

BIOSYNTHETICAL CAPACITY OF KAEMPFEROL FROM *IN VITRO* PRODUCED ARGEL (*SOLENOSTEMMA ARGEL* (DEL.) HAYNE) CALLUS

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ABSTRACT

Solenostemma argel (Del.) Hayne (family Asclepiadaceae) is an Egyptian natural perennial shrub. The study attempted to establish callus from *S. argel* and to investigate its biosynthetic potentiality to produce the flavonoid kaempferol, which has a wide range of pharmacological activities. Seeds and aerial parts were collected from naturally grown plants at Saint Catherine, Sinai. Callus from different explants of *in vitro* germinated seedlings (stem, leaf and root) was successfully initiated on Murashige and Skoog (MS) medium supplemented with each of 2,4-dichlorophenoxyacetic acid (2,4-D) and β -naphthalene acetic acid (NAA), separately, at concentrations of 1.0, 2.0 and 3.0 mg/L, in addition to 0.5 mg/L kinetin (KIN). However, the fresh weights of leaf-derived callus were the highest. Casein hydrolysate (CH) and yeast extract (YE), as elicitors, at concentrations of 0.5, 1.0 and 2.0 g/L, were examined for elevating the *in vitro* production of kaempferol in callus cultures. The total methanol extracts of the aerial parts of wild plants, four-week-old *in vitro* germinated seedlings' explants and their derived callus were analyzed qualitatively and quantitatively for kaempferol, using HPLC. A significant difference in the content of kaempferol was observed. Callus produced from tested explants showed a capacity for production and accumulation of kaempferol. The highest concentration of kaempferol was in callus obtained from seedlings' stem explants on MS medium containing 1.0 mg/L 2,4-D and 0.5 mg/L KIN, in addition to 2.0 mg/L YE. In conclusion, *S. argel* callus cultures showed a capacity for synthesizing kaempferol and could be an alternative renewable natural source for its large-scale production.

Keywords: Asclepiadaceae, *in vitro*, flavonoid, elicitor, casein hydrolysate, yeast extract

INTRODUCTION

Medicinal plants are the most exclusive natural source of life saving drugs for the majority of the world's population. Bioactive compounds currently extracted from plants are used as pharmaceuticals, food additives, pigments, dyes, insecticides, cosmetics, perfumes and fine chemicals, but unfortunately many are directed to extinction and should be conserved.

Solenostemma argel (Del.) Hayne (family Asclepiadaceae) is an Egyptian species, known as "Argal" or "Harghel" (El-Hadidi and Fayed, 1994), commonly growing in the Eastern Desert and along the Nile in South Egypt (Täckholm, 1974). It is a wild perennial shrub, with white flowers in auxiliary umbels, fruits ovate green-purple. *S. argel* leaves are used in Egyptian folk medicine as purgative, antipyretic, expectorant and as a remedy for

bronchitis, for gastrointestinal cramps, stomach ache, colic, cold and urinary tract infections (Hegazi *et al.*, 1994). In addition, this plant is used in herbal mixtures for the treatment of viral hepatitis B and C, as an immunostimulant and for hypercholesterolemia (Shawkat, 1997). Hegazi *et al.* (2006) investigated the hepatotoxic and nephrotoxic effect of *S. argel*. The plant also showed significant anti-cancer and anti-oxidant activities (Nassr-Allah *et al.*, 2009 and Hanafi and Mansour, 2011)

Phytochemical investigations on *S. argel* have led to the isolation of compounds belonging to several classes of secondary metabolites, such as flavonoid glycosides (El-Askary, 2003 and Heneidak *et al.*, 2006), monoterpenes (Kamel *et al.*, 2000 and El-Askary, 2003), acylated phenolic glycosides (Kamel, 2003), pregnane and secopregnane glycosides

