Antiviral Diversity of Compounds Derived From *Silybum marianum*

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ABSTRACT

Six genotypes of purple and white head flower varieties and one parent type of milk thistle (*Silybum marianum* L.*)* grow in the Egyptian desert. These are important plants used for treatment of various liver diseases. All the studied genetic parameters showed variations among milk thistle and parent varieties (plant height, main branch, total branches, head flower, fruit yield per plant). Concentration and yield amount of seven detected silymarin compounds using HPLC showed wide variations among varieties. They ranged from 9.2 to 34.78 mg/g fruits or 450.25 to 1250.25 mg/plant. The silymarin production has the same pattern of fruits yield. The methanolic solutions were extracted from milk thistle varieties then evaluated against HAV, HSV1 and COXB4 viruses. The cytotoxicity of different milk thistle methanolic extracts in Vero cells did not show any morphological difference compared with control. Antiviral affects of milk the most potent methanolic solutions of milk thistle that have antiviral activity with different degrees, line 32 with (HAV), line 9 with (COXB4) and, line 28 with (HSV1).

Key words: Antiviral activity, COXB4, Genetic diversity, HAV, HSVI, Plant antioxidant, TCID, Viral Cytotoxicity.

INTRODUCTION

There is increasing evidence that oxidestructive (redox) balance of cell is involved in viral infections and the antioxidant proteins have great interest because of their diverse activities. They are isolated from different living organisms like plants (Bazzoli *et al.*, 2002 and Sahar Shoman 2011). Plant antioxidant molecules are non toxic agents that exert potent antiviral activities *in vitro* and *in vivo* (Palamara *et al.*, 2005 and Gado, 2010). Concerning AIV infection, many studies considered the whey proteins as antioxidant, antihypertensive agents that have antiviral activity against *Poliovirus* type-1, COXB4 virus, B6, *Human herpes virus* (HSV), cytomegalovirus and human influenza virus subtype H3N1 and H1N1 (Chobert *et al.*, 2007).

Silybum marianum L. (Asteraceae) is an important medicinal plant. The plant fruits contain the 3-oxyflavones silymarin, an isometric mixture of three flavonolignans i.e silychristin, Silydianin and silybin (Cacho *et al.*, 1999). These compounds are of considerable pharmacological interest owing to their strong anti-hepatotxic and hepatoprotective activity (Sanchez sampedro *et al*., 2005). Silymarin is actually used for therapy of liver diseases and the flavolignan silybin is the most effective compound (Flory *et al*., 1980).

Two different varieties have been described under *S.marianum* species, purple flowers and *marianum albiflora* with white flowers (Tachholm, 1974). Ten wild genotypes were selected from the desert and variability of seven quantitative characters was evaluated between genotypes (Ottai *et. al*., 2010). Both varieties are distributed

widely and cultivated in the Mediterranean area and have similar ecological biosphere (Hetz *et al*., 1995). In Egypt milk thistle, *S. marianum*_grows widely as a winter crop on canal banks, while the plant cultivation in new reclaimed lands is more suitable for the plant growth traits and silymarin production. (Ezz El-Din, 1995).

The aim of this present study to evaluation of antiviral compounds derived from milk thistle, *S. marianum* varieties as well as pharmacy diversity of varieties against different viruses.

MATERIALES AND METHODS

Seeds of seven different varieties of two milk thistle, (*S. marianum*) purple and white head flower varieties were obtained from laboratory of cultivation and production of Medicinal and Aromatic plants, NRC, Egypt. Seeds were sown in October 2013 at the experimental station farm in shalakan, Qualiobia Governorate, Egypt.

Growth parameters: Five replication plants for 7 genotypes of purple and white varieties were selected. Six quantitative characters were investigated (plant height, number of main and total branches per plant, number of head flower per plant, fruit yield per plant and seed yield per plant).

