

EVALUATION OF ANTIVIRAL AND CYTOTOXIC ACTIVITIES OF *TAMARIX GALLICA* AND *SILYBUM MARIANUM* EXTRACTS

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Abstract

The aim of this study was to investigate the virucid and cytotoxic activity of two hydroalcoholic extracts from French tamarisk (*Tamarix gallica*) and Milk thistle (*Silybum marianum*), plants that have been used for their therapeutic effects from ancient times. Each of these extracts was extensively studied for their hepatoprotective and stimulant effects, sometimes with contradictory results.

The present study investigates for the first time the antiviral effect and cytotoxicity of these extracts using a set of European standardized procedures for the virucidal activity testing in human medicine. Antiviral activity demonstrated by the two extracts complete and sustain our previous results on the hepatoprotective effect of a pharmaceutical product that has both extracts in its composition.

Rezumat

Scopul prezentului studiu a fost acela de a investiga activitatea virucidă și citotoxică a două extracte hidroalcoolice obținute din cătină roșie (*Tamarix gallica*) și armurariu (*Silybum marianum*), plante utilizate în scopuri terapeutice încă din vechime. Fiecare dintre aceste extracte a fost intens studiat pentru efectele hepatoprotectoare și stimulative cu rezultate uneori contradictorii.

Acest studiu investighează pentru prima dată efectul virucid și citotoxicitatea acestor extracte, utilizând un set de proceduri standardizate la nivel european pentru testarea efectului virucid în medicina umană. Activitatea antivirală demonstrată de cele două extracte în acest studiu susține rezultatele noastre anterioare privind activitatea hepatoprotectoare a unui produs terapeutic care are ambele extracte în compoziția sa.

Keywords: hepatoprotective, virucidal activity, *Tamarix gallica*, *Silybum marianum*.

Introduction

Silybum marianum (*Asteraceae*) commonly known as Milk thistle and *Tamarix gallica* (*Tamaricaceae*) have been used for their therapeutical effects from ancient times as hepatic stimulants and tonics [1, 4, 13, 14, 24, 26, 27, 37]. Their antioxidant, antibacterial and antifungal effects were extensively studied, sometimes with contradictory results. On the contrary, the antiviral effect was insufficiently investigated even though the examination of the virucidal activity would help the better understanding of the therapeutic effects of these compounds. Usage of non-standardized methods for investigation of different effects was one important cause of controversy.

Silymarin, a polyphenolic complex extracted from Milk thistle, is a mixture of flavolignans such as silybine, isosilybin, silydianin and silychristin. Silybin is the main biologically active component from silymarin and the literature is abundant in data that sustain both liver cell membrane protection and restoration of damaged cells [15, 24, 27, 32, 38] and, on the other hand, there are data that claim an effect similar to *placebo* in clinical trials [9]. However, a comparative study of the different clinical trials on the effects of milk thistle for patients with alcoholic and/or hepatitis B or C virus liver diseases showed that most of them were not properly designed, therefore, a final conclusion on this topic cannot yet be drawn [28].

Nevertheless, silybin, tested on mice as an antidote to poisoning with phalloidin (*Amanita phalloides* mushroom toxin), proved to be a very good hepatoprotective, not only *in vitro* but also *in vivo*, circumventing poisoning by blocking the receptors on hepatocytes membrane and also by inhibiting other toxic peptides such as α -amanitine, this way, preventing them from reaching the cell nucleus [4, 8, 21, 38]. Other studies have reported that silymarin is able to inhibit the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pro-inflammatory cascade and increase the expression of interleukine-10 (IL-10), an anti-inflammatory cytokine [4, 10].

A recent study has confirmed that the silybin hepatoprotective actions are correlated with decreased membrane lipid peroxidation, reduced free radical release, and restoration of the glutathione levels [5]. *In vivo*, silybin stabilized mitochondrial membrane fluidity, decreased the level of serum alanine aminotransferase and hepatic malondialdehyde, and increased superoxide dismutase and glutathione, in rats. Furthermore, it was suggested that mitochondrial membrane stabilization, oxidative stress inhibition, and

improved insulin resistance might be the basic mechanisms for the hepatoprotective effect of silybin on nonalcoholic fatty liver disease [39].

