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Full Length Research Paper

Orthogonal test design for optimization of the isolation and purification of total favonoids from *Artemisia frigida* Willd using macroporous resin chromatography

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In the present study, the authors aimed to use an orthogonal test design to optimize conditions for isolating and purifying total flavonoids from *Artemisia frigida* Willd (*A. frigida*) using macroporous resins. An orthogonal L9(3)[4] test design was applied to select the optimum isolation and purification parameters, including the volume of sample solution, flow rate of adsorption and eluent concentration and volume. The efficiency of isolation and purification was evaluated by measuring the concentration of total flavonoids with an ultraviolet (UV) spectrophotometer. The qualitative analysis of the extracted flavonoids was carried out using infrared absorption spectroscopy (IR) and nuclear magnetic resonance (NMR). The crude extract from *A. frigida* was successfully isolated and purified by macroporous resin chromatography under the optimum conditions (the volume of sample solution, 40 mL; the flow rate of adsorption, 3.0 mL/min; the eluent concentration, 90% ethanol; the eluent volume, 40 mL). The positive reactions to Molish and HCl~Mg tests suggested that the extracted compounds were flavonoids. Furthermore, FTIR and NMR measurements also confirmed the presence of flavonoids in the extracts. The D101 macroporous resin is the most effective one for large-scale isolation and purification of flavonoids from *A. frigida*, and it meets industrial needs.

Key words: *Artemisia frigida* Willd, total flavonoids, orthogonal experiment design, ultraviolet (UV) spectrophotometer, macroporous resin.

INTRODUCTION

Artemisia frigida, Agi in Mongolian, which belongs to the composite family, is a medical material commonly used in Mongolian folk medicine (Wang et al., 2012). It is distributed throughout Inner Mongolia, occupying 10.38% of its steppe (Wang et al., 2011a). The aerial parts of *A. frigida* are used as a folk medicine to treat joint swelling, renal heat, abnormal menstruation and sore carbuncle (Wang et al., 2013a). Sesquiterpenoids (Liu and Mabry, 1981a), coumarins (Greger et al, 1983) and flavonoids (Liu and Mabry, 1981b; Liu and Mabry, 1981c) have been

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isolated from *A. frigida*. Recently, a series of flavonoids have been identified from the aerial parts of *A. frigida* (Wang et al., 2009; 2011b; 2010; 2013b) and these flavonoids (Shin et al., 2005) possess antioxidant and free radical scavenging activity. Epidemiological studies have indicated that consumption of these compounds is associated with a reduced risk of cancer and cardiovascular disease (Wang et al., 2009).

It will be of great significance to research the feasibility of extracting flavonoids from *A. frigida* since this plant is widely distributed in Mongolia. However, flavonoids from *A. frigida* are often isolated and purified by some conventional extraction and separation techniques, which involve the use of organic solvents and column chromatography including a silica gel and polymide. The organic solvents are unfriendly to our environment and the conventional separation methods have disadvantages like tediousness, time consuming and complex steps (Ramirez et al., 2004).

Macroporous resins (MRs) are made of porous high molecular material, which has porosity and biggish specific surface area, and adsorbs organic substance selectively from solutions through physical action (Liu et al, 2006). In recent years, MRs have become an effective means to extract and separate natural active constituents (Gao et al., 2012). The purification method using MRs is a new promising technology in pharmaceutical industry. It has the advantages of no irreversible adsorption, low risk of sample denaturation, total sample recovery, large load capacity and low cost, and has been successfully applied in the extraction, separation and purification of flavonoids, saponins and alkaloids (Liu and Guo, 2003; Xiang et al., 2003). However, it has never been reported on how to use macroporous resin chromatography (MRC) to isolate total flavonoids from *A. frigida*. Thus, it is imperative to investigate the possibility of isolation and purification of flavonoids from *A. frigida*.

In the present study, we aimed to use an orthogonal test design to optimize conditions for isolating and purifying total flavonoids from *A. frigida* using macroporous resins.

MATERIALS AND METHODS

The aerial parts of *A. frigida* were used as the experimental material and collected in Tongliao, Inner Mongolia of China in July 2013. They were identified by Prof. Buhebateer (Inner Mongolia University for Nationalities). A voucher specimen (No. 20130623) was deposited in the School of Traditional Mongolian Medicine of Inner Mongolia University for Nationalities.

