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Short Communication

In vitro biomass production of liver-protective compounds from Globe artichoke (*Cynara scolymus* L.) and Milk thistle (*Silybum marianum*) plants

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Abstract: The evolving importance of the plant secondary metabolites has in recent years resulted in a great interest of natural medicinal compounds, particularly their production by means of plant cell culture technology. The main advantage of this technology is that it provides continuous, reliable source of plant pharmaceuticals that could be beneficial in large-scale production. Globe artichoke and Milk thistle plants are considered important medicinal herbs producing active ingredients that treat liver diseases. Globe artichoke extract is recommended for liver damage disease and poor liver function. Cynarin, the main active compounds of artichoke has several pharmaceutical actions such as liver protection and re-growth of liver cells. Otherwise, silymarin extracted from Milk thistle has been used to treat hepatitis and liver damage. This work discusses different approaches of biotechnology used for biomass production of Globe artichoke and Milk thistle as sources of their active ingredients. Optimization of different physical conditions such as temperature, aeration, agitation light and chemical factors like addition of growth regulators and precursors to culture media for *in vitro* production of these active compounds have been described. Moreover, transformed hairy roots cultures, caused by infection of *Agrobacterium rhizogenes* has been discussed as an important technique for *in vitro* maximization of such secondary metabolites production.

Key words: Globe artichoke, Milk thistle, active ingredient, tissue culture

الانتاج المكثف فى المختبرات لمركبات واقية للكبد من نباتات الخرشوف وشوك الجمل

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قسم التكنولوجيا الحيوية النباتية - المركز القومى للبحوث – ش البحوث- ص ب: ١٢٦٢٢ - الدقى – القاهرة - جمهورية مصر العربية.

الملخص: الأهمية المتنامية للمركبات الثانوية النباتية فى السنوات الأخيرة أدت إلى إهتمام كبير بالمركبات العلاجية الطبيعية ولا سيما إنتاجها بوسائل تقنية الخلايا النباتية. الميزة الأساسية لهذه التقنية هو أنها توفر مصدر مستمر وموثوق للمواد الصيدلية ويمكن إستخدامها للإنتاج على نطاق واسع. تعتبر نباتات الخرشوف وشوك الجمل من الأعشاب الطبية الهامة المنتجة لمكونات نشطة تعالج أمراض الكبد. مستخلص نبات الخرشوف يوصى به لأمراض أو تلف الكبد وضعف وظائفه. السينارين وهو المركب النشط الرئيسى فى الخرشوف له العديد من التأثيرات الصيدلية مثل حماية وإعادة نمو خلايا الكبد. خلاف ذلك السليمارين المستخلص من نبات شوك الجمل تم إستخدامه لعلاج إلتهاب وتلف الكبد. فى هذا العمل يناقش مختلف أوجه التقنيات الحيوية المستخدمة لإنتاج الكتل الحيوية للخرشوف وشوك الجمل كمصادر لمكوناتها النشطة. وقد تم وصف تحقيق الأمثلية لمختلف الظروف الطبيعية للزراعة فى المختبرات مثل درجة الحرارة والتهوية والإضاءة والعوامل الكيميائية مثل إضافة منظمات النمو الى بيئة الزراعة من أجل إنتاج هذه المركبات الفعالة. علاوة على ذلك نوقشت مزارع الشعيرات الجذرية المعدلة وراثيا كتقنية هامة لتعظيم إنتاج مثل هذه المركبات الثانوية فى المختبرات.

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Introduction

Plants have been an important source of medicine for thousands of years. Pharmacopoeial plants have been used for a long time in folk medicine. Worldwide, between 50,000 and 80,000 flowering plants are used medicinally (Marinelli, 2005; IUCN Species Survival Commission, 2007). Over three-quarters of the world population relies mainly on plants and plant extracts for health care. Recently, several distinct chemicals derived from plants are important drugs currently used in most countries of the world. It was estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients (Sekar et al., 2010).

