

Green Extraction of Quercus Infectoria Gall With Supercritical CO₂ and Methanol Co-solvent

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Research Article

Keywords: Quercus infectoria, supercritical-CO₂-extraction, co-solvent-methanol, yield, reproducibility, and toxicity

Posted Date: February 14th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2431331/v1>

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Version of Record: A version of this preprint was published at Environmental Science and Pollution Research on June 28th, 2023. See the published version at <https://doi.org/10.1007/s11356-023-28047-1>.

Abstract

A comparative study of phenolic acid extract from *Quercus infectoria* Gall using supercritical fluid extraction of CO₂ (SCFE-CO₂) with co-solvent methanol can produce yields, phenolic acid compositions, and differences in the extract's toxicity. SCFE-CO₂ was carried out with the same weight of material (*Q. infectoria* gall) and run at a temperature of 60 °C and a pressure of 20 MPa with methanol as the co-solvent and an extraction time of 60 minutes. The extraction process extracts were identified by yield, phenolic acid composition using LC-MS/MS, total phenolic acid, and toxicity. The results of the SCFE-CO₂ extraction technique with co-solvent methanol do not produce extracts that are toxic but have high yields and tannic acid compound (TPC). The SCFE-CO₂ technique has small extract variability (div. sd. 0.1%), a high yield, a high tannic acid compound, a high TPC, and non-toxic, so SCFE produces extracts that have maintainable quality. The small amount of addition of methanol co-solvent can increase the quality of the extract using the SCFE-CO₂ technique, and increasing the addition of methanol cannot increase yield.

Introduction

Quercus infectoria is a plant that has been known to produce gall, where gall is a special product resulting from an interaction between plants and insects known as gallflies or *Cynips gallae-tinctoriae*. The gall of *Quercus infectoria* is called *Quercus infectoria* gall (*Q. infectoria* gall). Generally, *Q. infectoria* gall is popular in Southeast Asia, East Asia, and South Asia as a medicinal plant, for example, to treat inflammatory pain, stomach pain, diarrhea, postpartum care, etc. (Adi, 2016; Jamal et al., 2011). Apart from Southeast Asia, East Asia, and South Asia, this plant is also found in Turkey, Syria, Persia, Cyprus, and Greece (Barudin et al., 2015; Rahman et al., 2011). Then, further development of the use of *Q. infectoria* Gall revealed that this plant has anti-bacterial, anti-inflammatory, and strong antioxidant properties (Do et al., 2014; Dash et al., 2016).

Initially, *Q. infectoria* gall was consumed directly by the villagers with being soaked in hot water and drunk (Koddami et al., 2013). However, in its development, the use of extraction is carried out first to increase the concentration of the active substance and selective compounds (Hafizah et al., 2014; Arina and Harisun, 2019). In general, the extraction used conventional solvent extraction techniques (CSE) and increased the bioactive compound of phenolics with the main compounds of tannic acid (TA), gallic acid (GA), and ellagic acid (EA), along with the flavonoid compound (Askari et al., 2020; Ajilah et al., 2019; Radzali et al., 2014). The weakness of the *Q. infectoria* Gall extraction process is that the composition of the extract depends on the solvent used, the extraction temperature, and several other factors that also affect it (Nadia et al., 2018; Khemakhem et al., 2017). As a result of the extraction's weakness, bioactive components can degrade due to the use of high temperatures, reducing the efficacy of extract results if used as a drug (Manna et al., 2015). Furthermore, the resulting extract contains the solvent that was used, which can increase the toxicity of the extract and cause environmental problems (Hartanto et al., 2016; Hasim et al., 2015). Environmental issues due to the waste of the solution are not a simple matter

because the solvents used are generally organic solvents, which are toxic and can also be carcinogenic (Markom et al., 2013; Hamad et al., 2017; Wang and Weller, 2006).

Green extraction techniques have been developed for a long time, one of which is the supercritical fluid extraction (SCFE) technique (Zi et al., 2016; Sukor et al., 2020); in addition to that, there has also been the development of other extraction techniques (Singh et al., 2016; Kamarudin et al., 2021). In SCFE-CO₂, some researchers postulate that SCFE-CO₂ offers extraction packages that have high effectiveness (Shi et al., 2022; Yulianti et al., 2014), but since CO₂ is non-polar, it does not have excellent results for the extraction of polar compounds. This is due to the low solubility of CO₂. Therefore, the extraction efficiency and selectivity of SCFE-CO₂ can still be improved by modifying the addition of the corresponding joint solvent. In addition, SCFE-CO₂ uses very high pressure, so the combination of high pressure and polar co-solvent accretion is expected to improve the efficiency of a good extract (Wyepkowski et al., 2014; Hasimi et al., 2017; Fatima et al., 2008; Bouhafoun et al., 2018; Hartanto et al., 2016).