A very recent clinical trial, the first that systematically assessed the effects of silybin in non-alcoholic fatty liver disease (NAFLD) patients, with and without hepatitis C virus (HCV) infection, showed that patients treated for 12 months with an oral complex containing silybin, phosphatidylcholine, and vitamin E, improved the transaminases levels and gamma-glutamyl-transpeptidase (γ GT), insulin resistance and also several aspects of liver histology [15].

Few research centers investigated the effect of silybin and related compounds on hepatitis C virus (HCV) and their findings suggest a direct inhibition of the HCV RNA-dependent RNA polymerase and downregulation of HCV proteins [1, 18].

Tamarix gallica (*T. gallica*), was also used traditionally as hepatotonic and stimulant although in developed countries is still cultivated mainly for ornamental purposes. However, there are some scientific data that support the hepatoprotective action of *Tamarix gallica* extract, by normalization of serum proteins and cholesterol levels and document the effect on viral hepatitis [16, 19, 25]. The extract contains known antioxidant compounds such as tannins, flavanones, isoflavones, resveratrol, ellagic acid, syringic acid, isoquercetin, which exhibit a wide spectrum of medicinal properties [6,12, 22, 23, 30, 31]. It has been demonstrated *in vivo* that the extract of *T. gallica* inhibited cytotoxicity and hepatic oxidative stress by restoring hepatic antioxidant enzyme activities and, on the other hand, stopped chemically-induced liver tumor proliferation [20, 30]. Their data suggested that the chemopreventive effect of *T. gallica* is mediated by multiple mechanisms, which include restoration of cellular antioxidant defensive system and preventing mutagen uptake or DNA incorporation.

The aim of this study was to investigate the direct antiviral effect and cytotoxic activity, using a set of European standardized procedures for the virucidal activity testing in human medicine, of two hydroalcoholic extracts from French tamarisk (*Tamarix gallica*) and Milk thistle (*Silybum marianum*), in order to complete and sustain our previous results on hepatoprotective effect of a product that has both extracts in its composition.

Materials and Methods

Plant sampling and preparation for extract

T. gallica leaves and flowers and *S. marianum* seeds were harvested from the culture of S.C.Hofigal. S.A.. The harvested organs were rinsed with water, dried and extracts were obtained by maceration in 70% ethanol.

After filtration of extracts, ethanol was evaporated under *vacuum*. Then, they were stored at 4°C until analysis.

Cell culture preparation for virucidal testing

Cell lines and appropriate culture media were selected in accordance with their sensitivity to the test viruses. HeLa cells with known sensitivity to poliovirus/adenovirus have been used. Cells were plated in Dulbecco's modified Eagle (DMEM) medium supplemented with 10% Fetal Bovine Serum (FBS). Cell monolayers were more than 90% confluent before inoculation.

Stock virus suspension

The virus was multiplied on a large scale to obtain a virus suspension with the same characteristics as the reference virus suspension. For virucidal testing of compounds according to SR EN 14476+A1:2007, two viruses were used: one from *Picornavirus* group – poliovirus type 1, LSc-2ab and a Non-enveloped DNA virus from *Adenovirus* group – adenovirus type 5, strain Adenoid 75, ATCC VR-5. Third passage from the original seed virus was used for poliovirus.

Preparation of the test virus suspension

The stock virus suspension was multiplied in HeLa cells, which produce high titers of infectious viruses. The cellular debris was separated by low speed centrifugation. The minimum titer of the virus suspension was at least 10^8 50 % Tissue Culture Infection Dose (TCID₅₀)/mL or sufficiently high to enable a titer reduction of 4 lg to verify the method.

Preparation of the test mixture

Test mixture was obtained by combining 1mL of interfering substance (3g/L or 30g/L bovine serum albumin fraction V - BSA) with 1mL of the test virus suspension. 8 mL of the extract test solution was added, mixed, and placed in a water bath at the adequate test temperature (20°C ± 1°C). The activity of the product was determined for the appropriate contact times (0 min, 60 min). At the end of the contact time, tubes were mixed and 0.5mL of the test mixture were pipetted into 4.5mL ice-cold DMEM supplemented with 2% FBS.

