 60 min	1.4848±0.2522	0.1958±0.0333	86.8130%
F ratio	0.9767	1.7125	---
P value	NS	NS	---

NS= non significantly different at $p< 0.05$

ARF7 and ARF19, to activate the expression of LBD family transcription factors, LBD16, LBD17, LBD18 and LBD29. These LBDs in turn induce E2Fa, a transcription factor that plays a central role in cell cycle reentry. Cytokinin signaling is transduced via two-component regulatory pathway to activate the type-B ARR transcription factors. The expression of CYCD3; 1 is sharply up-regulated by cytokinin, but whether it is directly activated by type-B ARR is not known. The AP2/ERF transcription factor ESR1 is also up-regulated by cytokinin. Casein hydrolysate is a source of calcium, micronutrients, vitamins and a mixture of up to 18 amino acids, so that it played a very important role in callus initiation and growth [26].

***In vitro* Production of Secondary Metabolites:** Effects of physical and chemical elicitors on fresh and dry weight and moisture content of callus culture of *M. integrifolia*

The total quantity of the callus (57 g) obtained after three subcultures in callus mass production step, was cultured on the same media (Media no.12, Table (4) as 1 g of callus in 25 ml of media and divided into 12 different parts each of 4 g callus. Each part was exposed to different chemical and/or physical elicitors (including UV-C light, phenylalanine and silver nitrate) with different concentrations in addition to a control treatment which no elicitors were added as reference. For each treatment, callus fresh weight, dry weight and moisture content were measured after an incubation period of 6 weeks.

Table 5: Effect of pheylalanine, silver nitrate and ultraviolet irradiation treatments on total phenolics and flavonoids content of *M. integrifolia* callus cultures

Treatment	Total phenolics (mg GAE/100 g)	Total flavonoids (mg KE/100 g)
Control	439.77	87.95
Phenylalanine 100mg/L	450	90
Phenylalanine 200mg/L	398.86	79.78
Silver nitrate 100mg/L	245.45	49.09
Silver nitrate 200mg/L	347.73	69.55
Phenylalanine 100mg/L + 15W UV for 60 min	276.14	55.23
Phenylalanine 100mg/L + 30W UV for 60 min	347.73	69.55
Phenylalanine 200mg/L + 15W UV for 60 min	511.36	102.27
Phenylalanine 200mg/L + 30W UV for 60 min	511.36	102.27
Silver nitrate 100mg/L + 15W UV for 60 min	317.05	63.41
Silver nitrate 100mg/L + 30W UV for 60 min	378.41	75.68
Silver nitrate 200mg/L + 15W UV for 60 min	1196.59	239.32
Silver nitrate 200mg/L + 30W UV for 60 min	398.86	79.77
Mother plant kernel	254.45	49.09

As for fresh and dry weights, data showed that the highest fresh weight (1.72 g) was obtained when callus culture was supplemented with phenylalanine 200 mg/L and irradiated with ultraviolet at 15 W for an hour, whereas the lowest fresh weight 1.0 g was obtained when phenylalanine 100 mg/L was added to callus culture and irradiated with ultraviolet at 30 W for an hour. Meanwhile the highest callus dry weight was obtained when callus culture was supplemented with phenylalanine 100 mg/L and exposed to UV radiation at 15 W for an hour, whereas the lowest callus dry weight was obtained from callus culture containing phenylalanine 100 mg/L and exposed to UV radiation at 30W for an hour.

Moisture content was also affected by elicitation treatments used as the highest moisture content (89.5%) produced when callus culture was supplemented with silver nitrate 200 mg/L and irradiated with ultraviolet at 15 W for an hour. The lowest moisture content (82.5%) was observed when callus culture contained phenylalanine 100 mg/L and exposturd to ultraviolet irradiation at 15 W for an hour.

By comparing the effects of phenylalanine and silver nitrate concentrations on callus fresh weight, lower concentration of silver nitrate (100 mg/L) gave better results than higher concentration (200 mg/L), whereas phenylalanine had negative effect on callus growth compared to control where lower concentration (100 mg/L) was better than the higher concentration (200 mg/L).