The production of useful natural components form the plant by the conventional agricultural methods is met with several problems. The seasonal production, diseases, handling and storage hinder the availability of such demand compounds to pharmaceutical factories. The search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue culture systems represent a potential renewable source of valuable medicinal compounds (Tripathi and Tripathi, 2003). The main advantages of this technology are providing continuous, reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be extracted. The additional advantages of such processes include controlled production according to demand and reduced requirements of labor and space. Moreover, cell suspension cultures and bioreactor techniques could be used to regulate metabolic processes to maximize yields.

Liver viral diseases are considered a major health problem in Egypt, frequently causing cirrhosis and liver cancer. Clinical studies showed 70% to 90% of patients with chronic hepatitis, cirrhosis, or hepatocellular carcinoma had HCV infections (Strickland, 2006). However, liver medicines are very expensive and most poor people in developing countries such as Egypt cannot afford them. So, it is important and critical to develop alternative sources of such pharmaceutical products using indigenous natural resources and technology. Increasingly, medicinal species that reside in natural areas have received scientific and commercial attention (Abd El-Wahab et al., 2008). Globe artichoke and Milk thistle plants are considered important medicinal herbs producing active ingredients agents to treat liver diseases (Morazzoni and Bombardelli, 1995; Orlovskaya et al., 2007). The main actions of artichoke´s pharmacolgy are: liver and gallbadder bile stimulation, hepatoprotective (liver protector), antihepatotoxic (liver detoxifile) and hypocholestrolemic (lower cholesterol) (Gebbardt, 1997). The main active compounds in Globe artichoke are: phenolic acids particularly cynarin (Adzet, 1987). Artichoke dry extracts are currently commercialized as drugs mainly for treatment of liver diseases: these include Cynara (200 mg of artichoke extract; Vesta Pharmaceuticals, Inc.), Artichoke 500 mg (artichoke leaf extract; Jarrow Formula, Inc.). Otherwise, Milk Thistle Plus is a blend of natural ingredients that provides a safe and effective way to maintain liver health. Milk Thistle Plus effectively repairs liver cells, enhances the liver's ability to detoxify, restores liver functions, and strengthens the immune system (Pepping, 1999). It contains the optimal combination of ingredients for the detoxification and protection of the liver. Silymarin which is commonly used to treat liver diseases and promoting the growth of new liver cells is the main active compound of Milk theistle. The present article discusses different approaches of plant biotechnology used for biomass production of Globe artichoke and Milk thistle as sources of their active ingredients.

Medical uses and active ingredients of Globe artichoke

Globe artichoke (*Cynara scolymus* L.) is a perennial plant that belongs to the family Compositae and largely distributed in the Mediterranean region. In addition to its high nutritive value as food, artichoke is used as an important medicinal herb. Globe artichoke was reported as one of the oldest medicinal plants

used as a remedy, dating back to 4th century B.C. (Kraft, 1997). The plant has been appreciated by the ancient Egyptians, Greeks, and Romans, who used it both as a food and as a medicine (for its beneficial effects against hepato-biliary diseases and as a digestive aid). In the 16th century, medicinal use of the artichoke was documented for liver problems and jaundice (Panizzi and Scarpati, 1954). It has been known as an herbal medicine, the dried leaves of artichoke have long been used in folk medicine for their choleretic and hepatoprotective activities that are often related to the cynarin content (Preziosi et al., 1959).

Artichoke leaf extract has choleretic, lipid-lowering, antioxidant and hepato-protective effects and it has been shown to improve digestion, liver function, and help lower high cholesterol levels and prevent heart disease (Englisch et al., 2000). In this respect, dried or fresh leaves and/or stem Globe artichoke are used to increase bile production. The beneficial health side effects of artichoke leaf extract are due to the promotion of bile flow in the body. In various pharmacological test systems, artichoke leaf extracts have shown hepatoprotective (Eberhardt, 1973; Adzet et al., 1987), antioxidative (Gebhardt, 1997; Gebhardt and Fausel 1997; Brown and Rice-Evans, 1998), anticarcinogenic (Clifford, 2000) and the ability to inhibit cholesterol biosynthesis and LDL oxidation (Clifford and Walker, 1987; Englisch et al., 2000).

