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Extraction and identification of active ingredients in *Rhubarb* plant

Ramin Roohparvar[1,*]

[1]Cereal Research Department, Seed and Plant Improvement Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, IRAN

Email: r.roohparvar@areeo.ac.ir

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Abstract

Rhubarb is a plant from the Polygonaceae family, native to Asia (likely Siberia or the Himalayas). In this study, Rheum plants were dried in a cool and dark place, and then the stem and flower powders were extracted using hexane, chloroform, and methanol solvents at room temperature with a Soxhlet apparatus. Column and plate chromatography were then used for the main separation of the extract components. The hexane, chloroform, and methanol extracts of the Rheum stem were separated into 11, 8, and 7 parts, respectively, using the solvent systems of ethyl acetate/petroleum ether, chloroform/methanol/ethyl acetate, and methanol/ethyl acetate/water/acetic acid. The pure compounds were identified using FT-IR, ^{1}HNMR, and ^{13}CNMR spectroscopy.

Keywords: *Rhubarb* plant, Extraction, Steam evaporation, Spectroscopy investigation

Introduction

Rhubarb is a perennial plant, belonging to the family Polygonaceae and the genus Rheume, which grows in dry and semi-arid mountainous areas, and is usually well adapted to the slopes of the mountains, under the rocks. It grows in spring and early summer and has a common root that grows one to two meters in the ground and is resistant to cold and frost. It has two types of stems; one is an underground stem which is called a rhizome [1, 2]. After growing, the rhizome becomes fleshy and woody, and aerial stems and broad leaves grow from its buds. The second type is aerial stems that grow up to one and a half meters. In the years when the average annual rainfall is at least 130 mm. *Rhubarb* produces flowering stems 50 to 80 centimeters high. At the end of the flowering stem, the yellow, cream-colored flower is well visible in early spring (May). *Rhubarb* is a perennial herb that is widespread in southern and central parts of Asia. According to reports, it was used as a medicinal plant in China before the Christian era. *Rhubarb* grows in Iran in the foothills of the Alborz Mountains, the mountains of Azerbaijan, the Binaloud range north of Tehran, and in the mountainous village of Robat-e-Tork. The plant has 60 species [3-6] and is used to treat a variety of diseases such as liver disease, gastrointestinal bleeding, hepatitis, colds, hemorrhoids, coughs, anxiety, headaches, indigestion, stomach pain, constipation, and has antibacterial, anticancer, and antidiabetic properties [7]. *Rheum Ribes* tolerates very cold temperatures and survives even below 20 degrees Celsius. Leaves turn pink or red at low temperatures and dark green at temperatures above 30°C. *Rhubarb* is more productive in rainy areas and grows better in soft and fertile soils rich in potassium [8, 9]. This plant can be grafted to other species that are compatible with plants from other species. This plant prefers relatively neutral soils. But it can grow in acidic or alkaline soils. It can also tolerate harsh weather conditions and conditions with a lack of light and dry lands [10-12].

Out of 60 species of *Rheum*, 3 species grow in the climatic conditions of Iran, including *Rheum Persium*, *Rheum Ribe*, and *Rheum tarkestanicum* [13, 14]. In the years 1998 and 1995, five stilbene derivatives and six anthraquinone derivatives were extracted from the roots of Rheum. 57 different anthraquinone, stilbene, and flavonoid compounds have been identified from the roots and stems of *Rheum australe*. According to phytochemical studies carried out on *Rheum* ribes in the years 1961, 1973, 1978, 1982, 1988, and 1989, anthraquinone derivatives were discovered, and stilbene derivatives were also confirmed during studies conducted in 1984, 1971, and 1988 [14-17]. The effective compounds identified by HPLC analysis show that the most common components of anthraquinone and stilbene, which are shared by most genera of this plant. Different species of the *Rheum* genus contain polyphenolic compounds such as anthraquinones, anthocyanins, flavanols, stilbenes, and naphthalene. This plant contains vitamins A, B, and C. The content of phenolic and flavonoid compounds in stem and root extracts of Rheum obtained with methanol and chloroform solvents have also been determined.

Experimental

Materials

Methanol, ethanol, chloroform, diethyl ether, dichloromethane, ethyl acetate, hexane, petroleum ether, and chromatography silica gels including TLC Silica gel60 GF254 and column chromatography silica gel 60 (0.063-0.2) from Merck were used.

Charactrization

FT-IR spectra were recorded using a Bruker FT-IR instrument in the range of 400-4000 cm^{-1} with KBr pellets. For centrifugation, a Hettich D-785321 Tuttlingen centrifuge, a JENWAY1000 magnetic stirrer, a Sartorius LA1205 balance, 20 × 20 cm glass plates, and a Heidolph Heizbad Wb solvent evaporator were used. ^{1}HNMR and ^{13}CNMR spectra were recorded using a Bruker SP-100 AVANC (100MHZ) instrument.

Plant preparation and scientific verification

Rhubarb plant (*Rheum Ribes*) was collected from East Azarbaijan province, from the mountains around the city of Tasuj, and its scientific name was scientifically confirmed by members of the faculty of botany at Shahid Madani University. Then the plant was dried in a place away from light and with proper ventilation for 3 weeks. After complete drying, the stems and flowers were separated and packed in glass containers and paper boxes where air could enter and exit.

Methods of extracting the essence from the stem and flower of the plant

The stems and flowers are ground to a powder using a mill. The different parts of the plant should be powdered enough to ensure proper extraction. 15 grams of completely powdered fresh *Rheum Ribes* stems were placed in 280 mL of hexane solvent under the Soxhlet extraction system. After the extraction was complete (when the solvent became colorless on the system's finger), the flask containing the extract was subjected to solvent evaporation using a Heidolph Heizbad. The resulting extract is in hexane form, and in the next steps, chloroform and methanol extracts were prepared using this method of extraction. The extraction process was also carried out for the *Rheum Ribes* flower, and hexane, chloroform, and methanol extracts were obtained from the flower accordingly.