(Hamed, 2001; Hassan *et al.*, 2001; El-Askary, 2003; Plaza *et al.*, 2003, 2004, 2005a & b and Perrone *et al.*, 2006]. The pharmacological studies on *S. argel* evidenced spasmolytic and anaesthetic (El-Tahir *et al.*, 2005), fungitoxic (Abd El-Hady and Ouf, 1993 and Abd El-Hady *et al.*, 1994a & b) and antimicrobial activity against selected gram-positive and gram-negative bacteria (Hegazi *et al.*, 1994 and Tharib *et al.*, 1986). Topical anti-inflammatory activity of extracts of *S. argel* leaves were also reported (Innocenti *et al.*, 2005). Pregnane and secopregnane glycosides isolated from this species have shown interesting antiproliferative activity (Plaza *et al.*, 2005a & b). Fifteen new 14,15-secopregnane glycosides, namely argelosides A-J, and nine new 15-ketopregnane glycosides, namely stemmosides C-K, have been isolated from the pericarps, hairy seeds and leaves (Perrone *et al.*, 2008).

Kaempferol, kaempferol monoglycoside, and its derivatives, isolated along with other flavonoids from a preparation of the *S. argel*, were found to have a potent spasmolytic activity (Khalid *et al.*, 1992). Kaempferol derivatives inhibited oedema by 30-40%, these values were quite comparable to the activity of indomethacin (59% oedema reduction). This confirms and justifies the topical anti-inflammatory activity of this species in Egyptian folk medicine (Innocenti *et al.*, 2005). Also, Shafek *et al.* (2012) investigated the antibacterial and antioxidant activities of two new kaempferol glycosides isolated from *S. argel* stem extract.

Kaempferol is a natural flavonoid that has been isolated from many plant sources. It is a yellow crystalline solid with a melting point 267-278 °C. It is a strong antioxidant compound and helps to prevent oxidative damage of pure cells, lipids and DNA. It prevents arteriosclerosis by inhibiting the oxidation of low density lipoprotein and the formation of platelet in the blood; concomitantly it also inhibits the formation of cancer cell, thus meaning a reduced risk of developing several disorders such as cardiovascular diseases and cancer (Konam *et*

al., 2012). Numerous preclinical studies have shown that kaempferol and some of its glycosides have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, anti-osteoporotic, estrogenic/antiestrogenic, anxiolytic, analgesic and antiallergic activities (Calderon-Montaña *et al.*, 2011). Kaempferol consumption is correlated with a reduced lung cancer incidence (Irwin, 2008). It may be also a potent prophylactic against NOX-mediated neurodegeneration (Jang *et al.*, 2011).

In general, plants produce very small quantities of secondary metabolites and their production may only occur under certain circumstances. However, *in vitro* culture techniques could be the alternatives for the mass production of these metabolites, which is not season dependent. The principle advantage of this technology is that it provides a continuous, reliable source of pure and natural plant pharmaceuticals on a large scale, where plant cell cultures give metabolites that are easily extracted (Abraham *et al.*, 2011).

In plant tissue culture, accumulation of secondary metabolites could be enhanced by the treatment of various kinds of elicitors, which can be biotic and abiotic. Elicitors could be applied to unorganized plant cell and callus cultures for the production of specific compounds. They control the metabolism of receptive plant cells. Previous studies showed that the accumulation of different secondary metabolites can be efficiently induced by using elicitor (Ekiert and Gomolka, 2000 and Ekiert, 2001). Casein hydrolysate (CH) and yeast extract (YE) are well known as complex mixtures of organic compounds that have been used in plant tissue culture due to their ability to induce and promote secondary metabolism (Marsik *et al.*, 2014).

Callus cultures have been carried out in several plants for the production of flavonoids (Bharati and Bansal, 2014). However, few studies were found on the *in vitro* production of active compounds from *S. argel*. Amariei *et*

al. (1991) tested the biosynthetic capacity of the active principles of *in vitro* regenerated *S. argel* callus and shoots and found that both the callus and shoots showed a capacity for synthesizing some medicinal compounds. Also, El-Tigani and Ahmed (2009) showed that *in vitro* cultured tissues of *S. argel* are capable to produce and accumulate alkaloids, cardinolides and flavonoids. The present study aimed to produce kaempferol through *in vitro* callus cultures of *S. argel*, and to determine its yield in the callus tissue. CH and YE as elicitors were examined for the enhancement of kaempferol production in callus cultures of *S. argel*.