Silymarin components were determined for three selected varieties in each variety (purple and white) as well as parent Wiled One gram of air dried fruit were defatted in a soxhlet apparatus with 150 ml of petroleum ether (40-60˚C) for 12h. The residue were extracted with 50 ml of methanol at 65-70˚C over 8h. The extract was dissolved in 10 ml of methanolic solution (Cacho *et al*., 1999).

HPLC analysis was carried out shimaolzu HPLC, LC-6A. A phenomenex C-18 (250 X 4.6 mmlD) column was used, eluting with MeOH: H_2O: AcOH (40:60:5) at a flow rate of 1 ml min^{-1} and the detection at 280 nm according to **Alikaridis *et al*. (2000**). A commercially available (Aldrich 25492-4) mixture of flavonolignans was used as a reference standard for the identification and assay.

Estimation of antioxidant activity (AOA): The measurement of antioxidant activity was done according to the method of **Koracevic *et al* (2001).** The assay measured the capacity of the biological fluid to inhibit the production of thiobarbaitaric acid reactive substances (TBARS) from benzoate under the influence of free oxygen radical derived from Fenton's reaction (Fe-EDTA Complex react with hyotrogen peroxide) leading to formation of hydroxyl radicals (OH). Therefore, reactive oxygen species degrade benzoate and release TBARS. Antioxidants from plant samples cause suppression of production of TBARS. The reaction was measured spectrophotometrically and the inhibition of color development defined as AOA.

Cytotoxic assay: The soluble plant extracts were diluted using two fold dilution in PBS (500mg/ml). Each dilution was inoculated in each of 5 wells containing Vero cells (Moghannem 2009) and subjected to daily microscopic examination to assess Cytotoxic.

Antiviral activity: Tenfold dilutions of the tested extracts of seven milk thistle varieties were inoculated with AIV infected tissue culture (infectious dose, 100 TCID 50 of AIV) of 12 wells tissue plate. Where 25µl of each dilution was mixed with 25µl of 100 TCID 50 of AIV and allowed to stand 1h. at 37˚C then inoculated in Vero cells. The antiviral effect was determined by inhibition of the specific CPE of AIV cells. The test virus and cells control including two sets, First: Vero cells. Neither infected with virus nor treatment with milk thistle extract (well/ each plate).

Second: Virus infected cells with no treatments with any milk thistle extract to be used as control in the calculations of percentage reduction in plaque forming unit (PFU) due to different treatments.

The actual concentration of each crude methanolic extracts was not exactly determined but it was examined as follow:
100 ml of methanolic extracts were left to evaporate the solvent for concentration, then dissolved in small size of distaled water and used for antiviral activity.

RESULTS

Six varieties of *S. marianum* were evaluated for genetic variability and parent type based on their growth characters, phytochemical compounds and antiviral activities. Significant variation was detected of five growth characters among six *S. marianum* and between parent types.

Data presented in table (1) showed that the mean value of the six studied quantitative characters for the six genotypes and parent type of *S. marianum* varieties (purple and white head flowers). The varieties produced higher mean values than the parent type in all characters. White varieties had the higher mean values in all characters except fruit yield. The comparison among the parent and varieties, the mean values of plant height, 20.0, 130.7; 177.7; main branch, 7.5; 10.9; 11.06, total branches, 14.; 71.7;92.3; head flower 15.7, 96.5, 138.3 and fruit yield, 5.5, 43.4, 43.0, for parent type, purple and white varieties respectively.

Silymarin content in fruit of the parent type and (purple and white head fruits) varieties was determined by HPLC. All silymarin components both in terms of concentration (mg/g fruit) and accumulation (mg/ plant) revealed a high variability among parent type, purple and white varieties showed in table (2). All white genotypes produced higher components followed by purple varieties compared with parent type (table 2). White varieties 2 had higher silymarin content (concentration) and silymarin per yield (accumulation) as compared with corresponding purple varities, and parent type. Purple var 28 had higher silymarin content and silymarin per yield compared with other purple varieties and parent type. In varietal comparison among the parent type, purple and white varieties, the mean values of silymarin content (9.2; 18.7; 23.61 mg/ g fruits) , silymarin per plant (950.25, 832.8, 698.25) silychristin (7.5; 4.92; 5.76) ; silybin (1.22, 3.57; 5.26) silybin A(0.15; 3.87; 5.05) ; isosilybin A (0.04; 3.85; 4.17); silybin B (0.02, 2.1, 4.16) and isosilybin B (0.02; 2.24; 2.48 mg/g fruit) for parent type; purple and white genotypes respectively. Total silymarin and its components in most cases were varied between the purple and white varieties.

