Effects of Magnetic Field (M.F.) in Milk Thistle (*Silybum marianum* L.) Callus Cultures Induction and Accumulation of Silybin by Using (HPLC) Technique

Sabah A. Al-Badrani [(1)] and Rehab A. H. AL-Baker [(2)] *

(1). Qbat Al-Saskra School for Girls, General Directorate of Nineveh Education, Iraq.

(2). Dep. Of Environmental Sciences/ College of Environmental Sciences and Technologies/University of Mosul, Iraq.

(*Corresponding author: Rehab A. H. AL-Baker. E-Mail: rehsbio39@uomosul.edu.iq).

Received: 1/02/2022 Accepted: 14/10/2022

Abstract

The research was carried out in laboratories of the college science, University of Mosul between (2019-2020). The study aimed to detect the magnetic field (M.F.) activity in initiation and growth of *Silybum marianum* L. callus cultures from sterilized seedlings segments (roots, leaves, stems and nodes) which had been cultured on solid (MS) medium supplemented with (3.0)mg/L of Benzyl adenine (BA) and (5.0) mg/L of Naphthalene Acetic Acid(NAA). Besides measuring different callus biomarkers, and explaining the role of the (M.F.) in silybin accumulation in callus cultures, based on High Performance Liquid Chromatography (HPLC) Technique. The results indicated that the best response was for the roots callus cultures compared with the rest of the cultures used in the research, as the increase in fresh weight of roots callus after expose to magnetic field (M.F.), which reached (4.0) g fresh weight and (96%) viability, (1.85) mg/g protein concentration after 40 days. HPLC results revealed a dramatic increase in accumulation of active compound (silybin) by M.F. for all callus cultures depending on Retention time (R_t) and area under the curve for the standard sample (ST).

Keywords: (HPLC) Technique, Magnetic Field (M.F.), Silybin, *Silybum marianum* callus.

Introduction

Silybum marianum L. is an annual or biennial herb native (AbouZid, 2012), that belongs to the Asteraceae family (Cwalina-Ambroziak *et al*., 2012). It is a wild herb in Pakistan, Westen Asia and Europe and north and south America (Abenavoli *et al*,. 2010; Jabeen *et al*., 2019). It is commonly known as milk thistle due to presence of definite white veins in leaves (Abenavoli *et al*., 2010). Its main bioactive compound is silybin which is an isomeric combination with flavonolignans (Kurkin, 2003; Althagafy *et al*., 2013).It has been used widely as a natural medicine for liver and biliary tract disorders (Bahmani *et al*., 2015),anti-inflammatory, antioxidant (Lozano-Sepulveda *et al*., 2015) anticancer activity (Ramasamy and Agarwal, 2008). Also silybin can be used as protective agent in the management of asthmatic disorders (Breschi *et al*., 2002). Silybin has immunostimulatory effects (Ali *et al*., 2010).

The plant tissue culture technique, is an alternative source for production of secondary metabolites from medicinal plants (John and Koperuncholan, 2012; Lee *et al*., 2019). Magnetic fields (MF) are a type of abiotic stress, which can affect the growth and antioxidant efficiency of medicinal plants (Mansourkhaki *et al*., 2019). It can upgrade plant growth and development (Charlton, 2004), M. F. has positive influences on germination, growth, and evolution of cultivated strawberries (Althagafy *et al*., 2013). MF was used in practical application on seed germination, seedlings development and yields of different species such as field, fodder and industrial crop grass and medical plants, different fruits and vegetables and typical crops, has been massively studied through the last 80 to 90 past years (Teixeira and Dobranszki, 2014)

Research importance :

Due to the importance of plant tissue culture technology in the production of medicinal compounds, In a pure form and throughout the year, the Milk Thistle plant is an important economic and medicinal plants with high antioxidant properties, especially in the production of medicinal compound (silybin). This study aimed to exploit the efficiency of the magnetic field to stimulate the growth and development of different callus cultures. Thus increasing the production of medicinal compounds (silybin).

