applied sciences

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*Supplementary*

# Potential Role of Bioactive Compounds: In Vitro Evaluation of the Antioxidant and Antimicrobial Activity of Fermented Milk Thistle

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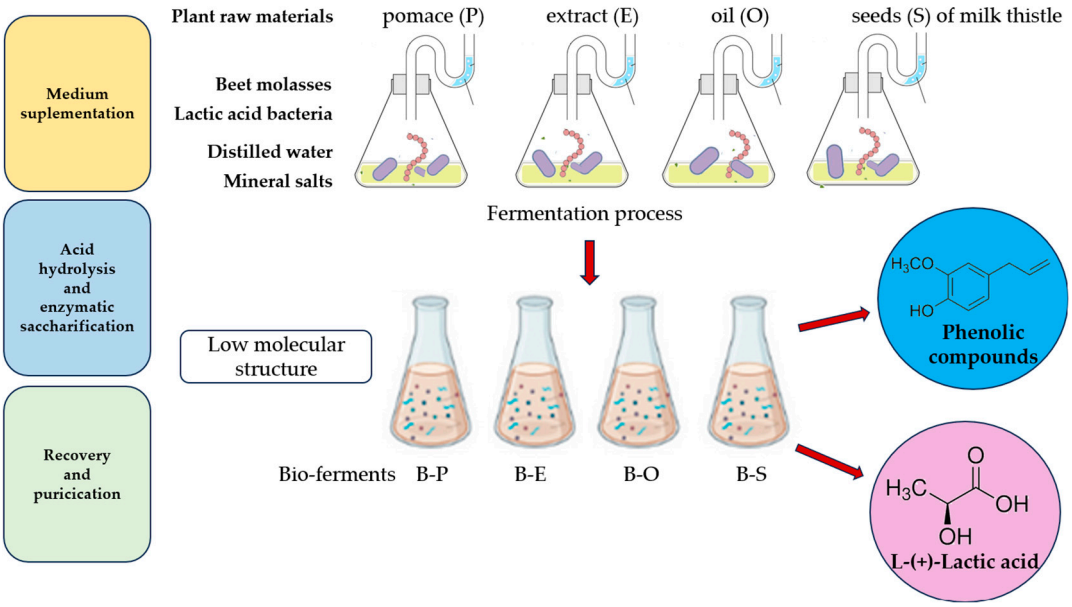



**Figure S1.** A scheme for obtaining bio-ferments from seeds (B-S), extract (B-E), oil (B-O), and pomace (B-P) of milk thistle.

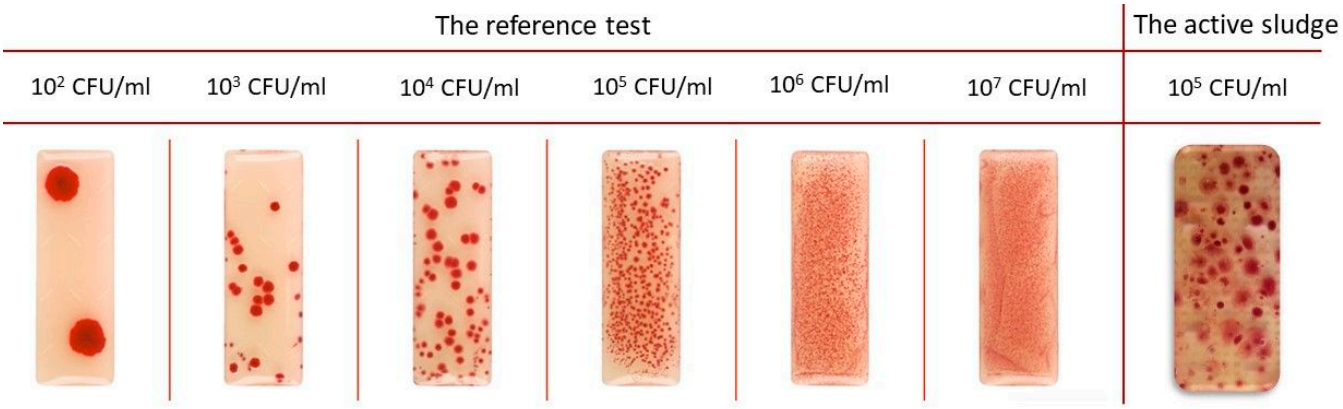



**Figure S2.** The appearance of the test obtained after immersion of the insert in the active sludge – rights, the appearance of the reference test - left.