Instrumentation and reagents

UV spectra were recorded by a Shimadzu UV-2201 spectrometer (Shimadzu, Japan). IR spectra were recorded in KBr discs by a Thermo Nicolet 200 double beam spectrophotometer (Shimadzu, Japan). NMR spectra were measured by a Bruker AVAIVCE Ш—500 NMR spectrometer with tetramethylsilane (TMS) as the internal reference, and chemical shifts were expressed in δ (ppm). MR AB-8, D101, NKA-9, ADS-7, ADS-17, HPD-100, D201 and HPD826 were provided by the Tianjin Bohong Rerin Technology Co., Ltd. Other chemicals used here were of analytical grade or better and purchased from Tianjin agent Co., Ltd., China. Rutin was purchased from National Institute for Control of Pharmaceutical and Biological Products, Beijing, China (No: 100080-200707).

Preparation of *A. frigida* extracts

The air-dried aerial parts of *A. frigida* (500 g) were crushed and extracted twice with 70% ethanol in a 10 L of solvent volume for 6 h under reflux. The ethanol solution was vacuum evaporated at 60°C. About 350 g of the residual ethanol was obtained. The residue was stored in a refrigerator (0-4°C) for further use.

Determination of total flavonoids in *A. frigida* extracts

Rutin was accurately weighed and dissolved in 250 mL volumetric flasks with 70% ethanol as stock standard solution (0.8 mg/mL). A series of standard solutions with a final concentration of 16, 32, 48, 64 or 80 μg/mL were prepared with the stock solutions as described previously (Hua et al., 2007). The absorbance of standard solutions was measured with a UV spectrophotometer at 350 nm. The calibration curves were constructed by the absorbance of the analytes (y) against the concentrations of the calibration standards (x). Equation $y = 11.156\ x + 0.0206$ ($r = 0.9997$) expressed the relationship between absorbance and concentration. All samples were quantified by using the same method and all measurements were performed in triplicate.

Screening of macroporous resins

Static adsorption and desorption: 20 mL of *A. frigida* extract solution (1.20 mg/mL) was added into each pretreated MR (400 mg) (Hou et al., 2013), followed by mixing in a thermostatic oscillator for 20 h at the speed of 180 revolutions/min. The resins were then filtered and the obtained filtrate was collected. In the end, the resins were desorbed with 20 mL of 70% aqueous ethanol.

Dynamic adsorption

Six grams of three kinds of pretreated resins were put individually into a glass column (1 × 20 cm) whose capacity was 3 mL. Next, the *A. frigida* extract solution (3.72 mg/mL) was added for adsorption dynamically at 3 mL/min. 3 mL of the eluting solution was each collected as one fraction. The content of total flavonoids was determined with a UV spectrophotometer at 350 nm.

Dynamic desorption

The resins were put into glass columns according to the method of dynamic adsorption and then 60 mL of the *A. frigida* extract solution was added for adsorption dynamically at 3 mL/min. The elution and desorption were performed with 200 mL water and 70% aqueous ethanol at 3 mL/min, respectively. The eluting solution (3 mL) was each collected as one fraction and detected as described above.

Revolutions

Optimization of A. frigida extraction

An orthogonal experiment $L9(3)^4$ test design was used to optimize

Table 1. The orthogonal experiment design (four factors and three levels).

Level	Factor			
	A, Volume of sample solution (mL)	B, Flow rate of adsorption (mL/min)	C, Concentration of the eluant (%)	D, Volume of the eluent (mL)
1	20	1	50	20
2	40	3	70	40
3	60	5	90	60