Table (1): Growth parameters of purple and white milk thistle marianum varieties

Growth parameters / Varieties and lines	Plant height	Main branch	Total branches	Head flower per Plant	Fruit yield Per plant
Wild (Parent)	**20.0**	**7.5**	**14.0**	**15.7**	**5.5**
Purple head flower 34	**142.0**	**10.5**	**73.5**	**95.5**	**42.5**
Purple head flower 22	**129.5**	**11.7**	**70.3**	**97.2**	**45.2**
Purple head flower 28	**120.7**	**10.5**	**71.4**	**96.7**	**43.5**
White head flower 9	**180.5**	**11.5**	**92.5**	**139.4**	**50.2**
White head flower 2	**179.2**	**10.5**	**97.1**	**134.5**	**37.3**
White head flower 13	**173.5**	**11.2**	**87.5**	**140.2**	**44.5**

Table (2): Silymarin composition of purple and white milk thistle varieties

Silymarin composition / Varieties and lines	Silymarin content (mg/g fruit)	Silymarin Yield per Plant (mg/plant)	Silychristitin	Silydianin	Silybin A	Isosilybin A	Silybin B	Isosilybin B
Wild (parent)	**9.20**	**950.25**	**7.50**	**1.22**	**0.15**	**0.04**	**0.20**	**0.02**
Purple head flower 34	**16.52**	**1250.25**	**4.25**	**2.75**	**2.12**	**3.50**	**1.15**	**1.75**
Purple head flower 22	**15.51**	**795.75**	**4.75**	**3.25**	**3.14**	**3.90**	**1.75**	**1.72**
Purple head flower 28	**23.94**	**450.25**	**5.77**	**5.25**	**6.35**	**4.15**	**3.17**	**3.25**
White head flower 9	**20.12**	**850.21**	**4.75**	**7.50**	**3.75**	**2.75**	**2.12**	**2.25**
White head flower 2	**34.78**	**725.25**	**6.60**	**9.72**	**7.25**	**3.25**	**6.26**	**2.71**
White head flower 13	**18.95**	**520.10**	**4.25**	**5.75**	**4.15**	**2.15**	**4.15**	**2.50**

Antioxidant activity (AOA): Methanolic extracts of parent type, purple and white varieties (milk thistle) were individually tested for their antioxidant activity. The results in table (4) showed increased AOA values for methanolic crude extracts. Purple varieties had higher AOA values followed by white varieties and parent type (mean 0.79, 0.63 and 0.25 respectively).

Cytotoxicity assay: It was imperative to determine the maximum non toxic level of these extracts tested. The maximum nontoxic dose (MNTD) was in the range of two 10^{-1} to 10^{-3} dilution of 100µl of different extracts with 1.0 ml medium. At MNTD, tested Vero cells did not show any morphological difference when compared with control as shown in table (3) and figure (1).

Antiviral activity: Cytotoxicity assay of methanolic extracts was performed before studying the antiviral activity. All methanolic extracts of milk thistle varieties did not reveal cytotoxic effects even crude extracts. These methanolic extracts were tested for their antiviral activity against HAV, HSVI and COXB4 viruses.

Table (3): Determination of non-toxic dose of different methanolic milk thistle extracts screened for antiviral activities.

Methamnolic extracts	MNTD
Parent (wild type)	10^{-1}
Purple head flower 34	10^{-1}
Purple head flower 32	10^{-3}
Purple head flower 28	10^{-3}
White head flower 9	10^{-2}
White head flower 2	10^{-2}
White head flower 13	10^{-2}

MNTD= Maximum non- toxic dose.