Method of identifying natural compounds present in *Rhubarb* plant

Identification tests for alkaloids, flavonoids, steroids, glycosides, anthraquinones, tannins, terpenoids, and saponins were performed on the species under study [18-20].

Method for separation of compounds in Rheum Ribes plant

In this study, to separate the compounds present in the *Rheum Ribes* plant, hexane, chloroform, and methanol extracts were prepared from the stem and flower of the plant using a *Soxhlet* apparatus. Then, a thin-layer chromatography (TLC) system was selected as a solvent system to separate the components of the extracts. Some of the components of the extracts were separated using column chromatography, and others were separated using plate chromatography under the selected solvent system. In cases where the number of spots observed in the thin-layer chromatography was not suitable, column chromatography was used for

separation. In this process, after the chromatography column was activated with silica gel, the extracts were mixed with some silica gel and slowly transferred to the column. Then, the column was gently washed with a suitable solvent system as the mobile phase. The resulting components were collected and concentrated (the separated components are recorded in the results section of the charts).

Method of purifying the separated extracts of hexane stem (H) and flower (GH), chloroform stem (2C) and flower (GC), methanol stem (M) and flower (GM) of the Rheum Ribes plant

Purification method for H4

H4 is a brownish-purple color and appears as a bright pink under a UV lamp at a wavelength of 366 nm. This component was dissolved in chloroform and then purified using TLC with pure methanol as the solvent.

Purification method for 2C7

2C7 is a cloudy green substance that appears as a bright blue under a UV lamp at a wavelength of 254 nm. It was dissolved in methanol and then further purified using the Nobel recrystallization method with chloroform as the solvent.

Purification method for 2C8

2C8 is a muddy green substance that appears pink under a UV lamp at a wavelength of 366 nm and dissolves in chloroform. The purification process continued by column chromatography using a system of methanol and chloroform, followed by recrystallization with methanol to obtain a white precipitate that dissolves in chloroform. The desired combination was separated by further purification using ethyl acetate and methanol as solvent.

M6 Purification Method

M6 is a golden orange color and appears dark under a UV lamp with a wavelength of 366 nm. It was dissolved in methanol and purified using a chromatography column with a chloroform and ethyl acetate solvent system. Finally, the pure compound was obtained by crystallization with chloroform.

GH9 Purification Method

GH9 is green and appears pink under a UV lamp with a wavelength of 366 nm. Methanol was added dropwise to the chloroform solution of GH9, resulting in white precipitates. The purification process was continued, leading to the pure compound.

GH10 Purification Method

GH10 is yellow and appears pink under a UV lamp with a wavelength of 366 nm. It was dissolved in hexane or chloroform and purified using a chromatography column with a dichloromethane and chloroform solvent system. Then, a mixture of methanol and water was added to the hexane solution of GH10, resulting in a two-phase mixture that was separated using a separatory funnel. Finally, the organic phase was concentrated using a pranayl solvent, resulting in white precipitates.

Result and discussion

Using the Soxhlet extraction method, higher amounts of hexane, chloroform, and methanol extracts were obtained from the

flower of *Rheum Ribes* compared to the stem extracts. The results are presented in Table 1.

Table 1. Amounts of hexane, chloroform, and methanol extracts from stem and flower *Rhubarb*

Name of extract (g)	**Amount of extract from stem (g)**	**Amount of extract from flower (g)**	**Initial powder weight (g)**
Hexane	0.26	0.67	15
chloroform	0.507	1.7	15
methanol	2.161	3.91	15

The thin-layer chromatography technique was also used to separate each extract into various solvent systems. In extracts made with hexane and chloroform, there were more separated components. In Figures 1 and 2, the separation images are displayed.

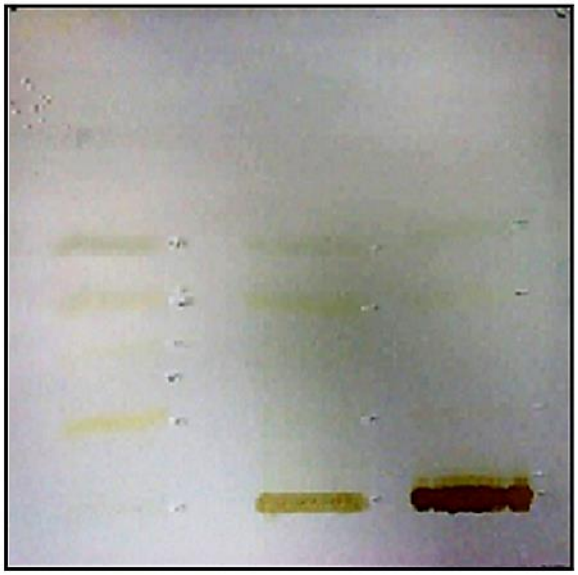

Figure 1. Separation of hexane, chloroform, and methanol extracts from flower (from left to right)

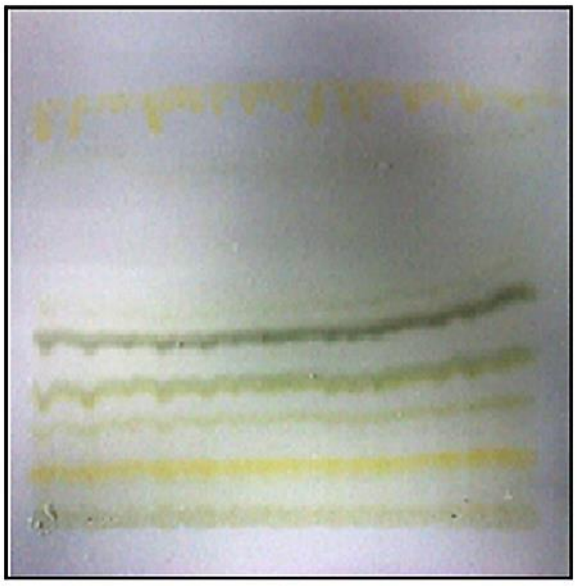

Figure 2. Separation of hexane extracts from stem

Identification tests were carried out to determine the kinds of natural compounds that were present in the stem and flower extracts of Rheum Ribes. Table 2 displays the outcomes of these tests. Solvents are required in addition to the column chromatography technique to dissolve the extracted materials on silica gel plates (in the TLC method). Table 3 includes a list of these solvents.

