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Phytochemical Composition and Anti-SARS-CoV-2 Activity of Leaves Methanolic Extract of *Silybum marianum*.

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Abstract

In line with the global interest and demand in finding sustainable ways to counteract the emerging viral pandemics, our study highlights the unique composition and anti- SARS-CoV-2 activity of a barely used and studied part of the famous medicinal plant *Silybum marianum* (L.)Gaertn.Various chromatographic and spectroscopic techniques used in the process of isolation and structure elucidation led to the identification of nine flavonoids for the first time from the methanolic leaves extract.Among them,the aglyconeacacetin (**2**)was isolated for the first time from the genus. The antiviral activity of the extract against SARS-CoV-2 main protease showed considerable activity with half-maximal inhibitory and cytotoxic concentrations; IC_{50}=80.5 µg/ml and CC_{50}=576.5 µg/ml, respectively indicating a relatively high selectivity; SI = 7.1.

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Keywords: *Silybummarianum, Asteracea, medicinal plant, leaves, flavonoids, anti-viral activity.*

Introduction

In an attempt to find effective, environment friendly and sustainable resources to face the emerging highly mutated viral pandemics that we face in the last years, our efforts were directed towards anti-SARS-CoV-2 (a positive-sense single-stranded RNA virus) activity screening of promising plants that were previously reported to have antiviral activities.

Silybummarianum(L.)Gaertnis valued medicinal plant belonging to the familyA steraceae and known as Milk Thistledue to the "milky veins"of its leaves, butin Arabic countries it is known as ShokEl-Gamal. It is an annual herb native to the Mediterranean and North African regions [1].The plant was reported to cure liver and biliary disorders with a number of other medicinal uses; as anti-diabetic, anti-hypertensive, anti-amnesia and antiviral [2-4]. In addition to its use as cancer therapy for prostate, skin, breast, cervix cancers and hepatocellular carcinoma [5,6].

The previously published research papers addressed the chemical composition of the flowers, seeds, stems and roots reporting the bioactive constituents flavonolignans which are known as silymarin [mixtures of Silybin A, Silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin (they are diastereomericisomers of each other) and taxifolin] [7-13].

The chemical composition of the leaves of *S. marianum* was barely subjected to any comprehensive chemical studyexcept for an articlein 2019 reporting the GC-MS of the leaves showing the presence of monoterpene, alcohols, alkane and carboxylic acids [14].

The present study aims fora comprehensive chemical investigation of methanolic leaves extract of the planttogether with detecting its activity against SARS-CoV-2 main proteasethat causes coronavirus diseases (COVID-19).

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Materials and Methods

Plant material

Leaves of *S. marianum*, were purchased from a herbalist, Harraz, Cairo, in May 2021and identified by Professor Dr. S.A. Kawashty, Department of Phytochemistry and Plant Systematics, National Research Centre. A voucher specimen (SM 211)was deposited in the herbarium of the National Research Centre (CAIRC) [15]. The plant was leftto dry in the shade at room temperature till reached a constant weight.

Extraction and isolation

Fine air-dried powder of *S. marianum* plant (500 g) was extracted with 70% methanol twice for two days, filtered and concentrated under reduced pressure. The obtained extract (60 g) was subjected to a Silica gel column using a chloroform-methanol system as an eluent in ascending sequence of polarity. Fractions eluted by 20%MeOH-$CHCl_3$ afforded the aglycones**1**(12 mg) and **2**(10 mg),while fractions eluted by 50% MeOH-$CHCl_3$ gavethe monoglycosides**3**(10 mg)**,4**(9 mg),**5** (12 mg),**6**(9 mg),**7**(11 mg) and **8**(9 mg). The triglycosidecompound **9**(5 mg) was eluted from the column using 80% MeOH-$CHCl_3$. The collected fractions were further purified on Whatman 3 MM paper chromatography using different solvent systems. Final purification was achieved by the Sephadex LH-20 column.