The antiviral effect of different milk thistle extracts on HAV, HSV and COXB4 viruses was carried out by plaque assay method using 1/1000 challenge dose virus (CDV) and viruses titer 50 x 10^3 PEU /ml. The results were shown in table (4) and figure (1).

It was found that Vero cells infected with viral isolates, and incubated at 37˚C started to show cytophathic effect (CPE) in the form of cell rounding at 24-48 hrs that was progressively increased by the 4th day post – infection (figure 2). The virus titer reached 10^{-4} TCID 50/ml (tissue culture infectivity dose). The virus titer was $5x10^5$ PFU/ml scored at 72 hrs. Post infection in Vero monolayer cell culture under agarose over layer as shown in figure (2). The antiviral effect of different milk thistle extracts on HAV, HSVI and COXB4 was carried out by plaque assay method using 1/1000 challenge dose of virus (CDV) and viruses titer $50x10^3$ PFU/ml. The results are shown in table (4) and figure (1). It was found that, the milk thistle extracts were different in antiviral effect on HAV, HSVI and COXB4. The most potent milk thistle varieties are purple head flower 32, with (HAV), white head flower 9 with (COXB4) and, purple head flower 28 with (HSVI) (table 4).

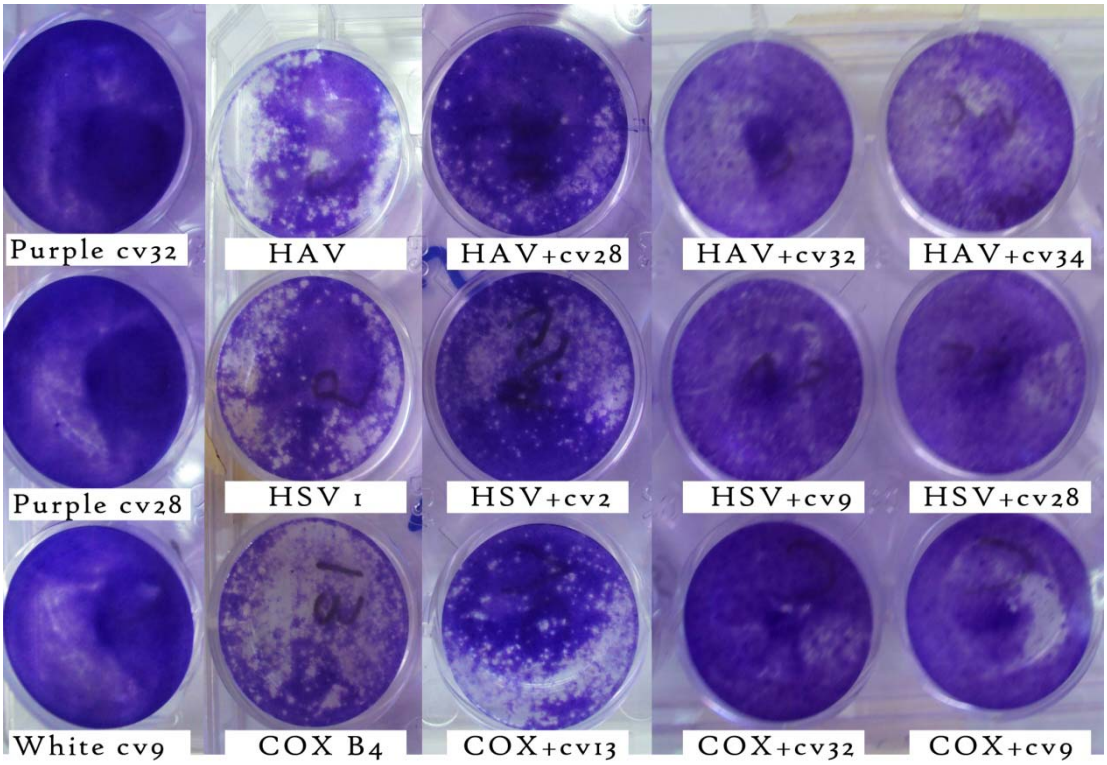

Figure (1): Photographs showing plaques assay (anti-infectivity effect) of different cultivates of milk thistles extracts on HAV, HSV and COXB4 viruses

Table (4): Antioxidant and antiviral activity evolution of purple and white flower milk thistle plants.