Table 2. Results of identification tests for natural compounds present in the *Rheum* plant

Flower	**Stem**	**Type of identification tests**
Observed	Observed	Tannin
Observed	Observed	Terpenoid
Observed	Observed	Anthraquinone
Observed	Observed	Saponin
Absent	Absent	Alkaloid
Observed	Observed	Flavonoid
Observed	Observed	Glycoside

Table 3. Required solvents for washing silica gel plates components

Used Extracted Components	Solvent	Used Extracted Components	Solvent	Used Extracted Components	Solvent	Used Extracted Components	Solvent
H1	Acetone and ethanol	2C1	ethanol and methanol	M4	Chloroform and ethanol and methanol	GH6	Chloroform
H2	Acetone and chloroform	2C2	Chloroform and methanol	M5	methanol/ ethanol (1:1)	GH7	Chloroform
H3	Acetone and chloroform	2C3	methanol	M6	methanol/ ethanol (1:1)	GH8	Chloroform
H4	chloroform	2C4	methanol	M7	Acetone and chloroform	GH9	Chloroform
H5	Acetone and chloroform	2C5	Acetone and chloroform	M8	Acetone and chloroform	GH10	Hexane
H6	chloroform	2C6	methanol	GH1	Acetone and chloroform	GC1	Ethyl acetate and chloroform,
H7	Acetone and chloroform	2C7	chloroform	GH2	Acetone and chloroform	GC2	Acetone and chloroform
H8	chloroform	M1	methanol/ ethanol (1:1)	GH3	chloroform	GC3	Acetone and chloroform
H9	chloroform	M2	methanol/ ethanol (1:1)	GH4	chloroform	GC4	Chloroform

H10	Hexane and chloroform	M3	methanol/ ethanol (1:1)	GH4	chloroform	GC5	Chloroform
H11	and chloroform	GM2	distilled water and methanol	GM4	Chloroform and ethanol and methanol	GC6	Chloroform
GM1	ethanol and methanol	GM3	Chloroform and ethanol and methanol	GM5	Chloroform and ethanol and methanol	GM6	Chloroform

Table 4. Components separated from the plate

Components	**R_F**	**Components**	**R_F**	**Components**	**R_F**
H1	0.055	M1	0.055	2C1	0.055
H2	0.083	M2	0.088	2C2	0.1
H3	0.11	M3	0.127	2C3	0.16
H4	0.16	M4	0.33	2C4	0.28
H5	0.22	M5	0.41	2C5	0.41
H6	0.24	M6	0.55	2C6	0.63
H7	0.2	M7	0.66	2C7	0.79
H8	0.32	M8	0.67	GC1	0.055
H9	0.33	GM1	0.055	GC2	0.072
H10	0.5	GM2	0.083	GC3	0.15
H11	0.8	GM3	0.16	GC4	0.36
GH6	0.37	GM4	0.33	GC5	0.44
GH7	0.47	GM5	0.47	GH1	0.055
GH8	0.58	GM6	0.5	GH2	0.11
GH9	0.69	GM7	0.055	GH3	0.22
GH10	0.80	GH5	0.3	GH4	0.27

The RF distances of the separated components of the hexane, chloroform, and methanol stem and flower extracts are given in Table 4.

FT-IR and ^{1}HNMR spectra of compound 2C7

A broad and strong absorption at 3418 cm^{-1} is related to the stretching vibrations of the acidic OH group, and the absorption at 1650 cm^{-1} is related to the carbonyl group of the acidic group. The absorption bands at 12953-2843 cm^{-1} are related to the stretching vibrations of the aliphatic side chain C-H, and the absorption band at 1660 cm^{-1} is related to the C=C group of the aromatic ring. The absorption band at 1113 cm^{-1} is related to C-O. In the ^{1}HNMR spectrum, the peaks at 10 ppm are related to the phenolic OH group, the peak at 11 ppm is related to the acidic OH group, and the peaks at 7.047-7.068 ppm are related to H1 in the ortho position of the acidic and phenolic groups on the aromatic ring. The peaks at 7.55-7.622 ppm are related to H2 and H3 on the aromatic ring. Based on the analysis, the aromatic nature of a part of the compound is confirmed. However, due to the complexity of the peaks in the aliphatic region, identification of this part of the molecule was not possible, and no structure was proposed for it (Figures 1 and 2).

