

## SUPPLEMENTARY MATERIAL

### **Silymarin from *Silybum marianum* by Naviglio's extractor: a new and very efficient approach**

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#### **ABSTRACT**

The aim of this work is to compare new and traditional extraction methods to obtain silymarin from *Silybum marianum*, a biennial herbaceous plant of the Asteraceae family, present throughout the Mediterranean basin and used to treat several diseases. Silymarin primarily contains flavonolignans and flavonoids and is used in some pharmaceutical preparations to improve of liver function and as a protective against some hepatotoxins. In six extracts obtained by new and traditional extraction methods, the total contents of silymarin and its main flavonolignans, total phenols and condensed tannins were evaluated in addition to their respective antioxidant capacities. By the Naviglio extractor, that is a rapid solid-liquid dynamic extraction method, it is possible to obtain a fraction quantitatively more abundant than other methods, and with a lower content of tannins and phenolic compounds but with a higher content of flavonolignans, rare and expensive, and therefore easier to separate and purify.

**Running headline:** Silymarin from *Silybum marianum*

## Experimental

### ***Plant Material and Experimental Design***

The plant material was provided by the Research Center *Tecno Bios* srl, Piazza S. Giuseppe Moscati, 8 - 82030 Apollosa – Benevento and identified by Prof. Antonino Pollio of the Department of Biology, University Federico II, Complesso Universitario Monte S. Angelo, IT-80126 Napoli, Italy. A sample specimen (HERBAZLS 317) has been deposited in the herbarium of *Tecno Bios* srl. The seeds were separated from the rest of the biomass and subsequently reduced to powder and stored at -4°C until use.

### ***Maceration Extraction***

In this experiment, 100 g of powdered seeds of *S. marianum* were first defatted with hexane (3x1000 mL), then extracted with ethanol (3x1000 mL, EtOH/ME), and subsequently with MilliQ water (3x1000 mL, H<sub>2</sub>O/ME), all in a dark glass container. Maceration was carried out for 3 days at room temperature with occasional stirring. Finally, the solutions were filtered (on Whatman No. 1 filter paper), dried under vacuum, weighed (Table 1) and kept at a constant temperature of 4°C until use.

### ***Decoction Extraction***

In this experiment, 100 g of powdered seeds of *S. marianum* were mixed with 1,000 mL of MilliQ water in a 2.0 L beaker and then heated in a 100°C water bath for 10 min. The fraction (H<sub>2</sub>O/DE) was separated from the solid material, filtered (on Whatman No. 1 filter paper), dried under vacuum, weighed (Table 1) and kept at a constant temperature of 4°C until use.

### ***Soxhlet Extraction***

In this experiment, 10 g of powdered seeds of *S. marianum* were defatted in a Soxhlet apparatus for 6 h with hexane (3x100 mL) and then extracted for 6 h with ethanol (3x100 mL, EtOH/SE). Finally, the solution was filtered (on Whatman No. 1 filter paper), dried under vacuum, weighed (Table 1) and kept at a constant temperature of 4°C until use.

### ***Rapid Solid-Liquid Dynamic Extraction***

The rapid cyclic solid-liquid dynamic extraction method utilizes a *Naviglio* Extractor (Figure S1), which is equipped with two extraction chambers and related steel pistons. The *Naviglio* Extractor is based on a suction effect generated by a compression of the extraction solvent (EtOH or H<sub>2</sub>O, 3000 mL) on the solid (300 g), at approximately 7 bar of pressure for a fixed time (2 min), which is then followed by a subsequent immediate decompression at approximately 0-1 bar of pressure (*Naviglio Principle*).

Due to the effect of the pressure gradient, there is a rapid release of the *extraction* liquid from the inside of the solid matrix, which releases the extractable substances (EtOH/RSLE and H<sub>2</sub>O/RSLE). An extraction cycle comprises a static phase and a dynamic phase. During the static phase, the system is maintained under pressure for 2 min, in this phase, the liquid penetrates inside the solid and permeates all empty spaces, also crossing the cell walls of the plants in a more effective manner than maceration. Once the static phase is complete, the dynamic phase (1 min) begins immediately and fulfils two objectives: precise generation of a negative pressure gradient between the inside and the outside of the solid matrix, which represents the driving effect of the extraction, and mixing of the liquid to avoid the formation of over-saturated areas of the compounds extracted near the surface of the solid.

### ***Total Phenols Content***

For the determination of the phenol content, the protocol performed was the modified method proposed by Serra et al. (Serra, 2008) with 5 µL of fraction, 120 µL of distilled H<sub>2</sub>O and 125 µL of Folin-Ciocalteu reagent added. The solution was first stirred and allowed to stand for 6 min; after that, 1.25 mL of 10% NaHCO<sub>3</sub> was added and then incubated at room temperature for 90 min. At the end of the incubation, the spectrophotometric determination at the wavelength of 760 nm was carried out. The total phenol content was expressed as mg equivalents of gallic acid (GA) per g of dry weight of the considered extract (mg GA/g DW)).

