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Original article

Himalayan Blackberry (*Rubus discolor*) Leaves and Stems: Phytochemical Analysis, Antioxidant, Antimicrobial and Cytotoxic Activities

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Abstract

Background and objectives: The fruits and leaves of *Rubus discolor* Weihe & Nees (Rosaceae) have been used for treatment of diabetes, diarrhea, prostatitis, and wounds and thus were evaluated in this study. **Methods:** The leaves and stems methanol extracts were prepared by maceration and different fractions were obtained. Total phenolics, tannins, and flavonoids contents were measured. The phenolic profiles were determined by HPLC/DAD analysis. The antioxidant activity was tested. The antiproliferative activity was evaluated against epidermoid carcinoma (A431) and normal (HU02) cells by MTT assay. The zone of inhibition and minimum inhibitory concentration (MIC) were determined against three pathogens. **Results:** The leaves ethyl acetate fraction showed the strongest antioxidant activity (IC_{50} 25.74±0.07 µg/mL) and the highest content of total phenolics (149.72±1.23 mg GAE/g). The antioxidant activity and phenolic content of leaves methanol extract were close to ethyl acetate fraction (IC_{50} 26.26±0.13 µg/mL and 140.63±1.32 23 mg GAE/g, respectively). The leaves methanol fraction and methanol extract showed the highest flavonoids (58.87±0.71 mg QE/g) and tannins content (314.4±0.11 mg TAE/g), respectively. The leaves and stems methanol extract and methanol fraction exhibited the highest antimicrobial activity against *Staphylococcus aureus* (MIC 37.5 mg/mL). The most potent cytotoxic effect was for leaves ethyl acetate fraction (369.6±56.1 µg/mL). HPLC analysis proved the presence of gallic acid, vanillic acid, ferulic acid, luteolin and rutin in leaves ethyl acetate fraction, and gallic acid and ferulic acid in leaves methanol fraction. **Conclusion:** The leaves ethyl acetate and methanol fraction showed potential to be investigated in further studies.

Keywords: antimicrobial; antioxidant; cytotoxicity; phytochemicals; *Rubus discolor*

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Introduction

Drugs of natural origin have been historically used for the treatment or prevention of health-threatening diseases. They are also at the center of attention for discovering new drug candidates. There is an upward trend in using botanical natural products around the world, and it is reported that nearly 80% of people in developing countries depend on traditional medicine for curing diseases [1,2]. Recently, natural antioxidants have attracted great attention

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because of their significant beneficial effects in preventing human diseases, such as rheumatoid arthritis, diabetes, cardiovascular diseases, and cancer [3]. Moreover, the discovery and development of effective and efficient antimicrobial drugs to fight against widespread antibiotic resistant bacteria is another field of interest [4]. Medicinal plants have also been used for their potential antibacterial effect against different food-borne and food spoilage pathogens to improve the safety of products and prolong their shelf life [5-7].

The genus *Rubus* (Rosaceae) includes over 750 species and is widely distributed throughout the world [8,9]. The berries are well-known as a healthy food. They are rich sources of polyphenolic compounds (tannins, flavonoids, and anthocyanins), vitamins (vitamins C and E), and dietary fibers [10,11]. In the leaves, the presence of flavonoids (such as quercetin and kaempferol) and phenolic acids (such as ellagic acid, gallic acid, chlorogenic acid, and p-coumaric acid) has been reported [3,12]. The leaves have been traditionally used for treating medical conditions like morning sickness, diarrhea, inflammation, preventing miscarriage, ameliorating labor pains, and as insecticidal agent [9,13-15]. Pharmacological studies have revealed the antioxidant, anti-inflammatory, chemo-preventive, antimicrobial, and anti-atherosclerosis activities of the *Rubus* berries [16].

In the flora of Iran, the *Rubus* species comprises eight species. Among them, *Rubus discolor* Weihe & Nees. (Syn: *R. armeniacus*) is well-known as the Himalayan blackberry and is a native species of the Caucasus region of Eurasia [17]. It is widely distributed in the North and Northwest of Iran [18]. The plant is a shrub growing to two meters, with reddish brown thick stems and straight needle spines. The leaves have 3-5 leaflets (about 80 mm in length), petals are pale pink, with white filament [19]. In folk medicine, the fruits and leaves of this species have been administrated for the treatment of diabetes, nephritis, diarrhea, prostatitis, and wounds [20,21]. Literature survey showed that studies have focused more on phenolic contents, and the biological activities of fruit extracts of *R. discolor* [22-24], while the phytochemical profile and biological activity of other parts, such as leaves and stems have been less investigated. In a study, the antimicrobial activity of leaves of *R. discolor*, growing in Turkey, was evaluated, but the phytochemical content remained unknown [25]. Also, the phenolic contents and antioxidant activity of leaves from *R. discolor*, growing in Serbia, were reported [26].