Materials and methods:

Seed sterilization and cultivation:

The research was carried out in laboratories of the college science, University of Mosul between (2019-2020).The seeds of *Silybum marianum* L. were soaked in water for 24 h then in 70% ethanol for 3 min and immersed then in 2.5% sodium hypochlorite (NaOCl) for 10 min, and finally in 0.1% tween20 for 10 min, (AL-Badrani and AL-Baker, 2021). The seeds were washed with sterile distilled water 4 times, then were placed on sterile filter paper, and cultured on solid (MS) medium (Mourashige and Skoog,1962), Then the seeds were incubated at 22±2C° with photoperiod at 16h light / 8h dark / day. After 20 days,the seedlings were cut of to (roots,leaves, shoots and nodes).

Culture medium:

The different segments were cultured on a solid (ms) medium supplemented with 3.0 mg/L of BA and 5.0 mg/L of NAA. The conical containers were incubated at 23°C with photoperiod of 16 h light / 8 h dark/day.

Explants exposing to magnetic field:

The Explants had been exposed to the magnetic field (M.F.) of strength (1500)Gauss generated by a winding coil magnetic, 1.5 cm from explants either side at 22 temperature for 5 days, Then incubated at 22±2C° with photoperiod at 16h light / 8h dark / day. A winding coil magnetic field device from the Physics department/ Sciences college, is shown in the figure (1).

Figure(1): Magnetic field device used for exposure Explant and Callus cultures

Callus induction and fresh weight estimate:

The response of callus induction from different seedlings segments (roots, leaves, stems and nodes), which exposed to M.F. (5 replications were considered for each explant) was recorded after 40 days, Also the response of callus induction from callus was recorded as fresh weight of callus after 40 days from culture.

Viability (Tetrazolium Assay) :

The milk thistle callus were taken with 0.5 g, for each sample, on the whole according to the methods of Towill and Mazur, The vitality of the callus exposed to the magnetic field for all treatments and control sample was measured based on the ability of the live callus cells to reduce triphenyltetrazolium chloride to red formazan according to (Towill and Mazur, 1975) method.

Protein estimate:

Protein had been determined according to the Lowry method (Lowery *et al*., 1951), based on the standard curve of bovine serum (albumin).

Silybin assessment by HPLC :

- **Extraction:** Silybin extraction was done according to the methods of (Cacho *et al*., 1999)
- Callus has been dried and powdered, then treated with ethyl acetate for 5h. Silybin (flavonolignan) was extracted from the dried residue with 10 ml of methanol at (40 ºC) for 8 h. and then 2 ml of methanol was added to the dried residue, and kept at (4 ºC) in the dark.
- Silybin standard solution : Reference standard of silybin was purchased as stock solution of the standard which was prepared by melting 0.1 g from sample powder in 500 mL acetonitrile 5%, and kept in the fridge until use.
- HPLC analysis: High performance liquid chromatography (HPLC)Japan /Shimadzu –used to determined the silybin components amount in callus cultures. The column was (phenomenex – C18,100A, 250 X 60mm), mobile phase(Methanol:water) (60:40) (vol:vol), flow rate :1.0 mL /minute,and detected by UV at 288nm. (Al-Hawamdeh *et al*., 2013). Identification was achieved by comparison of the sample retention time (Rt) with that of the standard. Quantification of silybin was accomplished using a known standard concentration and peak areas.

Statistical analysis :

Completely randomized design was used with 5 replicates. The data were subjected to the analysis of variance and mean values were compared using revised –LSD.

