

# Callus inductions and Phytochemical Determination of Artichoke (*Cynara scolymus*) growing in Iraq

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## ABSTRACT

The artichoke (*Cynara scolymus*) belongs to Asteraceae family. It is included in British and European Pharmacopeia and has long-used in traditional medicine in several countries. The plant primarily grows in North Africa and Mediterranean; in addition, it is cultivated around the world. Artichoke is widely distributed in Iraq at the watery lines and boundary of the field. The aim of study was to induce callus from leaves as explants, and to compare between contents of total phenolic and total flavonoids that extracted from the induced callus and from leaves. The induced callus was maintained on Murashige and Skoog (MS) medium supplemented with Naphthalene Acetic Acid (NAA) and Benzyl Adenine (BA). The highest percentage of callus (100%) was induced from the combination (5mg/l NAA plus 2 mg/l BA), followed by (86.67%) with the combination (3mg/l NAA plus 1mg/l BA). Two types of callus (friable and compact) were induced. The extract of friable callus produced 0.858 mg/g of total phenolic, while leaves extract produced 167.24 µg/ mg of total flavonoids. Auxins and cytokinins that added together in the culture medium seemed to be necessary for artichoke tissue culture.

## INTRODUCTION:

Medicinal plants have played an important roles in the treatment of diseases all over the world (1). They are important sources of medicine for thousands of years and are important for life saving of population around the world (2). Medicinal plants considered as important source of phytochemicals, which are synthesized from the secondary metabolisms and called secondary metabolites. These secondary metabolites are produced in valuable medicinal herbs with different pharmacological activity (3).

Artichokes *C. scolymus* that belongs to Asteraceae family are nutritional and medicinal plants; they were recognized by the Greeks, Romans, and ancient Egyptians (4). Artichokes used as edible material and medical purposes for its content of the polyphenolic constituent (5). *C. scolymus* is a small genus that includes 12 species spreading in the Mediterranean region. It is spiny perennial plant with large leaves lobed pinnatipartite spiny; heads flowers eaten and have medicinal uses (6). It is grown for its tender, eaten immature flower buds (7,8).

Callus cultures generally are induced from any explant structure such as leaves, stems, and roots that are kept on culture media containing a high level of

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auxin such as (NAA, IAA) and low level of cytokinin (BAP,BA). Callus initiation depends on concentration and type of growth regulators, nutrients, and vitamins in plant tissue culture media. Furthermore, response of explants on culture media depends also on the exposure to several lighting periods(9).

*C. scolymus* is a rich source of naturalist antioxidant such as vitamin C, flavones, and hydroxycinnamic acids (10). There was a considerable quantity of polyphenols in the fresh extract of *C. scolymus* that showed antioxidant activity (11). Plants are rich source of phenolic compounds, vitamins, carotenoids and terpenoids; these compounds have a potential antioxidant (12). Because *C. scolymus* leaves have a high level of phenolic compounds, it displays pharmacological activities such as antimicrobial and antioxidant (13). Artichoke extract contained high level of phenolic, flavonoid, and tannin contents and the strongest antioxidants activities including DPPH (94.23%) (14).

In the present investigation, friable and compact callus was induced from *C. scolymus*; the produced callus was compared with leaves regarding phytochemical contents including total phenolic and flavonoids.

## **MATERIALS AND METHODS:**

### **Plant material:**

Artichoke seeds were obtained from College of Pharmacy- University of Baghdad. During October 2016, the seeds were germinated in pots and grown in the green house; the plants were also grown in a private field at Al-Ramadi city. The leaves were collected during March and April 2017.

### **Preparation Of Culture Medium For Callus Induction:**

To prepare 1000ml MS medium, 34.1g of the ready made powder was dissolved in 800ml distilled water, then growth regulators and 3% sucrose were added. The pH was adjusted to 5.7-5.8 using 1N NaOH or 1N HCl, and the volume was adjusted to 1000ml. Eight g/l of agar was added to stiffen the medium. For callus induction, MS medium was supplemented with all the possible combinations for different concentrations 0.0, 1.0, 3.0 or 5.0 mg/l of naphthalin acetic acid (NAA), and different concentrations 0.0, 0.2, 0.5 or 1.0 mg/l of benzyl adenin (BA). The medium components were kept on a hotplate with magnetic stirrer close to boiling. The culture media were autoclaved at 121°C, 1.04 kg/cm<sup>2</sup> pressure for 15 min; then 10 ml were poured into glass vial, and placed at room temperature to cool and be ready for using (15).