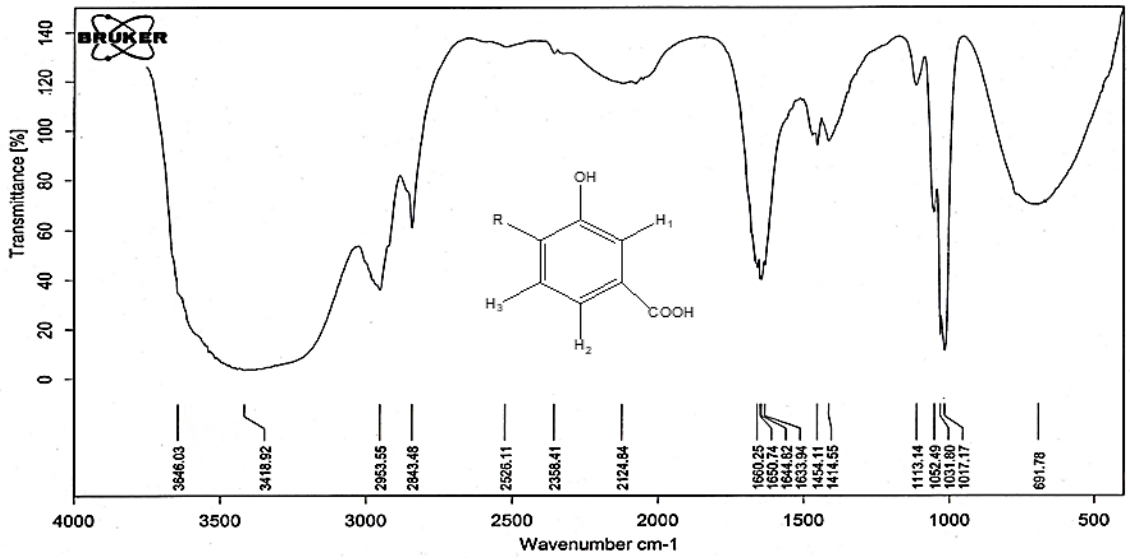

Figure 1. FT-IR spectrum of compound 2C7

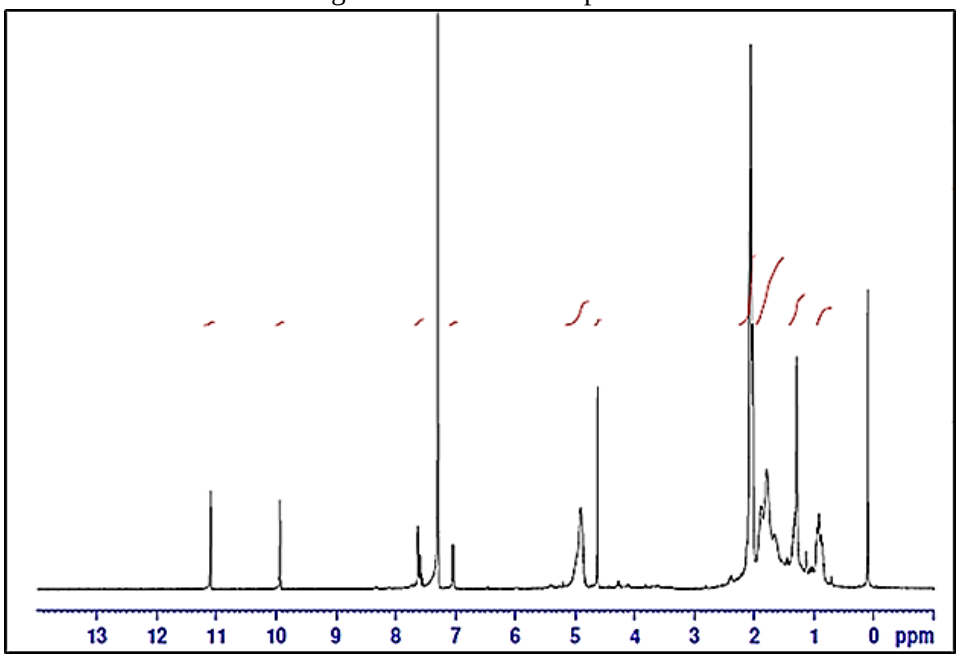

Figure 2. [1]HNMR spectrum of compound 2C7

FT-IR and [1]HNMR spectra of compound GH9

Strong absorptions in the range of 2920-2849 cm^{-1} are related to the stretching vibrations of aliphatic C-H, and the absorption at 1463 cm^{-1} is related to the vinyl C=C group. The absorptions at 1261 and 1096 cm^{-1} are related to C-O. In the [1]HNMR spectrum, the peaks at 4.714-4.616 ppm are related to H1 and H3, and the peak at 5.374 ppm is related to the CH_2 group. Again, due to the complexity of the side group structure, identification was not possible (Figures 3 and 4).