Antiviral activity / Varieties	Antioxidant activity Plaques formed Reduction (%)	Antiviral activity					
		HAV		HSV1		COXB4	
		Plaques formed	Reduction (%)	Plaques formed	Reduction (%)	Plaques formed	Reduction (%)
Parent (wild)	**0.25**	**125.00**	**7.4**	**14.25**	**81.00**	**85.27**	**28.94**
Purple head flower 34	**0.36**	**75.25**	**44.26**	**40.75**	**28.54**	**50.25**	**58.13**
Purple head flower 32	**0.92**	**25.10**	**81.41**	**49.20**	**34.40**	**115.71**	**3.57**
Purple head flower 28	**0.82**	**95.21**	**29.47**	**9.75**	**87.00**	**71.22**	**40.65**
white head flower 9	**0.72**	**130.75**	**3.15**	**10.25**	**86.33**	**45.25**	**62.29**
white head flower 2	**0.65**	**89.50**	**33.70**	**43.65**	**41.80**	**118.20**	**1.50**
white head flower 13	**0.50**	**130.25**	**3.51**	**50.72**	**32.37**	**82.75**	**31.05**

* Antioxidant activity AOA= m mol/L HAV infection = 135 PEU
** Mean of plaques formed number HSV infection = 75 PEU.
*** Reduction = Anti infectivity effect COX infection = 120 PEU.

DISCUSSION

The field of natural active plant anti oxidants and metabolites attracts the attention of many investigators all over the world. The present study is a continue of screening program for new active plant antioxidants and metabolites with biodiversal activities which was started at the virology lab. Agric. Microbiology Dept., Fac. Agric. Ain Shams Univ. 25 years ago (Othman *et al*., 1991; El-Dougdoug, 1997; and 2012).

Silymarin, an extract of milk thistle seeds and silymarin derived compounds are considered protective for liver since this plant was first described in ancient times. Hepatoprotection is a defined as several non-mutually exclusive biological activities including antiviral, antioxidant, anti-inflammatory and immunodulatory functions. (Polyak *et al.,* 2013)

Six genotypes and wild type of *S. marianum* varieties were selected from the desert and assessed for five growth characters to evaluate genetic variability among varieties and impact of environmental changes from a desert to an agricultural environment on plant characters. Significant variations were detected among varieties and revealed their considerable amount of genetic variability.

The results showed that the five studied quantitative characters for the six selected genotype of both milk thistle varieties (purple and white head flowers) produced higher mean values than the parent type in all characters.

Several authors (Ram *et al*., 2005; Ibrahim *et al*., 2007 and Ottai *et al*., 2010) found similar genotypic variation of *S. marianum*. The results indicated that all characters showed further improvement in the first adaptive season at the improved agricultural environment for all varieties (Ottai *et al*., 2009). They reported that the plant adaptation in agricultural environment (in old clay land) is an important mean for conservation and improvement. Other studies, Eslam (2004) found that high genetic variations for different characters between varieties of other crops like sun flower.