### **Explants Sterilization:**

Leaf explants washed with tap water and surface sterilized with 70% (v/v) ethanol for 10 seconds. After rinsing three times with sterile DH<sub>2</sub>O, the explants were dipped in 0.5, 1.0, 1.5 or 2% V/V of sodium hypochlorite solution containing 6.25% Cl<sub>2</sub> at three periods 5, 10 or 15 min for each concentration, and then rinsed three times with sterile DH<sub>2</sub>O. The sterilization was conducted inside the laminar air flow cabinet (16).

### **Preparation Of Methanolic Leaves And Callus Extract :**

The methanolic extract of leaves and callus were prepared by moistening 50 g of dried powdered samples in 250 ml of 80% methanol for 24h; the extract was filtered by using filter paper. The filtrate

was subjected to evaporation using a rotary evaporator in order to remove the solvent. The extract was used for phytochemicals screening (17).

### Determination Of Total Phenolic Content Of Leaves And Callus Extract:

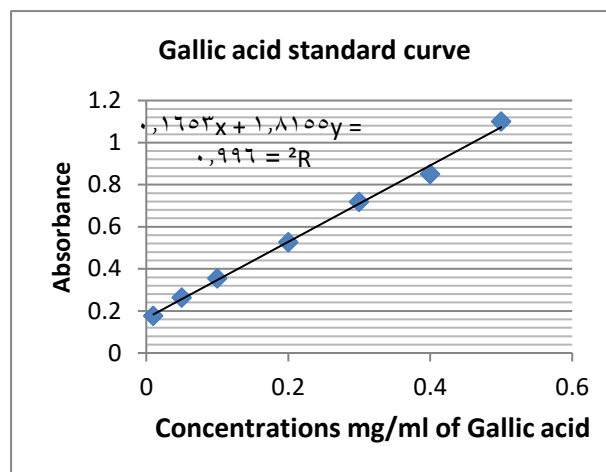
The total phenolic content of *C. scolymus* quantity in leaves and callus extracts was determined by the Folin-Ciocaliteu reagent. The standard of Gallic acid was used and the total phenolic was expressed as mg/g gallic acid equivalents (GAE). For this purpose, one ml of standard solution for the following concentrations of gallic acid (0.01, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) were prepared in methanol (Table 1), and the calibration curve of gallic acid was drawn (Figure 1).

Ten mg of each leaves extract, compact callus, and friable callus was solved in 1ml of methanol to product aw/v of 10 mg/ml. 50 µl aliquot was mixed with 1.25 ml of Folin–Ciocaliteu reagent (diluted 1:10 fold) and 1 ml of solution 7.5% Na<sub>2</sub>CO<sub>3</sub>. After 30min at room temperature, the absorbance was measurement by spectrophotometer a λ=765 nm (18).

**Table 1.** Concentrations of Gallic acid and absorbance at 765nm for determination of total phenolic content

Concentration Gallic acid mg/ml	Absorbance (Mean) λmax = 765nm
0.01	0.176
0.05	0.264
0.1	0.355
0.2	0.526
0.3	0.718
0.4	0.850
0.5	1.100

Replications = 3



**Figure 1:** Standard Curve of Gallic Acid

### Determination of the total flavonoid of leaves and callus extract:

In this method, Rutin was used as a standard. One ml of each standard solutions 5, 10, 30, 40 µg/ml or methanolic extract were taken into 10ml volumetric flask containing 4ml of distilling water; then 0.3ml of 5% NaNO<sub>2</sub> was added to the flask (Table 2). After 5min, 0.3ml (10% AlCl<sub>3</sub>) was added to the mixture. At the 6th min, 2ml of (1N NaOH) was added and the volume complete to 10ml with distills water. The absorbance was measured at 510nm using UV-Visible speictrrophotometer (19). The calibration curve of rutin was drawn (Figure 2), and the flavonoid contents were measured as rutin equivalent.