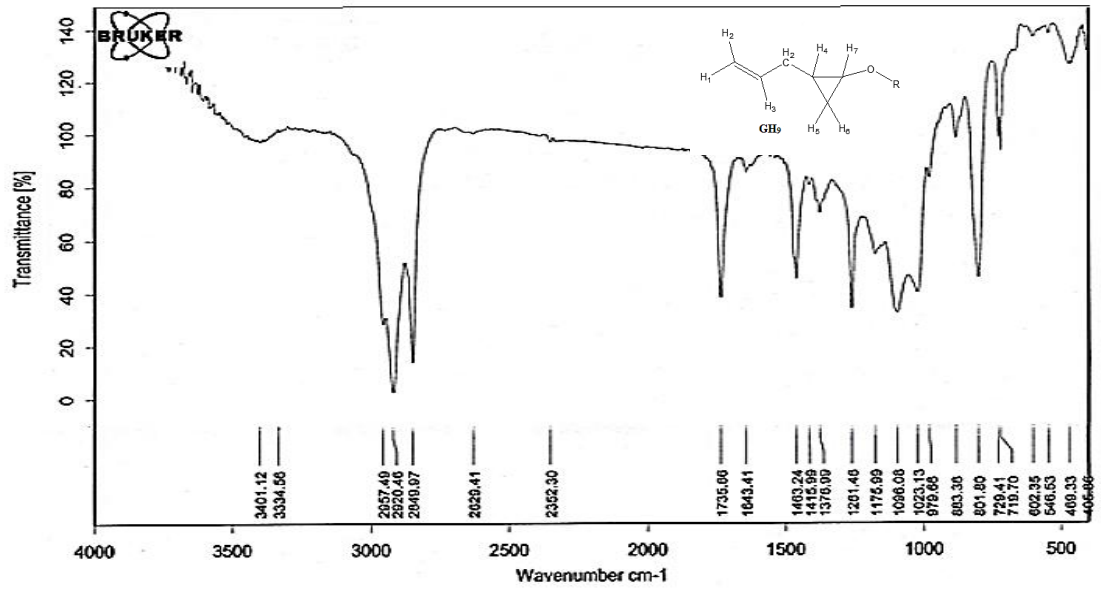

Figure 3. FT-IR spectrum of compound GH9

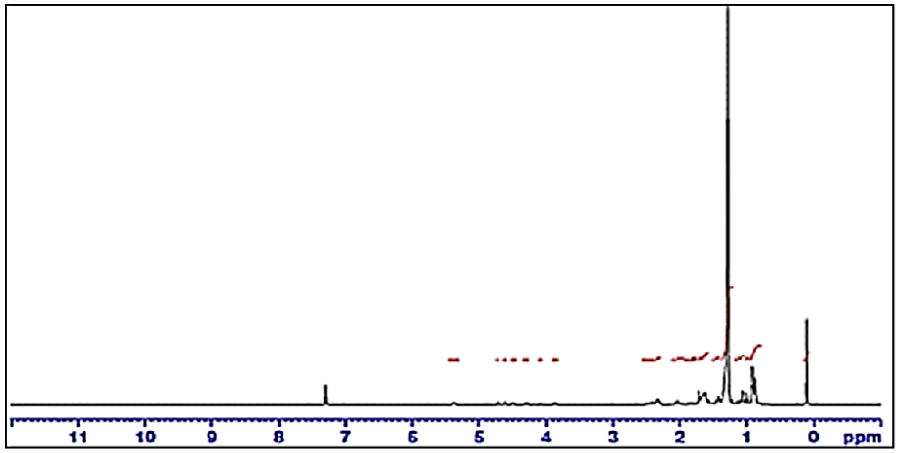

Figure 4. [1]HNMR spectrum of compound GH9

FT-IR and H1NMR spectra of GH10 compound

The absorption bands at 1460 cm^{-1} and 1265 cm^{-1} are related to the aliphatic C-H and C-O stretching vibrations, respectively. In the [1]HNMR spectrum, the peaks at 5.73-5.879 ppm are related to H5, the peak at 5.373 ppm is related to H4, and the peak at 2.917 ppm is related to H9. Due to the complexity of the side structure, no proposed structure was presented (Figures 5 and 6).

