

EXTRACTION, IDENTIFICATION AND DETERMINATION OF MOLECULAR STRUCTURE OF TWO NATURAL COMPOUNDS ALONG WITH PHYTOCHEMICAL STUDY OF *Hymenocrater calycinus* PLANT OF THE LAMIACEAE FAMILY COLLECTED FROM IRAN

Mehrnoosh Asadipour¹ and Mahboubeh Taherkhani^{2,*}

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The Iranian native plant called *Hymenocrater calycinus* (Boiss) Benth. of the Lamiaceae family, collected from the southern mountains of Khorasan Razavi, was evaluated and phytochemically studied in terms of natural compounds in the essential oil and extracts. The results of GCMS spectra from the essential oil of this plant showed that the highest concentration in this essential oil was related to sesquiterpene hydrocarbons. Oxygenated monoterpene 1,8-Cineole (12.54%) and hydrocarbonated sesquiterpene δ -cadinene (11.27%) had the highest concentrations in this essential oil. The plant extract was examined for the presence of flavonoids (Shinoda method), tannins (Ferric chloride method), alkaloids (Mayer test), glycosides (Keller-Kiliani test) and saponins (Froth method). Results showed that the plant extract contains large amounts of flavonoids (red color) and small amounts of saponins (foam layer), glycosides (reddish-brown color) and tannins (dark green for catechol tannin), but lacks alkaloids. The plant was extracted by soaking in a solvent and degreased to separate long-chain hydrocarbons. Then, the obtained components were examined and purified by column chromatography, plate chromatography and thin-layer chromatography (TLC) techniques. Natural compounds isolated from the extract were interpreted and identified using IR spectroscopy, ¹H and ¹³C NMR, and finally DEPT spectroscopy techniques. Two flavonols named santin and ermanin were identified. Since only few studies have been published on the medicinal effects of *H. calycinus*, results of this study provide a valuable report on phytochemical activities of this plant.

Keywords: *Hymenocrater calycinus*; santin; ermanin; chromatography; flavonoids, GC/MS.

1. INTRODUCTION

Hymenocrater is an ornamental and medicinal plant of the Lamiaceae family with more than 24 perennial species and shrubs in the world, particularly in Iran and Turan [1, 2]. *Hymenocrater* belongs to the genus of Lamiaceae (Labiatae or mint) and subfamily of Stachyioideae, which has twelve species in the world and are only scattered in the plateau region of Iran (Afghanistan and Turkmenistan) each with three species, and in Turkey, Pakistan, Iraq and Caucasus each with one species) [3].

In other words, the *Hymenocrater* genus belongs to the family Lamiaceae, a plant that includes eleven species of shrubs, among which the species *H. calycinus* grows widely in northeastern Iran [4]. *Hymenocrater calycinus* belongs to the genus *Hymenocrater*, the Lamiaceae family, and is endemic to Iran. This plant is used in traditional medicine as a painkiller, anti-skin allergies and burns, and has a fragrant essential with antibacterial properties [5]. This work was aimed at phytochemical study to determine the molecular structure of components or natural compounds isolated from the extract and to study the essential oil of one of the Iranian species called *Hymenocrater calycinus* from the Lamiaceae family collected in the south of Khorasan Razavi province near Kashmar.

¹ Department of Phytochemistry and Essential Oils Technology, Faculty of Pharmaceutical Chemistry, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Tehran, Iran.

² Department of Chemistry, Takestan Branch, Islamic Azad University, Takestan, Iran.

* e-mail: mahtaherkhani@yahoo.com

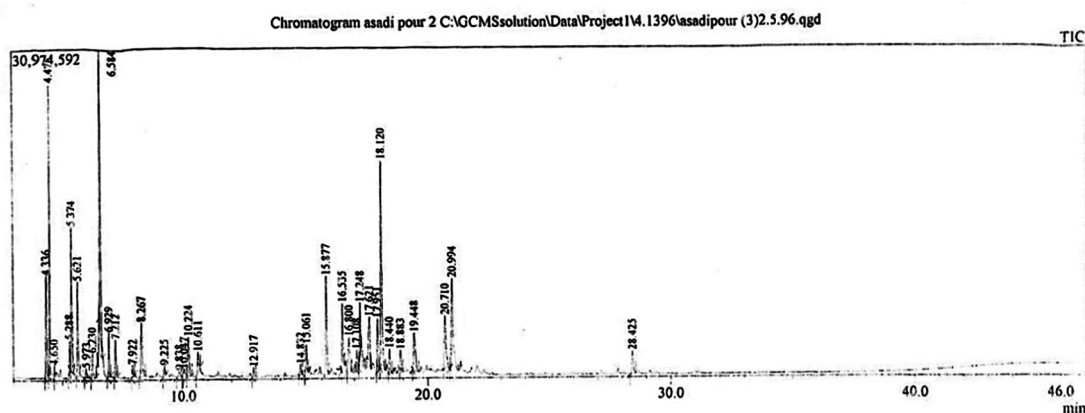


Fig. 1. General GC-MS chromatogram of *H. calycinus* essential oil.

2. EXPERIMENTAL CHEMICAL PART

2.1. Raw Material Collection and Sample Preparation

The aerial parts of *H. calycinus* plant were collected from mountainous areas located in the south of Khorasan Razavi province (Khorasan Razavi province, Road of Mashhad to Kalat, after Goojagi village). After transferring the collected samples to the laboratory, they were dried at room temperature and away from direct light for 14 days. In this study, the aerial parts (stems and leaves) of *Hymenocrater calycinus* (Boiss) Benth were used. The sample of the studied plant was collected in its natural vegetation areas (Khorasan Razavi province) using reliable botanical sources and was registered in the herbarium of Institute of Medicinal Plants of Academic Center for Education, Culture and Research on April 23, 2016 with the specialized code IMPH-7010.

