EXTRACTION, FRACTIONATION, AND SEPARATION OF CYNAROSIDE AND SCOLYMOSIDE FROM *Cynara scolymus* L. GROWN IN IRAQ

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ABSTRACT

Artichoke (*Cynara scolymus* L.) is a nutritious vegetable that grown all over the world. It is a promising herbal plant, rich in bioactive components. It is considered as medicinal plant due to its nutritional and phytochemical composition, especially high proportion of phenolic compounds. The primary aim of this study was to achieve chemical profile analyses of artichoke for different phytochemicals, especially Scolymoside and Cynaroside. Methanolic crude was extracted from Artichoke leaves by rotary evaporator and separated by column chromatography. The fractions monitored by Thin Layer Chromatography (TLC), and identified in High-Pressure Liquid Chromatography (HPLC). Two important compounds, Scolymoside and Cynaroside were separated and identified. In overall, Artichoke could be regarded as a rich source of biologically active compounds and considered as bio-functional with putative antioxidant effects.

Key words: *Cynara scolymus*, Artichoke, Flavonoids, Phytochemicals

إستخلاص وتجزئة وفصل مادتي سيناروسيد وسكوليموسيد من نبات الخرشوف المنزرع في العراق

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المستخلص

الخرشوف (*Cynara scolymus* L.) نبات غذائي يزرع في جميع أنحاء العالم، وهو نبات عشبي واعد غني بالمكونات النشطة بيولوجيًا. يعتبر من النباتات الطبية بسبب تركيبته الغذائية والكيميائية ، وخاصة إحتوائه على نسبة عالية من المركبات الفينولية. كان الهدف الأساسي من هذه الدراسة هو إجراء بعض التحليلات الكيميائية للخرشوف لمختلف المواد االنباتية وخاصة Scolymoside و Cynaroside. تم استخلاص المواد الميثانولية الخام من أوراق الخرشوف بواسطة المبخر الدوار وفصلها بإستخدام العمود الكروماتوجرافي. تمت متابعة هذه المواد بواسطة تقنية كروماتوغرافيا الطبقة الرقيقة (TLC) Thin Layer Chromatography، وتم تحديدها بإستخدام جهازالكروماتوغرافيا السائلة عالية الضغط (HPLC) High-Pressure Liquid Chromatography. لقد تم فصل وتحديد مركبين مهمين هما Scolymoside و Cynaroside. وبشكل عام، يمكن اعتبار نبات الخرشوف مصدرًا غنيًا للمركبات النشطة بيولوجيًا، كما يمكن اعتباره عاملًا حيويًا لإحتوائه على مضادات الأكسدة.

الكلمات المفتاحية: *Cynara scolymus*، نبات الخرشوف، الفلافونويد، الفايتوكيميكل

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INTRODUCTION

Artichoke (*Cynara scolymus* L.) is a nutrition and medicinal plant; the curative possibility of which was known to the Greeks, Romans, and ancient (18). This medicinal herb has been appropriately used in conventional medicine for a long and helps cure various ailments (25). Its leaf extracts are used as traditional herbal products for pharmaceutical preparations (17). Artichokes contain phenolic compounds such as Cynarin, Chlorogenic, and caffeic acid (9); also, its different plant parts are considered potential sources of valuable phytoconstituents, mainly polysaccharides, and polyphenols (27). Flavonoids include Cynaroside (luteolin-7-O-glucoside) and Scolymoside (luteolin-7-O-rutinoside) (11). Flavonoids are secondary metabolites of plants with polyphenolic structure. The flavonoid groups of poly phenolic compounds have low toxicity in mammals and are widely dispense in plant kingdom (20). Main dietary sources of flavonoids in the form of flavones, flavonols, flavonones, and isoflavones are found in tea, apple, cherry, tomato, onion, beetroot, thyme, parsley, grape fruit, lemon, orange, neem, and ginkgo (7) Flavonoids have gained attention because of their broad biological and pharmacological activities. As a result; many researches focused on increasing flavonoids in plants (4, 5, 6). In these order, flavonoids have been reported to exert multiple biological possession including cytotoxicity, antimicrobial, anti-inflammatory as well as antitumor activities. The best-described property of almost every group of flavonoids is their capacity to act as powerful antioxidants which can protect the human body from free radicals and reactive oxygen species (24). Scolymoside is a minor flavonoid found in the leaves and leaf extracts of artichoke (12), while Cynaroside is considered as essential artichoke compounds. Cynaroside is a flavone compound that can be found in artichoke (22). It is an abundant flavonoid produced in plants that has several health benefits, including neuroprotective properties (13). Cynaroside also possesses potential antibacterial, antifungal and antioxidant (14). The aim of this study was characterize the flavonoids and to separate and identifiy Scolymoside and Cynaroside from artichoke leaves by using High-Pressure Liquid Chromatography (HPLC).