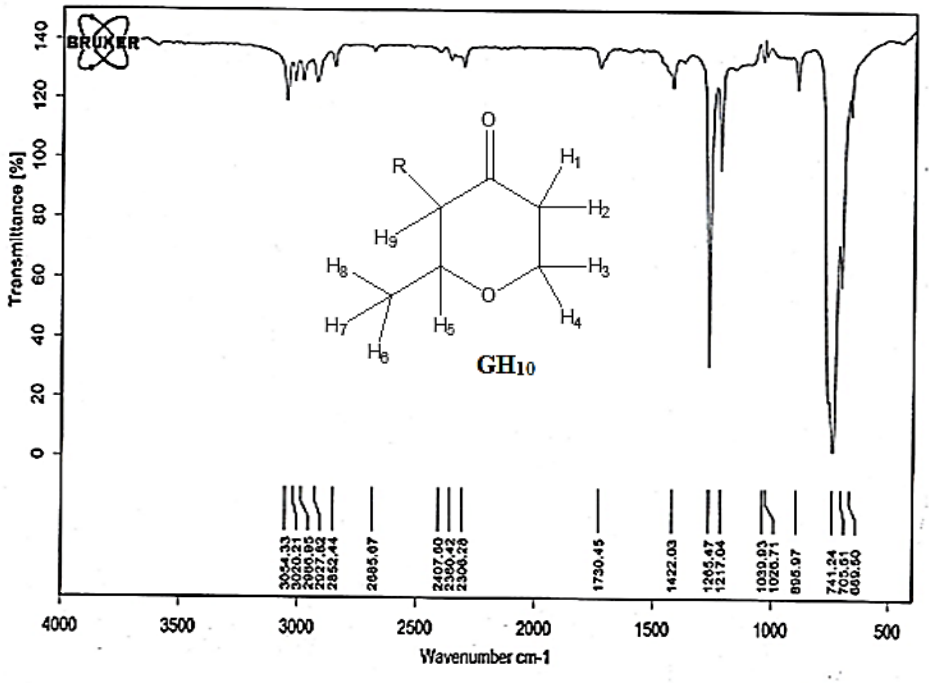

Figure 5. FT-IR spectrum of compound GH10

Extracti

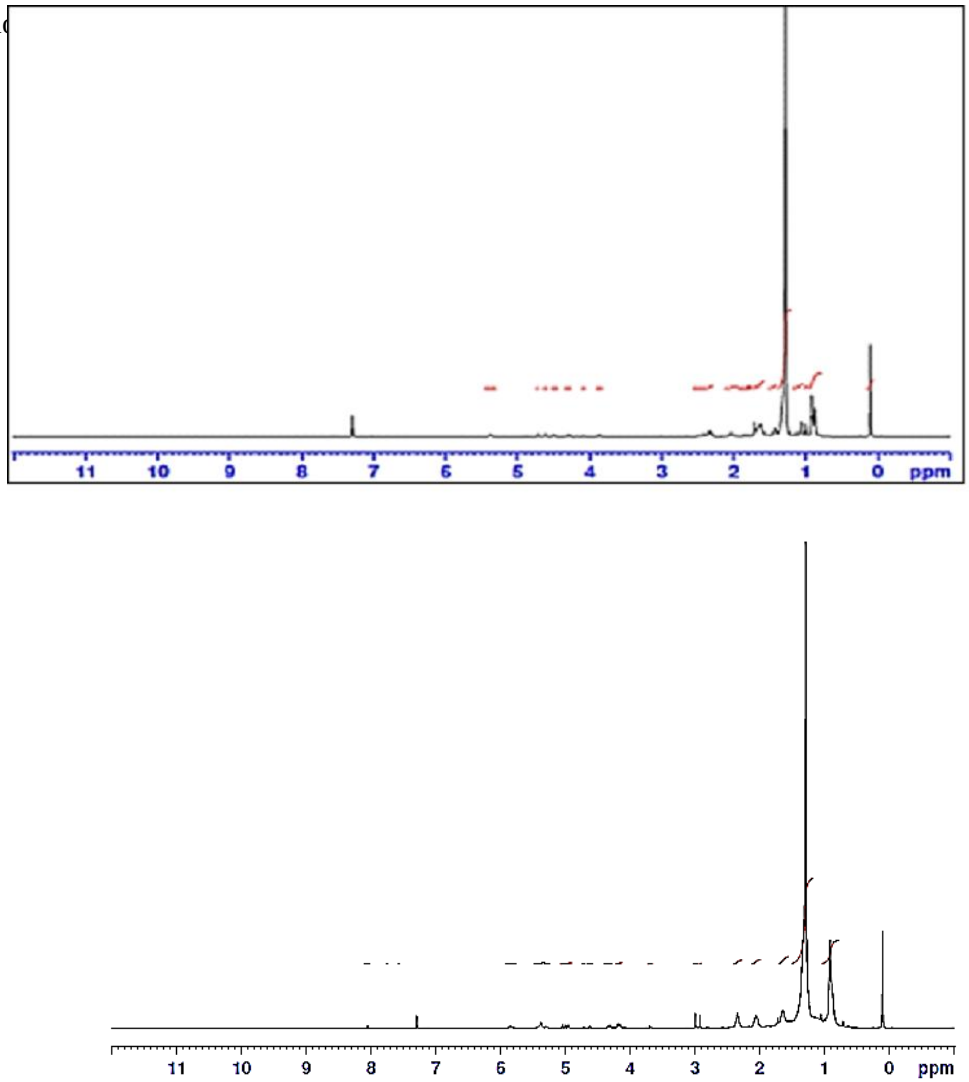

Figure 6. [1]HNMR spectrum of compound GH10

FT-IR and [1]HNMR spectra of M6 compound

The broad and strong absorption band at 3460 cm^{-1} is related to the stretching vibrations of the acid group OH, the absorption bands at 2843 cm^{-1} and 1644 cm^{-1} are related to the aliphatic C-H stretching and the C=C stretching of the aromatic group, respectively. The absorption band at 1016 cm^{-1} is attributed to the C-O stretching vibration. In the [1]HNMR spectrum, the peaks at 7.157-7.117 ppm are related to H2, and the peaks at 6.815-6.757 ppm are related to H5. Due to the complexity of the side structure, no proposed structure was presented (Figures 7 and 8).