Essential oil isolation. Aerial parts of the plant were extracted for 2 h by water distillation using Clevenger apparatus. Essential oils were then injected into a gas chromatograph coupled with mass spectrometer (GC/MS) and their components were identified. The sample was analyzed by GC/MS on HP-5MS column with a length of 30 m, internal diameter of 0.25 mm, and film thickness of 0.25 μ m using helium as carrier gas. The carrier gas flow velocity was 1 mL/min and the ionization energy in mass spectrometer was selected at 70 electronvolts. The temperature program of the device was set as follows: the temperature was first kept at 60°C for 3 min, then increased to 220°C at 5°C/min and remained at this temperature for 5 min; the speed of helium flow as the carrier gas was 1 mL/min. To identify the essential oil compounds of the plant according to suggestions provided by the GC-MS device library, each of them was examined separately. According to the retention time of each peak, the Kovats retention index (KI) was calculated through the corresponding equation and compared with the reference values (Adams Book and NIST site) for similar conditions

[6]. The obtained mass spectra were also compared with those mentioned in the Adams Book.

Preparation of samples for phytochemical analysis.

To prepare the ethanolic extract, 60 g of dry plant powder was poured into 300 mL of 98% ethanol, treated on a shaker for 48 h, passed through a filter and dried in rotary evaporator at 45°C.

2.2. Phytochemical Analysis

Determination of alkaloids. To detect alkaloids, one gram of dry plant extract powder was taken and 200 ml of 10% acetic acid solution in ethanol in a 250 ml Erlenmeyer flask was added. It was then kept in a sealed container at room temperature for 24 hours. After 24 hours, the solution was filtered and concentrated in a rotary at 45°C until its volume reached a quarter. Mayer's reagent (Potassium mercuric iodide) was then added dropwise until the solution became cloudy or alkaloid compounds were observed as a yellow precipitate at the bottom of the test tube. The amount of sediment formed indicates the relative content of alkaloid compounds in the sample [7].

Determination of glycosides. To detect glycosides, one gram of dry plant powder was taken and dissolved in 2 ml of glacial acetic acid, then 200 μ l of Iron(III) chloride was added. Then 1 ml of concentrated sulfuric acid was added to it. The formation of a reddish-brown color in the form of a ring between the two layers of acetic acid and sulfuric acid indicates the presence of glycosides or the formation of a blue-green layer above it indicates the presence of glycosides [8].

Determination of saponins. To detect saponins, 5 grams of dried plant extract powder was poured into 5 ml of distilled water. It was water bathed for 15 minutes and was then filtered after heating. Then 2 ml of the sample was mixed with 3 ml of distilled water. Observing the foam after heating and shaking indicates the presence of saponins. To confirm this point, 1 ml of edible oil is added to the sample and the emulsifying properties of saponins are thus proven [9].

Determination of flavonoids. To detect flavonoids, 1 gram of dried plant extract powder was poured into 10 ml of distilled water, then 4 ml of 50% methanol was added to it, then 100 μg of magnesium metal was added to it and was water bathed at 45°C for 15 minutes. The sample was then filtered and 4 ml of the filtered liquid was taken and 1 ml of concentrated hydrochloric acid was added. Observation of red color indicates flavonoid and orange color indicates the presence of flavon [10].

Determination of tannins. Pour half a gram of dry plant extract powder in 20 ml of distilled water and after boiling the sample, take it and by adding drop by drop of 0.1% iron

(III) chloride, a dark green color is formed, which indicates the presence of catechol tannins. If the sample is blue-black, it confirms the presence of gallic tannins in the sample [11].

2.3. Extraction of Natural Compounds from Plant Samples

In this research, extraction was performed by soaking in solvent. To prepare the main extract of this plant, about 700 grams of dried aerial parts of the plant were completely crushed. Then, to extract the plant components, it was soaked in a mixture of methanol – diethyl ether, hexane in a ratio of 1:1:1 in a percolator in a dark place for 48 to 72 hours. The resulting mixture was then filtered through a Buchner funnel

TABLE 1. Compounds Identified in the Essential Oil of *H. calycinus* (Boiss) Benth.

Percentage	Chemical structure of compound	Component	KI
4.50	Monoterpene hydrocarbons	α -Thujene	902
6.56	Monoterpene hydrocarbons	α -Pinene	948
1.22	Monoterpene hydrocarbons	Sabinene	972
6.59	Monoterpene hydrocarbons	β -Pinene	981
4.21	Monoterpene hydrocarbons	Myrcene	992
1.14	Monoterpene hydrocarbons	2-Carene	1004
12.54	Oxygenated monoterpene	1,8-Cineole	1030
1.82	Monoterpene hydrocarbons	Trans- β -ocimene	1038
1.64	Monoterpene hydrocarbons	γ -Terpinene	1074
0.48	Monoterpene hydrocarbons	Terpinolene	1095
3.14	Oxygenated monoterpene	Linalool	1100
0.91	Monoterpene hydrocarbons	Geijeren	1105
0.38	Oxygenated monoterpene	Pinocorvone	1114
0.59	Oxygenated monoterpene	Myrcenol	1137
2.24	Oxygenated monoterpene	4-Terpineol	1179
1.05	Oxygenated monoterpene	α -Terpineol	1195
0.48	Sesquiterpene hydrocarbons	Copaene	1221
1.47	Sesquiterpene hydrocarbons	β -Bourbonene	1339
5.00	Sesquiterpene hydrocarbons	Trans-caryophyllene	1414
2.95	Sesquiterpene hydrocarbons	β -Farnesene	1440
2.57	Sesquiterpene hydrocarbons	Aromadendrene	1475
0.82	Sesquiterpene hydrocarbons	γ -Muurolene	1475
2.08	Sesquiterpene hydrocarbons	Germacrene D	1487
4.17	Sesquiterpene hydrocarbons	α -Muurolene	1506
2.45	Sesquiterpene hydrocarbons	γ -Cadinene	1526
11.27	Sesquiterpene hydrocarbons	δ -Cadinene	1531
1.15	Oxygenated sesquiterpene	Longifolene aldehyde	1581
3.08	Oxygenated sesquiterpene	Spathulenol	1619
4.36	Oxygenated sesquiterpene	τ -Cadinol	1674
7.08	Oxygenated sesquiterpene	α -Cadinol	1676
97.94%	Total		