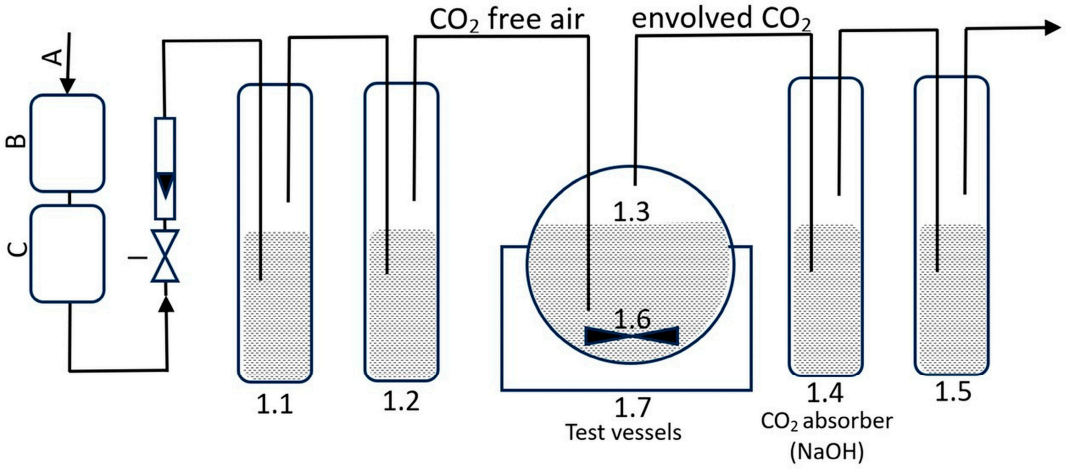



**Figure S3.** The carbon dioxide measurement method.

Figure S3 presents the arrangement of the test vessels (1.1, 1.2, 1.3, 1.4, and 1.5) and the placement of a magnetic stirrer (1.6) connected with tubes. The test apparatus was aerated by air (I) passing through carbon dioxide ($CO_2$) absorbers (B and C). The velocity of the air was regulated by a valve, which was routed to a carbon dioxide absorber (1.1), followed by a $CO_2$ indicator (1.2), with the intention of indicating the presence of carbon dioxide in the air via turbidity. A total of 250 mL of the test medium, 25 mg of dried active sludge, and bio-ferments corresponding to 40 mg/L of organic carbon were deposited in test vessel 1.3.

The bio-ferments capacity for biodegradation resulted in the production of $CO_2$ in vessel 1.3, which reacted with sodium hydroxide (NaOH) to produce sodium carbonate ($Na_2CO_3$):

$$2\ NaOH + CO_2 = Na_2CO_3 + H_2O \quad (1)$$

To determine the $CO_2$ concentration in vessel 1.3, 10 mL of the solution from vessel 1.4 was moved into a 25 mL volumetric flask. Deionized water was added to the flask until the designated maximum level was reached. Next, the sample was analyzed (three times) with total organic carbon analyzer as follows:

$$3\ Na_2CO_3 + 2\ H_3PO_4 = CO_2 + 2\ Na_3PO_4 + 3\ H_2O$$

$$3\ NaHCO_3 + H_3PO_4 = CO_2 + Na_3PO_4 + 3\ H_2O \qquad (2)$$

The test sample comprises carbonates ($Na_2CO_3$) and acidic carbonates ($NaHCO_3$) that were acidified with orthophosphoric acid ($H_3PO_4$) to achieve a pH of 2–3. $Na_2CO_3$ and $NaHCO_3$ acidified with $H_3PO_4$ are converted to $CO_2$. First, sodium bicarbonate ($NaHCO_3$) and sodium carbonate ($Na_2CO_3$) calibration curves were created. In order to achieve this, 4.415 g of $Na_2CO_3$ (which had been dried for two hours at 285 °C in a muffle furnace) and 3.500 g of $NaHCO_3$ (which had been dried for two hours over silica gel) were added to a 1000 mL flask. The flask was then filled with deionized water (previously boiled). From the starting solution thus prepared, dilutions of sodium carbonate and sodium bicarbonate were prepared in the concentration range of 0–100 mg/L inorganic carbon (IC), and a calibration curve was prepared. The volume of the dispensed sample was 50 µL.