The total phenolic content was significantly ( $p < 0.05$ ) affected by extraction procedure (Figure S2). The highest concentrations were found in ethanolic extracts obtained by RSLE, maceration and Soxhlet extraction (EtOH/RSLE, EtOH/ME and EtOH/SE), with values of 81, 70 and 56 mg GA/g, respectively).

The aqueous extracts are generally characterized by a lower content of phenolic compounds, but the one which contains the most is the extract obtained by decoction (H<sub>2</sub>O/DE), approximately 60% of the richest ethanolic extract obtained with the Naviglio extractor (EtOH/RSLE).

### ***Condensed Tannins Content***

In this experiment, 1.0 mL of the fraction was added to 2 mL of a 10% HCl solution in MeOH (v/v) and to 3 mL of a 5% vanillin solution in MeOH (w/v). The solution absorbance was measured at 500 nm in a UV/Vis spectrophotometer, after 30 min at 35°C in a water bath, using a catechin calibration curve. The total tannin content was expressed as mg equivalents of catechin (CE) per g of dry weight of the considered extract (mg CE/g DW)).

The condensed tannins ranged from 350 mg to 115 mg CE/g, with significant differences being observed among the different extraction techniques. The highest value was presented by the ethanolic extract obtained by maceration (EtOH/ME), followed by the water extract derived by decoction (H<sub>2</sub>O/DE) (350 and 258 mg CE/g, respectively, Figure S3), while the other extracts exhibit a quantity of tannins lower than 212 mg CE/g. It is interesting to note that the ethanolic extracts obtained by RSLE (EtOH/RSLE) and by maceration (EtOH/ME) (212 and 350 mg CE/g, respectively) contained almost twice as much as the corresponding aqueous extracts H<sub>2</sub>O/RSLE and H<sub>2</sub>O/ME (115 and 166 mg CE/g, respectively). These extracts, as rich sources of natural tannins, could find interesting applications in the pharmaceutical and food industries.

### ***DPPH-Based Assay to Estimate the Antioxidant Capacity***

The different extracts of *S. marianum* were tested for their scavenging effects towards the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical at different concentration levels. Tests have been carried out by performing three replicate measurements on three samples (n = 3) of each extract. The results are expressed as the mean ± SD values. The reaction is initiated upon mixing 3.9 mL of the radical DPPH solution with 0.1 mL of plant extract. After 30 min in the light and at room temperature, the absorption at 517 nm was measured by a Jasco UV-Vis V560 spectrophotometer in reference to a blank sample. The results (Figure S4) were expressed as mg TEAC g<sup>-1</sup> (Trolox Equivalent Antioxidant Activity) of dry weight, which was based on a calibration curve of Trolox.

The ethanolic extracts obtained by RSLE (EtOH/RSLE) and by maceration (EtOH/ME), and the aqueous extract obtained by decoction (H<sub>2</sub>O/DE), were the most active, with antioxidant capacities between 350 and 280 mg TEAC/g. A consistently lower antioxidant capacity was measured for the more polar extracts, namely, the ethanolic extract obtained by Soxhlet (EtOH/SE) extraction and the aqueous varieties obtained by RSLE (H<sub>2</sub>O/RSLE) and by maceration (H<sub>2</sub>O/ME), with values ranging between 159 and 121 mg TEAC/g. This means that the plant constitutes a possible raw material to obtain natural antioxidants for use in the food industry, considering that some synthetic antioxidants commonly used have been demonstrated to possess toxicity and threaten the risk of possible carcinogenic effects (Wojcik, 2010).

### ***Silymarin isolation***

Each extract was solubilized in methanol, slowly stirred, at room temperature for 20 minutes (3x30 mL). Finally, the suspension was centrifuged at 3000 rpm for 3 minutes, filtered, dried under vacuum, weighed (Table 1) and kept at a constant temperature of 4°C until use.