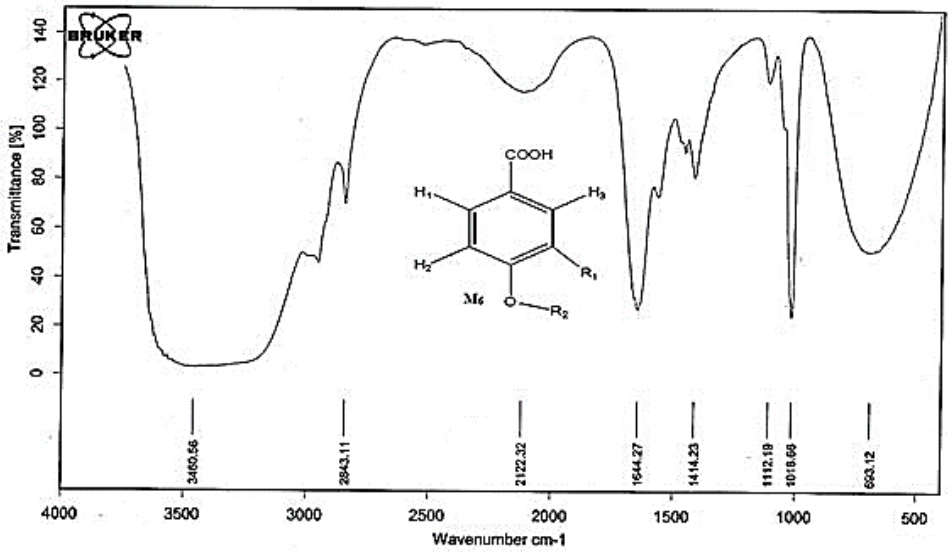

Figure 7. FT-IR spectrum of compound M6

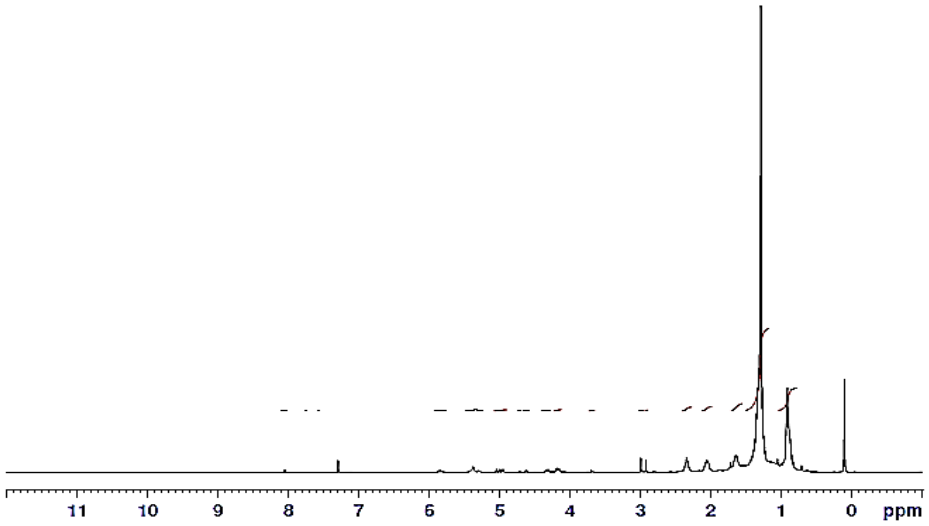

Figure 8. ^{1}HNMR spectrum of compound M6

FT-IR and ^{1}HNMR spectra of compound H4

FT-IR of H4 reveals absorptions in the region of 2881-2966 cm^{-1} corresponding to aliphatic C-H stretching vibrations, and an absorption at 1730 cm^{-1} corresponding to the carbonyl group of a ketone. In the ^{1}HNMR spectrum, peaks at 2.072 and 2.052 ppm are attributed to hydrogens of group a, while peaks in the region of 1.288-1.360 ppm are attributed to hydrogens of group b. Peaks at 0.907 and 0.876 ppm are attributed to H3, while peaks at 1.665 and 1.883 ppm are attributed to H4 and H5, respectively (Figures 9 and 10).

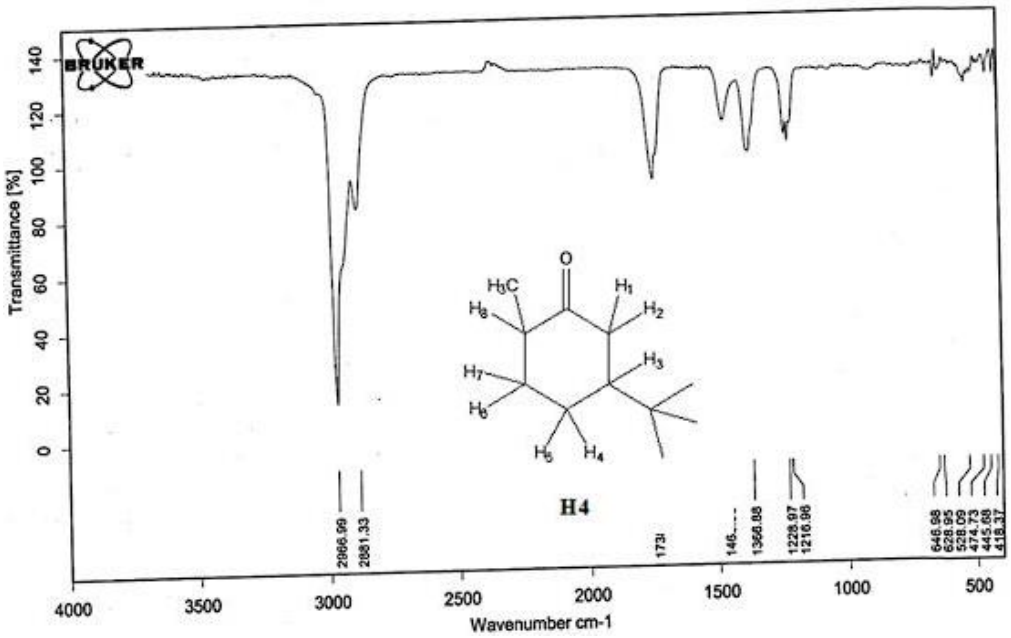

Figure 9. FT-IR Spectrum of compound H4

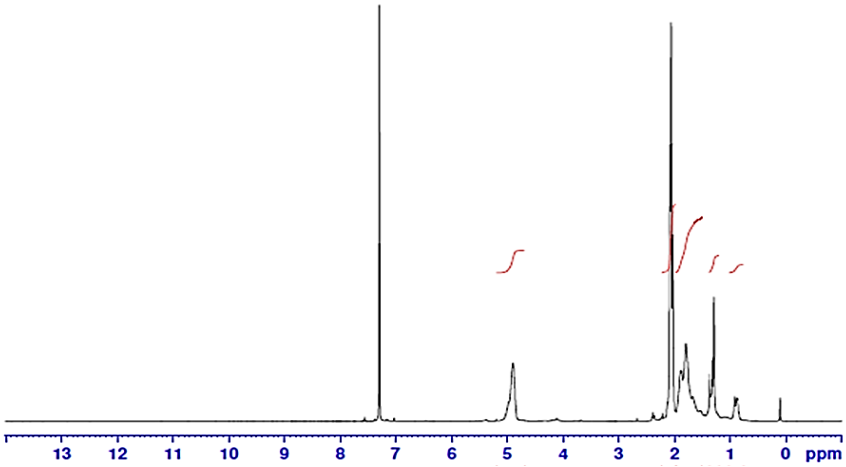

Figure 10. [1]HNMR Spectrum of compound H4

FT-IR and [1]HNMR spectra of compound 2C8

FT-IR of 2C8 reveals a sharp absorption in the region of 3083-3000 cm^{-1}, corresponding to the stretching vibrations of C-H bonds in the aromatic ring. The presence of a peak at 1622 cm^{-1} is attributed to the carbonyl group in the aromatic rings, while the peak at 1583 cm^{-1} corresponds to the C=C bond in the aromatic rings, and the peak at 1100 cm^{-1} is related to the C-O bond. In the [1]HNMR spectrum, the peaks in the region of 7.028-7.222 ppm are related to H1, H2, H3, and H4, while the peaks in the region of 6.602-6.484 ppm are related to H5-H6, and the peaks in the region of 5.408-5.332 ppm are related to H8. Due to the complexity of the R1 and R2 structures, a definite structure cannot be

proposed for the compound (Figures 11 and 12).