MATERIALS AND METHODS

Extraction and fractionation of flavonoids

Leaves powder (500 g) was free from fat (defatted) with hexane for 24 hours and then left to dry at room temperature. Defated leaves were extracted with 3L of 80% methanol (CH_3OH) in soxhlet appliance until complete consumption. The methanlic extract was evaporated under reduced pressure at a temperature not overriding $40C^o$ by using a rotary evaporator apparatus to deign a dark greenish-yellow sediments designated as a crude. The crude was acidified with 5% hydrochloric acid to pH 2 and divide with an egalitarian volume of ethyl acetate to get an aqueous acidic layer (AAL) and ethyl acetate layer (EAL). The AAL was then separated and basified with an equal volume of 5% NaOH to pH 10 and extracted with chloroform $CHCl_3$ in the separatory funnel to get a $CHCl_3$ layer and aqueous basic layer. The $CHCl_3$ layer evaporated under pressure at 40^oC to produce dark residue was specified as fraction1 (F-1), while the aqueous basic layer was specified as fraction2 (F-2). The EAL of the methanolic crude extract was evaporated under pressure at 40^oC and basified with 300ml of 5% NaOH to pH 10; then separated with $CHCl_3$ in the separatory funnel to get aqueous basic layer (ABL) and EAL. The ABL was separated, evaporated to dryness, and acidified with 5% HCl to pH 2; then extracted with ethyl acetate to get fraction designated as flavinoid fraction3 (F-3). Flavonoids extract was subjected to silica gel column chromatography (100 g, 4X60 cm), and eluted firstly with 100% hexane (C_6H_{14}), followed by increasing amounts of methylene chloride (CH_2Cl_2) in methanol (C_6H_{14}) as follows, C_6H_{14}: (CH_2Cl_2): (25:75), (50:50), (75:25), and (100%) CH_2Cl_2, then increase polarity with methanol CH_3OH in CH_2Cl_2 with an increment of 10% up-to CH_3OH (100%). One hundred fractions (10 ml each) were collected and observed on TLC silica gel plates, the platessilica gel F_{254} (Merck, Germany) were developed using solvents 1 to 5., and the spots were detected under UV light.

Solvent systems used for fractions

S_{1f}: Toluene $C_6H_5CH_3$: chloroform $CHCl_3$: acetone CH_3COCH_3 (40:25:35) (1).

S_{2f}: Ethylacetate $CH_3COOC_2H_5$: methanol CH_3OH: formic acid CH_2O_2 (50:50:1) (26).

S_{3f}: n-Butanol $C_9H_{19}NO_3$: glacial acetic acid CH_3COOH: water (40:10:50) (26).

S_{4f}: Hexane C_6H_{14}: Ethylacetate $CH_3COOC_2H_5$ (90:10) (10).

S_{5f}: Ethylacetate $CH_3COOC_2H_5$: glacial acetic acid CH_3COOH: formic acid CH_2O_2: water (30:0.8:1.2:8) (10).