Structure elucidation of the isolated compounds

NMR spectra were recorded on a Jeol EX-500 spectrometer: 500 MHz (^{1}H NMR), 125 MHz (^{13}C NMR). UV spectrophotometric analyses with Shimadzu UV-240.Negative Mass: ESI-MS negative ion acquisition mode was carried out on a XEVO TQD triple quadruple instrument (Waters Corporation, Milford, MA, USA).CC using Silica gel 60 (Merck, 0.063-0.2 mm) using $CHCl_3$/MeOH (9:1-9:9). For elution PC (descending) using Whatman No. 1 and 3 MM papers, and solvent systems:1)H_2O, 2) 15% HOAc (H_2O–HOAc 85:15), 3) 50%HOAc (H_2O–HOAc 50: 50), 4) BAW (n-BuOH–HOAc–H_2O 4:1:5, upper phase), 5) BBPW (C_6H_6–n-BuOH–pyridine–H_2O, 1:5:3:3, upper phase).Solvent 5 was used for sugar detection. SephadexLH-20 (Pharmacia) eluted with methanol. Acid hydrolysis for *O*-glycosides (2N HCl, 2 hrs,100°C) was carried out and followed by paperco-chromatography with authentic samples toidentify the aglycones and sugar moieties.

Biological activity

1- MTT cytotoxicity assay

To assess the half maximal cytotoxic concentration (CC_{50}), stock solutions of the test extract were prepared in 10 % DMSO in ddH_2O and diluted further to the working solutions with DMEM. The cytotoxic activity of the extract was tested in VERO-E6 cells by using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method with minor modifications. Briefly, the cells were seeded in 96 well plates (100 µl/well at a density of 3×105 cells/ml) and incubated for 24 hrs at 37 °C in 5%CO_2. After 24 hrs, cells were treated with various concentrations of the tested compounds in triplicates. 24 hrs later, the supernatant was discarded, and cell monolayers were washed with sterile 1x phosphate buffer saline (PBS) 3 times and MTT solution (20 µl of 5 mg/ml stock solution) was added to each well and incubated at 37 °C for 4 hrs followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 µl of acidified isopropanol (0.04 MHCl in absolute isopropanol = 0.073 ml HCl in 50 ml isopropanol). The absorbance of formazan solutions was measured at λ max 540 nm with 620 nm as a reference wavelength using a multi-well plate reader [16]. The percentage of cytotoxicity compared to the untreated cells was determined with the following equation.

The plot of % cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (CC_{50}).

% cytotoxicity = ((absorbance of cells without treatment-absorbance of cells with treatment) /(absorbance of cells without treatment) X 100).

2- Inhibitory concentration 50 (IC_{50}) determination

In 96-well tissue culture plates, 2.4×104 Vero-E6 cells were distributed in each well and incubated overnight at a humidified 37°C incubator under 5%CO_2 condition. The cell monolayers were then washed once with 1x PBS and subjected to virus adsorption(hCoV-19/Egypt/NRC-03/2020 (Accession Number on GSAID: EPI_ISL_430820)) for 1h at room temperature (RT). The cell monolayers were further overlaid with 100µl of DMEM containing varying concentrations of the test compounds. Following incubation at 37°C in 5%CO_2 incubator for 72 h, the cells were fixed with 100µl of 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet in distilled water for 15 min at RT. The crystal violet dye was then dissolved using 100µl absolute methanol per well and the optical density of the color is measured at 570 nm using AnthosZenyth 200rt plate reader (AnthosLabtec Instruments, Heerhugowaard, Netherlands). The IC_{50} of the compound is that required to

reduce the virus-induced cytopathic effect (CPE) by 50%, relative to the virus control [17].

Compounds characterization

Aglycones*:*

1- *Apigenin*

UV/Vis λ_{max} (MeOH): 266, 296sh, 335. MS: 269 (negative mode). ^{1}H-NMR in DMSO-d_6: δ 7.89 (d, *J*= 8.2 Hz, 2H, H-2', 6'), 6.90 (d, *J*=8.2 Hz, 2H, H-3', 5'), 6.80 (d,*J*=2Hz, 1H, H-8), 6.45 (d, *J*=2Hz, 1H, H-6), 6.20 (s, 1H, H-3).

2-*Acacetin*

UV/Vis λ_{max} (MeOH): 269, 301sh, 327. MS: 283 (negative mode). ^{1}H-NMR in DMSO-d_6: δ 7.70 (d, *J*=7.9 Hz, 2H, H-2', 6'), 6.80 (d, *J*=7.9Hz, 2H, H-3', 5'), 6.65 (s, 1H, H-3), 6.50 (d, *J*= 2Hz, H-8), 6.45 (d, *J*= 2Hz, H-6), 3.50, s, 3H, -OCH_3).

Mono-Glycosides:

3-*Apigenin 7-O-rhamnoside*

Acid hydrolysis:apigenin and rhamnose. UV/Vis λ_{max} (MeOH): 267, 332. ^{1}H-NMR in DMSO-d_6: δ 7.86 (d, *J*=8.1 Hz, 2 H, H-2', 6'), 6.88 (d, *J*= 8.1 Hz, 2H, H-3'. 5'), 6.82 (d, *J*=2 Hz, 1H, H-8), 6.79 (d, *J*= 2Hz, 1H, H-6), 6.30 (s, 1H, H-3), 5.15 (d, *J*=2 Hz, 1H, H-1rhamnose), 1.2 (d, *J*=6 Hz, 3H, rhamnose-CH_3).