The leaves of the artichoke contain a high content of pharmacologically active ingredients. The principle active compounds in artichoke are: phenolic acids mainly cynarin (1,5-di-O-caffeoylquinic acid), clorogenic acid (3-caffeolquinic acid) and caffeic acid and flavonoids including the scolymoside, luteolin glycoside and inulin (Lattanzio et al., 2005). Cynarin is considered the main biological active chemical in artichoke (Gebbardt, 1997). Cynarin was originally thought to be the single active component in artichoke leaf extract and was often used as a monosubstance. In this regard, synthetic cynarin preparations were used as a drug to stimulate the liver and gallbladder. However, further research observes that other active ingredients such as luteolin play a significant role in the effectiveness of artichoke leaf extract antioxidant (Kraft, 1997). It was found that the whole complex of compounds is considered just as active and aid in the many beneficial results.

Medical uses and active ingredients of Milk theistle

Silybum marianum (L.) Gaertn. is an annual herb or biannual herbaceous plant of family Compositae, commonly known as Milk-thistle or St. Mary's thistle. It is sometimes also called as silymarin, which is actually the herb's active component. Milk thistle is native to the Mediterranean, but is now widespread throughout the world usually in dry, sunny areas. It is recommended in traditional European and Asiatic medicine for more than 2000 years, mainly for treatment of liver disorders (Mossa et al., 1987). Milk Thistle protects and regenerates the liver in case of liver cirrhosis, jaundice, and chronic hepatitis. It cleans the liver of harmful substances such as alcohol, drugs, metals, anesthesia, etc. The plant and its extracts are reported to possess hepatoprotective, antioxidant (Morazzoni and Bombardelli, 1995), anticancer (Zi, 1997), anti-inflammatory (De La Puerta, 1996), and antidiabetic (Maghrani et al., 2004). A recent study found that Milk thistle also stimulates the immune system (Wilasrusmee et al., 2002).

The active ingredients of Milk thistle are chemicals called flavonoids. The flavonoids in Milk thistle are silybin, silydianin, and silychristin. Together, they are called silymarin. It is supported by numerous clinical studies (Feher et al., 1989; Ferenci et al., 1989; Sonnenbichler et al., 1999) for use as a liver protectant. Silymarin protects the liver by acting as an antioxidant and by promoting the growth of new liver cells (Barnes et al., 2002). It has been used (especially in Europe) to treat hepatitis and liver damage due to alcoholism (Blumenthal et al., 2000). The dried seeds contain 1–4% of silymarin flavonoids. Silymarin is a mixture of three flavonolignans, including silybin (silibinin), silidianin and

silichrystin (Wichtl, 1994). A standardized extract should be 80% silymarin (the active ingredient). The usual dosage of Milk thistle extract is between 300 milligrams (mg) and 600 mg daily. Silymarin promotes ribosomal RNA synthesis, which stimulates liver regeneration (Fraschini et al., 2002).

Biotechnological approaches

The recent advances in biotechnology particularly methods for culturing plant cell cultures, offer new means for the commercial processing of rare plants and the chemicals they provide. These new technologies will extend and enhance the usefulness of plants as renewable resources of valuable chemicals. There has been considerable interest in plant cell cultures as a potential alternative to traditional agriculture for the industrial production of secondary metabolites (Dicosmo and Misawa, 1995). The major advantages of cell cultures includes (1) synthesis of bioactive secondary metabolites is running in controlled environment, independently from climatic and soil conditions; (2) negative biological influences that affect secondary metabolites production in nature (microorganisms and insects) are eliminated; (3) it is possible to select cultivars with higher production of secondary metabolites; (4) with automatization of cell growth control and metabolic processes regulation, cost price can decrease and production increase (Mulabagal and Tsay, 2004). Cell suspension cultures are preferred for large-scale production due to its rapid growth cycles they have been used for generating large amounts of cells for quantitative or qualitative analysis of growth responses and metabolism of novel chemicals. Recently, plant cell and tissue cultures considered important sources of secondary metabolites such as medicinal substances, enzymes and natural colors and flavor. It is generally accepted that, differentiation in tissue cultures is associated with an increased in production of secondary products. Cell cultures in combination with biochemical and molecular biology techniques offer year round production of these compounds under controlled environmental conditions, thus securing a steady supply, as well as possibilities to regulate metabolic processes to maximize yields.