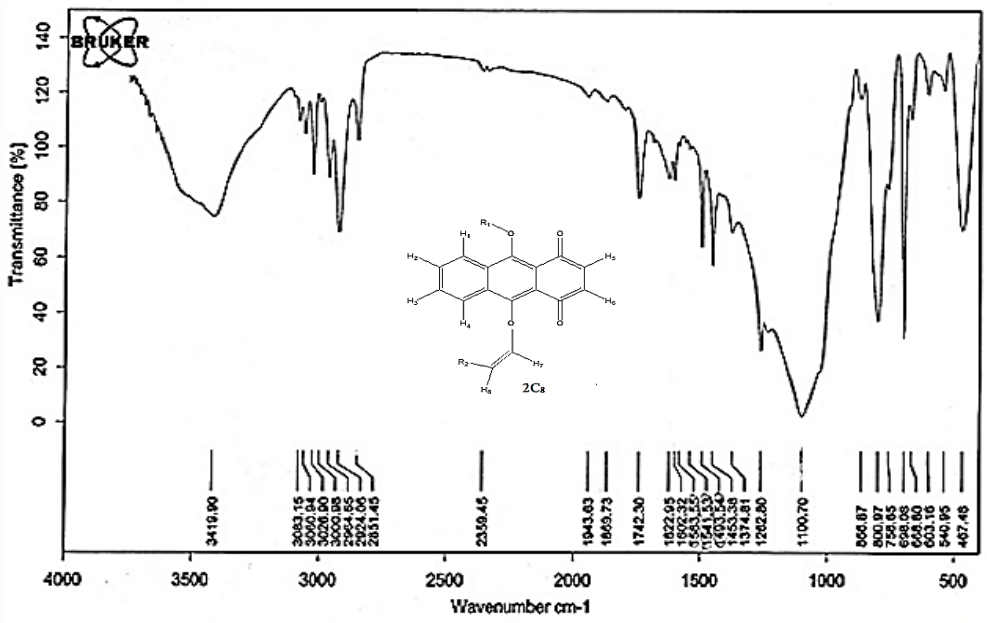

Figure 11. FT-IR Spectrum of compound 2C8

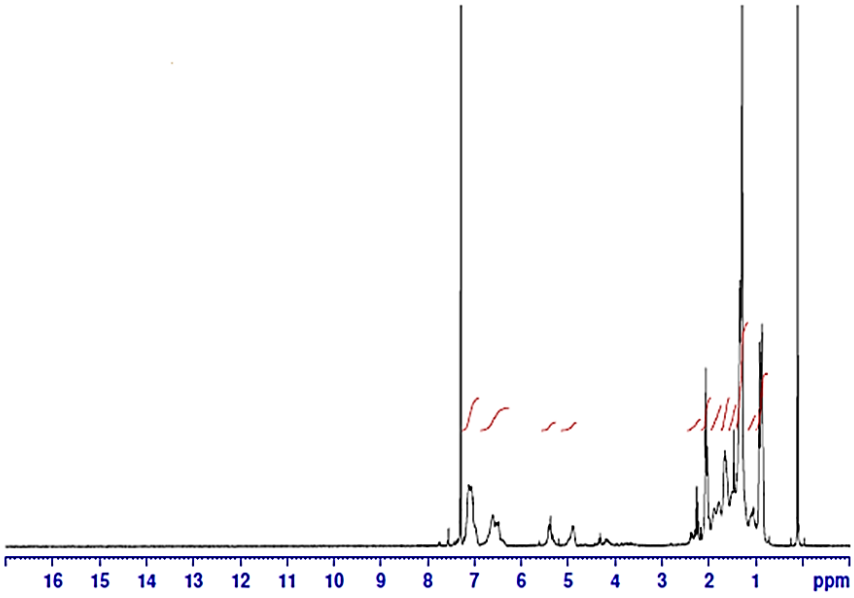

Figure 12. ^{1}HNMR Spectrum of compound 2C8

Based on the results shown in Table 1, the amount of extract obtained from 15 g of the flower and stem of the *Rhubarb* plant is higher in the methanol, chloroform, and hexane extracts of the flower. According to the identification tests performed on the *Rhubarb* plant (Table 2), it contains tannins, terpenoids, anthraquinones, saponins,

alkaloids, flavonoids, and glycosides. According to the results shown in Table 3, different solvents and single solvents can dissolve the components present in silica gel, and several compounds were purified using various methods such as crystallization, column chromatography, and thin-layer chromatography. Among these compounds, the ones with higher amounts were selected and identified using various methods, although some of the natural compound structures are too complex to be fully identified. This research on the *Rhubarb* plant's flower is innovative, and its purification method is cost-effective.

Conclusion

Using the Soxhlet extraction method, the amounts of hexane, chloroform, and methanol extracts of rhubarb flower were higher compared to the stem extracts. In order to identify the types of natural compounds present in the extracts of rhubarb plant stems and flowers, identification tests were performed. The results of these tests are shown in Table 2. Apart from the cases where the chromatography column is used for separation, to separate the extractable materials from the silica gel plate (in the plating method), solvents are needed to dissolve the desired extractable materials in itself, the natural extractable compounds include the following compounds: Tannins, terpenes, anthraquinones, alkaloids, flavonoids and glycosides.

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