Quantal tests (endpoint titration)

Virus titration was done on monolayers of cells on 96 wells microtiter plates. 0.1mL of each dilution of virus suspension was transferred into six wells of a microtiter plate containing an almost confluent cell monolayer without any medium. One row received 0.1mL culture medium and served as the cell controls. After 1h of incubation at 37°C, 0.1 mL of maintenance medium (DMEM + 2% FBS) was added to each well.

Cytotoxic effect

Morphological alteration of cells after the treatment with the extracts at different dilutions was verified every 24 h. Serial dilutions from a mixture of 2mL sterile water with 8mL of the extract were prepared and inoculated into cell cultures. Microscopic changes in the cells morphology are recorded when reading the tests for cytopathic effect (CPE). CPE was read by using an inverted microscope after the appropriate incubation time for each experiment. The results were recorded as "0" for no CPE, "1" (approximately 25% of the cells with CPE) to "4" (all of the cells with CPE).

Cell sensitivity to virus (check the reduction of the sensitivity to viruses)

0.1 mL of the lowest apparently non-cytotoxic dilution of the tested extract or PBS was distributed onto each of 6 established cell cultures in 96 wells microtiter plates. After 1h at 37°C the supernatant was discarded. The virus was diluted from 10^{-2} to 10^{-10} and titrated on the treated or untreated cells in parallel. There were used only those dilutions which:

- showed a low degree of cell destruction (< 25% of monolayer)
- produced a titer reduction of virus of < 1 lg.

Reference virus inactivation test

For the control of the system's validity, 2mL of the virus suspension with 8mL of PBS and 10mL of 1.4% (w/v) formaldehyde were mixed and after the contact times (5, 15, 30, and 60 min), further diluted 1:10 in ice-cold DMEM + 2% FBS. Dilutions up to 10-10 were prepared in ice-cold DMEM + 2% FBS and titrated on monolayer of HeLa cells.

For the cytotoxicity control of the formaldehyde test solution, 1mL of 1.4% formaldehyde was added to 1mL of phosphate buffer saline (PBS) and then serial dilutions were prepared.

Reduction of virus infectivity was calculated from differences of lg virus titers before and after treatment with the extracts.

Titration of the virus control

Instead of extract test solution, 8mL of water was added, mixed with 1mL of interfering substance (3 or 30 g/L BSA) and 1mL of the test virus suspension. The infectivity of the test virus suspension was determined under test conditions at contact times 0 min and 60 min. The viral CPE was read by using an inverted microscope after 72 h for poliovirus and after 120 h for adenovirus type 5.

Logarithmic titers $TCID_{50}$ were calculated according to nonparametric statistical procedure established by Spaerman and Karber [17, 35].

Results and Discussion

Cytotoxicity and virucidal effects on poliovirus of T. gallica extract and S. marianum extract or 1:1 combination of the two extracts

A suspension of a test virus was added to the product test extract under clean or dirty conditions and maintained at the adequate test temperature as mentioned in the methods. At least 6 cell culture wells were inoculated with each dilution. After the appropriate incubation time the viral cytopathic effect was evaluated, using an inverted microscope. Cytopathic effect (CPE) was noticed as the monolayer cells deteriorate due to the viral infection. This destruction of the cells in monolayer allowed monitoring and assessment of viral growth in the presence or absence of the *T. gallica* extract, *S. marianum* extract or both extracts (1:1). The results were recorded as “0” for no CPE, “1” (approximately 25% of the cells with CPE) to “4” (all of the cells with CPE).

The virus inactivation induced by the virucidal activity of each extract was calculated for each experimental point. Then the lg-reduction was determined by subtracting the logarithmic titer TCID₅₀ at any test point from the logarithmic titer TCID₅₀ of the virus control in similar experimental conditions. Results of the testing of virucidal effects of *T. gallica* extract and *S. marianum* extract or both extracts simultaneously on poliovirus type 1, LSc-2ab, are presented in Table I.