Establishment of callus and cell suspension cultures

Callus cultures usually are produced from any differentiated plant structure i.e. leaf, stem and root by placing explants on media containing relatively high level of auxin and low level of cytokinin. Callus initiation is affected by type and concentration of growth regulators in culture media. Furthermore, the response of explant on culture media also depends on the endogenous growth substances present at the time of excision. In this connection, callus formation in Globe artichoke had been studied by several researchers. Onisei et al. (1988) obtained callus cultures of Globe artichoke when leaf, cotyledon and mesocotyl explants were cultured on MS medium amended with different combinations of benzyladenine (BA), kinetin (kin) dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA). However, the most prolific callusing response of immature flower buds was obtained by Ordas et al. (1990, 1991) using MS medium supplemented with 2 mg/l BA + 5 mg/l NAA. In this concern, *in vitro* morphogenesis of Globe artichoke varieties e.g. Imperial Star, Green Globe and Balady were studied (El-Bahr et al., 2001). Calli were induced from cotyledon, leaf and stem explants using different combinations of BA, NAA, 2.4-D and gibberellic acid (GA_3). The best results of callus induction were observed when leaf explant was cultured on medium contained 10 mg/l BA+ 2 mg/l NAA + 0.1 mg/l GA_3. Moreover, morphogenic response as shoot organogenesis on the different types of calli was varied depending on genotype and type of callus. Otherwise, important study has been achieved on the evaluation of Globe artichoke varieties for their contents of some chemical and pharmaceutical compounds. Cynarin and inulin compounds were extracted and determined in leaves and flower buds and evaluated for response of some horticultural treatments (Sharaf-Eldin, 2002). It was found that the highest values of cynarin content in leaves and flower heads were recorded by Large Green cultivar.

However, Green Globe cultivar registered the highest values of inulin content.

Early, tissue culture protocols have been established for *Silybum marianum* from hypocotyl to induce callus on MS medium containing 0.8 mg/l NAA and 0.5 mg/l BA (Liu and Cai, 1990). However, optimization of the conditions of *in vitro* culture for callus production of *Silybum marianum* L. Gaertn has been achieved (Cimino et al., 2006). Cotyledons were used as explants and for its initial culture B5 medium supplemented with BA and 2,4-D was used. Recently, *in vitro* callus formation and plant regeneration of *Silybum marianum* L. Gaertn was reported (Manaf et al., 2009). Hypocotyl was the most convenient explant for callus formation. MS medium supplemented with 1 mg/l NAA + 0.1 mg/l BA showed the highest callus fresh weight in light condition (16 h/day at 6000 Lux). Direct regeneration (shoot formation) was achieved by cotyledonary node explant alone on MS medium supplemented with 2, 3, 4 mg/l BA in light condition. In this respect, we have developed protocols for in vitro culture of Globe artichoke and Milk thistle through an in-house project (NRC- Egypt- Ninth Research Plan –Project No, 9040101). Aseptic cultures were obtained using seeds materials (Figure 1). Calli cultures were established from cotyledon, leaf and stem explants on nutrient medium contained different types of growth regulators (Figure 2). The maximum cell growth rate was recognized by optimize some of the *in vitro* cultutre conditions. Moreover, cell cultures lines were obtained from friable callus using liquid medium.

Enhancement of active ingredients

For plant cell culture techniques to become economically viable, it is important to develop methods that allow for consistent generation of high yields of products from cultured cells (Berlin and Sasse, 1985). Careful selection of productive cells and cultural conditions resulted in accumulation of several products in higher levels in cultured cells. Plant cell cultures represent a heterogeneous population in which physiological characteristics of individual plant cells are different. Cell cloning methods provide a promising way of selecting cell lines yielding increased levels of product. This approach is useful when the precursors are expensive. In order to obtain yields in high concentrations for commercial exploitation, efforts have focused on the stimulation of biosynthetic activities of cultured cells using various methods (Dixon, 1999; Ramachandra Rao, 2000). Number of chemical and physical factors like media components, phytohormones, pH, temperature, aeration, agitation and light effects on production of secondary metabolites has been extensively studied (Fett-Neto et al., 1995, Goleniowski and Trippi, 1999; Wang et al., 1999; Lee and Shuler, 2000). In this respect, several products were found to be accumulating in cultured cells at a higher level than those in native plants through optimization of cultural conditions. The manipulation of physical aspects and nutritional elements in a culture is perhaps the most fundamental approach for optimization of culture productivity.