MATERIALS AND METHODS

Plant Material and Culture Conditions

S. argel seeds were collected from mature plants grown naturally in Saint Catherine, Sinai. Seeds were washed under running tap water for 30 min. Surface sterilization of seeds was carried out in a transfer hood by soaking them in a 50% (v/v) Clorox bleach solution (2.5% sodium hypochlorite) for 30 min, providing gentle agitation, followed by three sequential rinses for one min in sterilized distilled water. All seeds were placed directly on the surface of full-strength Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium (Duchefa, Haarlem, the Netherlands) supplemented with 100 mg/L myo-inositol and 30 g/L sucrose. The pH of the medium was adjusted to 5.7-5.8 before being solidified with 3.0 g/L phytigel (Duchefa, Haarlem, the Netherlands). Media were dispensed in large jars capped with autoclavable polypropylene lids, then autoclaved at a pressure of 1.06 kg/cm² and 121°C for 15 min. The cultures were incubated at approximately 25±2°C with a 16-h photoperiod under cool white fluorescent tubes (F140t9d/38, Toshiba). Four-week-old *in vitro* germinated seedlings of *S. argel* were used as sources of explants for initiation of callus cultures.

Callus Initiation and Proliferation

Leaves, stems and roots were excised from the seedlings of *S. argel* and cut into small pieces (0.5-1 cm), then cultured aseptically on solid MS medium supplemented with various

plant growth regulators (Sigma Cell Culture, min. 90%, St. Louis, USA). Naphthalene acetic acid (NAA) or dichlorophenoxy acetic acid (2,4-D), at concentrations of 1.0, 2.0 and 3.0 mg/L, as auxins, and kinetin (KIN), at a concentration of 0.5 mg/L, as a cytokinin, were used for callus initiation, in addition to MS medium without growth regulators. Callus tissues were transferred to freshly prepared medium for proliferation. Each treatment contained at least 10 replicates and was repeated twice. Percentage of callus initiation, fresh weight of callus (g) and callus color and texture were recorded after eight weeks of culture.

Elicitation

Two elicitors; CH (OXOID, OXOID Limited, Hampshire, England) and YE (Sigma Aldrich) at concentrations of 0.5, 1.0 and 2.0 g/L, were added to the most favorable callus initiation medium for each type of explants, in order to improve the yield of kaempferol in the callus cultures. The control medium was made without elicitors. Growth of the callus tissue (g fresh weight/jar) and kaempferol content (ng/g fresh weight) were measured. Callus tissues were oven dried at 35°C for 48 h for the quantification of kaempferol.

Extraction and Determination of Kaempferol

Kaempferol was extracted from dry powdered samples, of callus, tissue of *S. argel* seedling's explants (stem, leaf and root) and the aerial parts of wild plant. Extraction was carried out with methanol (HPLC grade), till exhaustion. The filtered extract was dried under vacuum, and a known weight of each of the dried extracts was diluted with methanol (1:1 w/v), into a round bottom flask, sonicated for five min. Four ml of conc. HCl (HPLC grade) was added and refluxed for 2.5 h, cooled to room temp. A known quantity was transferred to a volumetric flask and completed to a known volume with HPLC H₂O, then filtered over a 0.45 µm syringe.

A standard solution of kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one; Sigma Aldrich) was prepared by dissolving kaempferol in acetonitrile (2.0 mg in 10 ml), sonicated for

five min and filtered over a 0.45 µm syringe. Standard solutions were prepared in the range of 50-2000 ng/ml by serial dilution method. Presence and content of kaempferol was determined in the different methanolic extracts by RP HPLC column (phenomenex, ODS-3,5 micron; 4.6x250) using; mobile phase 50% acetonitrile : water, 50 µl injection volume, wave length of 365 nm, 1.5 ml/min flow rate, 40°C column temp, running time 4.5 min and PDA photodiode array detector. The retention time (R_t = 3.4 min) and online UV spectra of kaempferol was compared to those of the different samples.