### ***Identification and Quantification of Phenolic Compounds by LC-MS***

To complete the quantification of the compounds, each fraction obtained was dried, dissolved in tetrahydrofuran and passed through an MNYL type nylon membrane with 0.2 µm pore size (Kang et al. 2016). Identification and quantification of phenolic compounds was determined with a Shimadzu (Milan, Italy) LCMS-8040 Triple Quadrupole Liquid Chromatograph Mass Spectrometer (LC-MS/MS). Separations were accomplished on a Phenomenex Luna C18 column (100 mm x 2.1 mm, 1.6 µm) at a flow rate of 0.25 mL/min with H<sub>2</sub>O: CH<sub>3</sub>OH: CH<sub>3</sub>CN: TFA = 60: 35: 4.9: 0.1 as mobile phase (v/v/v/v). The column temperature was maintained at 27°C and the injection volume was 10 µL. The sample was dissolved in a 1: 1 (v/v) mixture of tetrahydrofuran and eluent mixture (Figure S5). Separation of compounds was monitored at 288 nm and with a mass spectrometer detector. Mass spectrometric analysis (ESI) was carried out on an LC-MS 8030 triple-quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The liquid chromatography–tandem mass spectrometer was utilized in both negative and positive ionization modes, with spectra acquired over a mass range of 50 to 700 m/z. The acquisition parameters were as follows: interface

voltage, 4.5 kV; interface temperature, 230°C; desolvation line temperature, 230°C; heat block temperature, 340°C; desolvation gas, nitrogen; desolvation gas flow rate, 3.5 L/min; drying gas, nitrogen; drying gas flow rate, 20 L/min; collision gas, argon; dwell time, 18 ms; and collision gas pressure, 230 kPa. The most appropriate precursor ions, daughter ions, cone voltages, and collision energies (CE) were adjusted according to the analyte of interest. The peaks of the present compounds were determined on the basis of their mass data and retention times and were quantified from the calibration curves (area under the peaks) obtained from the corresponding standards (Table 1).

### ***Statistical analysis***

At the end of the treatments, the data were processed using the Statistical Package for Social Sciences (version 10.0, SPSS Inc., Chicago, Illinois, USA). A regression binary logistic analysis was performed. The statistical significance level was established at  $P < 0.05$ .

### **References**

- Serra A.T, Matias A.A, Nunes A.V, Leitão M.C, Brito D, Bronze R, Silva S, Pires A, Crespo M.T, San Romao M.V, Duarte C.M. 2008. In vitro evaluation of olive- and grape-based natural extracts as potential preservatives for food. *Innov Food Sci Emer.* 9:311–319. DOI: [org/10.1016/j.ifset.2007.07.011](https://doi.org/10.1016/j.ifset.2007.07.011).
- Wojcik, M.; Burzynska-Pedziwiatr. I.; Wozniak, L.A. A review of natural and synthetic antioxidants important for health and longevity. *Curr Med Chem* 2010, 17, 3262–3288. DOI: [10.2174/092986710792231950](https://doi.org/10.2174/092986710792231950)

**Table S1.** Concentration of phenolic compounds found in *S. marianum* fractions. % respect to silymarin.

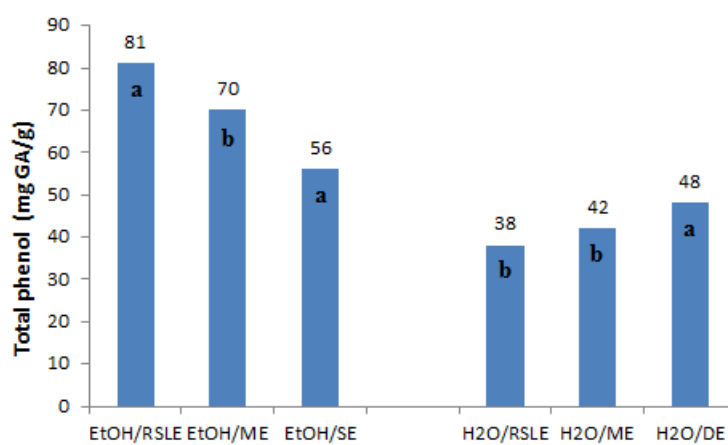
|                         | EtOH/RSLE                      | EtOH/SE           | EtOH/ME           | H <sub>2</sub> O/RSLE | H <sub>2</sub> O/ME | H <sub>2</sub> O/DE |
|-------------------------|--------------------------------|-------------------|-------------------|-----------------------|---------------------|---------------------|
| Mass yield <sup>a</sup> | 4.8±0.8                        | 4.1±0.6           | 5.5±0.9           | 4.0±0.5               | 3.8±0.5             | 2.6±0.2             |
| Silymarin <sup>b</sup>  | 1412±72                        | 1225±65           | 1452±60           | 1095±58               | 1108±55             | 1391±71             |
| Silybin A <sup>b</sup>  | 255±7.5<br>(18.1) <sup>c</sup> | 125±3.1<br>(10.2) | 175±8.0<br>(12.0) | 91±1.9<br>(8.3)       | 75±1.8<br>(6.8)     | 175±8.1<br>(12.6)   |
| Silybin B <sup>b</sup>  | 283±9.0<br>(20.0)              | 139±4.5<br>(11.3) | 193±8.1<br>(13.3) | 85±2.0<br>(7.8)       | 91±2.0<br>(8.2)     | 191±5.1<br>(13.7)   |
| Silychristin A          | 205±10.0<br>(14.5)             | 125±4.5<br>(10.2) | 175±6.6<br>(9.2)  | 133±5.5<br>(8.0)      | 90±1.8<br>(8.1)     | 146±4.5<br>(10.5)   |
| Silydianin              | 107±3.8<br>(7.6)               | 65±1.5<br>(5.3)   | 193±6.9<br>(4.3)  | 53±0.8<br>(4.8)       | 46±0.5<br>(4.2)     | 51±0.5<br>(3.7)     |
| Isosilybin              | 49±1.2<br>(3.5)                | 26±0.5<br>(2.2)   | 175±4.0<br>(2.0)  | 14±0.2<br>(1.3)       | 20±0.3<br>(1.8)     | 36±0.3<br>(2.6)     |
| Taxifolin <sup>b</sup>  | 75±2.2<br>(5.3)                | 131±3.5<br>(10.7) | 112±2.9<br>(7.7)  | 59±1.5<br>(5.4)       | 108±2.0<br>(9.7)    | 115±2.2<br>(8.3)    |