4-*Apigenin 7-O-glucoside*

Acid hydrolysis: apigenin and glucose. MS: 431 (negative mode).UV/Vis λ_{max}(MeOH): 267, 333. ^{1}H-NMR in DMSO-*d6*: δ 7.89 (d, *J*=7.8Hz, 2 H, H-2', 6'), 6.89 (d, *J*=7.8Hz, 2 H, H-3', 5'), 6.80 (d, *J*=2 Hz, 1H, H-8), 6.77 (d, *J*=2 Hz, 1H, H-6), 6.40 (s, 1H, H-3), 5.10 (d, J= 7.5, 1H, H-1 glucose).

5-*Apigenin4'-O-glucoside*

Acid hydrolysis: apigenin and glucose. UV/Vis λ_{max} (MeOH): 268, 302sh, 326. ^{1}H-NMR in DMSO-d_6: δ 7.92 (d, *J*=8 Hz, 2H, H-2', 6'), 6.88 (d, *J*= 8 Hz, 2 H, H-3', 5'), 6.85(d, *J*= 2 Hz, 1H,, H-8), 6.82 (d, *J*= 2 Hz, 1H, H-6), 6.40 (s, 1H, H-3), 5.1 (d, *J*= 7.6Hz, 1H, H-1 glucose).

6-*Apigenin7-O-glucuronide*

Acid hydrolysis: apigenin and glucuronic acid. MS: 445 (negative mode).UV/Vis λ_{max} (MeOH): 268, 332. ^{1}H-NMR in DMSO-d_6: δ 7.87 (d, *J*=8.5 Hz, 2H, H-2', 6'), 6.86 (d, *J*=8.5 Hz, 2H, H-3', 5'), 6.70 (d, *J*=2 Hz, 1H, H-8), 6.60 (d, *J*=2 Hz, 1H, H-6), 6.30 (s, 1H, H-3), 5.00 (d. 1H, *J*=7.5 Hz, H-1 glucose).^{13}C-NMR in DMSO-d_6:164.83 (C-2),103.48 (C-3), 182.48 (C-4), 161.56 (C-5), 100.13 (C-6), 162.10 (C-7), 95.12 (C-8), 157.47 (C-9), 103.48 (C-10), 121.31 (C-1'), 129.01 (C-2'), 116.53 (C-3'), 157.47 (C-4'), 116.53 (C-5'), 129.01 (C-6'),100.13 (C-1"), 73.49 (C-2"), 74.62 (C-3"), 72.39 (C-4"), 76.91 (C-5"), 172.07 (O=C-O, C-6").

7-*Luteolin 7-O-glucoside*

Acid hydrolysis: luteolin and glucose. UV/Vis λ_{max} (MeOH):255, 258sh, 347. ^{1}H-NMR in DMSO-d_6: δ 7.40 (m, 2H, H-2',6'), 6.85 (d, *J*= 8.4 Hz, 1H, H-5'), 6.85 (d, *J*= 2Hz, 1H, H-8), 6.75 (d, *J*= 2Hz, 1H, H-6), 6.40 (s, 1H, H-3), 5.00 (d, *J*=7.5 Hz, H-1 glucose).

8-*Kaempferol 3-O-rhamnoside*

Acid hydrolysis: kaempferol and rhamnose. UV/Vis λ_{max} (MeOH):267, 280sh, 295sh, 350. ^{1}H-NMR in DMSO-d_6: δ 7.70 (d, *J*=8.5 Hz, 2H, H-2', 6'), 6.88 (d, *J*=8.5 Hz, 2H, H-3', 5'), 6.33 (d, *J*= 2Hz, 1H, H-8), 6.14 (d, *J*= 2Hz, 1H, H-6), 5.25 (d, 1H, *J*=2 Hz, H-1rhamnose), 0.84 (d, *J*=6Hz, 3H, CH_3-rhamnose).^{13}C-NMR in DMSO-d_6: 157.14 (C-2), 131.05 (C-3), 178.00 (C-4), 161.77 (C-5), 99.60 (C-6), 166.00 (C-7), 94.40 (C-8), 157.14 (C-9), 104.14 (C-10), 121.31 (C-1'), 131.6 (C-2'), 115.93 (C-3'), 160.50 (C-4'), 115.93 (C-5'), 131.60 (C-6'), 102.30 (C-1"), 70.60 (C-2"), 70.80 (C-3"), 71.60 (C-4"), 70.80 (C-5"), 17.98 (C-6").