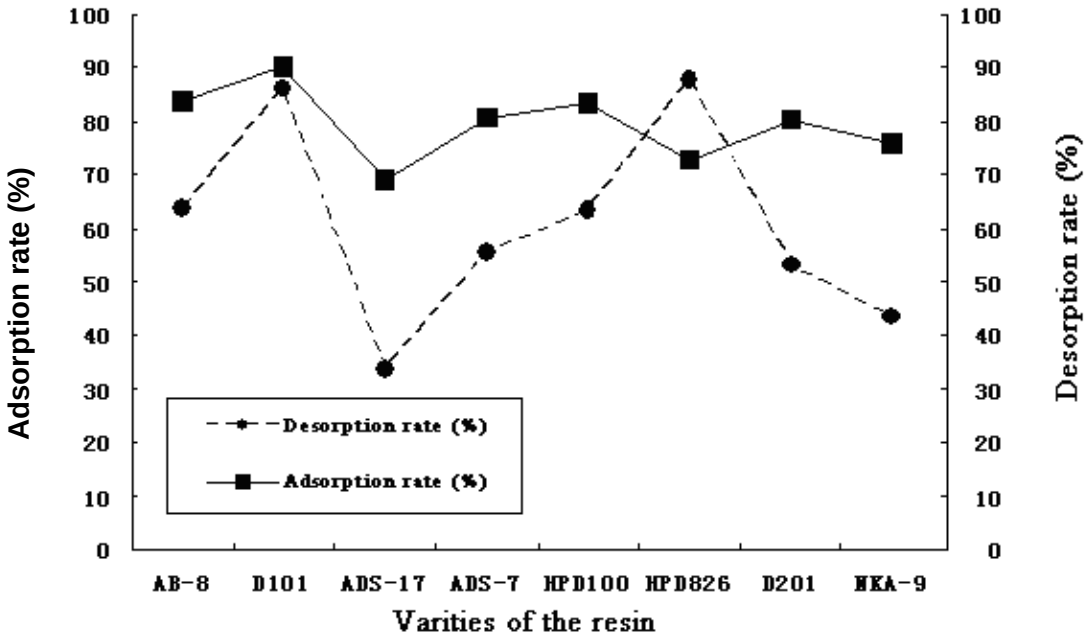

Figure 1. The adsorption and desorption rates of macroporous resins for total flavonoid extraction.

the conditions for isolating and purifying total flavonoids from *A. frigida* extracts. In this study, the isolation and purification was accomplished with MRs. The key parameters that influenced the flavonoid yield were analyzed, including the volume of sample solution (A), flow rate of adsorption (B), concentration of the eluant (C) and volume of the eluant (D). The factors and levels of L9(3)4 are listed in Table 1.

RESULTS AND DISCUSSION

Screening of macroporous resins

Adsorption followed by desorption of the filtrates was done to obtain total flavonoids from *A. frigida* extracts, and the flavonoid content was measured. As shown in Figure 1, the results indicated that eight MRs, including MR AB-8, D101, NKA-9, ADS-7, ADS-17, HPD-100, D201 and D101, showed an adsorption rate higher than 90% and a desorption rate higher than 80%. Among the tested resins, MR HPD826 had the best adsorption efficiency for total flavonoids and the D101 resin was the second (Figure 2). However, the desorbing process of MR D101 was better than that of HPD826 (Figure 3). The D101 resin was eventually selected for further study based on its regeneration property and price.

Optimized conditions for isolating and purifying total flavonoids from *A. frigida* extraction

Various parameters potentially affect the isolation and purification process using MRs, so it is critical to optimize experimental conditions when developing a MRC method. Hence, the related parameters in the present study were optimized to get efficient isolation and purification of total flavonoids from *A. frigida.* Generally, the volume of sample solution, flow rate of adsorption and eluent concentration and volume are all considered to be the most important factors. Optimization of the suitable isolation and purification conditions in a MRC method can be carried out step-by-step or by using an experimental design. In the present study, all selected

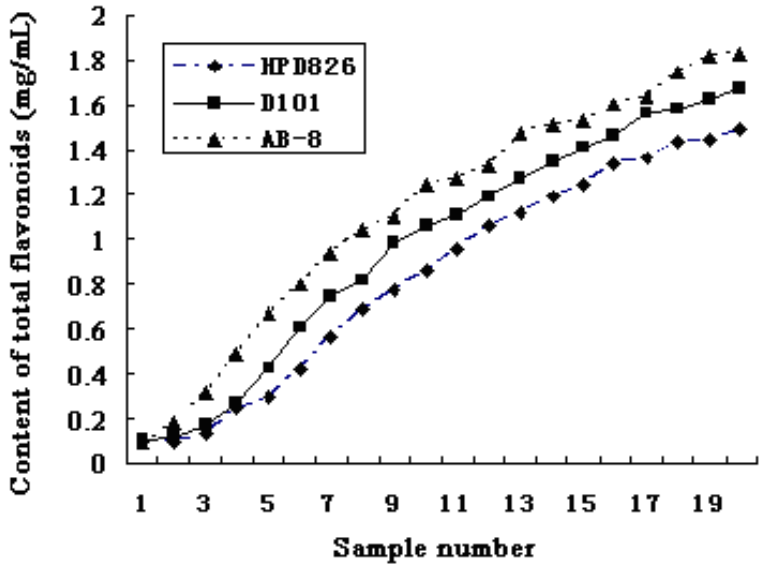

Figure 2. Results of dynamic absorption.