Results and discussion

Effect of (M.F) in callus induction and growth:

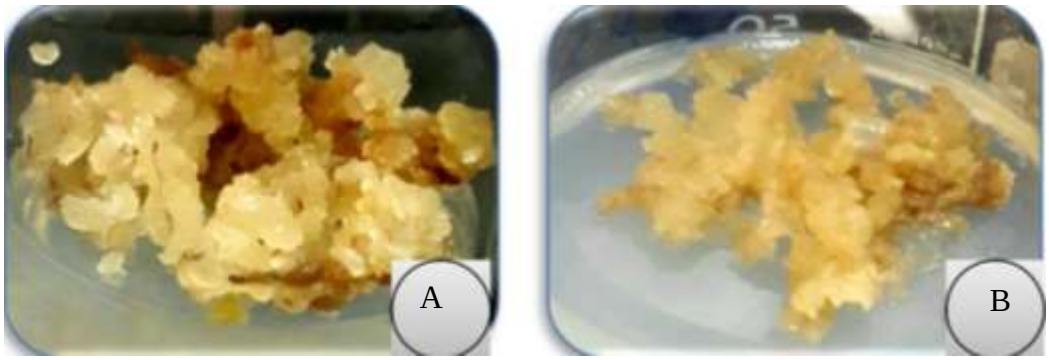
The different segments (roots, leaves, shoots and nodes) of seedlings which exposed to M.F. produced callus within (6-15) days after culture on MS medium supplied with 3.0 mg/L BA and 5.0 mg/L NAA.

The data (Table 1) showed that there is a clear enhancement and positive variation superior of M.F. in the biochemical activity (callus induction and fresh weight) in all segments to the control sample. It was observed the response of exposed segments to M.F. was rapid produced 100% callus after (6-15) days as compared to other not exposed segments to M.F that showed low rate of response to callus initiation. Data in (Table 1) showed that best treatment stimulate callus induction were observed in roots segments which exposure to M.F. with 100% callus induction after 6 days and 4.0 g fresh weight after 40 day compared to others in response to callus induction, Again it was noted an increase in fresh weight of root segments (with M.F.) which doubled to 4.0 gm after 40 days. These results confirm that, the magnetic field can alter plant growth and development through improve cell division and metabolism of exposed segments (Teixeira and Dobranszki, 2014). Also observed that fresh weight less than in the other explant (leaves, stems and nodes) (3.6, 1.9, 3.2) g respectively, This indicates that the response to callus initiation and growth, will change depending on the type of explant which used (Neumann *et al.*, 2009).It is worthy to mention, this callus was yellow color and its friable texture, figure (2). revealed that the M.F. had a stimulating effects in increasing callus growth and fresh weight, which may attributed to the M.F. role in stimulating the growth process, by reducing the resistance of cell walls to elongation of cell, where membranes systems are among the first parts of the plant affected by stress (Queen and Cosgrove, 1994).

Table (1): Effects of (M.F.) in callus induction and fresh weight which derivative from *Silybum marianum* L. seedlings.

Explant growth on (MS)*	Period of callus induction (days)		Callus induction (%)		Fresh weight (g)	
	Without (M.F.)	with (M.F.)	Without (M.F.)	with (M.F.)	Without (M.F.)	with (M.F.)
Roots callus	10 a	6.0 a	92 a	100a	2.7 a	4.0 a
Leaves callus	20 d	15 d	80 b	100a	2.0 b	3.6 b
Stems callus	15 c	10 c	75 c	100a	1.8 c	1.9 c
Nodes callus	13 b	7.0 b	90 a	100a	2.5 ab	3.2 b

*(MS) media supplemented with (3.0 and 5.0) mg/L BA and NAA after 40 dayes.



Figure(1): Effects of (M.F.) in induction and callus growth which derivative from *Silybum marianum* L. seedlings**. A:** Roots callus with (M.F.) **B:** Roots callus without (M.F.)