Regarding that geographical distributions have a significant impact on the levels of phytochemicals and their biological activities, in the present study, the antioxidant, antimicrobial, and cytotoxic activities of methanol extracts and fractions from leaves and stems of *R. discolor*, growing in Iran, were investigated. Also, the phytochemical profiles of fractions with the highest biological activities were determined and quantified using analytical HPLC, for the first time.

Material and Methods

Ethical considerations

This study was approved by the Ethical Committee of Guilan University of Medical Sciences (ID: IR.GUMS.REC.1398.215).

Chemicals

DPPH (2,2'-Diphenyl-1-picrylhydrazyl), polyvinylpolypyrrolidone (PVPP), gallic acid, quercetin, vanillic acid, ferulic acid, caffeic acid, luteolin, p-coumaric acid, rutin, and tannic acid were obtained from Sigma Chemical Company (USA). Folin–Ciocalteu's phenol reagent, Aluminum trichloride ($AlCl_3$), ammonium molybdate tetrahydrate, thiobarbituric acid, and sodium bicarbonate were purchased from Merck, Germany. Mueller Hinton Broth (MHB) and Sabouraud Dextrose Broth (SDB) were bought from Merk, Germany. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum were obtained from Gibco (USA) and Invitrogen (USA), respectively. HPLC-grade acetonitrile and acetic acid were purchased from DUKSAN, Korea. Other used reagents and solvents were of analytical grade.

Plant material

The aerial parts of *R. discolor* were collected from Foman-Saravan highway, Guilan province, in the North of Iran, in May 2021. The identification of plant species was performed by Dr. Fatemeh Yousefbeyk, Associate Professor of Pharmacognosy, School of Pharmacy, Guilan University of Medical Sciences (GUMS). The voucher specimen (113 HGUM) was deposited at the Herbarium of School of Pharmacy, GUMS.

The leaves and stems were separated and shade-dried at room temperature for two weeks.
The leaves and stems (300 g, each) were powdered in the mixer grinder and extracted with methanol (1000 mL) by maceration method for 24 h. The extraction was repeated three times. The solvent was evaporated using a rotary vacuum evaporator at 40 °C. The methanol extracts of each part were fractionated by hexane, ethyl acetate, and methanol, subsequently. All fractions were concentrated by a rotary vacuum evaporator (40 °C).

Cell lines
The A431 cancerous cell line was purchased from the Iranian Biological Resource Center (Iran). The Hu02 cell line was purchased from the National Cell Bank of Pasteur Institute (Iran).

Preliminary phytochemical assays
The preliminary qualitative phytochemical analysis was carried out to identify the presence of secondary metabolites, including tannins, flavonoids, alkaloids, steroids, and quinones, in the samples using the standard protocols described by Saeidnia and Ghohari [27].

Determination of total phenolics content (TPC)
The Folin-Ciocalteu method was used to measure total phenolics content in methanol extracts and fractions using a technique previously described [28]. All experiments were repeated three times. The total phenolics content was expressed as mg of gallic acid equivalents (GAE)/g extract.

Determination of total flavonoids content (TFC)
The measurement of flavonoids content was carried out by the Dowd method, as it was previously reported by Yousefbeyk et al. [4]. All experiments were done in triplicate. The total flavonoids content was expressed as mg of quercetin equivalents (QE)/g extract.

Determination of total tannins content (TTC)
All samples were investigated for the total tannins contents using a colorimetric method [29]. The total tannins content was expressed as tannic acid equivalent (TAE)/g extract.

DPPH radical scavenging activity
The 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay was carried out to investigate the radical scavenging activity of methanol extracts and fractions by a previous method [29]. The IC_{50} values (expressing the concentration of the extract (µg/mL) providing 50% inhibition) were calculated from the graph-plotted scavenging percentage against samples concentration.