Table I

Results of the testing of virucidal effects of *T. gallica* and *S. marianum* extracts or 1:1 combination of the two extracts on poliovirus after 60 min. contact time.

| Product | Interferent Substance | Cytotoxicity level | lgTCID ₅₀ after 60 min |
|--|-----------------------|--------------------|-----------------------------------|
| <i>T. gallica</i> extract | 0.3 g/L BSA | 5 | 5.5 |
| <i>T. gallica</i> extract | 3 g/L BSA | 5.2 | 5.2 |
| <i>S. marianum</i> extract | 0.3 g/L BSA | 3.8 | 6.2 |
| <i>S. marianum</i> extract | 3 g/L BSA | 3.2 | 6.2 |
| <i>T. gallica</i> extract + <i>S. marianum</i> extract | 0.3 g/L BSA | 4.5 | 5.0 |
| <i>T. gallica</i> extract + <i>S. marianum</i> extract | 3 g/L BSA | 4.5 | 5.0 |
| Formaldehyde 0.7% (w/v) | PBS | 3.2 | 5.2 |
| Virus control | PBS | n.a. | 8.8 |
| | 0.3 g/L BSA | n.a. | 9.5 |
| | 3 g/L BSA | n.a. | 9.2 |

Note: Cytotoxicity level was recorded as “0” for no cytopathic effect (CPE), “1” (approximately 25% of the cells with CPE) to “4” (all of the cells with CPE).

Logarithmic titers TCID₅₀ were calculated according to nonparametric statistical procedure established by Spaerman and Karber [17,35].

PBS - phosphate buffer saline; BSA - Bovine serum albumin, n.a. - not applicable.

Despite *T. gallica* extract had greater cytotoxicity on HeLa cells (see Table I), the reduction of the residual infectivity titer could be followed over a range of more than 4 lgTCID₅₀ without being necessary to employ other techniques to eliminate cytotoxicity. *T. gallica* extract reduced the poliovirus infectivity to a lgTCID₅₀ of 5.5 after 60 min of treatment and in the presence of a high protein content of the medium, 3g/L BSA, lgTCID₅₀ was 5.2. *T. gallica* extract was able to reduce significantly poliovirus infectivity.

S. marianum extract alone was less cytotoxic but also less active on poliovirus (Figure 1). When cells were infected with virus suspension treated with both extracts simultaneously, the effect on poliovirus was increased comparing to the effects of each individual extracts, resulting a lgTCID₅₀ of 5.

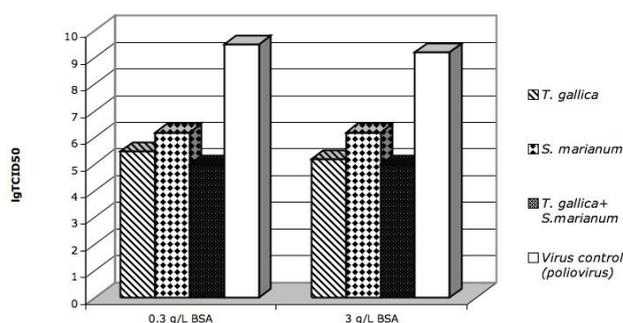


Figure 1.

Virucidal effects of *T. gallica* and *S. marianum* extracts on poliovirus type 1, LSc-2ab in the presence of the interferent substance, bovine serum albumin BSA 3g/L or BSA 0.3g/L. Logarithmic titers TCID₅₀ were calculated according to nonparametric statistical procedure established by Spaerman and Karber [17,35].

In the framework of the European Standard SR EN 14476+A1:2007 poliovirus was selected as a test virus because is highly resistant to chemicals, is acid-stable and is not affected by lipid solvents. Virucidal activity against poliovirus is therefore considered by this standard very relevant to consider the tested solution as a general virucide.