Effects of (M.F.) in callus viability and protein content :

The results in (Table 2) showed that the M.F. and type of explant had a clear effect on callus culture viability and protein content which exposed to M.F. especially the roots and leaves culture, with 96 and 90 % viability.As for the estimation of the amount of total protein in callus culture, it ranged between (0.79-1.85) mg\g. Also, it was observed that there is an increase in the protein content, of callus culture which exposed to M.F. with (1.85, 1.63) mg\g respectively,and the least response was in stem explant with 72% viability and 0.9 mg\g protein, and this appears clearly in the following figure(3). The protein concentration level calculated of cellular basis, and the increase in fresh weight of plant cell, it must be accompanied by an increase in other cellular components (Neumann *et al.*, 2009).

Results in figure (3) indicated that the important role of M.F. and type of explant in stimulation the biochemical activity, these results refer to the great role of M.F. in stimulate production of proteins and other components through the M.F. facilitating the movement of water inside the cells in contact with the medium, by improving the permeability of the cell membrane and increasing the ion exchange through it resulting from the change in osmotic pressure inside and outside the cell (Reina and Faschal,2001),and these results correspond with results of (Mansourkhaki *et al.*, 2019) showed that SMF up to 4 mT increased fresh weight, dry weight, leaf area, relative water content (RWC), root length significantly and then decreased these parameters at 6 mT. At 4 mT, SMF caused an 125, 132.73 and 52.19% increase in dry weight, leaf area and root length as compared to the control, respectively.

Table (2): Effects of (M.F.) in viability and protein content of *Silybum marinum* L. callus after (40) days.

Explant growth on (MS)*	Viability %		Protein concentration mg/g	
	Without (M.F.)	with (M.F.)	Without (M.F.)	With (M.F.)
Roots callus	85 a	96 a	1.25 a	1.85 a
Leaves callus	74 c	90 b	0.90 b	1.63 a
Stems callus	70 d	72 d	0.88 b	0.90 c
Nodes callus	78 b	82 c	0.79 ab	1.21 b

*(MS) media supplemented with (3.0, 50) mg/L BA and NAA

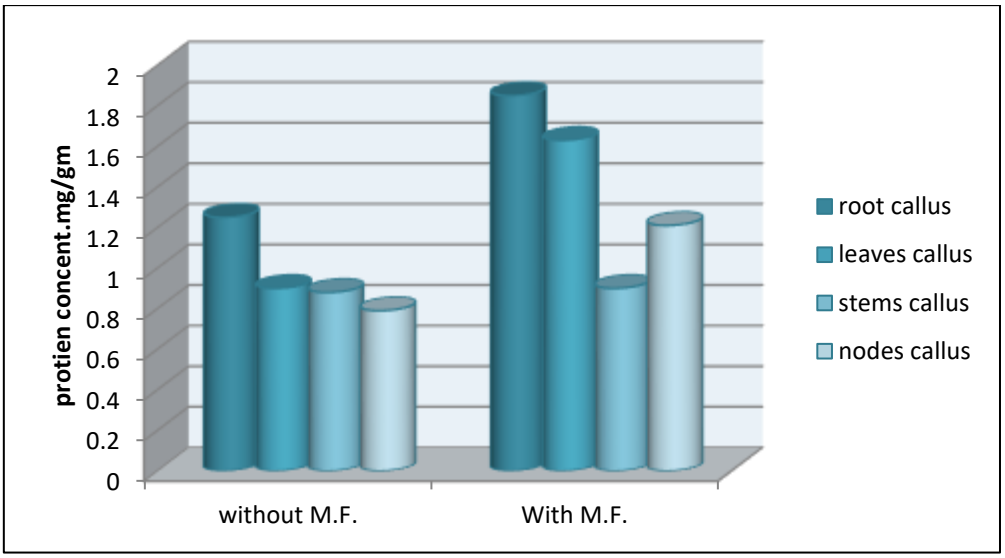


Figure (3): Comparison of protein concentration for *Silybum marinum* L. callus culture with and without (M.F.)