The carbon dioxide measuring apparatus as seen in Figure S3 made up of the following parts:

I: air with an aeration rate of 50–100 mL/min, used to aerate the all test systems;

B and C: $CO_2$ absorber (potassium hydroxide);

1.1: $CO_2$ absorber (potassium hydroxide at 5 mol/L concentration);

1.2: $CO_2$ indication (barium hydroxide at 0.01 mol/L concentration);

1.3: 500 mL capacity test vessels stirred with magnetic stirrer (1.6);

1.4: $CO_2$ absorber (sodium hydroxide at 0.05 mol/L concentration);

1.5: $O_2$ absorber (distilled water);

1.7: container filled with distilled water, inside which test vessels with a 500 mL capacity were placed;

1.8: cryostat that precisely sets the temperature of the distilled water in the container. Incubation was carried out at 23 ± 0.5 °C for 28 days.

Figure S4 shows a representative of chromatographs identifying individual phenolic acids in bio-ferments from seeds (B-S), extract (B-E), oil (B-O), and pomace (B-P) of milk thistle.

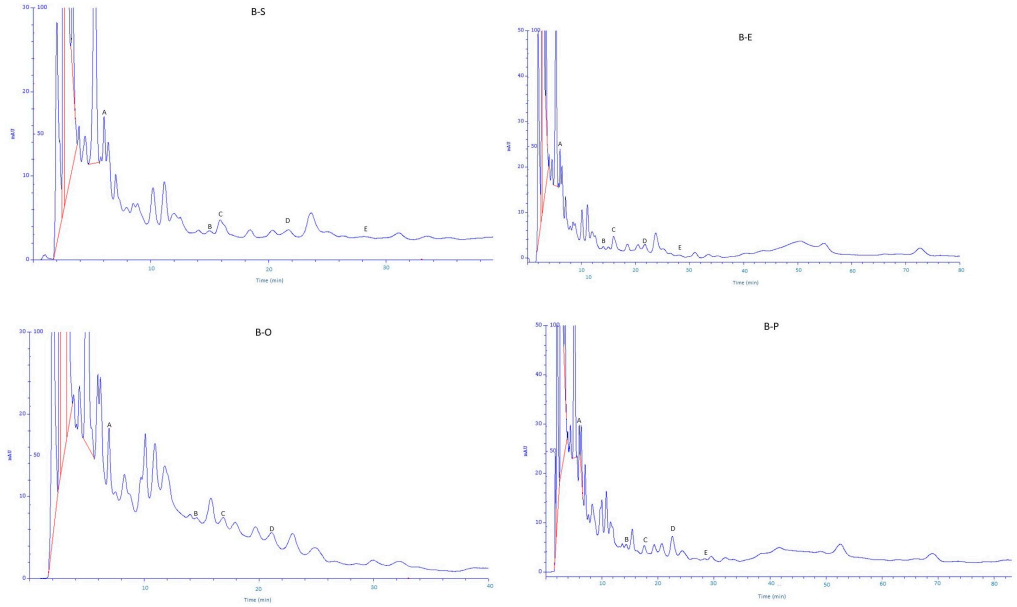



**Figure S4.** The representative of chromatographs identifying individual phenolic acids in bio-ferments from seeds (B-S), extract (B-E), oil (B-O), and pomace (B-P) of milk thistle: A - gallic acid,

RT=6.455 min; B - protocatechuic acid, RT=14.525 min; C - caffeic acid, RT=16.673 min; D - neochlorogenic acid, RT=21.027 min, and E - coumaric acid, RT=28.209 min.

Figure S5 shows an example chromatogram of a bio-ferment containing octane as an internal standard (RT=4.73 min) and lactic acid (RT=and 5.92 min).