Tri-glycoside:

9- *Kaempferol 3-O-(di-rhamnosyl)glucoside*

Acid hydrolysis: kaempferol, glucose and rhamnose. UV/Vis λ_{max} (MeOH):269, 283sh, 297sh, 353. ^{1}H-NMR in DMSO-d_6:7.70 (d, *J*=8.5 Hz, 2H, H-2', 6'), 6.90 (d, *J*=8.5 Hz, 2H, H-3', 5'), 6.40 (d, *J*= 2Hz, 1H, H-8), 6.10 (d, *J*= 2Hz, 1H, H-6), 5.25 (d, *J*= 7.5 Hz, 1H, H-1 glucose), 4.10 (d, 1H, *J*=2 Hz, H-1 rhamnose), 3.95 (d, 1H, *J*=2 Hz, H-1 rhamnose), 0.90 (d, *J*=6Hz, 3H, CH_3-rhamnose), 0.75 (d, *J*=6Hz, 3H, CH_3-rhamnose).

Results and Discussion

Compounds identification

The leaves methanolic extract of *S. maria*num yielded nine flavonoids isolated for the first time from the plant (Fig. 1). Two aglycones (**1**, **2**), six mono-glycosides (**3-8**) and one triglycoside (**9**). The aglycone**2** was reported here as a *Silybium* component for the first time.

Compounds**1** and **2** were isolated in free form and showed chromatographic properties, UV, MS and ^{1}H-NMR data similar to those reported for apigenin and acacetin [18-20].

The UV spectral data with diagnostic shift reagents of the compounds indicated flavones with occupations at position 7 for compounds**3**, **4**,**6** , **7**,position **4'**for **5**and a flavonol occupied at position 3 for **8** [18].

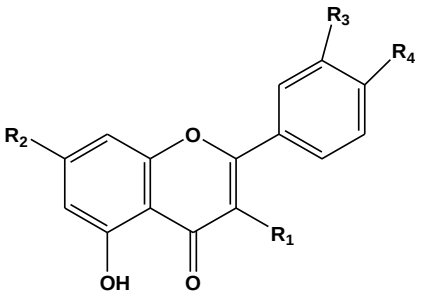
Compounds **3-8** on acid hydrolysis gave apigenin for **3-6**, luteolin for **7**, and kaempferol for **8**. The sugar moieties were identified as rhamnose for **3**and **8**;glucose for **4**, **5**, **7,** and glucuronic acid for **6** (co-chromatography with authentic samples).

The ^{1}H-NMR spectra of **3**, **4**, **5**, and **7** showed the

characteristic protons pattern of apigenin. It showed the pattern of AB system of ring B, in addition to the signals of H-3, H-6, and H-8 (experimental). Thus compounds **3**, **4**, **5**, **6** were identified as :apigenin 7-O-rhamnoside, 7-O-glucoside, 4'-O-glucoside and 7-O-glucuronoide.

The ^{1}H-NMR of **7** and **8** showed the ABX system and ABC system for the proton of ring B. The sugar anomeric protons of **7** and **8** were located at δ 5.00 and 5.25 ppm in the ^{1}H-NMR spectra. These chemical shifts confirmed the direct attachment of the sugars to the aglycone. The rhamnose was confirmed by its $-CH_3$ doublet with *J*= 6Hz atδ 0.048. The chromatographic properties and the ^{1}H-NMR of **7** are similar to those reported forluteolin 7-O-glucoside [18-21]. The ^{13}C-NMR of **8** confirmed that it is a monoglycoside of kaempferol. The ^{13}C-NMR chemical shift corresponded well with the shift of kaempferol. The only difference is theupfield shift of the signal assigned to C-3 by 2 ppm (133 to 131.0). This shift is an analogue to those reported for the glycosylation of flavonoids at C-3 [18-21]. Thus **8**is identified as kaempferol 3-O-rhamnoside.