Material And Methods

Materials

Q. infectoria gall was purchased from a local market in Indonesia, and then *Q. infectoria* galls were washed with distilled water and dried in an oven at 40°C for approximately 24 hours. After that, it is cooled, and when it is cold (room temperature), it is ground to a size of around 0.30 mm. Galls with a size of 0.3 mm are put into plastic bags and sealed and stored in the freezer, and this material is used for extraction.

Solvent Extraction as Control

Ten g of 0.3 mm *Q. infectoria* Gall was placed in a beaker glass with a volume ratio of 1:10 solvent, and the mixture was heated on a hotplate at 50°C for 8 hours while being stirred with a magnetic stirrer at 250 rpm (Slimen et al., 2017). During the extraction process, the beaker glass was closed to reduce evaporation. The results were filtered, and the filtrate was evaporated until the solvent ran out. It measured yield, total phenolic compound, composition, and toxicity.

Supercritical fluid extraction of CO₂ with co-solvent

One hundred grams of *Q. infectoria* The extraction vessel was filled with 0.3 mm galls, and the thermal jacket was filled with hot water (60 oC). Liquid CO₂ and co-solvent are pumped into the extraction tube with a ratio of CO₂ to methanol of 500:1 and a pressure of 20 MPa. The extraction was carried out for 60 minutes, and the output flow rate was 25 mL/min. The extract was collected in an extract storage tube, which was also cooled with dry ice, and the extracted results were weighed gravimetrically. Extraction was spared for other ratios of CO₂ and methanol (Al-Matari et al., 2021; Qi et al., 2020). The research data scheme can be seen in Fig. 1.

Analysis of Extract Compounds with LC-MS/MS

The extract from *Q. infectoria* Gall's composition was determined using an LC-MS/MS with a Phenomenex Prodigy 5. The detection of gallic acid, ellagic acid, and quinic acid were set at 270 nm, and tannic acid was set at 280 nm. In an isocratic program, the mobile phase is 1 ml/min (phosphoric acid: acetonitrile = 9:1). The standard curve was prepared at concentrations of 10 ppm–60 ppm (Ayed et al., 2021; Sun et al., 2014; Kaur et al., 2016; Aras et al., 2016).

Analysis of the total phenolic compound (TPC) of the *Q. infectoria* gall extract used The Folin-Ciocalteu Method

This method uses the Folin-Ciocalteu reagent, where the Folin-Ciocalteu reagent is prepared by diluting Folin-Ciocalteu with distilled water at a ratio of 1:10 v/v. 0.5 mL of *Q. infectoria* Gall extract was mixed with 5 mL of Folin-Ciocalteu reagent and put into a flask. After 5 minutes, 4 ml of sodium carbonate (Na_2CO_3) was added to the flask and stirred until homogeneous. UV spectrophotometer was used to compare the solution to a blank solution without extract at 765 nm. The TPC compound is expressed as a mass equivalent of gallic acid (equivalent to mg of gallic acid per gram of extract) (Slimen et al., 2017).

The MTT assay was used to determine the toxicity of *Q. infectoria* gall extract.

The toxicity test of *Q. infectoria* Gall extract was carried out using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) reagent. Before the test was carried out, Vero cell seeding was carried out first on 96-well plates with a density of 2.4×10^4 per well.

The test was carried out by adding *Q. infectoria* Gall extract to Vero cells and then incubating them in an incubator for 48 hours at 37 °C and with 5% CO_2 compound. After the incubation, the old medium was removed and a medium containing 10% MTT was added. They then incubated for another 4 hours. The precipitate was added to DMSO to dissolve it, and it formed from the MTT reaction. The absorbance at 560 nm and 750 nm of the MTT reaction results with *Q. infectoria* Gall extract was measured using the Glo-Max Microplate Multi-Detection Reader (Dos Santos et al., 2018).

Result And Discussion

Yield and Composition

The results of supercritical CO_2 extraction (SCFE-CO_2) of *Q. infectoria* gall with changes in co-solvent methanol flowing between 0–6% against the flow of CO_2 (25 ml/min) are shown in Table 1. For comparison, the results of extraction using standard procedures (CSE) and methanol solvents were also given.