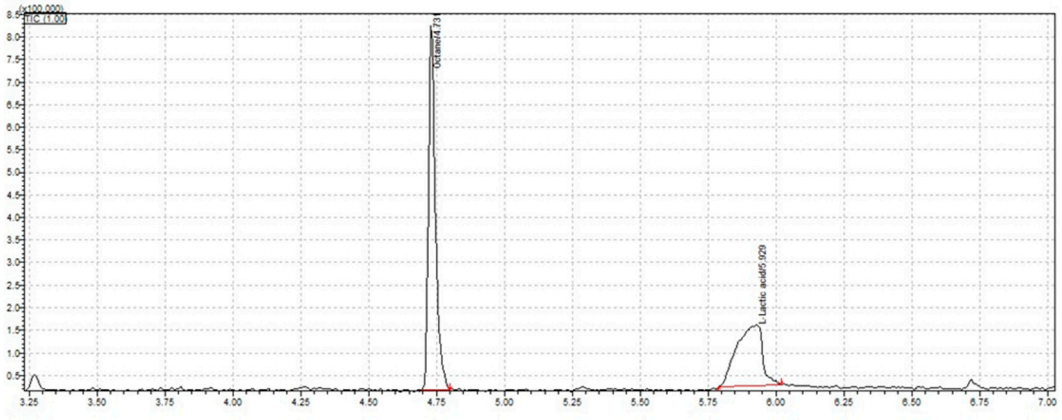


**Figure S5.** The representative chromatogram of a bio-ferment containing internal standard (octane RT=4.73 min) and lactic acid (RT=and 5.92 min).

**Table S1.** Table S1. Presents the results of monitoring the total polyphenols content production of the tested bio-ferments.

| **Day** | **Bio-ferments** | | | |
|---|---|---|---|---|
| | B-P | B-E | B-O | B-S |
| Total polyphenols content (TPC) | mg GA/L B | mg GA/L B | mg GA/L B | mg GA/L B |
| 1 | 679.11 | 601.07 | 682.09 | 599.01 |
| 2 | 778.12 | 701.02 | 782.09 | 794.00 |
| 3 | 872.10 | 891.01 | 881.44 | 899.22 |
| 4 | 998.14 | 901.01 | 982.39 | 992.00 |
| 5 | 1072.16 | 1001.02 | 1088.09 | 1099.00 |
| 6 | 1278.15 | 1103.21 | 1182.49 | 1279.03 |
| 7 | 1475.10 | 1301.01 | 1495.09 | 1394.01 |
| 8 | 1578.110 | 1409.11 | 1582.22 | 1499.45 |
| 9 | 1589.17 | 1467.61 | 1605.00 | 1588.05 |
| 10 | 1733.15 | 1629.01 | 1798.01 | 1607.00 |
| 11 | 1932.15 | 1827.01 | 1972.07 | 1808.44 |

| | | | | |
|---|---|---|---|---|
| 12 | 2111.13 | 2049.02 | 2198.04 | 2016.05 |
| 13 | 2533.11 | 2429.01 | 2592.09 | 2308.00 |
| 14 | 2546.69 | 2439.52 | 2599.43 | 2306.82 |

**Table S2.** Presents the results of the monitoring of the phenolic compound production of the tested bio-ferments from pomace (B-P), extract (B-E), oil (B-O), and seeds (B-S) of milk thistle.

| **Phenolic acid** | B-P | B-E | B-O | B-S |
|---|---|---|---|---|
| | (mg/L B) | | | |
| gallic acid | 9.01-44.25 | 6.98-17.76 | 3.99-17.83 | 6.97-34.26 |
| protocatechuic acid | 2.045-16.44 | 3.33-9.30 | 1.02-4.59 | 2.87-6.21 |
| caffeic acid | 10.22-41.42 | 7.01-19.17 | 4.33-23.29 | 4.98-15.98 |
| neochlorogenic acid | 1.44-7.12 | 1.01-5.20 | 0.00-0.19 | 2.22-6.21 |
| coumaric acid | 2.09-10.97 | 1.98-7.15 | n.d. | 3.09-10.13 |