Determination of total antioxidant capacity by phosphomolybdenum reduction assay (PRA)
The antioxidant activity of each sample was determined by the phosphomolybdenum reduction assay (PRA) using a method previously described by [7]. The total antioxidant capacity of each sample was expressed as mg of α-tocopherol equivalent (αTE)/ gram extract.

Cell cultures and cytotoxicity assay
The methanol extracts and fractions prepared from leaves and stems of *R. discolor* were tested for their anti-proliferative activities against A431 cell line and a normal cell line (Hu02) by methyl thiazol tetrazolium (MTT) assay using a method previously described by Azmian Moghadam et al. [30,31]. The IC_{50} of samples against each cell line was defined as the concentration in which only 50% of cells were alive and computed using GraphPad Prism (Version 8, GraphPad Software, USA) [32,33].

Antibacterial assay
Antimicrobial activities of extracts and fractions were tested against a Gram-positive (*Staphylococcus aureus* ATCC 6538) and a Gram-negative (*Escherichia coli* ATCC 8739) bacteria as well as a fungal strain (*Candida albicans* ATCC 10231) by agar disc diffusion method. Minimum inhibitory concentration (MIC) was measured by the broth microdilution method using 96 U-shaped well plates according to a method previously described [34].

HPLC analysis of *R. discolor*
The quantitative analysis of phenolic compounds was performed in the fractions with the highest biological activity, including leaves methanol fraction) and ethyl acetate fraction, using the HPLC apparatus (Waters Aliance e2695). It was equipped with the photodiode array (PDA) detector (Waters 2998) and C_{18} reversed-phase column (Waters spherisorb, S5 ODS2, 4×250 mm, 5 µm). The absorbance changes were monitored at 210 nm to 400 nm. Empower 3

software (Waters, USA) was used for data acquisition and quantitation. The mobile phase consisted of a time-varying ratio of H_2O-acetic acid (1%) (solvent A) and acetonitrile (solvent B). The flow rate was set at 1 mL/min, and the total run time was 60 min.

The following gradient program was used for elusion: 1-10 min: 10% B, 10-20 min: linear gradient from 10% to 30% B, 20-60 min: linear gradient from 30% to 100% B. The peak related to each compound was identified by comparison of DAD absorbance spectra with external standards. Gallic acid, vanillic acid, ferulic acid, caffeic acid, luteolin, p-coumaric acid, and rutin were used as standard phenolic compounds. The peak areas of each standard were plotted against the corresponding standard concentrations (µg/mL) using linear regression to create standard curves. The determination coefficient (R^2) was calculated by employing the least-square analysis.

Results and Discussion

According to the results, the yields of extraction were 22% and 10% for leaves and stems, respectively. The results of the preliminary phytochemical investigation are depicted in Table 1. Phytochemicals such as flavonoids, tannins, steroids, quinones, and alkaloids were detected in the samples. Methanol extract, ethyl acetate fraction, and methanol fraction of leaves and stems showed the highest amounts of steroids, flavonoids, and tannins.

The total phenolic, tannin, and flavonoid contents of leaves were higher than those of stems. The results indicated that TPC ranged from 78.25±0.67 to 149.72±1.23 mg GAE/g extract (Table 2). The leaves ethyl acetate fraction had the highest content of phenolic compounds, while hexane fractions of leaves and stems had the lowest concentrations.

The TFC of samples ranged between 4.85±0.01 and 58.87±0.71 (mg QE/g extract). Generally, the flavonoid contents of leaves fractions were higher than stems fractions. Among samples, leaves methanol fraction showed the highest amount of flavonoids. Also, leaves samples showed higher TTC than stems (Table 2). Among leaves fractions, ethyl acetate fraction had the highest level of tannin contents (270.4±0.71 mg TAE/g extract).

In a study conducted by Yousefbeyk et al. the TPC of leaves extract and fractions of *R. hyrcanus* ranged between 68.30 and 101.82 mg GAE/g [3]. Veljkovic et al. reported that the TPC in leaves of different populations of *R. idaeus* ranged from 59.7 to 96.8 mg GAE/g [35]. Asnaashari et al. showed that in leaves of *R. fruticosus*, the TPC varied from 57.6 to 108.6 mg GAE/g. The results of our study are in the range of these studies.