Table 1. The characteristic of the extract results in the extraction of conventional solvent extraction (CSE) and Supercritical extraction of CO_2 (SCFE) with a Flow rate of CO_2 25 ml/minute

Extraction Methods	Solvent/ Co-Solvent Methanol (ml/minute)	Yield (%)	Tannic acid		Gallic acid		Ellagic acid		Quinic acid		TPC mGAE / 100gr	Toxicity LC ₅₀ ± sd
			wt. %	sd	wt. %	sd	wt. %	sd	wt. %	sd		
CSE	100	4.29 ± 2.1	67.46	6.64	2.08	0.63	0.15	0.07	2.77	1.80	1596 ± 41	713 ± 86
SCFE-CO ₂	-	7.18 ± 0.17	67.08	0.72	2.57	0.10	0.18	0.01	6.41	0.67	1638 ± 22	2945 ± 92
SCFE-CO ₂	0.05	7.95 ± 0.11	67.18	0.82	2.50	0.10	0.19	0.01	7.18	0.60	1608 ± 23	2685 ± 51
SCFE-CO ₂	0.10	8.01 ± 0.12	67.31	0.65	2.46	0.10	0.20	0.01	7.35	0.63	1756 ± 20	2303 ± 55
SCFE-CO ₂	1.5	8.09 ± 0.10	67.28	0.62	2.35	0.11	0.23	0.01	7.99	0.67	1781 ± 18	2085 ± 75
SCFE-CO ₂	3	8.11 ± 0.10	67.20	0.63	2.18	0.12	0.23	0.01	8.38	0.67	1799 ± 13	1502 ± 81
SCFE-CO ₂	6	8.02 ± 0.13	67.14	0.61	2.03	0.12	0.23	0.01	8.98	0.67	1768 ± 11	1335 ± 82

Table 1 shows the SCFE-CO₂ results of *Q. infectoria* Gall both without and with co-solvent and CSE as controls. The use of the CSE technique as a control only obtained a yield of 4.29%, and with 5 repetitions, a standard deviation of + 2.1% was obtained. While the results of the yield using the SCFE-CO₂ technique produced *Q. infectoria* gall extracts of 8–10%, or at least 3% above the CSE technique, with a standard deviation of around 0.1%, a very significant difference from the standard deviation indicates that the *Q. infectoria* gall extract using the CSE technique has a high yield variation, so the yield produced is not always the same or close to each extraction repetition. Then, for the SCFE-CO₂ technique, the results of the *Q. infectoria* Gall extract were obtained with a yield that had a low standard deviation, namely around 0.1%, both with and without co-solvent; this indicated that the continuity of the *Q. infectoria* Gall extract had a yield that was approximately the same for each extraction repetition, and the SCFE-CO₂ extraction technique produces extracts with a high yield, or around 7–8%, in each extraction repetition. The results of the *Q. infectoria* Gall extract with CSE as a control had a lower yield and a higher standard deviation, while the SCFE-CO₂ technique showed that the extract obtained had a high yield and a low standard deviation so that the SCFE-CO₂ performance obtained a higher yield. high, and the standard deviation is low, so repeated extractions will also produce extracts with almost the same yield. so that the composition of the compounds that are made should always give the same results, both in terms of quality and quantity.

Based on the above extract results using the SCFE technique, the four compounds contained in the extract can be seen in Table 1, namely tannic acid, gallic acid, ellagic acid, and quinic acid. Further qualitative analysis of the *Q. infectoria* gall extract using LC-MS/MS reveals many compounds in the extract. Based on the qualitative analysis of the MS spectroscopy results from *Q. infectoria* gall extract, more than twenty compounds were found. The LC-MS/MS chromatogram is illustrated in Fig. 2, and the qualitative tabulation of the compounds that make up the *Q. infectoria* Gall extract can be seen in Table 2.

Table 2. Characteristics of the composition of the *Q. infectoria* Gall extract extracted using the SCFE technique at a CO₂ speed of 25 ml/minute and with a co-solvent of 0.05 ml/min