The fraction was taken (1mg dissolved in 1ml solvent) and utilized with TLC plates, using pipette in form of spots and allowed to dry and then developed by ascending technique. After imbibition and ascension of the solvent system, the plates of Thin Layer Chromatography (TLC) were exposed to UV light at 254 and 366 nm.=

Fractionation and identification of flavonoids: The polyphenolic compounds of artichoke extracts were fractionated and identified for flavonoids by HPLC, according to the method described by (2). The eluted peaks were monitored by UV-Vis 10A-SPD spectrophotometer. The main compounds were separated on FLC (Fast Liquid Chromatographic) column under the optimum conditions. Column: phenomenex C-18, 3µm using a hypersil C18 reversed- phase column with 5µm particle size (100 X 4.6 mm I.D) column. The mobile phase was linear gradient of solvent A (0.1% trifluoroacectic acid) in deionized water and solvent B (100% of acetonitrile). The program was started with elution with 95% A (0-1min); a linear gradient was used until 10 min, lowering A to 20%; from 17 min to 24 min, and A decreased to 0%. The flow rate was 1.3 ml/ min, and the runs were integrated at 280 nm for each phenolic derivative. Scanning was performed from 200 to 600 nm. Phenolic compounds were identified by comparing retention times and UV-VIS spectra with those of pure standards and the range of calibration curves. The repeatability of the quantitative analysis was ±4%. The results were expressed as microgram/ml of each compound per total phenolic compounds. The penolic compounds of extract calculated according to the following equation:

Concentration of sample (µg/ml) = $\frac{\text{area of sample}}{\text{area of standard}}$ X conc. of standard X dilution

RESULTS AND DISCUSSION

Fractionation and Separation of Flavonoids

The flavonoid extract was produced from the extraction and fractionation process of the active compound ethyl acetate to get fraction3 (flavonoid). It was subjected to column chromatography on column No.1 Silica gel (100g, 60 X 3cm), using a C_6H_{14} and CH_2Cl_2 increase polarity with CH_3OH. Sub-fractions (1-10) were collected and monitored by TLC silica gel plates using different solvent systems as mobile phases. Result of TLC Sub–fractions (8, 9, and 10) showed different spots pattern after the chromatoplates were air-dried and visualized under daylight and UV lights at 366 nm (Figure 1). Sub-fraction 8 was separated in C_6H_{14}-CH_2Cl_2 (20-80); the S1f solvent $C_6H_5CH_3$-$CHCl_3$–aceton (40-25-35) detected five spots. While Sub-fraction 9 was separated in C_6H_{14}- CH_2Cl_2 (10-90) and produced seven spots with S1f solvent. At the same time, Sub-fraction 10 was separated in C_6H_{14}- CH_2Cl_2 (0-100), and revealed six spots by S1f solvent. Retardation factor (R*f*) of sub-fraction 8 ranged between (0.21-0.70), Sub-fraction 9 ranged between (0.21-0.78), as well sub-fraction 10 ranged between (0.15-0.74) **(**Table 1). Sub-fractions (1-7) did not give clear patterns through identification by TLC. Therefore, the powders of sub-fractions 8, 9, and 10 with 1.75, 1.63, and 1.30 g, respectively were used to complete the separation phases by column chromatography No.2 Silica gel and Sephadex G50 (30g, 40 X 1cm) and column chromatography No.3 Sephadex G50 (10g, 20 X 0.5cm).

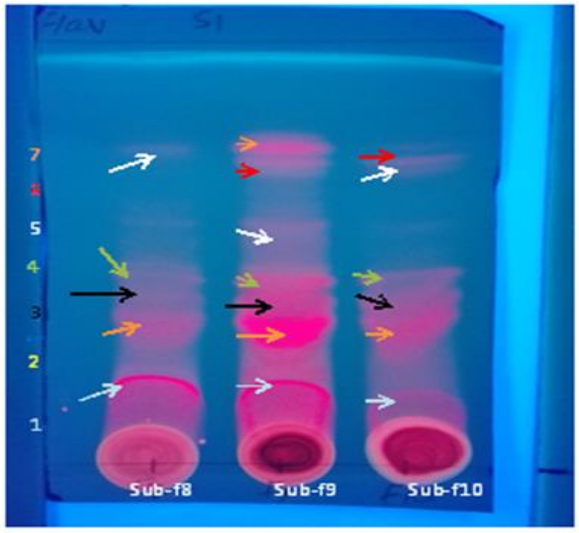

Figure 1. TLC of flavonoid extract subtractions (8, 9, 10), column No. 1 using S1f $C_6H_5CH_3$-$CHCl_3$ – CH_3COCH_3 (40-25-35), UV light (366nm).