Quantitative and qualitative estimate of silybin compound in *Silybum marianum* L. callus cultures by using (HPLC) Technique:

Silybin was detected and quantified by HPLC for different samples of *S.marianum* callus cultures exposed to M.F. (Table 3). To standardize the fingerprints of tissue cultured materials through HPLC, identical peaks present in all samples appeared as" common peaks" for the silybin at (2.57) min (retention time) **Figure 4and 5**. The chromatographic data along with silybin standard representing valuable information based on the comparative peak area and R_t for silybin assessment and quantification in all callus cultures.

Generally, Table 3 illustrated that the callus culture exposure to (M.F) of strength 1500 gauss for 5 days, caused an increase in silybin concentration in all callus cultures compared with the standard. Data in (Table 3) indicated that best treatment stimulate accumulation silybin in nodes callus culture,since the (62.23%) peak area,when exposed the culture to (M.F), an increase was observed in silybin concentration, depending on the peak area since the (100 %) peak area.There are several studies indicate similar role of (M.F) enhanced the biochemical and enzymatic activity. (Asgher *et al*., 2016 and Chen *et al*., 2017).

Table(3):Estimate of silybin compound in *Silybum marianum* L. callus cultures by using (HPLC) Technique.

Extraction of	(80) dayes			(80) dayes+ M.F.		
	Retention Time (minute)	Peak area	% Peak area	Retention Time (minute)	Peak area	% Peak area
Roots callus	2.54	3503529	37.20	2.66	9633149	66.81
Leaves callus	2.68	7529126	53.43	2.45	19020987	100
Stems callus	2.74	11951726	45.11	2.68	20506001	99.97
Nodes callus	2.76	7826918	62.23	2.32	18608714	100
St.(Silybin)	2.57	2596305	100			

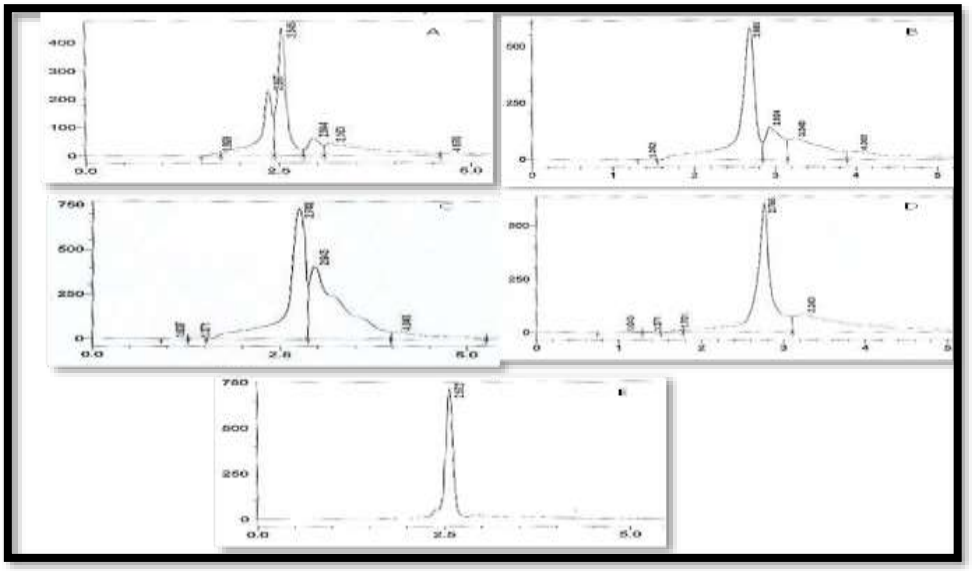


Figure (4): Retention Time(minute) of silybin compound in *Silybum marianum* L. callus cultures (80) dayes by using (HPLC) Technique
A - Leaves callus. B- Roots callus. C- Stems callus.
D- Nodes callus. E- St.(Silybin).