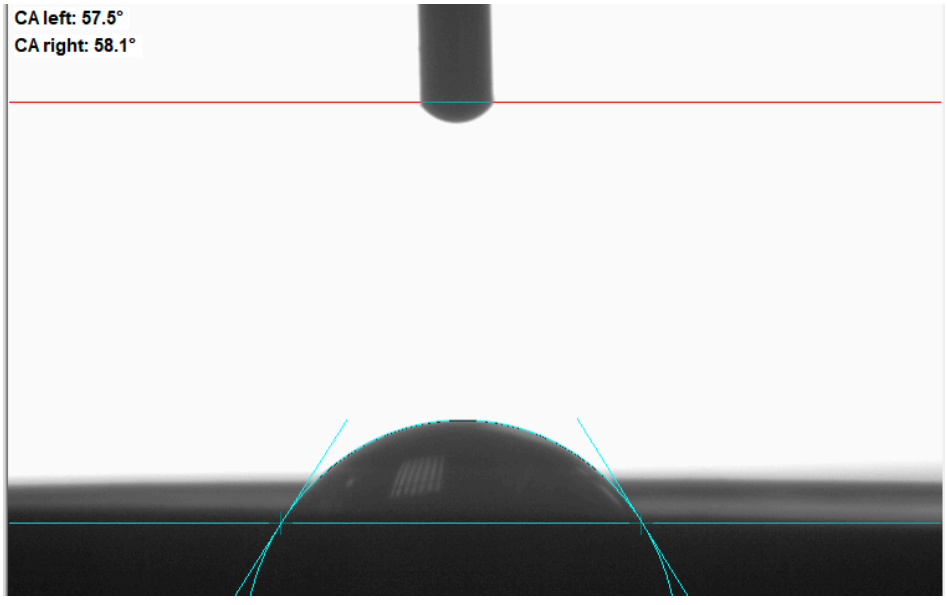
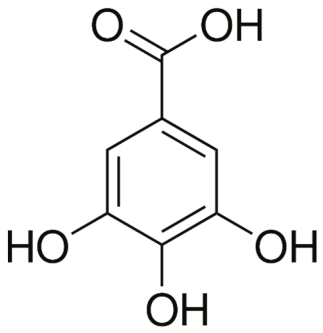
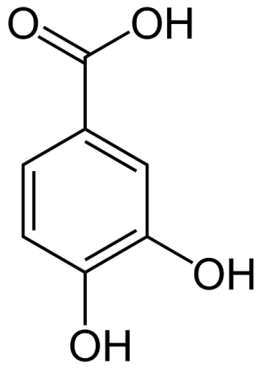



**Figure S6.** Contact angle (c.a.$_{av.}$) of the control samples (distilled water): c.a.$_{av.}$ = 57.8° ± 1.0.

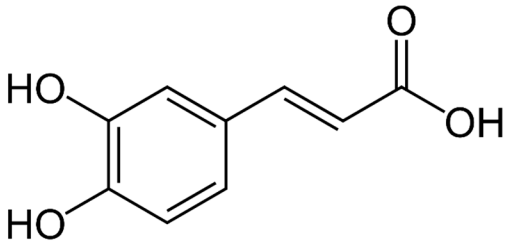
A - gallic acid:



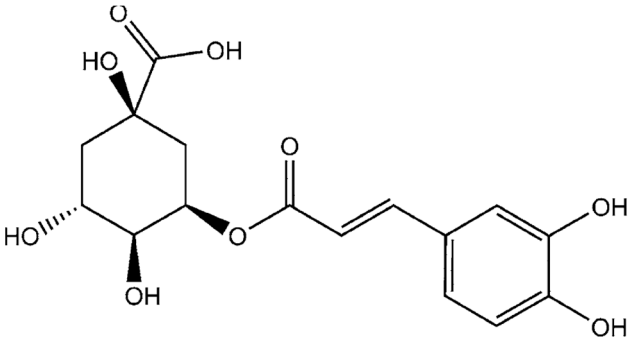

B - protocatechuic acid:



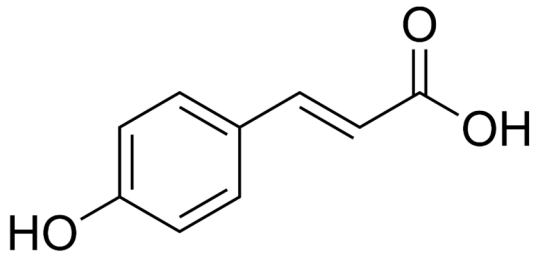

C - caffeic acid:




D - neochlorogenic acid:




E - coumaric acid:




F - lactic acid:

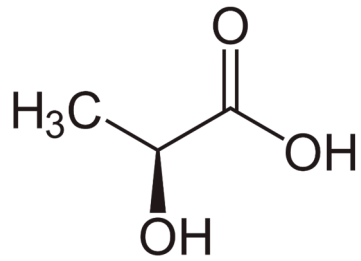



**Figure S7.** The structure of identifying individual phenolic acids and lactic acid in bio-ferments from seeds (B-S), extract (B-E), oil (B-O), and pomace (B-P) of milk thistle: A - gallic acid, B - protocatechuic acid, C - caffeic acid, D - neochlorogenic acid, E - coumaric acid, and F - lactic acid.