**Table 2.** Concentrations of standard (Rutin) and absorbance at 510nm for determination of total flavonoid content

Concentration Rutin µg/ml	Absorbance (Mean) λmax = 510nm
5	0.053
10	0.061
20	0.093
30	0.110
40	0.141

Replications =3

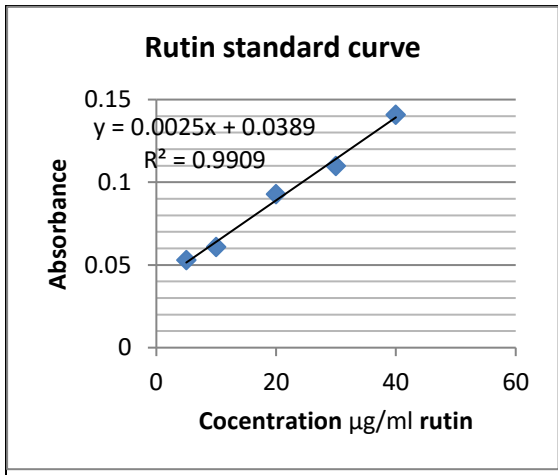


Figure 2: Standard Curve of Rutin

**Results and Discussion:**

**Surface Sterilization of Explants:**

Sodium hypochlorite (6.25% NaOCl), 70% ethanol, and distilled water were applied to sterilize the *Cynara scolymus* explants parts that used for plant tissue culture. The main solution of the sterilization was NaOCl, where the concentrations and durations were changed to obtain parts free of bacterial and fungal infections and the tissues are able to continue growing and producing callus. The percentage of survival using 0.5% NaOCl for 5, 10, and 15 minutes was 81.25, 68.75, and 56.25, respectively; while the percentage of contamination was 18.75, 12.5, and 12.5, respectively. The results of the concentrations and durations are presented in (Table 3). As the NaOCl concentration was increased, the percentage of contamination decreased, but the rate of explants death increased. For example, when applying 0.5% NaOCl for 5 min, the contamination was 18.75% and the plant death was 0.0%. On the other hand, using 2% NaOCl for 15 min, the contamination was 0.0% and the plant death was 93.75 that resulting 6.25% survival only. The best concentration of NaOCl was 1% for 10 min as the highest survival was 93.75%, while the lowest survival (6.25%) produced from 2% concentration for 15 min.

For sterilizing buds of artichoke, Brutti *et al.*(20) used 5g/l mercury dichloride for 5min and 1g/l sodium hypochlorite for 10 min. Shawky and Aly(21) used 70% ethanol for 5 seconds followed by 1.25% NaOCl for 20 min to sterilize shoot tips explants that excised from selected globe artichoke plants. This was the most effective sterilizing and disinfectant treatment for surviving the majority of meristem tip after 5 weeks of culturing.

The importance of NaOCl was reported by Firoz *et al.*(22) who mentioned that increasing the surface sterilization period and the concentration cause increase in survival rate. It has also been reported that when NaOCl diluted with water, the hypochlorite salts lead to formation of Hypochlorous acid (HClO), which is negatively correlated with bactericidal activity, perhaps in part due to lethal DNA damage (23). HClO causes dissolvent of organic tissue, because of chlorine that combined with protein (amino acid ) (24).

**Table 3.** Survival % of *Cynara scolymus* leaf explants after sterilization with different concentration of NaOCl and three time durations

NaOCl %	Time (min) duration	Survival		Contamination		Death		Total No. of explants
		No.	%	No.	%	No.	%	
0.5	5	13	81.25	3	18.75	0	0.00	16
	10	11	68.75	2	12.50	3	18.75	16
	15	9	56.25	2	12.50	5	31.25	16

1.0	5	12	75.00	3	18.75	1	6.25	16
	10	15	93.75	0	0.00	1	6.25	16
	15	10	62.50	0	0.00	6	37.50	16
1.5	5	12	75.00	0	0.00	4	25.00	16
	10	8	50.00	0	0.00	8	50.00	16
	15	7	43.75	0	0.00	9	56.25	16
2.0	5	10	62.50	0	0.00	6	37.50	16
	10	5	31.25	0	0.00	11	68.75	16
	15	1	6.25	0	0.00	15	93.75	16