Table 1. TLC sub-fractions (8, 9, 10), R*f* values, UV light (366nm).

Fractions	Spot No.	R*f* values	Color UV light at 366nm
Sub-fraction 8	1	0.21	Red
C_6H_{14}- CH2Cl2	2	0.35	Pink
(20-80)	3	0.41	light pink
	4	0.47	light pink
	5	0.70	light pink
Sub-fraction 9	1	0.21	Red
C_6H_{14}- CH2Cl2	2	0.31	Red
(10-90)	3	0.38	Pink
	4	0.45	Pink
	5	0.58	light pink
	6	0.74	light pink
	7	0.78	Pink
Sub-fraction 10	1	0.15	Pink
C_6H_{14}- CH2Cl2	2	0.31	Pink
(0-100)	3	0.38	Pink
	4	0.45	light pink
	5	0.70	light pink
	6	0.74	light pink

Isolation of Fractions

Sub-fraction 8, column No.1, (1.75g), (C_6H_{14}: CH_2Cl_2- CH_3OH) (20:80)

Sub-fraction 8 was subjected to column No.2 (phase-1) packed by C_6H_{14} only. Elution was started with C_6H_{14} and polarity was gradually increased with CH_2Cl_2 by CH3OH. Ten ml of each fraction was collected. The collected fractions (100 fractions) were then concentrated by rotary evaporator, and the powder was detected by TLC using solvent system. Fractions (10-20) gave 84mg powder that treated with solvent (C_6H_{14}: CH_2Cl_2-CH_3OH) (90:10) and showed three spots by TLC using mobile phase $S3_f$ ($C_9H_{19}NO_3$: CH_3COOH: water) (40:10:50). They visualized under UV lights at 366 nm (Figure 2)**;** R*f* of spots was 0.64, 0.84, and 0.90 (Table 2).

Figure 2. TLC of flavonoid extract sub-fraction 8, column No. 2, using S3f ($C_9H_{19}NO_3$: CH_3COOH: water) (40:10:50), UV light (366nm).

Table 2. TLC for sub-fraction No. 8, R*f* values, color UV light (366 nm).

Spot No.	R*f* values	Color UV light 366nm
1	0.64	Light pink
2	0.84	Pink
3	0.90	Pink

Sub-fraction 10, column No.1, (1.30g), (C_6H_{14}: CH2Cl2- CH_3OH) (0:100)

Sub-fraction 10 was subjected to column No.2 (phase-1) packed by C_6H_{14} only. Elution was started with C_6H_{14} and polarity was gradually increased with CH_2Cl_2 by CH3OH. Ten ml of each fraction was collected. The collected fractions (100 fractions) were then concentrated using rotary evaporator, and the powder was detected by TLC using solvent system. Fractions (70-80) gave 83mg powder that treated with solvent (C_6H_{14}: CH_2Cl_2- CH_3OH) (0:100) and showed four spots by TLC using mobile phase $S1_fC_6H_5CH_3$-$CHCl_3$–CH_3COCH_3 (40-25-35), they visualized under UV lights at 366 nm (Figure 3); R*f* spots range was 0.22- 0.58 (Table 3)

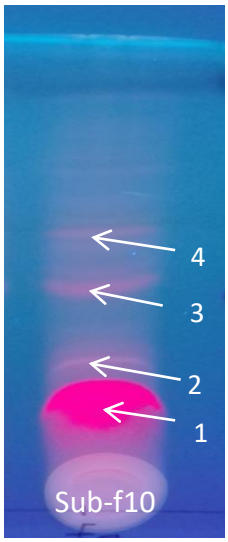

Figure 3. TLC of flavonoid extract sub-fraction 10, column No.2 (phase-1), using $S1_fC_6H_5CH_3$-$CHCl_3$ – CH_3COCH_3 (40-25-35), UV light (366nm).

Table 3. TLC for sub-fraction No. 10, R*f* values, color UV light (366 nm).