Statistical Analysis

Experiments were subjected to the completely randomized design. Variance analysis of data was carried out using ANOVA for statistical analysis. The differences among means for all treatments were tested for significance at 5% level, by using Duncan's multiple range test (Duncan, 1955), as described by Snedecor and Cochran (1990). Means followed by the same letter are not significantly different.

RESULTS AND DISCUSSION

Callus Initiation

Hundred percent of explants of *S. argel* produced callus on media containing all the growth regulators' combinations examined in the present study. As presented in Table 1, comparing the effect of different treatments on each explant type, the maximum callus fresh weight from seedlings' stem explants was obtained on MS medium containing 1.0 mg/L 2,4-D and 0.5 mg/L KIN (Figure 1a), while, seedlings' leaf explants initiated callus of the highest biomass on MS medium containing 3.0 mg/L 2,4-D and 0.5 mg/L KIN (Figure 1b). However, seedlings' root explants produced the maximum biomass of callus on MS medium containing 2.0 mg/L NAA and 0.5 mg/L KIN (Figure 1c). Comparing between explants, it was found that the ability of seedlings' leaf explants to produce and proliferate callus was higher than that of the other tested explants (Figure 1d), followed by the seedlings' root tissue. Moreover, 2,4-D was found to stimulate callus formation from

both seedlings' stem and leaf explants, while seedlings' root explants responded better in the presence of NAA. The results show also that callus on the most favourable medium for each explant had a yellowish green color and a friable nodular desirable appearance.

In general, all explants had the capability to initiate callus, and this callus successfully proliferated on the same medium, which is promising for the production of high yield of tissue and consequently secondary metabolites. The capability to cultivate plant callus cells and produce homogeneity of an *in vitro* cell population, with large availability of material, and high rate of cell growth provide a valuable platform for the production of high-value secondary metabolites and other substances of commercial interest (Moscatiello *et al.*, 2013).

Effect of Elicitors on Callus Growth and Kaempferol Content

Two elicitors; CH and YE were added, separately, to the culture medium and their influence on the growth of callus cultures of *S. argel* and kaempferol content was studied (Table 2). Generally, CH negatively affect callus biomass and had non-significant effect on kaempferol accumulation, compared to the control, except the concentration of 0.5 mg/L, it increased the kaempferol production in callus derived from seedlings' root to 276.050 ng/g dry weight, but this amount decreased with the increase of CH concentration. Treatment with YE also resulted in a lower callus biomass, compared to the control, for both callus derived from seedlings' stem and root, but it gave higher biomass of callus derived from seedlings' leaf over the control at concentrations of 0.5 and 1.0 mg/L. The results in Table 2 show that there were significant differences in the content of kaempferol between the induced callus and the wild and seedlings' explants. The highest content of kaempferol was in callus derived from seedlings' stem on medium containing 2.0 mg/L YE (624.4356 ng/g dry weight) with a significant difference with all the other treatments, it enhanced kaempferol content to about 13-fold over the control. Followed by

Table 1. Callus initiation from seedlings' explants of *S. argel* on MS medium containing 0.5 mg/L KIN in addition to different concentrations of 2,4-D or NAA (5 explants/jar) after 8 weeks of culture with 100% callus initiation on all tested growth regulators' combinations.