## Callus Induction Culture

For callus induction, leaves were used as explants to obtain callus from globe artichoke. The auxin, Naphthaleneacetic acid (NAA), and the cytokinin, Benzyladenine (BA) were used in different combinations. The data showed statistically significant differences among the means (Table 4). Callus induction culture was calculated according to the following equation:

$$\text{Callus induction culture \%} = \frac{\text{Number of explant produced callus}}{\text{Total number of explants cultured}} \times 100\% \quad (25)$$

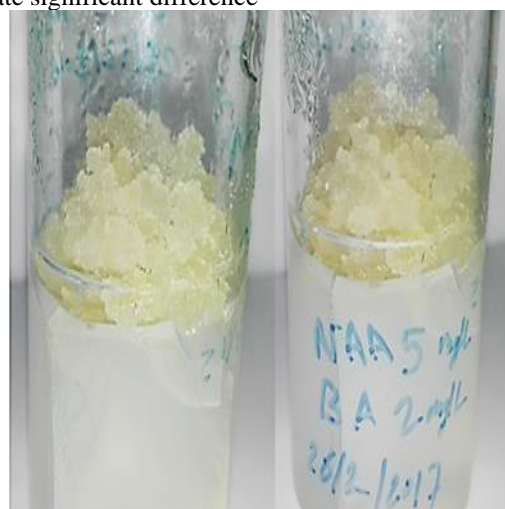
The combination (NAA 5.0 mg/l + BA 2.0 mg/l) produced 100% response for callus induction that appeared as friable after 4 weeks in culture (Figure 3); while the combination (NAA 3.0 mg/l + BA 1.0 mg/l) produced 86.67% friable callus (Figure 4).

**Table 4.** Effect of various concentrations of NAA, BA, and their combinations on the response of callus induction from *Cynara scolymus*

Mean (BA)	Concentration of BA (mg/l)											
	0.0			1.0			3.0			5.0		
	Mean +SD	%	Mean (NAA)	Mean +SD	%	Mean (NAA)	Mean +SD	%	Mean (NAA)	Mean +SD	%	Mean (NAA)
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5.25±3.36 <sup>a</sup>	8.33±1.15 <sup>a</sup>	83.33	6.00±1.0 <sup>c</sup>	6.67±0.57 <sup>b</sup>	66.67	3.33±1.15 <sup>c</sup>	8.67±0.57 <sup>b</sup>	86.67	10.00±0.0 <sup>a</sup>	63.33	100	6.16±3.99 <sup>a</sup>
4.58±3.44 <sup>b</sup>	6.33±0.58 <sup>b</sup>	63.33	3.33±1.15 <sup>c</sup>	8.67±0.57 <sup>b</sup>	86.67	3.33	46.67	46.67	46.67	46.67	46.67	3.50±2.43 <sup>c</sup>
5.50±3.91 <sup>a</sup>	10.00±0.0 <sup>a</sup>	100	4.67±0.57 <sup>c</sup>	7.33±1.52 <sup>b</sup>	73.33	46.67	46.67	46.67	46.67	46.67	46.67	3.50±2.43 <sup>c</sup>
-	-	-	3.50±2.43 <sup>c</sup>	5.67±3.57 <sup>b</sup>	56.67	3.50±2.43 <sup>c</sup>	5.67±3.57 <sup>b</sup>	56.67	3.50±2.43 <sup>c</sup>	5.67±3.57 <sup>b</sup>	56.67	3.50±2.43 <sup>c</sup>

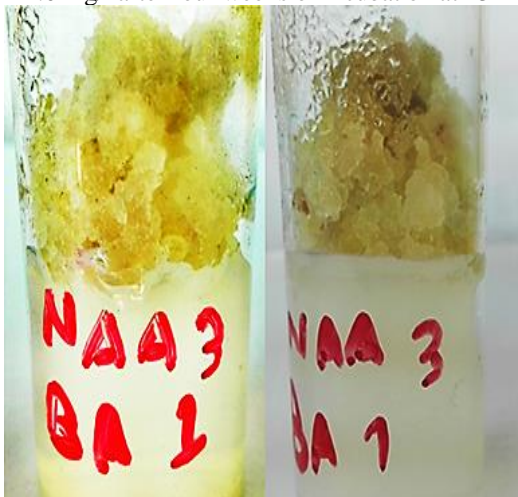
Replications =10

LSD (p≤0.05) = 0.617 Different letters within each row indicate significant difference