Cytotoxicity and virucidal effects on Adenovirus type 5 of T. gallica extract and S. marianum extract or 1:1 combination of the two extracts

The European Standard SR EN 14476+A1:2007, included also Adenovirus type 5, a non-enveloped DNA virus, as a test virus and *T. gallica* extract was able to decrease the infectivity of this virus from 9.2 to a lgTCID = 5.5 in the presence of low amount of interfering protein (0.3% BSA) while in the presence of high protein concentration (3% BSA) lgTCID

decreased from 9.5 to 5.2 which qualifies *T. gallica* as an effective virucid (Table II).

Table II

Results of the testing of virucidal effects of *T. gallica* and *S. marianum* extracts or 1:1 combination of the two extracts, on Adenovirus type 5 after 60 min. contact time.

| Product | Interferent Substance | Cytotoxicity level | IgTCID ₅₀ after 60 min |
|--|-----------------------|--------------------|-----------------------------------|
| <i>T. gallica</i> extract | 0.3 g/L BSA | 5 | 5.5 |
| <i>T. gallica</i> extract | 3 g/L BSA | 5.2 | 5.8 |
| <i>S. marianum</i> extract | 0.3 g/L BSA | 3.8 | 7.2 |
| <i>S. marianum</i> extract | 3 g/L BSA | 3.2 | 8.5 |
| <i>T. gallica</i> extract + <i>S. marianum</i> extract | 0.3 g/L BSA | 4.5 | 5.0 |
| <i>T. gallica</i> extract + <i>S. marianum</i> extract | 3 g/L BSA | 4.5 | 5.0 |
| Formaldehyde 0.7% (w/v) | PBS | 3.2 | 6.2 |
| Virus control | PBS | n.a. | 8.8 |
| | 0.3 g/L BSA | n.a. | 9.5 |
| | 3 g/L BSA | n.a. | 9.2 |

Note: Cytotoxicity level was recorded as "0" for no cytopathic effect (CPE), "1" (approximately 25% of the cells with CPE) to "4" (all of the cells with CPE).

Logarithmic titers TCID₅₀ were calculated according to nonparametric statistical procedure established by Spaerman and Karber [17, 35].

PBS - phosphate buffer saline; BSA - Bovine serum albumin; n.a. - not applicable.

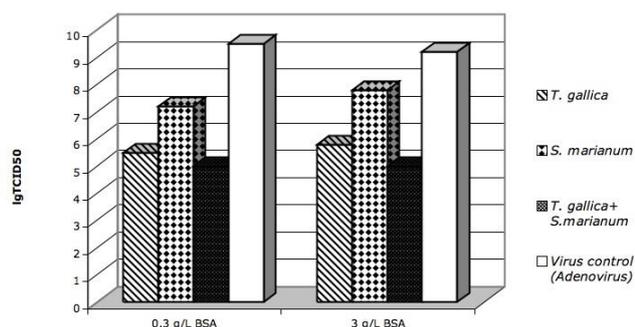


Figure 2.

Virucidal effects of *T. gallica* and *S. marianum* extracts or 1:1 combination of the two extracts, on Adenovirus type 5, in the presence of the interferent substance bovine serum albumine BSA 3% or BSA 0.3%. Logarithmic titers TCID₅₀ were calculated according to nonparametric statistical procedure established by Spaerman and Karber [17, 35].

S. marianum extract decreased the infectivity of Adenovirus type 5 only to a $\lg\text{TCID} = 7.2$ in the presence of 0.3% BSA, while in the presence of 3% BSA, $\lg\text{TCID}$ was 8.5. When Adenovirus type 5 was treated with both extracts simultaneously, infectivity was reduced to a $\lg\text{TCID} = 5$ which show that the 1:1 combination of the two extracts is more effective as virucid than each individual extract (Figure 2).

Verification of the methodology

Validity of the test was checked according to the European Standard SR EN 14476+A1:2007 and all criteria were fulfilled. Test virus suspensions had a $\text{TCID} > 10^{8.8}/\text{mL}$ in the absence or presence of BSA as interfering substance. Difference of the logarithmic titer of the control virus and of the test virus in the reference inactivation test (Formaldehyde 7% (w/v) final concentration) was 2.3 after 30 min and 3.6 after 60 min contact time (Table I), for poliovirus, as required by the standard. Cytotoxicity, in spite of being higher than expected on this tumoral cell line (HeLa), did not affect cell morphology, growth or susceptibility for the test viruses in the dilutions of the test mixture that demonstrated 4 lg reduction of the virus titer. Comparative virus titration on cells treated with the herbal extracts dilutions or on cells treated with PBS, resulted in a difference <1 lg of virus titer.