and a vacuum pump, and the resulting solution was evaporated and concentrated by a rotary apparatus. At the end, the extract was obtained as a thick juice and viscous dark green color and dried.

Defatting of extract. Degreasing was performed to separate fats and saturated compounds with long chains. To do this, first 250 ml of distilled water was added to a 2-liter decanter and then the resulting extract was dissolved with a very small amount of methanol and transferred to the decanter. Then 150 mL hexane was poured on it and the decanter was shaken well. After complete separation, the upper phase, which contains the hexane phase and the dissolved fats and hydrocarbons, was separated. Again, the lower phase was transferred to the decanter, to which 150 mL hexane was added, and the previous step was repeated. This operation was repeated until finally the whole extract would be washed with 400 mL hexane and the consumed hexane phase was discolored. At this stage, the hexane phase, which contains non-polar material and fats, was discarded and the lower phase or main phase was transferred back to the decanter. This was similar to the previous step, except that 450 mL of ethyl acetate solvent was used to dissolve the polar material instead of hexane. First, 200 mL of ethyl acetate was added to the decanter, which contained lower phase of the previous step, and the decanter was shaken until it was completely mixed. The decanter was then placed in a fixed position. After sufficient time to separate phases, the upper phase (ethyl acetate and polar phase) was separated. This operation was repeated until the consumed ethyl acetate phase became colorless. Finally, the ethyl acetate phase, which is the main phase, has been collected. To make sure, similar to washing with ethyl acetate, the extract is additionally washed with 250 mL of n-butanol. In the next step, the ethyl acetate phases from the degreasing step were concentrated on a rotary apparatus to obtain a viscous extract. The temperature was set at 45°C and the rotation speed was set at 25°C.

Column preparation. To the concentrated extract, some silica gel for column chromatography was added with a small amount of ethyl acetate, and evaporation on the rotary apparatus was continued until the extract would be completely dry and the silica gel was adsorbed so that the extract became a dark green powder. It is checked by not sticking to the balloon, that is dry and powdered, and was prepared to be applied to the chromatographic column. To prepare the col-

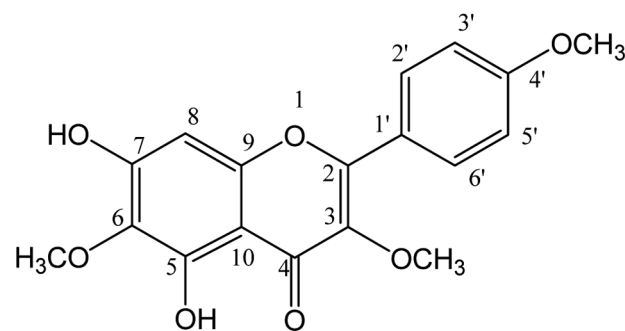


Fig. 2. The chemical structure of santin.