**Figure 3.** Callus induction of *Cynara scolymus* from leaf explants grown on MS medium containing NAA 5.0 mg/l +

BA 2.0 mg/l after four weeks of incubation at  $23\pm 1C^{\circ}$



**Figure 4.** Callus induction of *Cynara scolymus* from leaf explants grown on MS medium containing NAA 3.0 mg/l + BA 1.0 mg/l after four weeks of incubation at  $23\pm 1C^{\circ}$



**Figure 5.** Callus induction of *Cynara scolymus* from leaf explants grown on MS medium containing NAA 5.0 mg/l + BA 0.5 mg/l after 4 weeks of incubation at  $23\pm 1C^{\circ}$

Several researchers studied callus formation in Globe artichoke. Ordaset *et al.*, (26) mentioned successful callusing response from bracts globe artichoke using MS medium containing a combination of 5 mg/l NAA and 2 mg/l BAP. Bruttiet *et al.*, described a micropropagation protocol for *Cynara scolymus*; shoot apices were established on basic culture medium consisting of MS medium containing 3mg/l NAA and 4mg/l cyclodextrins. Leaf segments, taken from *in vitro* grown shoots were sub-cultured on MS medium contained 2 mg/l BA and 5 mg/l NAA according to (27) and (21). In another study, a protocol for *in vitro* multiplication of globe artichoke (*Cynara scolymus* L.) using auxiliary buds was established using medium

containing 1mg/l kinetin and 0.1 mg/l NAA after removal of apical buds, leaves and roots from seedlings (28). Leaf explants were cultured on MS medium, for callus induction added with 5 mg/l NAA, 2 mg/l Kin, and 0.1 mg/l GA3 (29). The results presented in Table 4 indicated that callus was induced only when using different combinations of the auxin NAA and the cytokinins BA. When only one of growth regulators was used in the treatment, the explants produced no callus.

Cell division appears to be regulated by the joint effect of auxins and cytokinins. Auxins seems to influence DNA replication, while cytokinins influence the control over the proceedings that leading to mitosis. In general, auxin is demanded for induction of callus from explants. Cells that responding to auxins start dedifferentiated state and begin to divide (30).

Lo Schiavoet *et al.*, (31) revealed that auxins cause DNA to shows more methylated than usual and indicated that this could be necessary for the reprogramming of differentiated cells. Cytokinins seem to be indispensable that for plant cell division of plant tissue. They proposed that cytokinins may be necessary to streamline the synthesis of proteins that involved in the function and structure of the mitotic spindle apparatus. Callus is produced when cytokinin and auxin are available together in the medium (32). Adding auxins and cytokinins together seem to be necessary for plant tissue culture, this explains why there is no callus induction when growth regulators are absent in the medium.

### **Determination of Total Phenolic Content of Leaves and Callus Extract**

The total phenolic content of compound in mg/gm in the methanol extract of leaves, friable, and

compact callus was determined using calibration curve ( $R^2= 0.996$ ), ( $y=1.8155x + 0.1653$ ). The equation was expressed as gallic acid equivalents (GAE)/dry weight of extract (DWE) in gm. The results indicated that total phenolic was  $0.858\pm0.008$  mg/gm in friable callus extract, and was  $0.518\pm0.007$  mg/gm in leaves extract, and was  $0.423\pm0.006$  mg/gm in compact callus extract; the differences were statistically significant (Table 5).

The results revealed high level of total phenolic in friable callus compared to the leaf and this is in agreement with Giri *et al.* (33) who indicated that the increasing of antioxidant activity in callus cultures could depends on the influence of various plant growth regulators with an accompanying increase of phenolics in callus. Phenolics are often produced in plant tissues exposed to stress and pathogen (34). It is believed that the type and period of lighting is one of the factors that stimulate the increase of phenolic production; photo inhibition and nutrient stresses are also trigger the synthesis of phenolic compounds (35).