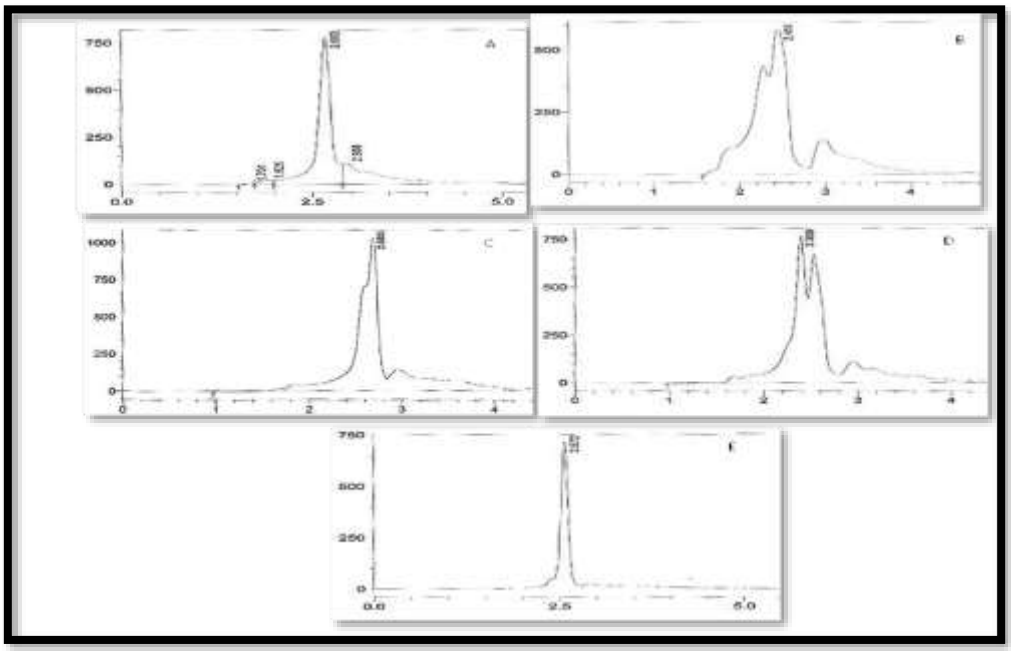


Figure (5): Retention Time (minute) of silybin compound in *Silybum marianum* L. callus cultures (80) dayes (exposing to magnetic field) by using (HPLC) Technique
A - Leaves callus. B- Roots callus. C- Stems callus.
D- Nodes callus. E- St.(Silybin)

Conclusion:

In the present study, the results confirm there is a clear enhancement of M.F in the biochemical activity (callus induction, fresh weight, viability and protein content) in all callus cultures. It is worthy to mention, that the M.F was of a stimulating effects in the increase of silybin accumulation in all callus cultures.

Acknowledgement:

The authors would like to express their thanks to the University of Mosul, College of Science, Biology Department for supporting this research work.

References:

Abenavoli, L.; Spagnuolo, R.; Luppino, I. and Luzza, F. (2010). "Recent Progress in Medicinal Plants". Spllc Press., 387 - 409.

AbouZid, S. (2012). Silymarin, natural flavonolignans from milk thistle. Phytochemicals-A Global Perspective of Their Role in Nutrition and Health. Rijeka: Croatia InTech., 255-272.

Al-Badrani,S.A. and Al-Bakr,R.A.(2021).Initiation Callus Cultures from the Seedlings of *Silybum Marianum* and Estimate their Protein Content. Rafidain Journal of Science.30(1):21-37.

Al-Hawamdeh, Fayha M.; Shibli, Rida A. and Al-Qudah, Tamara S. (2013).*In vitro* Production of Silymarin from *Silybum marianum* L. Med Aromat Plants., 4: 1-4.

Ali, S.; Khan, M.S. and Iqbal, J. (2010). Genotype independent in *vitro* regeneration system in elite varieties of sugarcane. Pak J. Bot., 42(6):3783-3790.