Compound **9** gave kaempferol, glucose and rhamnose on acid hydrolysis which were identified by co-chromatography with authentic samples. The **^{1}**H-NMR spectrum confirmed that **9** is a triglycoside of kaempferol on the basis of H-1 of glucose and rhamnose moieties. Thus the doublet at δ5.25 was assigned to the anomeric proton of glucose. This chemical shift confirms that glucose is directly attached to the aglyconekaempferol. The coupling constant *J*= 7.5 Hz indicates the B–configuration [20]. Two rhamnose H-1swere located as two doublets (*J*= 2Hz) at δ 4.10 and 3.95 and the coupling constant confirming L-configuration. The two rhamnose $-CH_3$ groups resonated as two doublets at δ 0.90 and 0.75 with *J*= 6Hz. The spectrum also showed the characteristic pattern of kaempferol 3-*O*-glycoside. The B-ring protons showed the AB system for 2', 6' at δ 7.70 and 3'. 5' at δ 6.90. H-8 and H-6 appeared as two doublets with *J*=2 Hz at δ 6.40 and 6.10. The UV, H-NMR and chromatographic properties are similar to those reported for kaempferol3-*O*-triglycoside [18-20]. However, the low concentration of **9** prevents any further investigation to identify the inter-glycosidic linkage between the two rhamnose moieties and the glucose. Thus **9**is identified as kaempferol 3-*O*-(di-rhamnosyl)-glucoside.

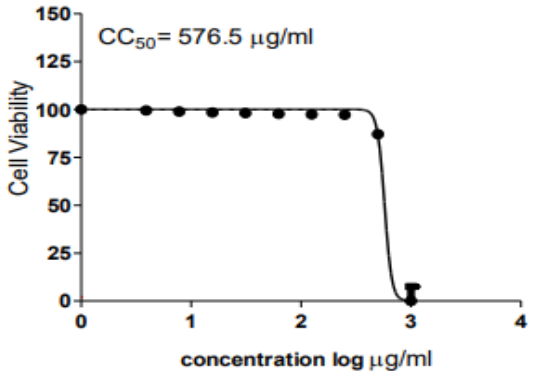


Compd.	R_1	R_2	R_3	R_4
1	H	OH	H	OH
2	H	OH	H	OCH_3
3	H	O-rhamnosyl	H	OH
4	H	O-glucosyl	H	OH
5	H	OH	H	O-glucosyl
6	H	O-glucuronyl	H	OH
7	H	O-glucosyl	OH	OH
8	O-rhamnosyl	OH	H	OH
9	O-di-rhamnosylglucosyl	OH	H	OH

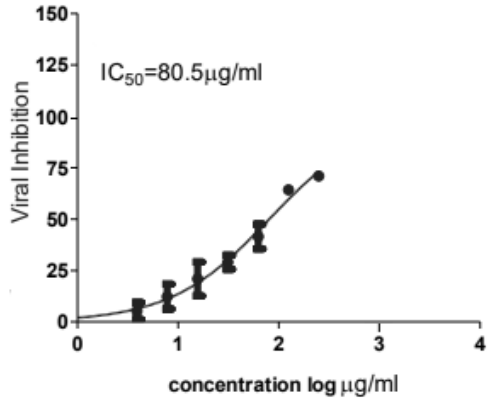
Fig (1): Chemical structure of the isolated compounds from *S. marianum*leaves.

Antiviral activity

The methanolic extract of the leaves of *S. marianum* was evaluated for the first time against SARS-CoV-2 main protease. The methanolic extract showed considerable antiviral activity with a 50% inhibitory concentration; IC_{50}=80.5 µg/ml and 50% cytotoxicity concentration; CC_{50}=576.5 µg/ml against SARS-CoV-2 indicating a relatively high selectivity; SI = 7.1 (Fig.2).



(a)



(b)

Fig. 2.Anti-SARS-CoV-2 activities of the extract

(a) Half-maximal cytotoxic concentrations (CC_{50}) on Vero E6 cells,

(b) half-maximal inhibitory concentrations (IC_{50}) against (hCoV-19/Egypt/NRC-03/202) in Vero E6. Inhibitory concentration 50% (IC_{50}) values were calculated using nonlinear regression analysis ofGraphPad Prism software (version 5.01) by plotting log inhibitor versus normalized response (variable slope).

Conclusion

Nine flavonoids were isolated and identified fromthe leaves extract of *S. marianum,*for the first time, indicating the presence of apigenin and its derivatives as the major components of the extract. This is the frist report of the aglyconeacacetinin the genus *Silybum*. The methanolic extract was evaluated for the first time against SARS-CoV-2 main protease. The results showed considerable activity with a relatively high selectivity; which may be attributed to the phenolic content of the extract; a fact that encourages further investigation of the plant to put a hand on the active leads and check for agonists and antagonists in the extract components in an approach of trying to find more sustainable ways to counteract highly mutated viruses.

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