<sup>a</sup> in g/100 g of dry plants; <sup>b</sup> expressed in mg; <sup>c</sup> mg/100 g of Silymarin (mean ± SD).

**Figure S1.** Naviglio 's extractor.

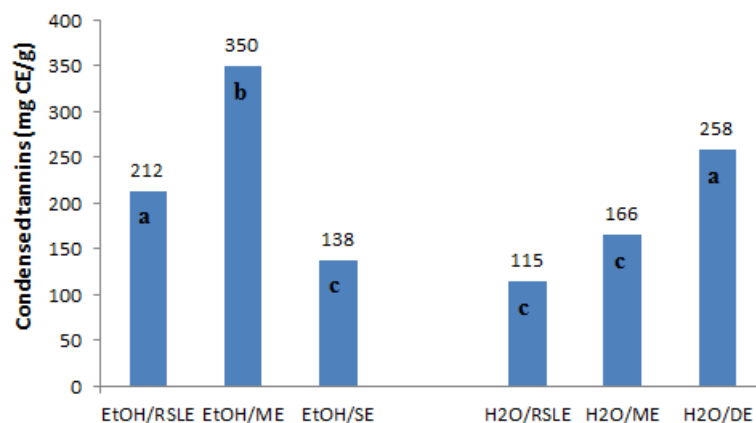


**Figure S2.** Total phenol concentration in different extracts of *S. marianum*. a-c  
Different letters indicate significant difference ( $p \leq 0.05$ ).

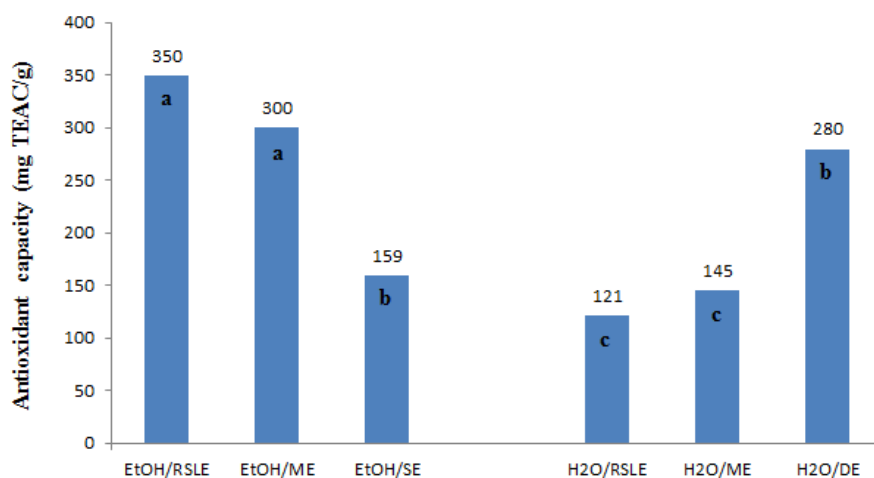




**Figure S3.** Concentrations of condensed tannins in different extracts of *S. marianum*. <sup>a-c</sup> Different letters indicate significant differences ( $p \leq 0.05$ ).



**Figure S4.** Antioxidant capacities among different extracts of *S. marianum*. <sup>a-c</sup> Different letters indicate significant differences ( $p \leq 0.05$ ).



**Figure S5.** HPLC profile of a solution of EtOH/RSLE in methanol.