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No	Co-solvent Methanol 0.05 ml/minute		Parent ion m/z	Fragment ion (MS)(CE)
	Item	RT		
1	Quinic Acid	2,938	191.0	85 (22), 93 (22)
2	Gallic Acid,	3,256	169.1	125(14), 79(25)
3	Malic Acid,	3,565	133.1	115 (14), 71 (17)
4	Ellagic Acid,	3,755	301	257, 229, 185
5	Aconitic Acid,	4,111	172.9	85 (12). 129 (9)
6	Tannic Acid,	4,412	183.0	757, 713, 633, 631, 613
7	Salicylic acid	4,846	137.0	93 (16), 65 (31), 75 (30)
8	Chlorogenic Acid,	5,546	353.0	357,195, 179, 125
9	Benzoic Acid,	6,711	137.0	93 (17), 65 (27)
10	Tran-Caffeic Acid,	7,628	179.0	135 (15), 134 (24), 89 (31)
11	Vanillin,	8,591	151.1	136 (17), 92 (21)
12	P-Coumaric Acid,	9,219	163.0	119 (15), 93 (31)
13	Rosmarinic Acid,	9,352	358.9	161 (7), 133 (42)
14	Rutin,	9,428	609.0	300 (37), 271 (51), 301(38)
15	Hesperidin,	9,999	611.0	303(24, 465(12)
16	Hyperoside,	10,228	463.1	300(27), 301(26)
17	Myricetin,	11,818	317.0	179(19), 151(23), 137 (26)
18	Fisetin,	12,493	285.0	135(22)121(27)
19	Quercetin,	13,155	300.9	179(19),151(21),121(28)
20	Coumarin,	14,282	147.0	103(17), 91(26), 77(27)
21	Naringenin,	14,434	271.0	151(18), 119(24), 107(26)
22	Hesperetine,	15,094	301.0	164(25), 136(33), 108(42)
23	Luteolin,	15,100	285.0	217(25)199(28),175(29)
24	Kaempferol,	15,253	285.0	217(29)133(32)151(23)
25	Apigenin,	17,156	269.0	151(25) 133(32),175(29)
26	Rhamnetin,	18,853	315.0	165(23),121(28), 300, (22)
27	Chrysin.	21,039	253.0	143 (29), 119 (32), 107(26)

Effect of addition of methanol as a co-solvent in SCFE-CO₂

The co-solvent in supercritical CO₂ extraction is expected to help increase or decrease the polarity of CO₂ so the optimal extract can be produced. This extraction uses a pressure of 200 MPa, an operating temperature of 60 °C, and 3 mm of *Q. infectoria* gall material. The addition of co-solvent methanol was carried out at 0–6 ml/minute. The yield results of changes in cosolvent flow during the extraction of *Q. infectoria* gall can be seen in Fig. 3.

Meanwhile, in SCFE-CO₂, with the addition of co-solvent methanol of 0.05 ml per minute, there is an increase in yield in the range of 1%, but if the co-solvent flow is increased by more than 0.05 ml per minute, there is almost no increase in yield anymore, which means that there is no significant change in the yield results obtained from the increase in co-solvent flow. This pattern can be seen in Fig. 1, where the yield of SCFE-CO₂ is shown in the chart with a horizontal response at flow speeds ranging from 0.1 ml/min to 6 ml/min. So, it can be said that the addition of co-solvent methanol from 0.1 ml per minute to 6 ml per minute produces several extracts that have approximately the same yield.

Furthermore, the study of extracts obtained by the SCFE-CO₂ technique included an analysis of the quality of the extract, which was observed with LC-MS-MS to obtain the composition of the extract. Based on the results of the LC-MS-MS analysis, the composition of some phenolic compounds was determined, such as tannic acid, quinic acid, gallic acid, and ellagic acid (Table 1). In general, the SCFE-CO₂ technique extracts without the use of co-solvent methanol and yields tannic acid levels in the 67% range. Other than tannic acid, compositions from the extract *Q. infectoria* Gall SCFE-CO₂ technique, either without or with co-solvent methanol, contain phenolic acids, namely tannic acid, gallic acid, ellagic origin, and quinic acid, with varying compositions. While the amount is extracted, *Q. infectoria* gall contains the four phenolic compounds: tannic acid, ellagic acid, gallic acid, and quinic acid, which are the largest after tannic acid. In the SCFE-CO₂ technique for the extraction of *Q. infectoria* gall without the addition of co-solvents, the composition of the extract is approximately the same as in SCFE-CO₂ with co-solvent methanol. The difference in the extract results is an increase of approximately 1% between the treatments without and with co-solvent. Meanwhile, the variability of the extracted compound was roughly the same across treatments, namely a range of 0.1%. *Q. infectoria* gall extract differs by 3% and varies by 2.1% when compared to the control using the CSE technique. The quantity and technique of SCFE-CO₂ are very stable and quite profitable for further application, either with the addition of co-solvent or not.

Toxicity of infectoria gall extract and its impact on the environment

The addition of co-solvent to the extraction process of *Q. infectoria* Gall with the SCFE-CO₂ technique turned out to be only binding to the yield, but there was no correlation with the changes and composition

of the extract obtained, as illustrated in Fig. 4. The graph shows that adding co-solvents increases the number of extracts of *Q. infectoria* gall, which results in an increase in the yield obtained, but the magnitude of the increase in yield quantity is not proportional to the amount of co-solvent methanol added. The addition from 0.05 to 6 ml per minute only increases the yield by 1% and does not show any increase in yield quantity even though the addition of co-solvents improves the extraction process. Meanwhile, phenolic compositions have the same compound composition and the same quantity.