			Seedlings' stem derived callus			Seedlings' leaf derived callus			Seedlings' root derived callus		
2,4-D (mg/L)	NAA (mg/L)	KIN (mg/L)	Fresh weight of callus (g/jar)	Colour of callus	Shape	Fresh weight of callus (g/jar)	Colour of callus	Shape	Fresh weight of callus (g/jar)	Colour of callus	Shape
0.0	0.0	0.0	-	-	-	-	-	-	-	-	-
1.0	0.0	0.5	1.633 ^a	Yellowish white	Friable nodular	1.623 ^b	Yellowish green	Friable nodular	0.847 ^d	Whitish creamy	Compact nodular
2.0	0.0	0.5	0.993 ^b	Yellowish creamy	Friable nodular	1.443 ^b	Yellowish green	Friable nodular	2.797 ^c	Whitish creamy	Compact nodular
3.0	0.0	0.5	1.527 ^{ab}	Creamy	Friable nodular	9.03 ^a	Yellowish green	Friable nodular	2.300 ^c	Whitish creamy	Compact nodular
0.0	1.0	0.5	1.433 ^{ab}	Whitish green	Compact nodular	2.723 ^b	Green	Compact nodular	3.743 ^b	Yellowish green	Compact nodular
0.0	2.0	0.5	1.230 ^{ad}	Whitish green	Compact nodular	1.577 ^b	Whitish creamy	Compact nodular	4.920 ^a	Yellowish green	Friable nodular
0.0	3.0	0.5	1.3633 ^{ab}	Green	Compact nodular	2.643 ^b	Whitish green	Compact nodular	0.847 ^d	Creamy	Friable nodular

its content in the callus derived from the same explants treated with 0.5 g/L YE (307.211 ng/g dry weight). However, the content of kaempferol was the highest in the wild plants (11885.125 ng/g dry weight) than in the seedlings' explants and in the callus. In general, the highest contents of kaempferol were in callus treated with YE. On the other hand, CH had the lowest effect on kaempferol accumulation, except for callus derived from seedlings' root at the concentration of 0.5 mg/L. It is noticed that there is a reverse correlation between cell growth and kaempferol production. This represents a general

complication found in *in vitro* secondary metabolites production (Maharik *et al.*, 2009). Difference in contents of kaempferol between callus cultures and wild and seedlings' explants could be explained by the influence of overlapping growth regulators depending on the type and concentration of the used auxin and cytokinin. They could increase or decrease the amount of accumulated compound, due to the fact that growth regulators increase the absorption of minerals and accumulation of carbohydrates and amino acids (Besher *et al.*, 2014).

Table 2. Fresh weight and kaempferol concentration in callus of *S. argel* after 8 weeks of culture on MS medium supplemented with the best callus induction medium for each explant type in addition to different concentrations of CH and YE as elicitors (5 explants/jar).

Explant type	CH (g/L)	YE (g/L)	Fresh weight (g/jar)	Kaempferol (ng/g dry wt.)
Callus derived from seedlings' stem ¹	0.0	0.0	3.593 ^b	48.322 ^{ef}
	0.5	0.0	0.758 ^k	11.561 ^f
	1.0	0.0	1.055 ^{ij}	55.758 ^{ef}
	2.0	0.0	1.010 ^{ijk}	5.830 ^f
	0.0	0.5	1.190 ^{hi}	307.211 ^b
	0.0	1.0	1.705 ^{ef}	55.416 ^{ef}
	0.0	2.0	1.623 ^{efg}	624.435 ^a
Callus derived from seedlings' leaf ²	0.0	0.0	1.880 ^e	45.426 ^{ef}
	0.5	0.0	1.220 ^{hi}	47.814 ^{ef}
	1.0	0.0	1.520 ^{fg}	20.775 ^f
	2.0	0.0	1.215 ^{hi}	47.896 ^{ef}
	0.0	0.5	2.150 ^d	15.065 ^f
	0.0	1.0	2.750 ^c	62.354 ^{ef}
	0.0	2.0	1.820 ^e	102.946 ^{de}
Callus derived from seedlings' root ³	0.0	0.0	4.055 ^a	120.820 ^d
	0.5	0.0	0.875 ^{jk}	276.050 ^b
	1.0	0.0	2.210 ^d	7.143 ^f
	2.0	0.0	1.390 ^{gh}	57.700 ^{ef}
	0.0	0.5	0.812 ^{jk}	Not detected
	0.0	1.0	2.585 ^c	169.060 ^c
	0.0	2.0	2.715 ^c	130.245 ^d
Seedlings' stem				8790.520
Seedlings' leaf				4002.226
Seedlings' root				2061.622
Wild plant				11885.125

¹Callus derived from seedlings' stem cultured on MS medium+1.0 mg/L 2,4-D+0.5 mg/L kinetin supplemented with elicitors.