Table 1. Qualitative analysis of phytochemicals in methanol extract and fractions from leaves and stems of *Rubus discolor*

Samples		Tannin	Flavonoid	Steroid	Alkaloid	Quinon
ME	Leaves	++	++	++++	++	+
	Stems	++	++	++++	++	+
HF	Leaves	-	-	+	++	-
	Stems	-	-	++	++	-
EF	Leaves	++	++	+++	-	+
	Stems	++	++	++	+	-
MF	Leaves	++	++	++++	+	+
	Stems	++	++	++++	++	+

ME: methanol extract; HF: hexane fraction; EF: ethyl acetate fraction; MF: methanol fraction; (−) absent; (+) present; (++) moderate concentration; (+++) abundant concentration; (++++) very abundant concentration

Table 2. Total phenolics, flavonoids, and tannins contents of methanol extracts and fractions of *Rubus discolor* leaves and stems

Sample	TPC (mg GAE/g extract)		TFC (mg QE/g extract)		TTC (mg TAE/g extract)	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
ME	140.63±1.32	127.54±1.11	45.93±0.68	26.72±0.21	314.4±0.11	224.4±0.21
HF	78.27±1.14	78.25±0.67	7.64±0.21	4.85±0.01	-	-
EF	149.72±1.23	130.84±0.98	30.76±0.32	12.79±0.07	270.4±0.71	61.8±0.12
MF	138.36±0.79	122.07±1.01	58.87±0.71	22.6±0.05	144.4±0.56	52.13±0.62

TPC: total phenolics contents; TFC: total flavonoids contents; TTC: total tannins content; ME: methanol extract; HF: hexane fraction; EF: ethyl acetate fraction; MF: methanol fraction; -: not detected; the results are expressed as mean ± standard deviation of three independent experiments.

Moreover, Velickovic et al. reported that the TPC on leaves of *R. discolor*, growing in Serbia, ranged from 250.05 to 446.61 mg GAE/g [26], which indicated the effect of geographical distribution in levels of polyphenols in plants. So far, flavonoids such as hyperoside, quercetin, rutin, kaempferol, and quercetin 3-O-β-d-glucopyranoside have been reported in the leaves of *Rubus* species [14,36]. In both antioxidant assays, leaves samples showed more potent activities than stems (Table 3). In DPPH radical scavenging assay, the IC_{50} of the samples in leaves extracts ranged from 25.74±0.07 to 76.84 ±0.11 µg/mL. The highest antioxidant activity was observed from ethyl acetate fraction, methanol extract, and methanol fraction of leaves (25.74±0.07, 26.26±0.13, and 26.91±0.10 µg/mL, respectively). The IC_{50} of the standard antioxidant agent, BHA, was 8.01±0.16 µg/mL.

Previously, Yousefbeyk et al. indicated that in *R. hyrcanus*, the antioxidant activities ranged from IC_{50} 30.15 to 122.87 µg/mL [3]. These results indicated that the leaves of *R. discolor* showed better antioxidant activity than *R. hyrcanus* leaves. Moreover, Muniyandi et al. reported that the DPPH antioxidant activities of fractions from the fruits of *R. niveus*, *R. ellipticus*, and *R. fairholmianus* ranged from 11 to 406.8 µg/mL [37]. They also showed that methanol and ethyl acetate were the best solvents for extracting antioxidant compounds [37]. Grochowski et al. revealed that in DPPH assay of *R. caesius* fractions, the IC_{50} values ranged between 44.5 and 240.9 µg/mL, and the ethyl acetate fraction showed the best activity [2].

In PRA, α-Tocopherol was used as the standard substance, and the following calibration curve was plotted: $y= 0.0024x + 0.004$; $R^2 = 0.998$. The results revealed that the leaves ethyl acetate fraction, methanol extract, and methanol fraction showed the highest antioxidant activities (783.62±0.24, 772.00±0.16, and 769.95±0.15 mg αTE/g extract, respectively). This result is in line with the study of Yousefbeyk et al. that reported the antioxidant activity of *R. hyrcanus* in range of 447.14-1010.5 mg αTE/g extract [7].

The *R. discolor* samples were investigated for antimicrobial activities against three common pathogens. The results are depicted in Table 4. The methanol extract and methanol fraction of leaves and stems showed the highest antibacterial activities on *S. aureus*, with zone of inhibitions ranging between 13 and 12 mm, respectively, and MIC of 37.5 mg/mL.

The sample did not show antibacterial activities against *E. coli* except for methanol fraction of leaves and stems (zone of inhibitions 5 and 4 mm, respectively). None of the samples showed anti-*candida* activity in this study.