In Fig. 2, the correlation between the yield of the extract, the total phenolic compound, and the toxicity properties of the SCFE-CO₂ extract of *Q. infectoria* Gall is figured out. The things that can be observed from the graph are: (1) the yield of the extract of *Q. infectoria* Gall obtained was not increased with the addition of co-solvent flow during extraction, while the total phenolic compound increased significantly with increasing co-solvent flow. (2) The total phenolic compound increased with the toxicity of *Q. infectoria* gall extract. (3) The increase in the addition of co-solvent methanol affects the toxicity of the extract. From the three things above, referring to the results in Fig. 2, it can be seen that increasing the quantity of TPC and yield has less effect on toxicity, as evidenced by data extraction using the CSE technique (as a control), even though the TPC is the lowest but has the highest toxicity. This is related to the extraction process with the CSE technique used as a control using 100% methanol solvent; if this pattern can be applied, it will produce *Q. infectoria* gall extract obtained from SCFE-CO₂ with the addition of large amounts of methanol, which will have a high toxicity. Therefore, an extraction with low toxicity and a high yield can be said to be a good extraction.

The result of the high compound of TPC was associated with the magnitude of the toxicity of the extract of *Q. infectoria* Gall, where the method of determining the total phenolic was determined by the Folin-Ciocalteu method as a standard of gallic acid. Based on the phenolic determination method above, if the extract has other fractions and is mixed with the extract, it can contribute to increased toxicity. As a result, the toxicity of *Q. infectoria* gall extract can be attributed to several factors, including the use of solvents, the extraction of highly toxic compounds, and the total phenolic compound. In the SCFE-CO₂ method with co-solvent, methanol extracted by *Q. infectoria* Gall does not have toxic properties because when the extract is separated from CO₂, the methanol is carried by CO₂, so that the extract of *Q. infectoria* Gall obtained is not contaminated with methanol. Conversely, by increasing the flow rate of the co-solvent methanol, the *Q. infectoria* gall extract will increase the TPC compound. This is understandable because the co-solvent methanol can bind phenolic compounds and produce more yield in the extract. The highest TPC compound of the extract was 1756 mGAE, which was an SCFE-CO₂ technique extract with methanol as the co-solvent. Meanwhile, the MTT test was used to determine the toxicity of the extract. The most toxic extract was shown in the CSE technique with methanol as a solvent and an LC₅₀ of 713. The extract with the SCFE-CO₂ technique did not show a small LC₅₀ number, all above 1000, even though the co-solvent used was methanol.

Based on the improvement of the extract from the SCFE-CO₂ technique for the extraction of *Q. infectoria* gall with co-solvent methanol, whose addition is very small (0.05 ml/minute) and produces very little

toxicity, this extraction technique is very good for the environment. Even with no methanol flow, the yield obtained only decreased by 1% and was of good quality.

Conclusion

The supercritical CO₂ or SCFE-CO₂ extraction technique with cosolvent methanol obtained a good *Q. infectoria* gall extract with high yields and tannic acid, as well as high TPC and non-toxicity, and produced more than 20 compounds in the extract. In the flow of methanol added as a cosolvent, it can increase the yield, but the increase in the addition of methanol does not increase the yield obtained. The addition of this very small cosolvent causes the SCFE-CO₂ technique to be classified as an environmentally friendly process or green extraction.

Declarations

Acknowledgment The authors would like to thank Prof. Dr. Djoko Hartanto for his excellent assistance in the Laboratory of Material and Energy, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia.

Author Contribution Prof. Dr. Achmad Syahrani conceived the ideas and designed the experiments. Retno Purbowati conducted the experiments, analyzed the data, and wrote the manuscript. Prof. Dr. Titik Taufikurohmah revisited the manuscript.

Funding The research was supported by the Indonesian Ministry of Research, Technology and Higher Education (RISTEK DIKTI) under the Doctor Dissertation support program (Grant Number: 085/E5/PG.02.00.PT/2022 and 884/UN3.15/PT/2022).

Availability of data and materials Not applicable.

Ethics Approval and Consent to Participate Not applicable.

Consent to Publish The Authors approve the manuscript and give their consent for submission and publication.

Competing Interests The Authors declare no competing interest.