Irradiation of the media with different ultraviolet powers (15 and 30 W) for an hour had changed this effect, as culture supplemented with phenylalanine (100 or 200 mg/L) showed better increase in callus growth when exposed to low power of ultraviolet irradiation (15 W) than when exposed to high power of ultraviolet (30 watt). On the other hand, culture supplemented with silver nitrate (100 or 200 mg/L) showed a bigger increase in callus fresh weight when exposed to high power of ultraviolet irradiation (30 W) when it is exposed to low power of ultraviolet irradiation (15 W).

Comparing the result obtained, no effect can be noticed on moisture content with increasing the concentrations of phenylalanine or silver nitrate, but this was totally changed when cultures were exposed to ultraviolet irradiation of different powers (15 and 30 W), as for phenylalanine at low concentration (100 mg/L), increasing the power of ultraviolet irradiation from 15 to 30 watt had increased the moisture content by 5.5%, while when high concentration of phenylalanine (200 mg/L) was used, increasing the ultraviolet power from 15 to 30 watt had decreased the callus moisture content by 0.895%.

For cultures supplemented with silver nitrate and exposed to ultraviolet irradiation, increasing the power of ultraviolet irradiation had decreased the callus moisture content for both concentrations of silver nitrate used (100 and 200 mg/L), as increasing the ultraviolet power from 15 to 30 W in culture containing silver nitrate (100 mg/L) had decreased the moisture content by 1.04% and for culture containing silver nitrate (200 mg/L), increasing the ultraviolet power irradiation from 15 to 30 W had decreased the moisture content by 2.68%.

Effects of Physical and Chemical Elicitors on Total Phenolic and Total Flavonoid Contents of Callus Culture of *M. integrifolia:* As shown in Table (5) total phenolics and total flavonoids content were measured in each of the tested callus treatment and compared to total phenolics and flavonoids contents in mother plant kernels. Total flavonoids were expressed as mg kaempferol equivalents per 100 g dry weight (mg KE/100g) and total phenolics were expressed as mg gallic acid equivalents per 100 g dry weight (mg GAE/100g).

Table 6: Concentrations of identified phenolics and flavonoids expressed as mg/100 g dry powder of different calli of *M. integrifolia*

	Phenolics (mg/100 g)			Flavonoids (mg/100 g)	
Treatment	Gallic acid	Dhurrin	Protocatechuic acid	Hespiridin	Kaempferol
Rt min	3.56±0.5	3.92±0.5	4.50±0.5	6.29±0.5	10.27±0.5
Control	20.798	2842.05	30.682	0.184	--
Phe. 100mg/L	17.83	2121.99	51.34	0.048	0.008
Phe. 200mg/L	5.89	499.06	16.14	0.011	0.001
Silver nitrate 100mg/L	8.09	448.26	9.36	0.049	--
Silver nitrate 200mg/L	7.11	655.85	28.35	0.02	0.004
Phe. 100mg/L + 15W UV for 60 min	15.15	1482.59	43.58	0.125	0.007
Phe. 100mg/L + 30W UV for 60 min	12.13	819.93	20.16	0.025	0.002
Phe. 200mg/L + 15W UV for 60 min	15.03	1652.79	83.52	0.056	0.003
Phe. 200mg/L + 30W UV for 60 min	8.29	1018.03	21.79	0.012	0.008
Silver nitrate 100mg/L + 15W UV for 60 min	12.35	964.93	41.03	0.020	0.003
Silver nitrate 100mg/L + 30W UV for 60 min	28.54	2081.9	64.35	0.056	0.008
Silver nitrate 200mg/L + 15W UV for 60 min	29.71	4268.25	148.7	0.162	0.044
Silver nitrate 200mg/L + 30W UV for 60 min	15.72	1491.71	42.28	0.036	0.006
Mother plant kernel	75.23	2107.5	136.14	0.088	0.004

The presented data indicated variation in total phenolic and total flavonoid contents as a result of the different treatments used where the highest total phenolic contents was estimated in callus treated with silver nitrate 200 mg/L + 15W UV for 60 min at 1196.59 mg/100 g and as a consequence this treatment also showed the highest flavonoid content at 239.32 mg/100 g.