Silymarin content in the fruits of the purple and white varieties was determined by HPLC. Six Silymarin compounds Silychristin, Silydinin, Silybin A, isosilybin A, silybin B and isosilybin B were detected in the methanolic extracts of all milk thistle varieties. All Silymarin components showed a high variability among milk thistle varieties. These results are in agreement with the

findings of Ram *et al*. (2005). Although, the selfing mating improved the content of Silymarin in the purple varieties, the contents of parent was the best value in the white varieties confirming that each variety has different physiology and heredity behavior systems as mentioned by Hetz *et al*. (1995). The results are important for genetic improvement program for growth trails and Silymarin production of the milk thistle plant. Further improvement was also achieved in Silymarin yield plant and its constituents from wild up to the first up to the second adaptive season with different constituent values for different genotypes. This may be attributed to delete the stress conditions of desert land by cultivation in old land which riches in water. Organic matter, micronutrients with suitable direct heat and humidity therefore, help the plants to increase the synthesis of secondary metabolites (Omer, *et al*., 1994). They concluded that these results are phenotypic relatively and not necessary of genetic origin and these relation are influenced by environmental factors limiting yield.

The potential of silymarin derived natural products for hepatitis C originated from cell culture based showing that silymarin blocked HCV infection. (Wagoner *et al.*, 2010)

Milk thistle, *S. marianium* (L), is an important medicinal annual or biennial plant belonging to family Asteraceae. The plant fruits contain the 3-Oxyflavone silymarin, an isomeric mixture of three flavonolignans i.e. Silychristin, Silydionin and silybin(Cacho *et al*. (1999). These compounds are of considerable pharmacological interest owing to their strong anti-hepatotoxic and hepatoprotective activity (Soamchez-Sampedro *et al*., 2005). Silymarin is actually used for therapy of liver diseases and the flavolignan silybin is the most effective compound. The seeds are used to treat toxic liver damage, supportive treatment in chronic inflammatory liver disease, cirrhosis, and help maintain healthy liver function. The active chemical component of milk thistle is silymarin (Henywood *et al*., 1977)

Milk thistle extracts were investigated for antiviral activity against HAV, HSV-1 and COXB4. Firstly, the toxicity of different crude extracts was high on Vero cells (10^{-2} to 10^{-4}/ml) compared with control. They showed that no change in the morphology of Vero cells. The antiviral result indicated that the most active antiviral extracts of varieties are purple head flower 32, purple head flower 28 and white head flower 9 of milk thistle. The antiviral activity of parent type, purple head flower 28, and white head flower 9 were about 81.0; 87.0 and 86.33% reduction respectively against HSV-1. Purple head flower 32, and 34 were about 81.41 and 44.26% reduction respectively against HAV. White head flower 9 and, purple head flower 34 were about 62.29 and 58.13% reduction respectively against COXB4.

Despite clear evidence for silymarin induced hepatoprotection in cell culture and animal models, evidence for beneficial effects in humans has been equivocal. (Polyak *et al*, 2013)

Plant antioxidant molecules are non toxic proteins that exert antiviral activities *in vivo* and *in vitro* (Palamara *et al*., 2005 and Sahar Shoman, 2011). Concerning AIV infection many studies reported that the whey proteins as antioxidant, antihypertensive agents showed antiviral activity against *Poliovirus* type1, Coxsackie virus B6, cytomegalovirus, *Human herpes virus* and human influenza virus sub type H3N1 and H1N1 (Chobert *et al*., 2007).

Lazrak *et al*. (2009) found that, the M2 Protein is part of viral envelope which interferes with the cell ion channels of hydrogen ions flow and control of pH within the virus where the process of uncoating is started. Antioxidants act by blocking the virus ion channels disturbing the virus life cycle. While, Beck (2001) suggested that the

increase of oxidative stress status of the host may cause direct damage to the viral RNA itself resulting in new mutations that lead to enhanced pathogenesis.

Many plants were found to have proteins with both antioxidant and antiviral activities. These proteins can be explored as a very potential candidate for biotechnological manipulation. *Chenopodium album* and *Bougainvillea xbuttiana* are plants that grow wild as well as cultivated have been exploited for their and antiviral and antioxidant properties (Dutt *et al*., 2003; Bhatia *et al*., 2004 Mahdy *et al*.(2011).

Silymarin has liver regenerative effects by stimulating the nuclear enzyme RNA polymerase of liver cells .This leads to increased synthesis of ribosomal protein which helps to regenerate hepatocytes (Gruenwald, 2004).

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