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Figures

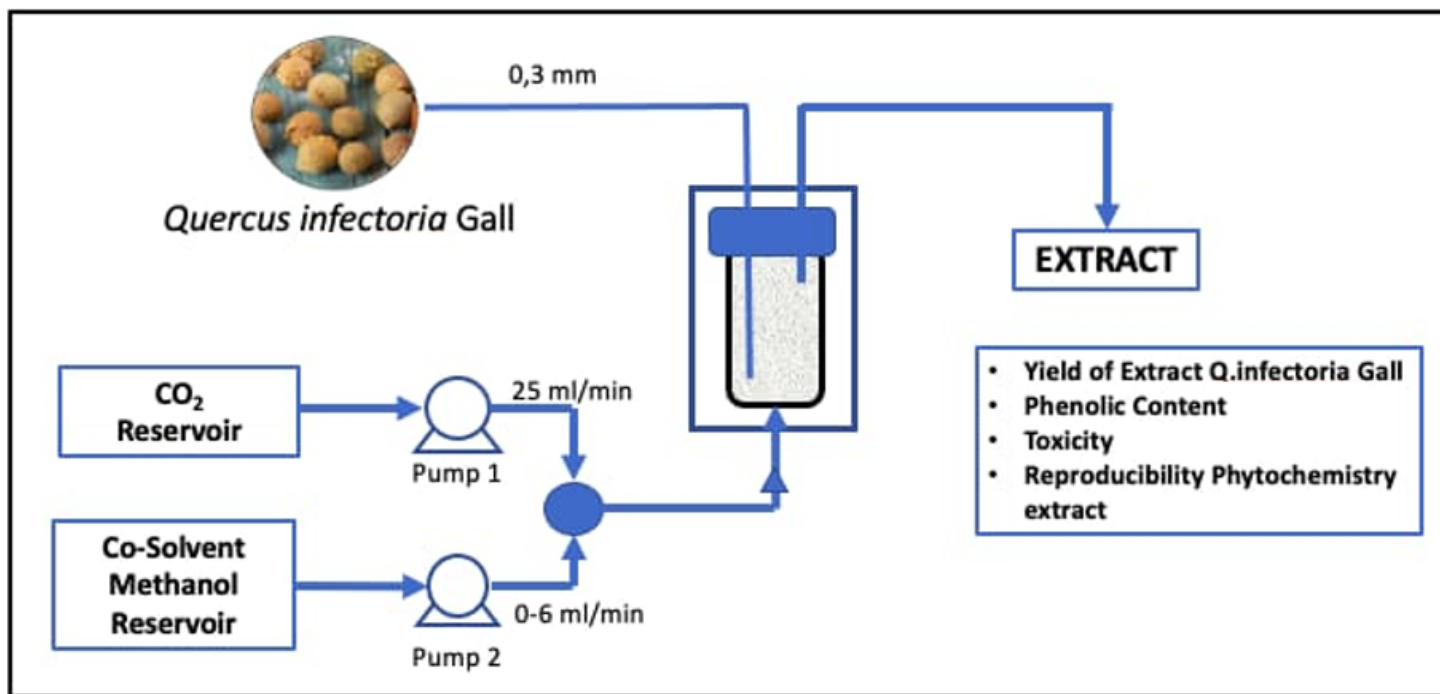


Figure 1

Graphical abstract of *Q. infectoria* gall extraction with supercritical fluid extraction (SCFE).