Comparing with control, callus cultures supplemented with phenylalanine showed unnoticeable decrease in phenolics and flavonoids contents with values at 450 mg and 90 mg /100 g for phenolics and flavonoids, respectively for the low concentration of phenylalanine (100 mg/L) and 398.86 mg and 79.78 mg /100 g for phenolics and flavonoids for the high concentration of phenylalanine (200 mg/L) respectively. Exposing the callus culture to UV irradiation at 15 W for an hour decreased the total phenolic and flavonoid contents to 276.14 mg and 55.23 mg /100 g for phenolics and flavonoids, respectively and 347.73 mg and 69.55 mg/100 g for phenolics and flavonoids when treatment included supplementation with low concentration of phenylalanine (100 mg/L). Treatments supplemented with high concentration of phenylalanine (200 mg/L) showed different pattern when exposed to UV irradiation where total phenolics and flavonoids contents were increased to 511.36 mg and 102.27 mg/100 g when exposed to UV irradiation at 15W and 30W for an hour, respectively.

Callus cultures supplemented with silver nitrate showed decreased total phenolics and flavonoids contents compared to control except the treatment containing combination of high silver nitrate concentration (200 mg/L) and UV irradiation at 15 W for an hour where the highest phenolic and flavonoids contents were obtained among all of the tested treatments.

Upon comparing the total phenolics and flavonoids contents in different calli obtained with mother plant kernel as being the source of callus in all treatments tested, all of the callus treatments showed higher contents of phenolics and flavonoids except the treatment supplemented with low concentration of silver nitrate (100 mg/L) without exposure to UV irradiation, where this treatment showed the lowest phenolics and flavonoids content among all of the tested treatments at 245.45mg and 49.09 mg/100 g for total phenolics and flavonoids respectively.

Determination of Individual Phenolics and Flavonoids by HPLC: From the results shown in Table (6), treatment containing a combination of silver nitrate at 200 mg /L and exposure to UV light at 15 W for 60 minutes resulted in the highest concentration of dhurrin, protocatechuic acid and kaempferol in all of the measured samples and almost the same concentration for hespiridin when compared to callus conrol sample. However, gallic acid concentration was the highest among all of the tested treatments but still lower than its concentration in mother plant kernels. The lowest concentration of gallic acid, hespiridin and kaempferol where obtained in treatment containing phenylalanine 200 mg/L and the lowest concentrations of dhurrin and protocatechuic acid were obtained in treatment containing silver nitrate 100 mg/L. All of the

treatments tested had positive effect in increasing biosynthesis of hesperidin compared to control callus sample except treatment containing silver nitrate 100 mg/L where hesperidin was undetectable. Increase in phenolic content is an adaptive response, acting as a screen that provides protection against UV-B penetration in leaf tissues and as antioxidants protecting cells from reactive oxygen species (Rozemaand Bjorn,2002). Phenolic compounds of plants have been generally reported to be responsiblefor different biological properties. Hence, enhancement in the level of phenolics in UV- B-exposed plants plays a significant role in plant functions. The activation of the genes of phenyl propanoid pathway producing phenolic compounds is a common response to UV-B stress in plants. These phenolic compounds are produced from aromatic amino acids (phenylalanine and tyrosine) via the phenyl propanoid pathway. These compounds can mitigate the UV-induced damage by protecting the photosyntheticpathway and cellular components. Increase in concentrations of UV-B absorbing compounds including phenolic acid and flavonoids are the most consistent response of plants to UV-B supplementation. Increased amounts of UV protective compounds have been commonly shown in the literature of Santos *et al*., [27], while stimulation in leaf respiration has previously been observed [28] but not discussed [29]. But now it is hypothesized that a stimulation of leaf respiration represents increased resource demands for protection and repair (cuticular thickening, flavonoid biosynthesis and photoreactivation) [30]**.** According to Günter *et al*. [31], the irradiation of *Silene vulgaris* callus with UV-B and UB-C, the antioxidant activity of the silenan and the relative content of phenolic compounds in it, increased, the highest increase was observed after irradiation of callus by UV-B. The increased production of secondary metabolites after the silver ions elicitation was demonstrated also at other medicinal plant [32].

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