Table 3. Antioxidant activities of methanol extracts and fractions from leaves and stems of *Rubus discolor*

Sample	DPPH (IC_{50} µg/mL)		PRA (mg αTE/g extract)	
	Leaves	Stems	Leaves	Stems
ME	26.26±0.13	32.86±0.11	772.00±0.16	551.12±0.13
HF	76.84±0.11	82.33±0.14	621.12±0.32	432.05±0.11
EF	25.74±0.07	30.73±0.23	783.62±0.24	483.23±0.31
MF	26.91±0.10	41.74±0.40	769.95±0.15	564.42±0.41

DPPH radical scavenging assay; PRA: phosphomolybdenum reduction assay; ME: methanol extract; HF: hexane fraction; EF: ethyl acetate fraction; MF: methanol fraction; Values of the results are expressed as mean ± standard deviation of three independent experiments.

Table 4. Antimicrobial activities of methanol extracts and fractions from leaves and stems of *Rubus discolor*

Sample		*Staphylococcus aureus*		*Escherichia coli*		*Candida albicans*	
		ZI (mm)	MIC (mg/mL)	ZI	MIC	ZI	MIC
ME	Leaves	13±0.1	37.5	-	-	-	-
	Stems	12±0.1	37.5	-	-	-	-
HF	Leaves	10±0.12	75	-	-	-	-
	Stems	3±0.1	150	-	-	-	-
EF	Leaves	7±0.21	75	-	-	-	-
	Stems	5±0.1	150	-	-	-	-
MF	Leaves	12±0.23	37.5	5±0.1	150±2.56	-	-
	Stems	12±0.22	37.5	4±0.2	150±1.14	-	-

ME: methanol extract; HF: hexane fraction; EF: ethyl acetate fraction; MF: methanol fraction; ZI: zone of inhibition; MIC: minimum inhibitory concentration; - no bacterial activity found; the results are expressed as the mean ± SD

Table 5. Cytotoxic activities of methanol extracts and fractions from leaves and stems of *Rubus discolor*

Cell line / Sample	A431 (IC_{50} µg/mL)		HU02 (IC_{50} µg/mL)	
	Leaves	Stems	Leaves	Stems
ME	> 500	> 500	> 1000	> 1000
HF	> 500	> 500	> 1000	> 1000
EF	369.6±56.1	> 500	> 1000	> 1000
MF	> 500	> 500	> 1000	> 1000

ME: methanol extract; HF: hexane fraction; EF: ethyl acetate fraction; MF: methanol fraction

Studies on *R. hyrcanus* revealed that leaves methanol extract and methanol fraction showed antibacterial activities against *S. aureus* with MIC of 3.12 mg/mL. These samples showed weak antibacterial activity against *E. coli* (MIC of 100 mg/mL) [3]. Moreover, the *R. fairholmianus* root acetone extract exhibited antibacterial effect against *S. aureus* with MIC of 337.86 µg/mL [38].

In another study, Thiem et al. reported that the butanol fraction from *R. chamaemorus* had antibacterial effect on Gram-positive and Gram-negative bacteria (MIC from 0.58 to 2.33 mg/mL) [39]. Also, Jang et al. reported antibacterial activity from different fractions of *R. coreanus* roots against *B. subtilis* and *S. aureus* (MIC between 5 and 30 mg/mL) [40].

The investigation of cytotoxic activity of samples against epidermoid carcinoma (A431) cell line was carried out by MTT assay. HU02 cell line was used as normal cell line. Both cell lines were treated by each plant sample at different concentrations for 48 h. The results are presented in Table 5. Among the samples, ethyl acetate fraction of leaves showed the highest cytotoxicity on A431 cell line with the IC_{50} of 369 µg/mL. The anti-proliferative activity of other samples on A431 cell line were > 500 µg/mL, and samples showed no significant activity on the normal cell line (>1000 µg/mL).

Previously, Veljkovic et al. reported that the leaves methanol fraction of *R. idaeus* had cytotoxicity on HCT-116 (Human colorectal cancer) cell line with IC_{50} of 95.7 µg/mL [35]. Also, Yousefbeyk et al., indicated that the methanol extract prepared from leaves and roots of *R. hyrcanus* had cytotoxicity on MCF-7 (breast cancer) cell line with the IC_{50} of 392 and 414 µg/mL, respectively [7].