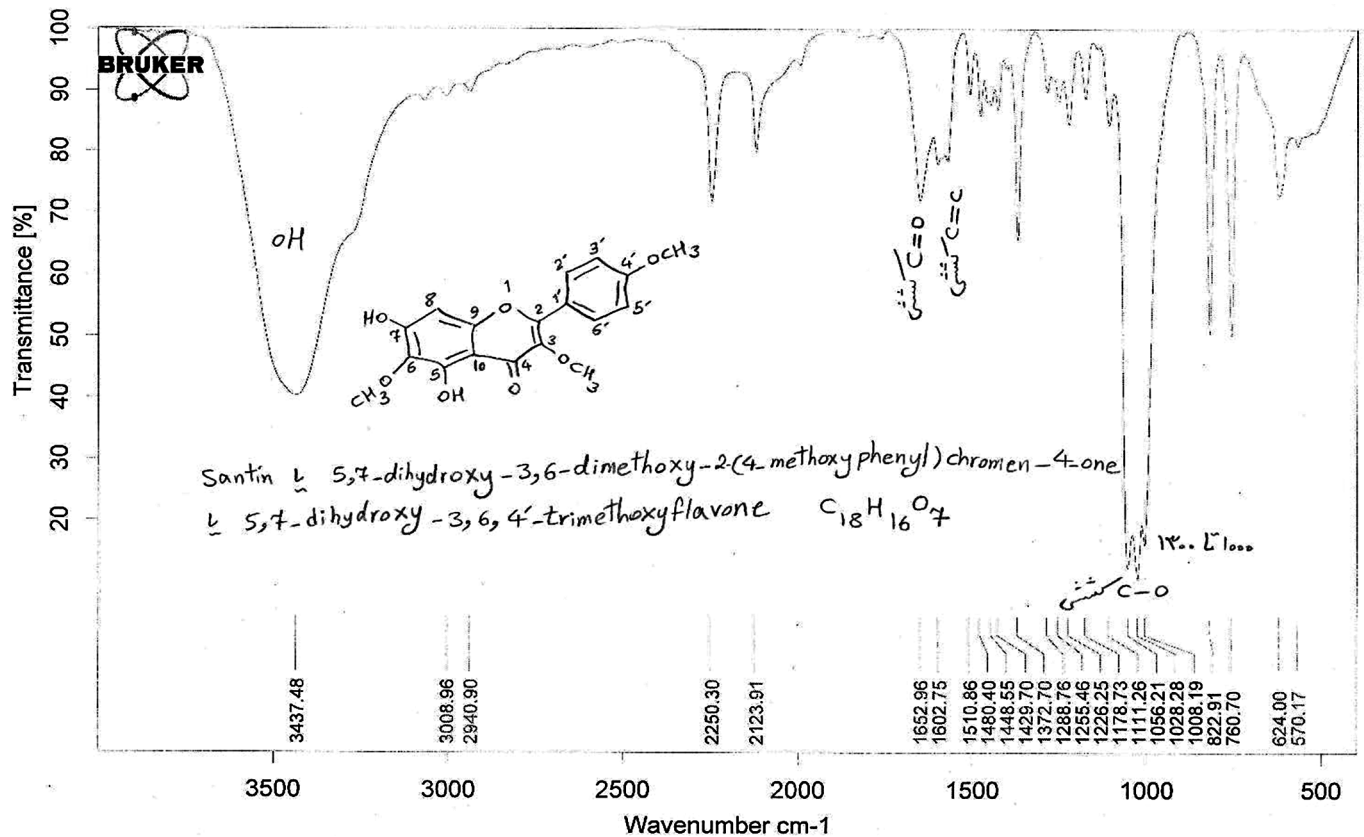
umn, the required amount of silica gel for column chromatography was mixed with a sufficient amount of hexane and the resulting slurry was slowly applied on the column.

Separation of compounds into fractions. After preparing the chromatographic column, the extract adsorbed on silica gel was added to the chromatographic column. To separate the extract components with different polarities, the column was first washed with hexane, which is a non-polar solvent. Gradually, by adding ethyl acetate, the polarity was reduced to semi-polar. At the next step the polarity was increased by adding certain amounts of methanol. The polarity and the volume of solvents used to wash the column were gradually changed to higher polarization. Finally, the column was washed with 100% methanol. Fractions removed from the column were separated in respect of color and numbered. Then, fractions were kept in the dark for 4 days.

Identification of compounds in fractions. Thin layer chromatography (TLC) was performed to determine the number of compounds in each fraction by changing solvents from diethyl ether to hexane and methanol with different ratios poured into the TLC tank. Each TLC paper sheet was then scored with four consecutive fractions. The TLC plates were placed in the TLC tank and then viewed under a UV lamp. Similar fractions were mixed and again placed in the dark for 4 days. The obtained crystals were separated from all fractions with ethyl acetate and their ^1H and ^{13}C NMR spectra were taken to identify natural compounds.

TABLE 2. Nature and Identification Signs of Compounds in Aerial Parts of *H. calycinus*

Chemical class	Identification sign	Detection tests	Result
Alkaloids	Yellowish white deposit	Mayer test	-
Glycoside	Formation of a red-brown solution or a blue-green layer	Keller-Kiliani test	+
Saponin	Formation of a stable foam as well as oil emulsifying	Froth test	+
Flavonoid	Red (flavonoid) and Orange (Flavon)	Shinoda test	++
Tannin	Blue (Gallic tannin) and Dark green (Catechol tannin)	Ferric chloride	+



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F5+6-DMSO

sample form

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Fig. 3. FTIR Spectrum of santin extracted from *H. calycinus*.

3. RESULTS AND DISCUSSION

3.1. Analysis of *H. calycinus* Essential Oil

According to interpretation of the GCMS spectra obtained from the essential oil of *H. calycinus* (Boiss) Benth. (Fig. 1), the percentage of components in the essential oil was determined as shown in Table 1. As can be seen from this table, the percentage of compounds in the essential oil (97.94% totally) is expressed as hydrocarbonated monoterpenes with a total concentration of 29.07%, oxygenated monoterpenes (19.94%), hydrocarbonated sesquiterpenes (34.31%) and oxygenated sesquiterpenes (15.67%). The highest concentration in this essential oil was related to sesquiterpene hydrocarbons, followed by 1,8-cineole (12.54%) as an oxygenated monoterpene, and δ -cadinene (11.27%) as a sesquiterpene hydrocarbon.