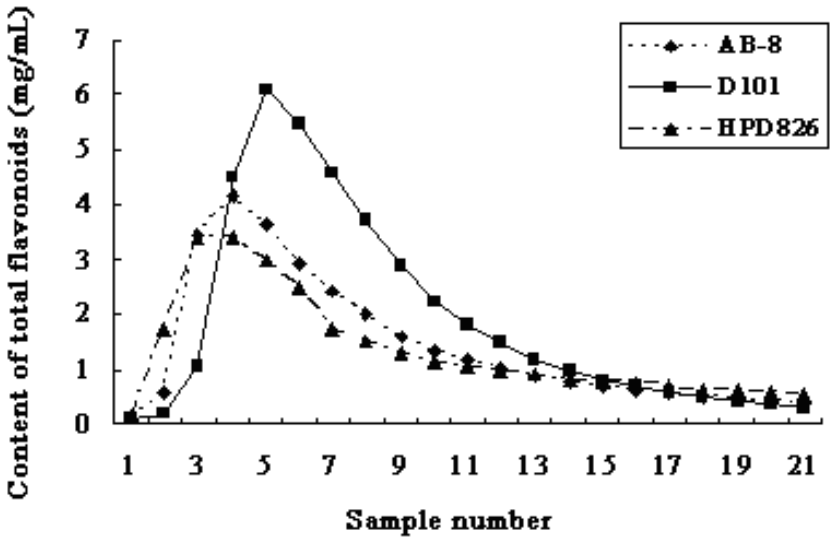

Figure 3. Results of dynamic desorption.

factors were examined using an orthogonal $L9(3)^4$ test design.

The results of the orthogonal test and the extreme difference analysis are presented in Table 2. However, the best extraction conditions cannot be determined only based on the outcomes in Table 2 and further orthogonal analysis is needed. Thus, the K, k and R values were calculated and listed in Table 3. As shown in Table 3, the yield and purity of total flavonoids were decreased in the order of B>C>D>A according to the R values. The flow rate of adsorption was found to be the most important factor determining the yield. In other words, the maximum yield of the flavonids was obtained, when the flow rate of adsorption was 3 mL/min, the eluent concentration was 90% aqueous ethanol, the eluent volume was 40 mL and the volume of sample solution was 40 mL, respectively.

Verification test

The isolation and purification was scaled up by 50 times using a preparative-scale MRC system. The yield of total

Table 2. Orthogonal array of the experiments extracting flavonids from *A. frigida.*

Experiment No.	Factor				Yield of total flavonoids (%)
	A	B	C	D	
1	1	1	1	1	40.41
2	1	2	2	2	90.63
3	1	3	3	3	84.54
4	2	1	2	3	71.59
5	2	2	3	1	87.95
6	2	3	1	2	70.91
7	3	1	3	2	69.70
8	3	2	1	3	67.73
9	3	3	2	1	60.76

Table 3. Analysis of $L9(3)^4$ test results.

*	Yield of total flavonoids (%)			
	A	B	C	D
K1	215.58	181.7	179.05	189.12
K2	230.47	246.31	222.98	231.24
K3	198.19	216.21	242.19	223.86
k1	71.86	60.57	59.68	63.04
k2	76.82	82.10	74.33	77.08
k3	66.06	72.07	80.73	74.62
R	10.76	21.53	21.05	14.04
Optimal level	A2	B2	C3	D2

* K1: Σ A1, Σ B1 and Σ C1; K2: Σ A2, Σ B2 and Σ C2; K3: Σ A3, Σ B3 and Σ C3. k1: K1/3; k2: K2/3 and k3: K3/3. R: Range.

Table 4. The purification results of total flavonoids from *Artemisia frigida* by different methods.