Althagafy, H. S.; Meza-Avina, M. E.; Oberlies, N. H.and Croatt, M. P. (2013). Mechanistic study of the biomimetic synthesis of flavonolignan diastereoisomers in milk thistle. J. Org. Chem. 78:7594–7600.

Asghar, T. ; Jamil, Y. ; Iqbal, M. ; Haq, Z. and Abbas, M. (2016). Laser light and magnetic field stimulation effect on biochemical, enzymes activities and chlorophyll contents in soybean seeds and seedlings during early growth stages. J. Photochemistry and Photobiology, B:Biology.165:283-290.

Bahmani,M.; Shirzad,H.; Rafieian,S.;and Rafieian-Kopaei,M.(2015).*Silybum marianum*:beyond hepatoprotection. J. Evidence-based Complementary and Alternative Med., 20(4): 292-301.

Breschi, M. C., Martinotti, E., Apostoliti, F., and Nieri, P. (2002). Protective effect of silymarin in antigen challenge-and histamine-induced bronchoconstriction in *in vivo* guinea-pigs. European J. Pharmaco., 437:91-95.

Cacho, M.; Moran, M.; Corchete, P.; and Fernandez-Tarrago, J. (1999).Influence of medium composition on the accumulation of flavonolignans in cultured cells of *Silybum marianum* (L.). Gaertn. Plant Sci., 144(2): 63-68. 22.

Charlton, M. (2004). Nonalcoholic fatty liver disease: a review of current understanding and future impact. Clin. Gastroenterol. Hepatol., 2(12): 1048-1058.

Chen, Y. ; Chen, D. and Liu, Q. (2017) Exposure to a magnetic field or laser radiation ameliorates effects of Pb and Cd on physiology and growth of young wheat seedlings.. J. Photochemistry and Photobiology, B:Biology.169:171-177.

Cwalina-Ambroziak, B.; Wierzbowska, J.; Damszel, M. and Bowszys, T.(2012). The effect of mineral fertilization on achenes yield and fungal communities isolated from the stems of milk thistle *Silybum marianum* (L.). Gaertner. Acta Sci. Pol.Hortorum Cul., 11(4): 157 - 168.

Jabeen, Z.; Rahman, K. U.; Zia, M. A. and Jhan, N. (2019). Establishment of an efficient and reproducible *in vitro* protocol for callogenesis of *Silybum marianum*. Inter. J. Biosciences., 14(1): 402-410.

John, A. S. and Koperuncholan, M. (2012). Direct root regeneration and indirect organogenesis in *Silybum marianum* and preliminary phytochemical, antibacterial studies of its callus. Inter. J. Pharm., 2(2): 392-400.

Kurkin, V. A. (2003). Phenylpropanoids from medicinal plants: distribution, classification, structural analysis, and biological activity. Chem. Natural Compounds., 39(2): 123-153.

Lee, T. J.; Zobayed, S. M. A.; Firmani, F. and Park, E. J. (2019). A novel automated transplanting system for plant tissue culture. Biosystems Eng., 181: 63-72.

Lowery, O. H.; Rosebrough, N. J.; Farr, A. L. and Randall, R. J. (1951).Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193:265-275.

Lozano-Sepulveda, S. A.; Bryan-Marrugo,O. L.; Cordova-Fletes,C.;Gutierrez-Ruiz, M. C.and Rivas-Estilla,A.M.(2015). Oxidative stress modulation in hepatitis C virus infected cells. World J. Hepatology., 7(29): 2880-2889.

Mansourkhaki, M.; Hassanpour, H.; and Hekmati, M. (2019). Effect of static magnetic field on growth factors, antioxidant activity and anatomical responses of *Silybum marianum* seedlings. J. Plant Process and Function. Seven (28): 10-15.

Murashige,T. & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia plantarum, 15(3), 473-497.