Generally, it is considered that the antiviral actions of the compounds from the two extracts tested here are related mainly to their antioxidant properties. Most of the previous studies showed hepatotonic, stimulant or hepatoprotective action of different components of *T. gallica* or *S. marianum* extracts and these were explained mainly by the ability of these compounds, to restore hepatic antioxidant enzyme activities and on the other hand to stop chemically-induced liver tumor proliferation.

Few studies to date, showed a direct anti-viral effect for silybin, and even this effect was considered to be *via* reducing the levels of some free radical species produced during viral gene expression, and in this manner it interferes with the regulation of some proteins necessary for virus replication [18].

Our study showed for the first time a general virucidal activity when testing the extracts according to the European Standard SR EN 14476+A1:2007, that specifies the test methods and the minimum requirements for virucidal activity of chemical disinfectants or antiseptic products. The standards impose strict testing methods on two non-enveloped viruses, one DNA and one RNA, highly resistant and stable in different

conditions, that qualified them as representative for the viruses that may contaminate human hand, medical instruments and surfaces.

Virucidal effect and effective stimulative actions on the immune system might assist the body in fighting off infection before the virus has had a chance to invade living cells. The herbal extracts used in this study might disrupt the life cycle of the viruses and inhibit further replication and infection of the cells. These extracts used in conjunction, have synergistic virucidal effect and also are able to promote detoxification and elimination enhancing action of the immune system.

Conclusions

The combination of the two-plant extracts subject of this study is valuable for general virucidal and the ability to sustain liver and biliary tract health. Association of this two plants containing bioactive substances with a pronounced hepatoprotective activity, anti-inflammatory and antioxidant effects led to a combination with synergistic activity between silymarin, bioactive component of milk thistle seeds (*Silybum marianum*) and French tamarisk (*Tamarix gallica*). Analysis of antiviral activity of the two extracts, complements and supports the positive results previously obtained on improving activities of "marker enzymes" - aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) - enzymes important in hepatocellular health.

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References

1. Ahmed-Belkacem, A.; Ahnou, N.; Barbotte, L.; Wychowski, C.; Pallier, C.; Brillet, R.; Pohl, R. T.; Pawlotsky, J. M. Silibinin and related compounds are direct inhibitors of hepatitis C virus RNA-dependent RNA polymerase. *Gastroenterology*. 2010; 138: 1112–1122.
2. Barbarino F., Neumann E., Deaciuc I., Ghelberg N.W., Suciuc A., Cotuțiu C., Effect of silymarin on experimental liver lesions, *Med. Interne*. 1981; 19: 347-357.
3. Bosisio E., Benelli C., Pirola O., Effect of the flavanolignans of *Silybum marianum* L. on lipid peroxidation in rat liver microsomes and freshly isolated hepatocytes, *Pharmacol. Res*. 1992; 25: 147-154.
4. Comelli M.C, Mengs U., Schneider C., Prosdociami M., Toward the definition of the mechanism of action of silymarin: activities related to cellular protection from toxic damage induced by chemotherapy, *Integr. Cancer Ther*. 2007, Jun; 6(2): 120-129.
5. Colturato, C. P.; Constantin, R. P.; Maeda Jr., A. S.; Constantin, R. P.; Yamamoto, N. S.; Bracht, A.; Ishii-Iwamoto, E. L.; Constantin, J. Metabolic effects of silibinin in the rat liver. *Chem. Biol. Interact*. 2012; 195: 119–132.