²Callus derived from seedlings' leaf cultured on MS medium+3.0 mg/L 2,4-D+0.5 mg/L kinetin supplemented with elicitors.

³Callus derived from seedlings' root cultured on MS medium+2.0 mg/L NAA+0.5 mg/L kinetin supplemented with elicitors.

Although, CH did not enhance kaempferol production in the present study, it is well known that CH is a source of calcium, several micro-nutrients, vitamins and a mixture of up to 18 amino acids. It was

investigated that CH contains some unknown growth promoting factors (Khaleda and Al-Forkan, 2006). Thus, the response of callus to a particular elicitor may vary between different plants and cell lines, therefore, it becomes crucial to determine the suitable type and concentration of elicitors for product optimization.

Currently, YE is commonly employed as a biotic elicitor for the induction and enhancement of secondary metabolites production. Reports suggest that YE is used as a supplement in order to promote plant growth, due to its high amino acid content (George *et al.*, 2008). However, YE did not affect the fresh biomass of callus, the addition of YE into the culture medium increased kaempferol. This indicates that YE did not perform as nutrient supplement for the growth of callus. This might be the result of stress response of the callus to the accumulation of secondary metabolites due to the YE that acted as elicitor (Abraham *et al.*, 2011).

Generally, elicitors could be used to enhance plant secondary-metabolite synthesis and could play an important role in biosynthetic

pathways. Elicitation is one of the important strategies to get better productivity of bioactive secondary products and to lower production costs. The secondary metabolites are released due to defense responses, which are triggered and activated by elicitors (Bharati and Bansal, 2014).

In conclusion, medicinal plants are the main source of secondary metabolites, which are industrially important as they constitute pharmaceutically important drugs. As a result, there is a huge demand for these plants, which are overexploited from their natural habitat. Therefore, plant tissue culture is being potentially used as an alternative strategy for production of secondary metabolites. Flavonoids are important secondary metabolites having immense medicinal properties ranging from anti-oxidant, anti-cancerous, anti-inflammatory etc. To enhance the rapid and controlled, continuous production of this class of compounds, *in vitro* culture strategies application would ensure large scale and stable production throughout the year, under controlled environmental conditions. The present study is a stepping stone in *in vitro* production of the flavonoid kaempferol from callus cultures of *S. argel*. Callus showed a potentiality for accumulation of kaempferol, especially from seedlings' stem explants.

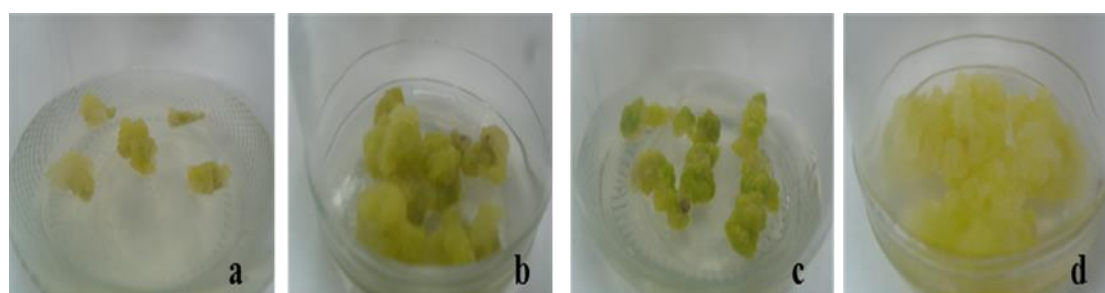


Figure 1. Callus of *S. argel*. (a) Callus derived from seedlings' stem cultured on MS medium+1.0 mg/L 2,4-D+0.5 mg/L kinetin. (b) Callus derived from seedlings' leaf on MS medium+3.0 mg/L 2,4-D+0.5 mg/L kinetin. (c) Callus derived from seedlings' root on MS medium+2.0 mg/L NAA+0.5 mg/L kinetin. (d) Proliferated callus of *S. argel*.

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