#### Determination of Total Flavonoids Content of Leaves and Callus Extract:

The total flavonoids was determined in  $\mu\text{g}/\text{mg}$  in the methanol extract of leaves, friable callus, and compact callus content using a calibration curve ( $R^2= 0.990$ ) ( $y=0.0025x + 0.0389$ ) and expressed as Rutin equivalents (RE)  $\mu\text{g}/\text{mg}$  dry weight of extract (DWE). The results were confirmed by statistical analysis, and the differences showed statistically significant differences ( $p<0.05$ ). The results indicating high total flavonoids content  $167.24\pm11.47$   $\mu\text{g}/\text{mg}$  in leaves extract,  $124.3\pm18.64$   $\mu\text{g}/\text{mg}$  in compact callus extract, and  $76.57\pm7.61$   $\mu\text{g}/\text{mg}$  in friable callus extract (Table 5). The results are in agreement with (36) who indicated that flavonoids content of callus extract from

*Decalepis hamiltonii* was less than of plant extract. Other results on *Oroxylum indicum* (L.) indicated high flavonoids content in leaves; when the age of callus increase, the production of flavonoids also increase (37). The production of phytochemicals (phenols and flavonoids) in leaves were increased comparing to callus because the phytochemicals are accumulated in different parts of leaves (38).

**Table 5.** Dry weight of total phenol and flavanoid content of leaves, compact callus, and friable callus

Concentration 10 mg/ml	Total phenolic Gallic acid mg/g	Total flavonoid Rutin $\mu\text{g}/\text{mg}$
	Mean $\pm$ SD	Mean $\pm$ SD
Leaves	$0.518\pm0.007^b$	$167.24\pm11.47^a$
Compact callus	$0.423\pm0.006^c$	$124.3\pm18.64^b$
Friable callus	$0.858\pm0.008^a$	$76.57\pm7.61^c$
LSD	0.052	26.73

Replications=3

LSD  $p<0.05$ , different letters within each row indicate significant difference

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## انتاج الكالس وتحديد المركبات الكيميائية النباتية لنبات الخرشوف (*Cynarascolymus*) النامي في العراق

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### الخلاصة:

نبات الخرشوف (*Cynara Scolymus*) ينتمي إلى العائلة النجمية، وهو مسجل ضمن دستور الادوية البريطانية والأوروبية، ولقد تم استخدامه في الطب التقليدي في دول مختلفة. ينمو الخرشوف بشكل أساسي في شمال أفريقيا ومنطقة البحر الأبيض المتوسط بالإضافة إلى ذلك فهو يزرع في العديد من دول العالم. ينتشر الخرشوف في العراق على حواف قنوات الري والحقول الزراعية. كان الهدف هذه الدراسة هو إنتاج الكالس من الأوراق، وإجراء مقارنة بين الكالس والأوراق من خلال قياس محتواها من مركبات الفينول والفلافونويد. تم استحضات الأوراق على إنتاج الكالس بواسطة حفظ القطع الورقية في وسط Murashige and Skoog (MS) بعد إضافة Naphthalene Acetic Acid (NAA) و Benzyl Adenine (BA) إلى الوسط. كانت أعلى نسبة لإنتاج الكالس هي 100% باستخدام التراكيز 5mg/l NAA و 2 mg/l BA، وكانت النسبة إنتاج الكالس 86% باستخدام التراكيز 3mg/l NAA, 1mg/l BA. لقد تم إنتاج نوعين من الكالس وهي الكالس الهش والكالس الصلب. أنتج مستخلص الكالس الهش أعلى تركيز (0.858 ملجم/ غرام) من محتوى الفينول الكلي، وأنتج مستخلص الأوراق أعلى تركيز (167.24 ميكروغرام/ ملغم) من محتوى الفلافونويد الكلي. أظهرت الدراسة أنه من الضروري إضافة الأوكسينات والسيتوكينينات معاً في الوسط الزراعي للحث على إنتاج الكالس من الخرشوف.