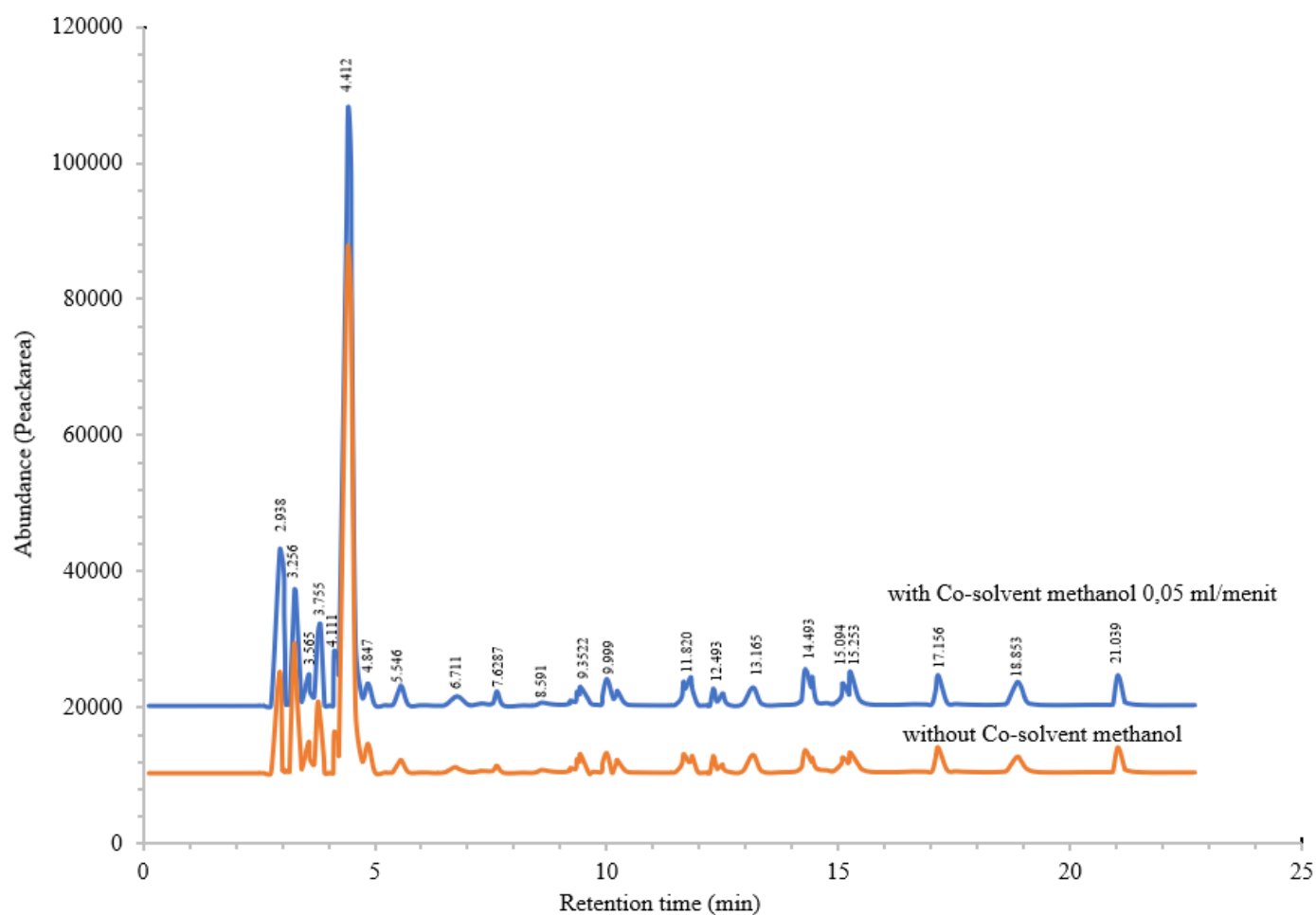


Figure 2

Chromatograms of *Q. infectoria* Gall extracts extracted using the SCFE-CO₂ technique with and without co-solvent methanol.