Method No.	70% ethanol extract (g)	Total flavonoids purified (g)	Purity of the total flavonoids (%)	Yield of the total flavonoids (%)
Macroporous resin chromatography (1)	5.0	1.48	65.54	85.46
Silica gel chromatography (2)	5.0	1.17	66.02	67.55
Polyamide chromatogtaphy (3)	5.0	0.98	71.24	56.58

flavonoids reached 85.34% (n = 5) and the purity of total flavonoids in the purified products was 65.32% (n = 5), indicating that this method was reliable and could be used for the industrialized production.

Check test

The 70% ethanol extract of *A. frigida* were isolated, purified and determined by the reported methods in the literature (Xu et al, 2011; Cai et al., 2009) and the presented optimized method, respectively. The results are listed in Table 4. The results show that the yield of the total flavonoids with the method 1 is more than that of the methods 2 and 3, and the purity of the total flavonoids is similar to that of methods 2 and 3.

UV/Vis absorption and FT-IR spectra of total flavonoids after the resin treatment

The positive reactions to Molish and HCl-Mg tests suggested that the extracted compound was flavonoid. UV and FTIR spectroscopy are effective tools for the semi-quantitative estimation of structural information on functional groups in complex solids (Cai et al., 2011;

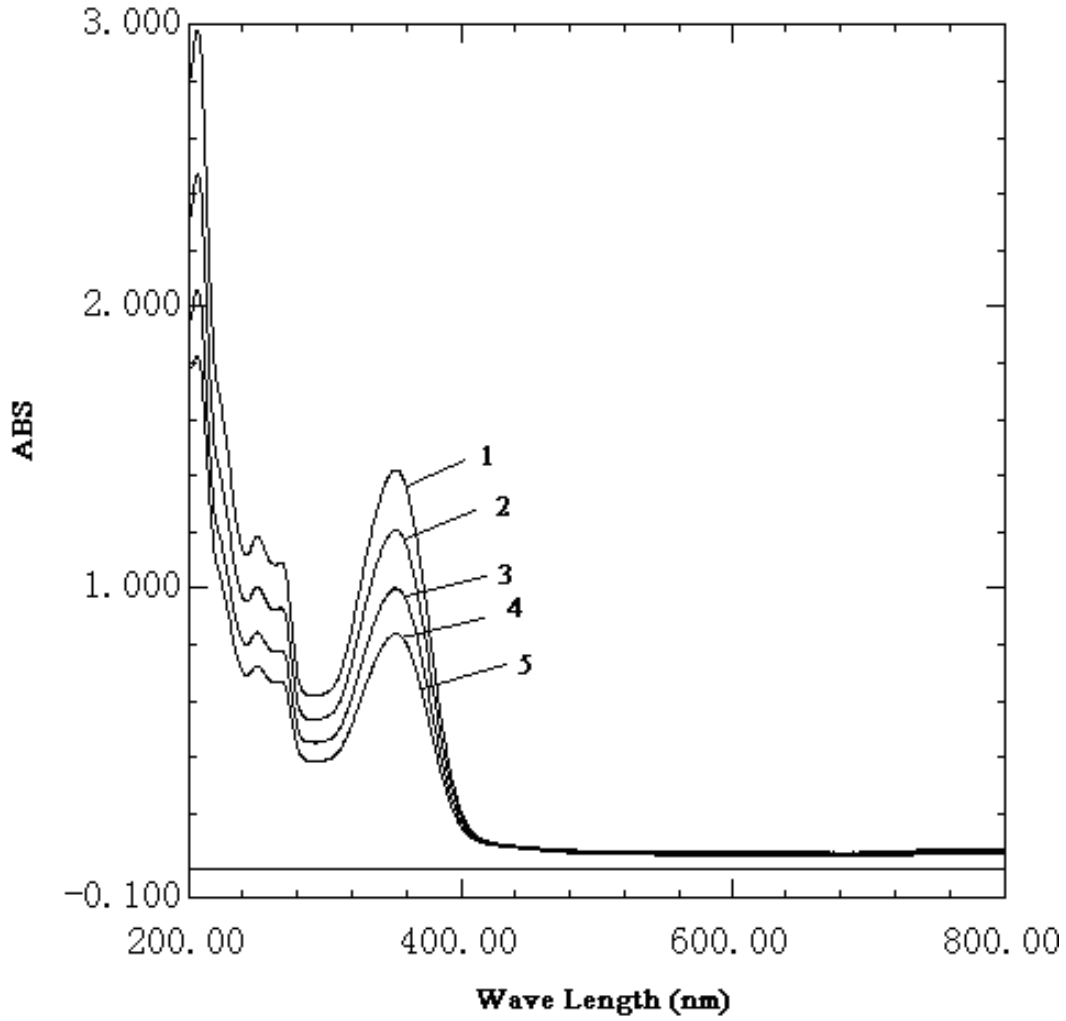

Figure 4. UV spectra of total flavonoids from *A. frigida* extraction (1. 50 µg/mL; 2. 40 µg/mL; 3. 30 µg/mL; 4. 20 µg/mL; 5. 10 µg/mL).