Figure 1. Aseptic seedlings of Globe artichoke (A) and Milk thistle (B) grown on MS- hormone free medium.

Exogenous supply of a biosynthetic precursor to culture medium may also increase the yield of the desired product. Attempts to

induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases (Moreno et al., 1993; Whitmer et al., 1998; Silvestrini et al., 2002). For example, amino acids were added to cell suspension culture media for the production of tropane alkaloids, indole alkaloids etc. Addition of phenylalanine resulted in the stimulation of taxol production in Taxus cultures (Fett-Neto et al., 1995). Moreover, the effect of coniferyl alcohol as a precursor of flavonolignan biosynthesis on silymarin components production in *Silybum marianum* suspension culture was investigated (Tůmová et al., 2006). A significant increase of silydianin was observed only after 72 h of the application of 46 µM coniferyl alcohol.

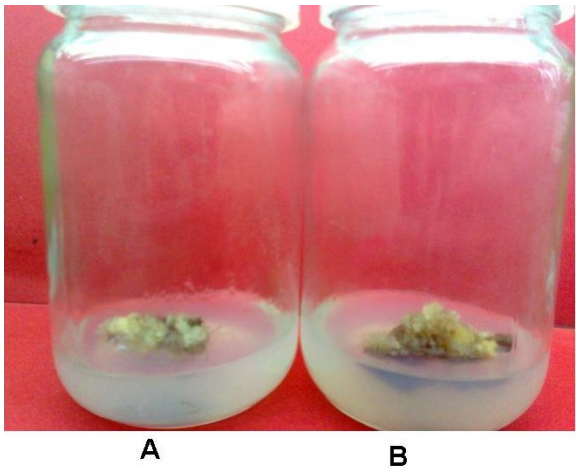

Figure 2. Callus cultures of Globe artichoke (A) and Milk thistle (B) proliferated from leaf explants.

Genetically transformed hairy roots, caused by infection of *Agrobacterium rhizogenes* suggest attractive properties for secondary metabolite production such as fast growth, greater biosynthetic capacity for secondary metabolites production. Hairy roots are genetically stable and not repressed during the growth phase of its culture (Bourgaud et al., 1999). They often grow as fast as or faster than plant cell cultures (Srivastava and Srivastava, 2007). The greatest advantage of hairy roots is that their cultures often exhibit approximately the same or greater biosynthetic capacity for secondary metabolite production compared to their mother plants (Kim et al., 2002). In this connection, Silymarin production by hairy root culture of Milk thistle (*Silybum marianum* L. Gaertn) was reported using *Agrobacterium rhizogenes* AR1583 (Rahnama et al., 2008). Moreover, enhancement of silymarin accumulation using precursor feeding in *Silybum marianum* hairy root cultures was study (Rahimi et al., 2011). In this study, the effect of different concentrations of L-phenylalanine (0, 1, 10 and 100 µM) as the precursor, on the phenylalanine ammonia-lyase activity, naringenin content, root biomass and silymarin production in *Silybum marianum* hairy roots were investigated. Phenylalanine 100µM after 72h was found to be the optimal feeding condition for producing silymarin.

Conclusion

Globe artichoke and Milk theistle are considered important medicinal plants that produce active ingredients to treat liver diseases. Biotechnology can be employed in the production of their active compounds i.e., Cynarin and Silymarin. This technology provides continuous, reliable source of such compounds and could be used for the large-scale production under controlled conditions. Moreover, cell suspension cultures and bioreactor techniques could be used for to regulate metabolic processes to maximize yields. For the establishment of *in vitro* cultures of Globe artichoke and Milk theistle, several factors such as type of explant, growth regulators added to culture medium and environmental conditions i.e., light, temperature and pH should be optimized. Otherwise, exogenous supply of a biosynthetic precursor to culture medium can be used to increase the yield of the desired products.

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