Neumann, K. H.; Kumar, A. & Imani, J. (2009)."Plant cell and tissue culture-A Tool in Biotechnology. Basics and Application" Springer-Verlag Berlin Heidelberg.

Queen, M. and Cosgrove, S. (1994). Disruption of hydrogen bonding between plant cell polymers by proteins that induce wall extension. Proc. Nat. Acad. Sci., USA. 91: 6574 6578

Ramasamy, K. and Agarwal, R. (2008). Multitargeted therapy of cancer by silymarin. Cancer Letters., 269(2): 352-362

Reina, F. G., & Pascual, L. A. (2001). Influence of a stationary magnetic field on water relations in lettuce seeds. Part I: Theoretical considerations.Bioelectromagnetics: Journal of the Bioelectromagnetics Society, The Society for Physical Regulation in Biology and edicine, The European Bioelectromagnetics Association, 22(8), 589-595.

Teixeira da Silva, J. A. and Dobranszki, J. (2014). Impact of magnetic water on plant growth. Environ. Exp. Biol., 12(4): 137-142.

Towill, L. E. and Mazur, P. (1975). Studies on the reduction of 2, 3, 5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures. Can. J. Bot., 53(11): 1097-1102.

تأثير المجال المغناطيسي (M.F.) في استحداث مزارع كالس نبات شوك الجمل (*Silybum marianum* L.) Milk Thistle ومستوى تراكم مركب السلبن silybin فيها بأستخدام تقنية HPLC

صباح البدراني [1] ورحاب البكر * [2]

(1). مدرسة قبة الصخرة للبنات ، المديرية العامة لتربية نينوى، العراق.

(2). قسم علوم البيئة ، كلية علوم البيئة وتقاناتها، جامعة الموصل، العراق.

(*للمراسلة الباحث: د. رحاب البكر ، البريد الإلكتروني: rehsbio39@uomosul.edu.iq)

تاريخ الاستلام:2022/02/1 تاريخ القبول: 2022/10/14

الملخص:

أجري البحث في مختبرات كلية العلوم / جامعة الموصل للفترة مابين (2019–2020)، هدفت الدراسة إلى الكشف عن فاعلية المجال المغناطيسي (MF) في استحداث ونمو مزارع الكالس لنبات شوك الجمل *Silybum marianum* L من قطع البادرات المعقمة (الجذور والأوراق والسيقان والعقد) المزروعة على وسط (MS) الصلب والمدعم بـ (3.0) ملغم / لتر من (BA) و (5.0) ملغم / لتر من (NAA). إلى جانب قياس المؤشرات الحيوية المختلفة لمزارع الكالس المعرضة للمجال المغناطيسي، وبيان دور المجال المغناطيسي (MF) في تراكم السبلين في مزارع الكالس ، بالاعتماد على تقنية الكروماتوكرافيا السائلة عالية الأداء (HPLC). أشارت النتائج إلى أن افضل استجابة كانت لمزارع كالس الجذور مقارنة ببقية المزارع المستخدمة في البحث، حيث بلغت الزيادة في الوزن الرطب لكالس الجذور بعد التعرض للمجال المغناطيسي (MF) (4.0) غرام وزن رطب, وكانت الحيوية (96)% و البروتين كان بمقدار (1.85) ملغم / غم بعد مرور 40 يوم من التعرض للمجال المغناطيسي. كشفت نتائج تقنية الـ HPLC عن زيادة كبيرة في تراكم المركب الفعال (silybin) في مزارع الكالس بعد تعرضها للمجال المغناطيسي (M.F.) اعتمادًا على (Rt) والمساحة تحت المنحنى للعينة القياسية (ST).

الكلمات المفتاحية: تقنية كروماتوكرافيا السائل ذو الاداء العالي (HPLC) ، المجال المغناطيسي (M.F.) ، السلبن , كالس شوك الجمل *Silybum marianum*