3.2. Phytochemical Analysis

In the alkaloid detection test, a black deposit was formed instead of the yellow one, which confirmed the absence of al-

kaloid compounds in the plant extract. In the glycoside detection test, the green-blue layer on top of the sample indicates some glycoside in the sample. In the saponin detection test, a small amount of foam was formed on the surface of the sample, which indicated a small amount of saponin in the extract of this plant. In the flavonoid detection test, an orange color appeared that indicated the presence of a very large amount of flavonoids. The formation of a dark green

TABLE 3. IR Spectrum of Santin Extracted from *H. calycinus*

Functional group	Wavelength, cm^{-1}
OH	3437.48
=C-H and -C-H	2940.90
C-C	1372.70
C=O	1652.96
C-O	1028.28
C=C	1602.75

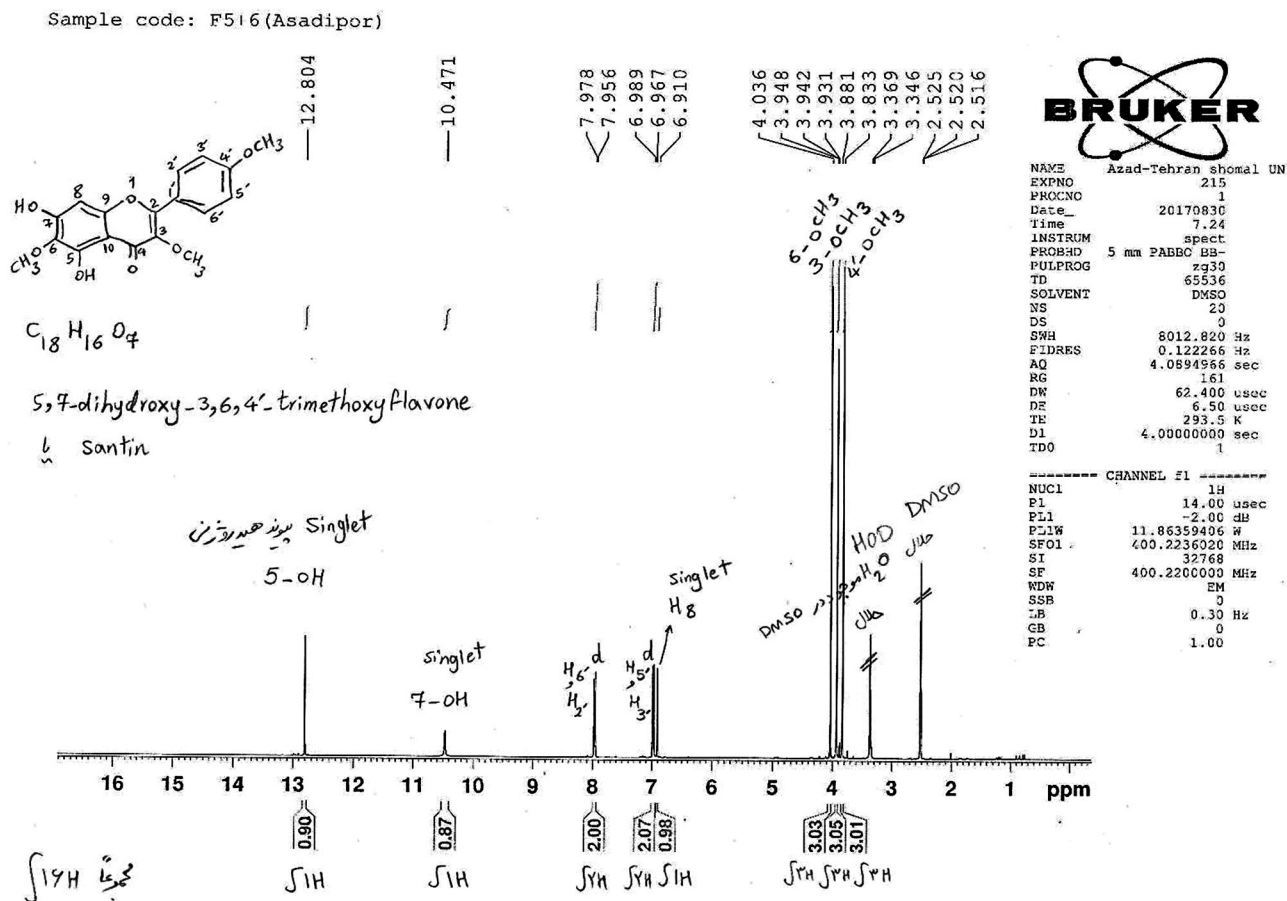


Fig. 4. ^1H NMR spectrum of santin extracted from *H. calycinus*.

color in the tannin test confirmed the presence of tannin catechol. The obtained results are presented in Table 2.

TABLE 4. ^1H NMR Spectrum of Santin Extracted from *H. calycinus*

Number of proton	Chemical shift δ , ppm	Splitting	Coupling constant J , Hz	Remarks	Integral
OH on C-5	12.804	S	–	OH Hydrogen Bonding	1
OHs on C-7	10.471	S	–	OH Group	1
H-3',5'	6.967 & 6.989	d	8.8	Aromatic Region	2
H-2',6'	7.956 & 7.978	d	8.8	Aromatic Region	2
H-8	6.910	S	–	Aromatic Region	1
Methoxy 4'	3.833	S	–	4'-OCH ₃	3
Methoxy 3	3.931	S	–	3-OCH ₃	3
Methoxy 6	4.036	S	–	6-OCH ₃	3

Natural compounds present in the aerial parts of *H. calycinus* were identified based on the analysis of IR, ^1H and ^{13}C NMR, and DETP spectra of compounds extracted from various fractions isolated as crystals from the aerial parts of *H. calycinus*.

3.3. Santin: The First Compound

Analysis of the FTIR, ^1H and ^{13}C NMR, and DETP spectra of natural compounds extracted from 5+6 fractions isolated as crystals confirmed the presence of santin as first compound with the corresponding structure (Fig. 2).

FTIR spectrum of santin extracted from *H. calycinus*.