Spot No.	R*f* values	Color UV light 366nm
1	0.22	Pink
2	0.30	Light pink
3	0.47	Light pink
4	0.58	Light pink

Separation of Flavonoids: Flavonoid sub-fractions 8, column No.2 (C_6H_{14}: CH_2Cl_2- CH_3OH) (90:10): Sub-fractions 8, column No.2 was submitted through column chromatography No.3 Sephadex G50 (10g, 20 X 0.5cm) filled with 100% C_6H_{14}. Elution was started with C_6H_{14} and polarity was gradually increased with CH_2Cl_2 by CH3OH. Ten ml of each fraction was collected; the collected fractions (100 fractions) were then concentrated using rotary evaporator, and detected by TLC using solvent system. Fractions (80-90) produced 35mg powder; when treated with solvent (C_6H_{14}: CH_2Cl_2- CH_3OH) (20:80) gave one spot with TLC using S2f ($CH_3COOC_2H_5$: CH3OH: CH_2O_2) (50:50:1). It visualized under UV lights at 366nm (Figure 4); R*f* spot was 0.91 (Table 4).

Figure 4. TLC of flavonoid fraction 8, column No.3 (phase-2), using S2f ($CH_3COOC_2H_5$: CH_3OH: CH_2O_2) (50:50:1), UV light (366nm).

Table 4. TLC for fraction No. 8, R*f* values, color UV light (366 nm).

Spot No.	R*f* values	Color UV light 366nm
1	0.90	Pink

Flavonoid sub-fractions 10, column No.2 (phase-1) (C_6H_{14}: CH_2Cl_2- CH_3OH) (0:100)

Sub-fractions 10, column No.2 was submitted through column No.3 filled with 100% C_6H_{14}. Elution was started with C_6H_{14} and polarity was gradually increased with CH_2Cl_2 by CH3OH. Ten ml of each fraction was collected; the collected fractions (100 fractions) were then concentrated using rotary evaporator, and detected by TLC using solvent system. Fractions (100-110) produced 19mg powder; when treated with solvent (C_6H_{14}: CH_2Cl_2- CH_3OH) (10:90) it gave one spot with TLC using S3f ($C_9H_{19}NO_3$: CH_3COOH: water)

(40:10:50). It visualized under UV lights at 366 nm (Figure 5); R*f* spot was 0.71 (Table 5).

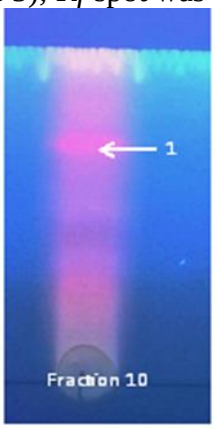

Figure 5. TLC of flavonoid fractions 10, column No.3 (phase-2), using S3f ($C_9H_{19}NO_3$: CH_3COOH: water) (40:10:50), UV light (366nm).

Table 5. TLC for fraction No. 10, R*f values*, color UV light (366 nm).

Spot No.	R*f* values	Color UV light 366nm
1	0.71	Pink

Identification of the flavonoid fractions by HPLC : The fractions from flavonoids extract of Artichoke leaves were identified by HPLC. The flavonoid compounds include glycosidesluteolin-7-β-rutinoside (Scolymoside), luteolin-7-β-D-glucoside, luteolin-4-β-D-glucoside, and luteolin 7-glucoside (Cynaroside). The results of the retention time and area (**µ**volt) are shown in (Table 6); the flavonoids compounds standard presented in (Figure 6).

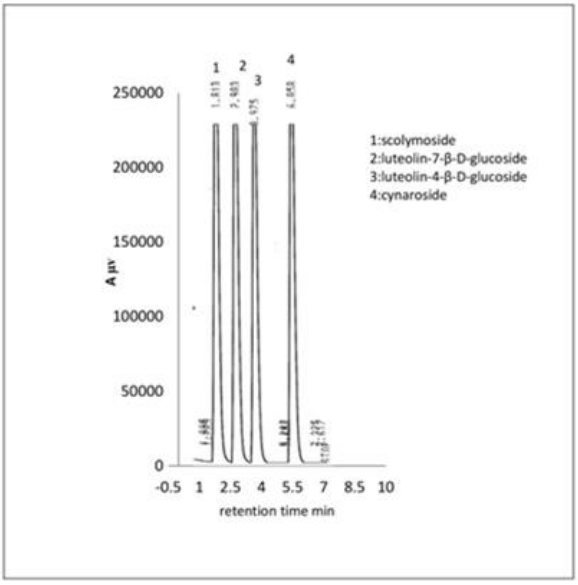

Figure 6. Flavonoid compounds, retention time (min), and area (µvolt) for the standard analysis by HPLC