Huang et al., 2007). They were used to investigate the functional groups on the flavonoids after the treatment. The UV and FTIR spectrum of the flavonoids are shown in Figures 4 and 5. Two broad ultraviolet absorption peak appeared (Figure 4). One band indicated cinamyl (300~400 nm), and the other one was benzoyl (240~285 nm). As shown in Figure 5, the strong band appearing at 681.3 cm^{-1} was related to the expansion vibration of two isolated hydrogens in a benzene ring (Cai et al., 2011). The band at 822.4 cm^{-1} was related to the ortho-position hydrogen. Two bands at 1324.3 and 1263.1 cm^{-1} showed the anti-symmetry and the symmetrical expansion vibration related to the C-O-C ether. Two wide and strong absorption peaks appearing at 1725.6 and 1685.4 cm^{-1} were mainly produced by the C=O stretching vibration. The IR spectrum of total flavonoids also indicated the presence of aromatic groups (1601.5 and 1497.4 cm^{-1}). Based on the UV–VIS spectra of flavonoids at different concentrations in Figure 4, they all have the same UV absorption peaks of 350 nm, so that the content of total flavonoids was determined with UV spectrophotometer at 350 nm.

^{1}H-NMR and ^{13}C-NMR spectra of total flavonoids after the resin processed

The ^{1}H-NMR (500MHz, DMSO-d6) and ^{13}C-NMR (125MHz, DMSO-d6) spectra of the flavonoids are shown in Figure 6. In the ^{1}H-NMR spectrum, the characteristic resonances for HO-5 of the flavonoids exhibited at δH 13.6-12.0, and the downfield signals of aromatic protons at δH 8.0-6.0 belonged to the aromatic rings (A, B and C rings). Furthermore, the 1H-NMR spectrum showed signals for the methoxyl groups at δH 3.75-3.90 and for anomeric protons at δH 4.80-5.00. The ^{13}C-NMR signals also proved the presence of the flavonoids. Moreover, the characteristic resonances for C-4 of the flavonoids were