As can be seen from the FTIR spectroscopy data (Fig. 3 and Table 3) the absorption band in the region of 3437.48 cm^{-1} is related to tensile vibrations of the hydroxyl group (OH) in the first natural compound, which appears wide due to the formation of hydrogen bond. Also, the band at 2940.90 cm^{-1} is related to the tensile vibration $=\text{C}-\text{H}$ and $-\text{C}-\text{H}$ in the desired compound. The absorption band 1372.70 cm^{-1} is related to the tensile vibration of C-C and the band 1652.96 cm^{-1} is related to the tensile vibration of C=O carbonyl. The absorption band 1028.28 cm^{-1} is also related to

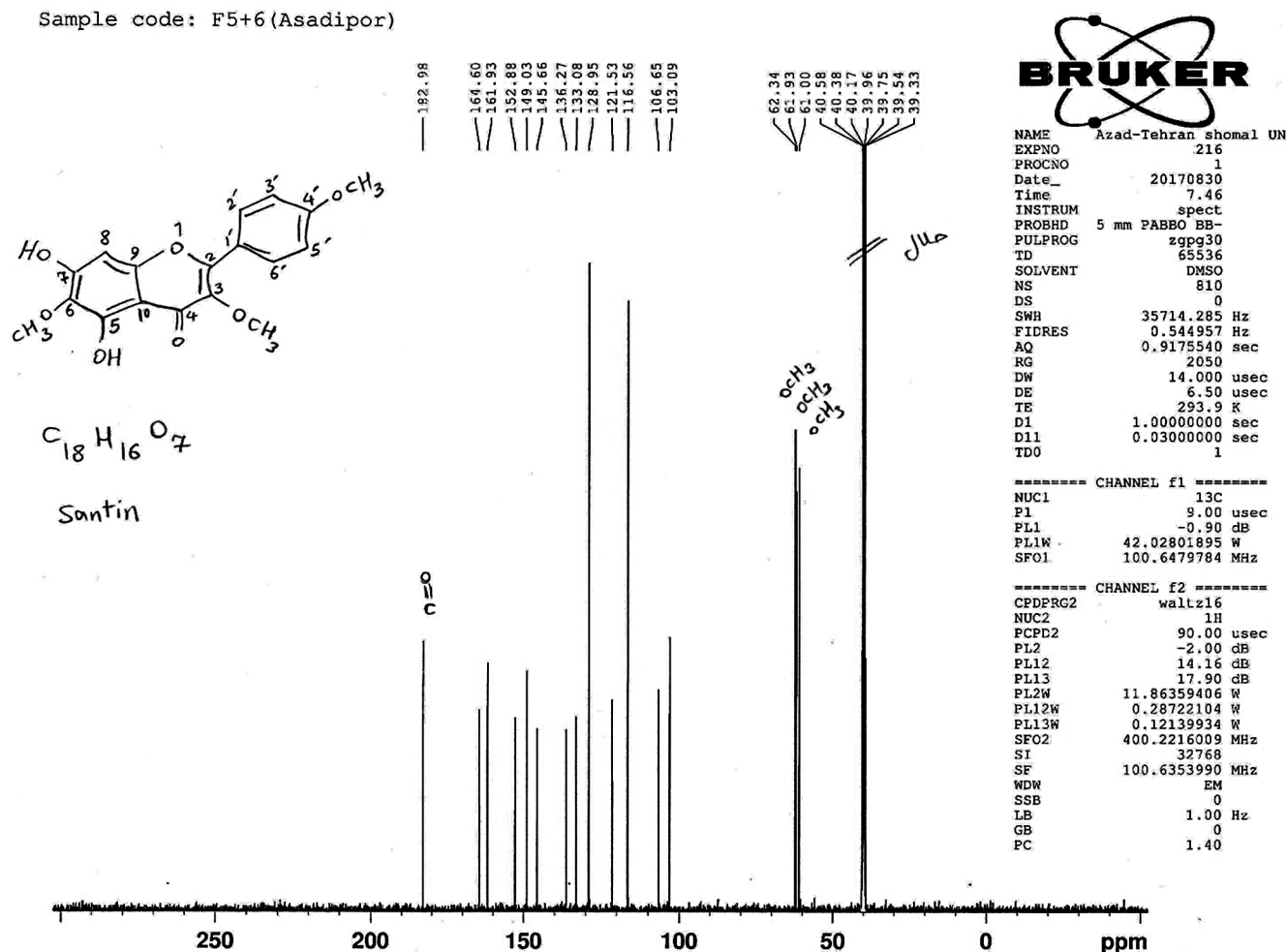


Fig. 5. ^{13}C NMR spectrum of santin extracted from *H. calycinus*.

the tensile vibration of C-O and 1602.75 cm^{-1} is related to the tensile vibration of C = C in the compound.

1H NMR spectrum of santin extracted from *H. calycinus*. Parameters of the 1H NMR spectrum are presented in Table 4. There are a total of 18 hydrogens in the santin molecule according to integrals determined for the spectrum. The solvent peak of DMSO in the region of 2.520 and the H_2O in the solvent (as HOD) has shown its peak in the delta of 3.369 and both without integrals. As can be seen in the 1H NMR spectrum (Fig. 4), five aromatic hydrogens numbered 8, 3' and 5', as well as 2' and 6' have appeared in the range from 6.91 to 7.97 ppm, due to the high chemical shift of aromatic hydrogens – the anisotropic effect. Peaks of hydrogens 3' and 5', as well as 2' and 6', due to their symmetry, appear in pairs and have the form of two doublets with a coupling constant of $^3J = 8.8\text{ Hz}$, which is due to the presence of a neighboring hydrogen plus number one. Aromatic hydrogen no.8 has appeared as a singlet due to the lack of a neighbor. Hydrogen of the hydroxyl group at carbon 7 appeared at 10.471 ppm. Hydrogen belonging to the hydroxyl group on carbon number 5

is affected by hydrogen bonding with the carbonyl group and appeared at a higher chemical shift of 12.804 ppm. In the structure of santin, hydrogens belonging to three methoxy groups (OMe) are not covered due to binding to the electronegative atom and appear in the range from 3.5 to 5 ppm as three singlets with integral 3.