Table 6. Flavonoid compounds, retention time (min), area (µvolt) for the standard

Flavonoids compounds	Standard	
	Retention time(min)	Area (µvolt)
glycosidesluteolin-7-β-rutinoside (SC)	1.813	247519
luteolin-7-β-D-glucoside	2.903	232260
luteolin-4-β-D-glucoside	3.975	200789
Luteolin 7- glucoside (CN)	6.058	219838

Fraction 8 showed one peak for the retention time (1.808) and this was identical to the standard of Scolymoside (Table 7) and (Figure 7); the concentration for the fraction was separated by HPLC (Table 8). Fraction 10 showed one peak (6.062) and was identical to the standard of Cynaroside (Table 9) and (Figure 8); the concentration for the fraction was separated by HPLC (Table 10). Sub-fraction 9 showed two unclear mixed spots that are difficult to be identified by HPLC; for this reason, this fraction was ignored.

Table 7. Comparison of retention times of fraction No. 8 with standard flavonoid after separation in HPLC

Peak No.	Retention time (min)	identical standard	Retention time (min)
1	0.068	Unknown	
2	1.338	Unknown	
3	1.808	glycosidesluteolin-7-β-rutinoside (SC)	1.813
4	3.827	Unknown	
5	3.258	Unknown	
6	3.457	Unknown	
7	8.437	Unknown	

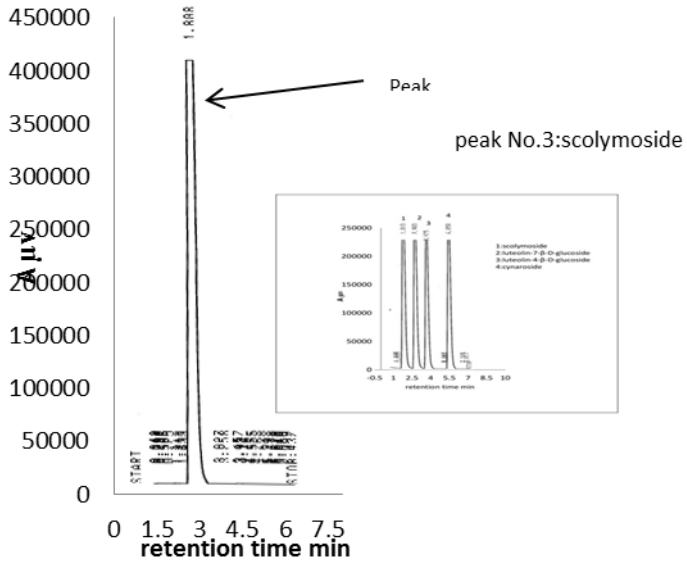

Figure 7. Glycosidesluteolin-7-β-rutinoside (Scolymoside), retention time (min), area (µvolt) for the fraction 8 separation in HPLC

Table 8. Glycosidesluteolin-7-β-rutinoside (Scolymoside), retention time (min), area (µvolt) and concentration for fraction No.8 after separation in HPLC

Fraction	Retention time(min)	Area (µvolt)	Concentration %
8	1.808	439175	100
Total	-	439175	100