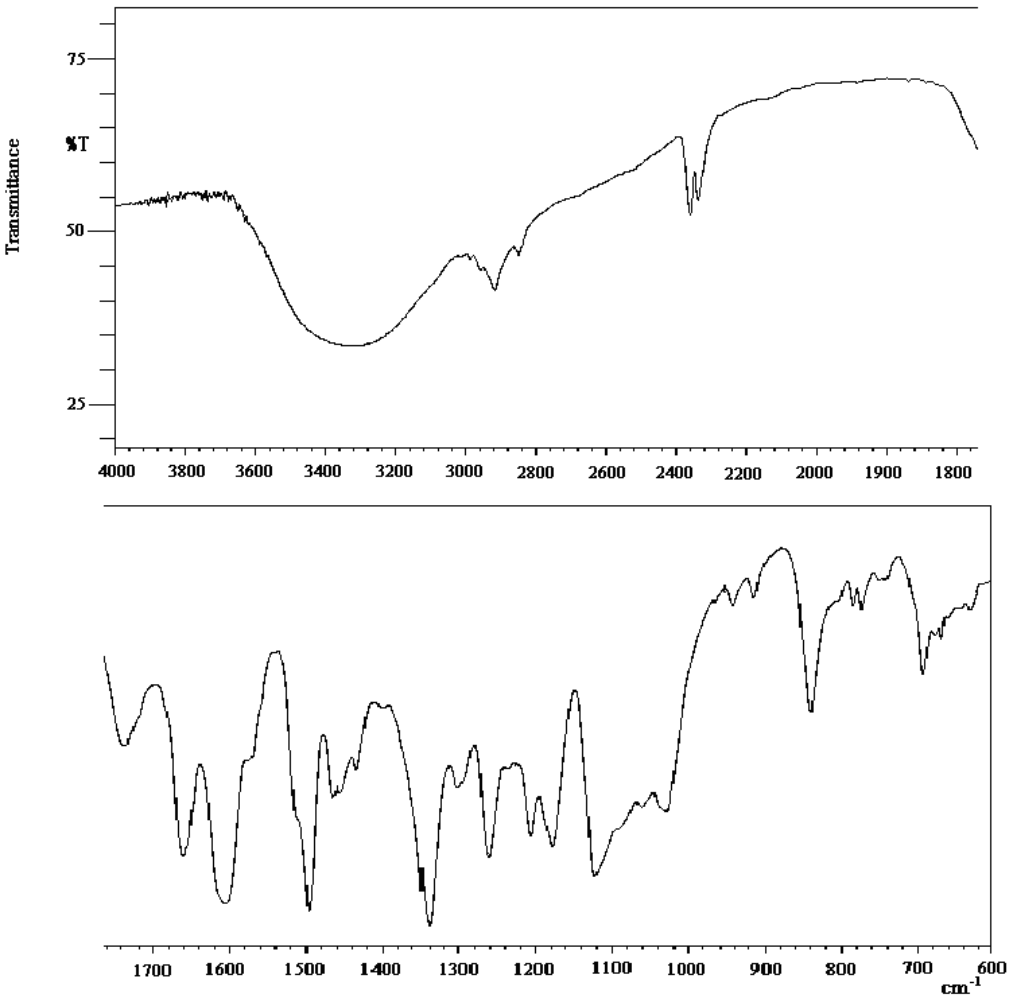

Figure 5. FTIR spectra of total flavonoids from *A. frigida* extraction.

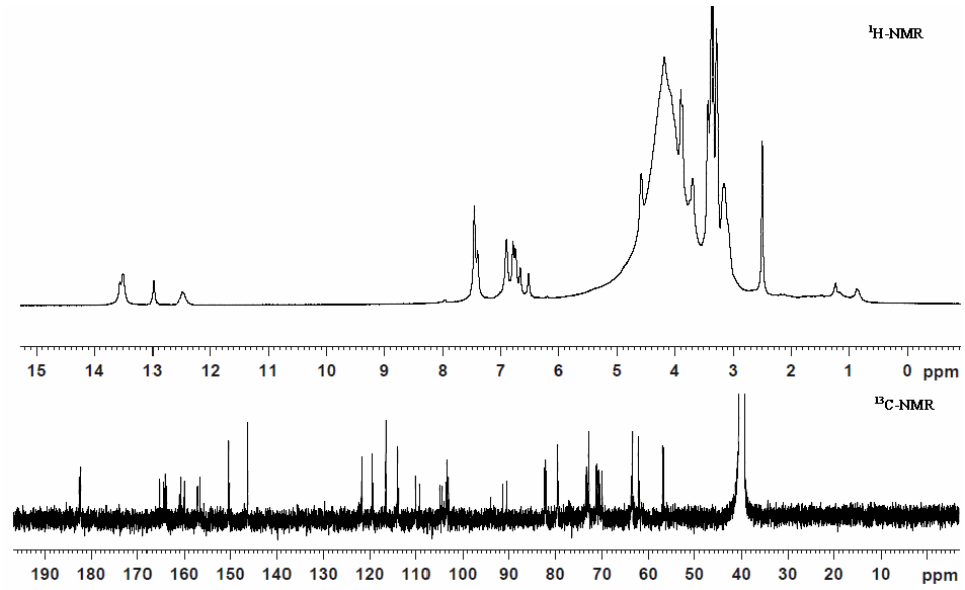

Figure 6. ^{1}H-NMR and ^{13}C-NMR spectra of total flavonoids from *A. frigida* extraction.

exhibited at δC 185.0-181.0, and the aromatic carbon signals of the aromatic rings (A, B and C rings) were displayed at δC 168.0-100.0. In addition, the remaining carbon signals belonged to the sugar moieties and methoxyl groups (Figure 6).

Conflict of interest

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

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