In another study, Jazić et al. represented that the *R. fruticosus* fruit extract had anti-proliferative effect on MCF-7 and Hela (cervix epithelioid carcinoma) cell lines with IC_{50} of 306.7 and 315.5 µg/mL, respectively [41]. Moreover, the root extract of *R. fairholmianus* was investigated by Plackal Adimuriyil et al. The results showed that the extract had cytotoxicity against human colorectal cancer cells (Caco-2), reducing the cell viability and inducing apoptotic activity [42]. These findings are in line with the results of the present investigation. Recently, some researches proved the correlation between the antioxidant activity and the anticarcinogenic properties of polyphenols [42,43]. The results of this study showed that the leaves ethyl acetate fraction, which contained the highest amount of total phenolic compounds, had also the higher IC_{50} values against A431 cell line. However, more investigations need to clarify the mechanism of cytotoxicity.

Among the prepared fractions, two fractions of leaves with the most significant biological properties (ethyl acetate fraction and methanol fraction) were selected for identification and quantification of phenolic compounds using DAD-HPLC analysis. The focus of this study was on the evaluation of phenolic compounds. As a results, eight standards were used. The identification of compounds was performed according to the retention times (RT) and absorbance spectra. The HPLC chromatographic profiles are depicted in Figure 1. The calibration characteristics of detected compounds are represented in Table 6.

Table 6. The calibration characteristics of the reference compounds in HPLC-DAD

Phenolic compounds	Calibration curve equation	Linearity*
Gallic acid	$Y = 1.1\times10^{3}X + 1.3\times10^{5}$	0.9999
Vanillic acid	$Y = 2.72\times10^{5}X + 7.5\times10^{6}$	0.9993
Ferulic acid	$Y = 7.32\times10^{4}X + 1.34\times10^{5}$	0.9966
Luteolin	$Y = 7.95\times10^{6}X + 3.01\times10^{4}$	0.9991
Rutin	$Y = 3.32\times10^{5}X + 1.14\times10^{6}$	0.9997

*The linearity was given over the five-point calibration curve

As is presented in Table 7, luteolin had the highest concentration in leaves ethyl acetate fraction (25 mg/g), followed by gallic acid and vanillic acid (11.25 and 10.1 mg/g, respectively). Rutin and ferulic acid had the lowest concentration (8 and 7.5 mg/ g, respectively).

Table 7. Phenolic compounds in leaves of *Rubus discolor*

	Amount (mg/g extract)						
Sample	**Gallic acid**	**Vanillic acid**	**p-Coumaric acid**	**Caffeic acid**	**Ferulic acid**	**Luteolin**	**Rutin**
LEF	11.25±0.4	10.1±0.23	-	-	7.5±0.11	25±0.51	8±0.15
LMF	6.12±0.15	-	-	-	12.1±0.26	-	-

LEF: leaves ethyl acetate fraction; LMF: leaves methanol fraction; -: not detected; results are expressed as mean ± SD.