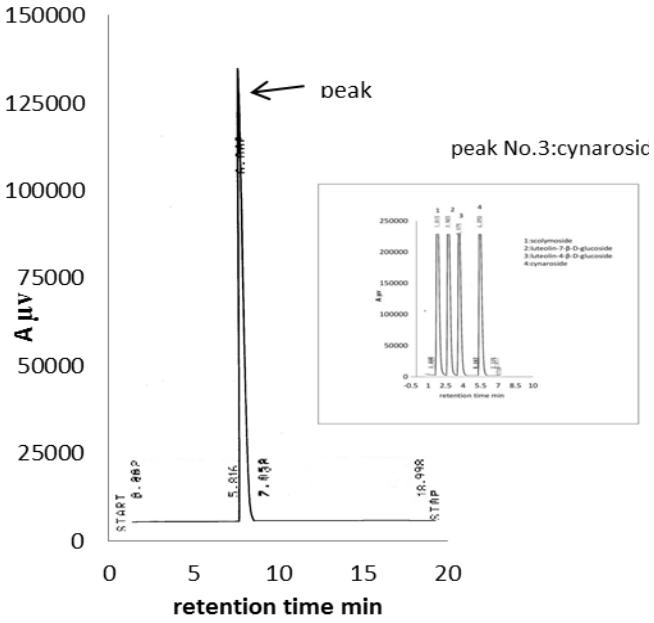

Figure 8. Luteolin 7- glucoside (Cynaroside), retention time (min), area (µvolt) for the fraction 10 separation in HPLC

Table 9. Comparison of retention times of fraction No. 10 with standard flavonoid after separation in HPLC

Peak No.	Retention time (min)	identical standard	Retention time (min)
1	0.382	Unknown	-
2	5.816	Unknown	-
3	6.062	Luteolin 7- glucoside (CN)	6.058
4	7.058	Unknown	-
5	18.998	Unknown	-

Table 10. Luteolin 7- glucoside (Cynaroside), retention time (min), area (µvolt) and concentration for fraction No. 10 after separation in HPLC

Fraction	Retention time(min)	Area (µvolt)	Concentration %
10	6.062	134907	100
Total		134907	100

Artichoke has been well known as a traditional medicine because of its phenolic compounds (18). The pharmaceutical properties of this plant are linked to their special chemical composition, which includes high levels of polyphenols. (The application of biotechnology tools such as in vitro culture techniques could be used for the controlled production of artichoke secondary metabolites (8). The results of this study revealed that Artichoke leaves possess important flavonoid compounds that may improve its quality as a functional food; this in agreement with (6). The process of separation and purification using the column chromatography method, which used the flavonoid extract of Artichoke proved efficient. The Scolymoside and Cynaroside were separated and identified by HPLC. This result is similar to (23) and (16) who mentioned that Artichoke is containing flavonoid compounds, including luteolin, luteolin-7-O glucoside, luteolin-7-rutinoside, and Cynaroside. (12) used liquid chromatographic isolation techniques (Sephadex LH-20) in combination with analytical methods (HPLC-DAD-MS and HPLC-DAD) to separate, characterize, and quantify minor Artichoke compounds. The most important flavonoid compounds were Scolymoside, Cynaroside, and cynaropicrin. Flavonoid compounds separated from Artichoke included glycosides luteolin-7-β-rutinoside (Scolymoside), and luteolin 7-glucoside (Cynaroside) their weight was 84 and 83 mg, respectively. The method of separation by column chromatography was efficient as indicated by the produced amount of Scolymoside and Cynaroside. The Scolymoside and Cynaroside were separated after the extraction of flavonoids from Artichoke. It is difficult to separate and purify these materials directly from the extract by column chromatography because of the abundance of other secondary metabolic materials. All chromatography separation methods are mainly aimed at separating two or more of the material. The separation process depends on the reality that one of the materials in the stationary phase (Silica gel) is attached compared to other materials that tend to move with the mobile phase (solvent). Extended studies on the chemical components of the Artichoke detected that flavinod substances are considered to be as the active ingredients of the plant and included 0.1–1.0 % flavone glycosides (e.g. luteolin-7-β-rutinoside (Scolymoside), luteolin-7β-glucoside, and luteolin-4β-glucoside (19). Five compounds have been recognized in Artichoke as Cynaroside, apigenin-7-O-glucoside, Scolymoside, sesquiterpens viz. grosheimin, and solstitalin. Their structures were elucidated by spectral methods (10). Moreover, Ultrasound-Assisted Extraction technique proved to be more appropriate and effective for the extraction of antiradical and phenolic compounds (15). In conclusion, Scolymoside and Cynaroside can be isolated from Artichoke; they are futuristically favorable biologically active compounds. They are separated from flavonoid by column chromatography because it did not separate directly from methanolic crude of Artichoke.

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