^{13}C NMR spectrum of santin extracted from *H. calycinus*. Figure 5 shows the ^{13}C NMR spectrum of santin extracted from *H. calycinus*. Results of analysis of this spectrum are given in Table 5. As can be seen from Fig. 5, the ^{13}C NMR spectrum of santin contains 16 peaks corresponding to 16 carbons according to the molecular formula. Alkane carbons are manifested at δ values below about 63 ppm. The most uncoated carbons in this compound are first carbonyl carbon, then olefinic carbons, and then the carbon attached to the electronegative oxygen atom.

Due to the symmetry and equivalence of carbons 6' and 2' with each other and also carbons 5' and 3' with each other, both carbons have appeared together in the same place (Figure 5). The rest of the peaks are not symmetrical and each one gives its own individual peak. The peak of septet of sol-

طیف ^{13}C و Dept CH_2 در سانتین

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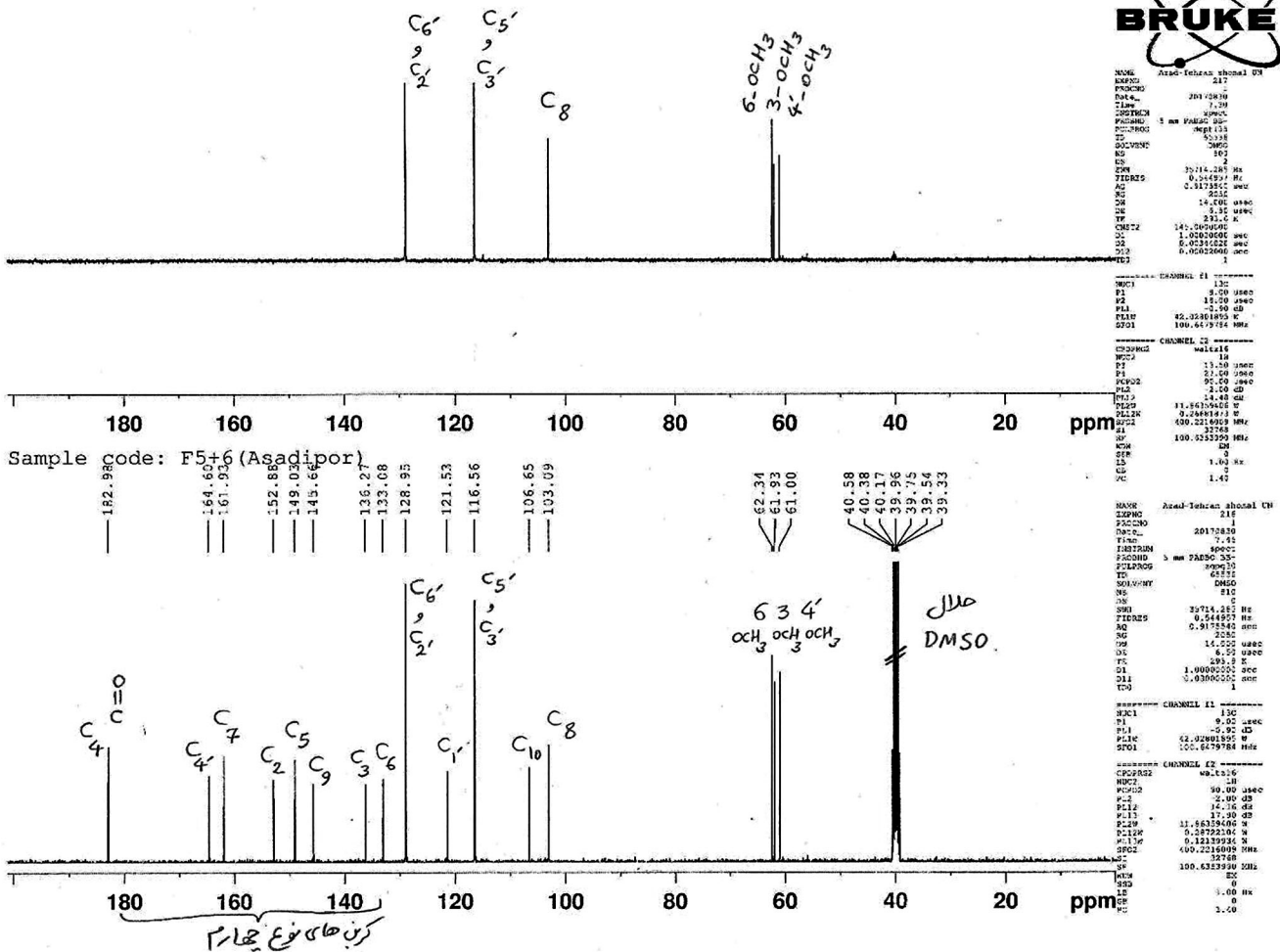


Fig. 6. DEPT spectrum along with ^{13}C NMR spectrum of santin extracted from *H. calycinus*.

vent dimethyl sulfoxide (DMSO) appeared in the region of 39.33 to 40.58. The high electronegativity of oxygen in the carbonyl group causes the carbon 4 signal, which belongs to the carbonyl group, to appear in a higher chemical shift, in the region of 182.98 ppm. After carbonyl, oxygen-bound carbons such as 7, 5, 6, 9, 2, 3 and 4' show similar behavior. As can be seen from Table 5, the peaks of aromatic carbon in the ^{13}C NMR spectrum range from 103.09 to 164.60 ppm, which can be slightly shifted under the influence of the corresponding substituent. Three different methoxy groups have also appeared separately in the region from 61 to 62.34 ppm due to the binding of methyl carbon to oxygen.