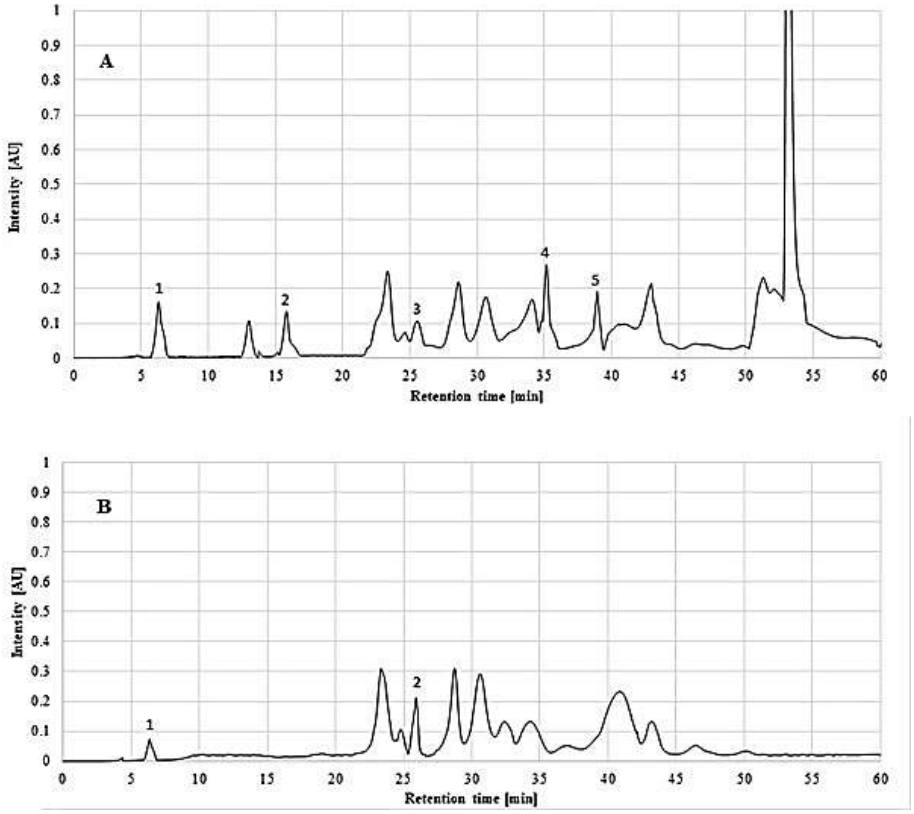

Figure 1. HPLC chromatograms of *Rubus discolor*; A) leaves ethyl acetate fraction, gallic acid (1), vanillic acid (2), ferulic acid (3), luteolin (4), and rutin (5); B) leaves methanol fraction, gallic acid (1), ferulic acid (2)

Caffeic acid and p-coumaric acid were not detected in leaves ethyl acetate fraction. The rest of the peaks could not be identified. Furthermore, in the leaves methanol fraction, gallic acid, and ferulic acid were detected at the concentrations of 6.12 ± 0.15 and 12.1±0.26 mg/g, respectively, but other compounds were not detected.

Previously, El Cadi et al. reported the presence of fourteen phenolic acids (such as gallic acid, ferulic acid, p-coumaric acid, and vanillic acid) and flavonoids (such as luteolin, rutin, and quercetin-3-O-glucosid) from leaves of *R. fruticosus* [44]. In another study, Martini et al. reported flavonoids (including rutin, and quercetin) and phenolic acids (like caffeic acid, ferulic acid, and gallic acid) in *R. ulmifolius* leaves [36]. In our study, quercetin was not detected in tested fractions, but luteolin, and rutin were identified. The phytochemical investigation of other compounds especially steroids that were abundant in ethyl acetate and methanol fractions could be carried out in the future studies.

Conclusion

In general, the total phenolics, tannins, and flavonoids contents of leaves were higher than stems. Also, samples from leaves showed higher antioxidant activities. The highest antioxidant activity was reported from leaves ethyl acetate fraction, which introduced ethyl acetate as the best solvent for extracting antioxidant compounds. All samples showed antibacterial activity against *S. aureus*, while they were inactive on *E. coli* (except methanol fraction) and *C. albicans*. The most potent cytotoxic effect on A431 was seen in leaves ethyl acetate fraction.

HPLC analysis detected the presence of three phenolic acids, including gallic acid, vanillic acid, and ferulic acid, as well as two flavonoids, luteolin and rutin in leaves ethyl acetate fraction. Also, gallic acid and ferulic acid were detected in leaves methanol fraction. The leaves of *R. discolor* are a considerable source of phenolic compounds with antioxidant, cytotoxic, and antibacterial activities.

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Author contributions
Saeed Ghasemi managed the research project, supervised the study and interpreted the HPLC data; Mehdi Evazalipou performed cell culture and the acquisition of cytotoxicity data; Elaheh Khateri prepared the extract and fractions from leaves; Meysam Rostampour prepared the stems extract and fractions; Diba Eghbali Koohi contributed to the acquisition of antioxidant assay data; Omid Goudarzvand performed the antimicrobial tests; Fatemeh Yousefbeyk designed and supervised the study, managed the methods of phytochemical analysis and antioxidant assays, data analysis, and drafted the manuscript.

Declaration of interest
The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

DPPH: 2,2'-Diphenyl-1-picrylhydrazyl; MHB: Mueller Hinton broth; SDB: Sabouraud dextrose broth; GUMS: Guilan University of Medical Sciences; TPC: total phenolics content; GAE: gallic acid equivalents; TFC: total flavonoids content; QE: quercetin equivalents; TTC: total tannins content; TAE: tannic acid equivalent; PRA: phosphomolybdenum reduction assay; αTE: α-tocopherol equivalent; MTT: methyl thiazol tetrazolium; MIC: minimum inhibitory concentration; PDA: photo diode array; ME: methanol extract, HF: hexane fraction; EF: ethyl acetate fraction; MF: methanol fraction