6. Costea T., Istudor V., Fierăscu R., Fierăscu I., Botez A., Researches upon indigenous herbal products for therapeutic valorification in metabolic diseases, note I. *Betulae folium* and *Rubi idaei folium*, sources of micro- and macroelements, *Farmacia*. 2013; 61(1): 162-169.
7. Deak G., Muzes G., Lang I., Nekam K., Gonzalez-Cabello R., Gergely P., Effects of two bioflavanoids on certain cellular immune reactions *in vitro*, *Acta Physiol. Hung.* 1990; 76: 113-121.
8. Desplaces A., Choppin J., Vogel G., Trost W, The effects of silymarin on experimental phalloidine poisoning, *Arzneimittelforschung*, 1975; 25: 89-96.
9. Fried M. W., V. J. Navarro, N. Afdhal, S. H. Belle, A. S. Wahed, R. L. Hawke, E Doo, C. M. Meyers, K. Rajender; Reddy Effect of Silymarin (Milk Thistle) on Liver Disease in Patients With Chronic Hepatitis C Unsuccessfully Treated with Interferon Therapy a Randomized Controlled Trial, *JAMA*. 2012; 308(3): 274-282.
10. Hanje AJ, Fortune B, Song M, Hill D, McClain C. The use of selected nutrition supplements and complementary and alternative medicine in liver disease. *Nutr. Clin. Pract.* 2006; 21: 255-272.
11. Ionescu D., Mihele D., Rizea G. D., Manea S., Research on hepatoprotective effect of a new herbal product, 8th International Conference of PHD students, University of Miskolc, Hungary, 6-10 August 2012.
12. Kamel Chaieb d, Amina Bakrouf d, Christian Magné c, Chedly Abdelly Mohamed A.A. Orabi a,c, Shoko Taniguchi a, Susumu Terabayashi b, Tsutomu Hatano, Hydrolyzable tannins of tamaricaceous plants. IV: Micropropagation and ellagitannin production in shoot cultures of *Tamarix tetrandra*, *Phytochemistry*. 2011; 72: 1978-1989.
13. Kim Y.C., Kim E.J., Lee E.D., Kim J.H., Jang S.W., Kim Y.G., Comparative bioavailability of silibinin in healthy male volunteers, *Int. J. Clin. Pharmacol. Ther.* 2003; 41: 593-596.
14. Luper S., A review of plants used in the treatment of liver disease, Par.1, *Altern. Med. Rev.* 1998; 3: 410-421.
15. Loguercio C., P. Andreone , C Brisc, M. C. Brisc, E. Bugianesi, M. Chiaramonte, C. Cursaro, M. Danila, I. de Sio, A. Floreani, M. A. Freni, A. Grieco, M. Groppo, R. Lazzari, S. Lobello, E. Lorefice, M. Margotti, L Miele, S. Milani, L. Okolicsanyi, G. Palasciano, P. Portincasa, P. Saltarelli, A Smedile, F Somalvico, A. Spadaro, I Sporea, P Sorrentino, R Vecchione, C. Tuccillo, C. Del Vecchio Blanco, A. Federico, Silybin combined with phosphatidylcholine and vitamin E in patients with nonalcoholic fatty liver disease: A randomized controlled trial. *Free Radical Biology & Medicine*. 2012; 52: 1658-1665.
16. Fallah Huseini F., Alavian S.M., Heshmat R., Heydari M.R., Abolmaali K.. The efficacy of Liv-52 on liver cirrhotic patients: a randomized, double-blind, placebo-controlled first approach. *Phytomedicine*. 2005; 12(9): 619-624.
17. Finney, D.J. Statistical method in biological assay. Third Edition. Charles Griffin & Co. Ltd, London, England, 1978, 508 pp.
18. Mehrab-Mohseni, M.; Sendi, H.; Steuerwald, N.; Ghosh, S.; Schrum, L. W.; Bonkovsky, H. L. Legalon-SIL downregulates HCV core and NS5A in human hepatocytes expressing full-length HCV. *World J. Gastroenterol.* 2011; 17: 1694-1700.
19. Mendal JN, Roy BK. Studies with Liv.52 in the treatment of infective hepatitis, chronic active hepatitis and cirrhosis of the liver. *Probe*. 1983; 22: 217.
20. Morazzoni P., Bombardelli E., *Silybum marianum* (*Cardus marianus*), *Fitoterapia*, 1994; 66: 3-42.
21. Muriel P., Mourelle M., Prevention by silymarin of membrane alterations in acute carbon tetrachloride liver damage, *J. Appl. Toxicol.* 1990; 10: 275-279.
22. Nencu I., Vlase L., Istudor V., Duțu L.E., Gîrd E.C., Preliminary research regarding the therapeutic uses of *Urtica dioica* L. note I. the polyphenols evaluation, *Farmacia*. 2012, 60(4): 493- 500.
23. Nurzyńska-Wierdak R., Bogucka-Kocka A. ,Sowa I.,Szymczak G.,The composition of essential oil from three ecotypes of *Origanum vulgare* L. ssp. *vulgare* cultivated in Poland, *Farmacia*. 2012; 60(4): 571-577.