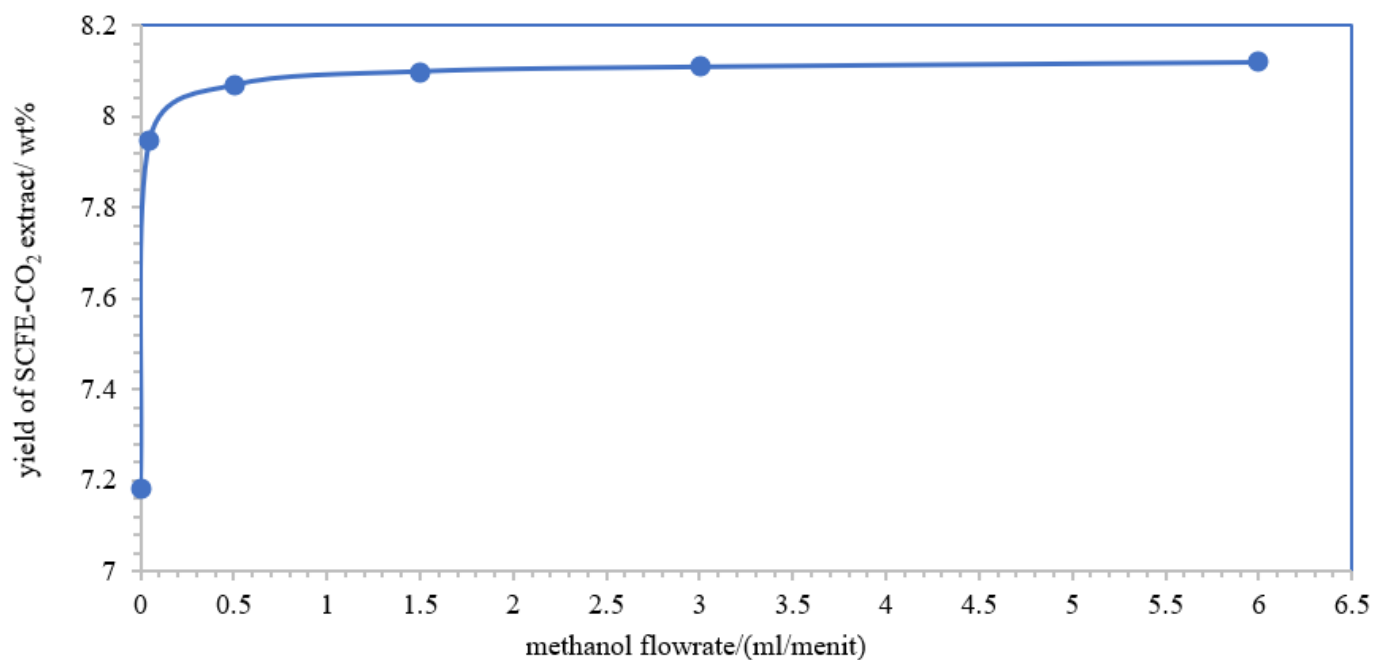


Figure 3

The relationship between co-solvent methanol flow rate and extract yield obtained using the SCFE-CO₂ technique.

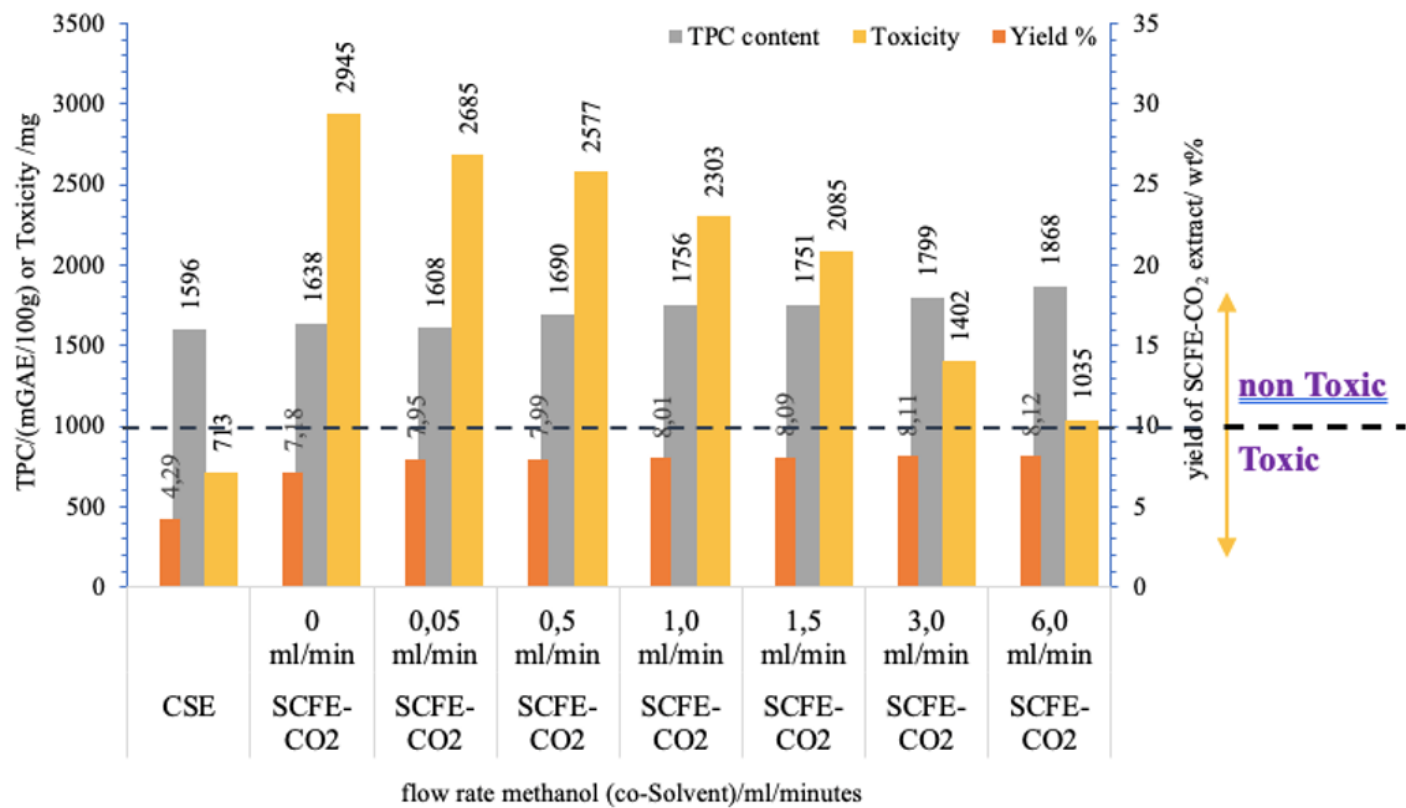


Figure 4

The comparison between yield, TPC, and toxicity of the *Q. intectoria* Gall extract using the SCFE-CO₂ technique and with the addition of methanol as a co-solvent.