24. Nitin Dixit, Sanjula Baboota, Kanchan Kohli, S. Ahmad, Javed Ali, Silymarin: A review of pharmacological aspects and bioavailability enhancement approaches, *Indian Journal of Pharmacology*. 2007; vol. 39, Issue 4: 172-179.
25. Parimala Devi B., Manoharan K., Antiviral medicinal plants - an ethnobotanical approach - review article, *Journal of Phytology*. 2009; 1(6): 414-421.
26. Pepping J., Milk thistle: *Silybum marianum*, *Am. J. Health Syst. Pharm.* 1999; 56: 1195-1197.
27. Pradhan S.C., Girish C., Hepatoprotective herbal drug, silymarin from experimental pharmacology to clinical medicine, *Indian J. Med. Res.* 2006; 124: 491-504.
28. Rambaldi A, Jacobs BP, Gluud C. Milk thistle for alcoholic and/or hepatitis B or C virus liver diseases. *Cochrane Database of Systematic Reviews* 2007, Issue 4. Art. No.: CD003620.
29. Ramellini G., Meldolesi J., Liver protection by silymarin: *In vitro* effect on dissociated rat hepatocytes, *Arzneimittelforschung*. 1976; 26: 69-73.
30. Riadh Ksouri, Antioxidant and antimicrobial activities of the edible medicinal halophyte *Tamarix gallica L.* and related polyphenolic constituents, *Food and Chem. Toxicol.* August 2009; Vol. 47, Issue 8: 2083-2091.
31. Robu S., Aprotosoae A.C., Miron A., Cioancă O., Stănescu U., Hăncianu M., *In vitro* antioxidant activity of ethanolic extracts from some *Lavandula species* cultivated in Romania, *Farmacia*. 2012; 60(3): 394-401.
32. Saller R., Meier R., Brignoli R., The use of silymarin in the treatment of liver diseases, *Drugs*. 2001; 61: 2035-2063.
33. Sehrawat Anuradha and Sultana Sarwat, *Tamarix gallica* ameliorates thioacetamide-induced hepatic oxidative stress and hyperproliferative response in Wistar rats, *J. of Enzyme Inhibition and Med. Chem.* 2006; Vol. 21, 2: 215-223.
34. Sehrawat A, Sultana S, Evaluation of possible mechanisms of protective role of *Tamarix gallica* against DEN initiated and 2-AAF promoted hepatocarcinogenesis in male Wistar rats, *Life Sci.* 2006 sept. 5; 79(15): 1456-1465.
35. European Standard EN 14476+A1:2007, European Committee for Standardization, Bussels, 2007.
36. Sonnenbichler J., Zetl I., Biochemical effects of the flavonolignane silibinin on RNA, protein and DNA synthesis in rat livers, *Progr. Clin. Biol. Res.* 1986; 213: 319-331.
37. Thakur S.K., Silymarin - A hepatoprotective agent, *Gastroenterol. Today*. 2002; 6: 78-82.
38. Valenzuela A., Garrido A., Biochemical bases of the pharmacological action of flavanoid silymarin and its structural isomer silibinin, *Biol. Res.* 1994; 27: 105-112.
39. Yao, J.; Zhi, M.; Minhu, C. Effect of silybin on high-fat-induced fatty liver in rats. *Braz. J. Med. Biol. Res.* 2011; 44: 652-659.

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