DEPT spectrum of santin extracted from *H. calycinus*. As can be seen from the DEPT spectrum (Fig. 6), the CH and CH_3 carbons are visible upward, CH_2 carbon is present downward in the DEPT spectrum, and the fourth type of carbon does not appear in this spectrum. Ten cases of the

fourth type carbon with the numbers 7, 6, 5, 9, 10, 2, 3, 4, 1' and 4' do not appear in the DEPT spectrum. In addition, the lack of downward peak in the DEPT spectrum is the reason for the lack of CH_2 in santin.

3.4. Ermanin: The Second Compound

The IR ^{13}C -NMR, 1H -NMR and DETP spectra obtained for the natural compound extracted from fractions no. 13 + 14 in crystalline form and the results of their analysis proved the presence of ermanin as the second compound in *H. calycinus* (Fig. 7).

IR spectrum of ermanin extracted from *H. calycinus*. As can be seen from Table 6 and the FTIR spectrum in Fig. 8, the absorption band in the region of 3420.92 cm^{-1} is related to tensile vibrations of the hydroxyl group (OH) in the natural compound of ermanin, which appears wide due to the formation of hydrogen bonds. The band at 1654.95 cm^{-1}

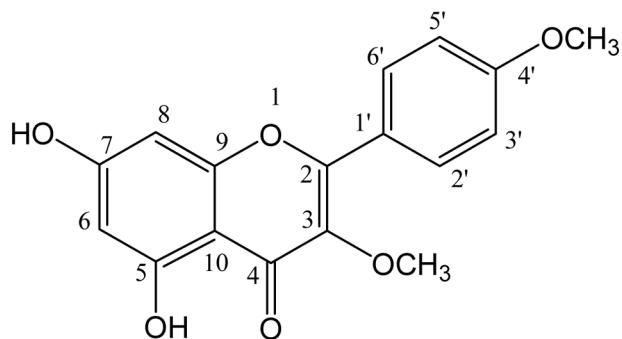


Fig. 7. The chemical structure of ermanin.

is related to the tensile vibrations of C=O carbonyl group, which probably overlaps with the C=C vibration according to the wide spectral band that appears. The absorption band at 1026.82 cm^{-1} is also related to the tensile vibrations of C-O in this compound.

^1H NMR spectrum of ermanin extracted from *H. calycinus*. Figure 9 shows the ^1H NMR spectrum of ermanin extracted from the plant. The results of analysis are presented in Table 7. There are a total of 17 hydrogens in the Ermanin molecule according to the given integral in the spectrum. The peak of DMSO solvent in the region of 2.522 ppm and the peak of D_2O solvent were observed in the region of $\delta = 3.369$ ppm, both without integrals. According to the ^1H NMR spectrum (Fig. 9), six aromatic hydrogens with numbers 6, 8, 3' and 5', as well as 2' and 6' have appeared in the

TABLE 5. ^{13}C NMR spectrum of Santin Extracted from *H. calycinus*

Number of carbon atom	δ , ppm
1'	121.53
2	152.88
3	136.27
4	182.98
4'	164.60
5	149.03
5' and 3'	116.56
6	133.08
2' and 6'	128.95
7	161.93
8	103.09
9	145.66
10	106.65
4'-OCH ₃	61.00
3-OCH ₃	61.93
6-OCH ₃	62.34

TABLE 6. IR Spectrum of Ermanin Extracted from *H. calycinus*

Wavelength, cm^{-1}	Functional group
3420.92	OH
1654.95	C=O
1026.82	C-O

region from 6.899 to 8.073 ppm due to the high chemical shift of aromatic hydrogens and the related anisotropic effect. According to Fig. 9, hydrogens 3' and 5', as well as 2' and 6' are due to symmetry, appear in pairs, and have the form of two doublets with a coupling constant of $^3J = 8.8$ Hz, and this split is due to the presence of a neighboring hydrogen plus number one. Aromatic hydrogens no. 6 and 8, due to the lack of neighbors, appeared at $\delta = 6.899$ and 6.940 ppm, respectively, as singlets and each with an integral of one hydrogen. Hydrogen of the hydroxyl group on carbon 7 appeared at 8.755 ppm. Hydrogen belonging to the hydroxyl group on carbon number 5 appeared at a higher chemical shift of 12.629 ppm because it is affected by hydrogen bonding with the carbonyl group. In the ermanin structure, hydrogens belonging to the two methoxy groups (OMe) are not shielded due to binding to the electronegative atom, and in the region of $\delta = 3.5$ to 5 ppm appear as two singlets (due to the lack of neighboring hydrogen), each with integral 3 in such a way that methoxy groups no. 3 and 4' at $\delta = 3.874$ have their peaks at $\delta = 3.938$ ppm.

^{13}C NMR and DEPT spectra of ermanin extracted from *H. calycinus*. The ^{13}C NMR spectrum of ermanin in Fig. 10 can be considered in comparison with the DEPT spectrum in Fig. 11. Parameters of the ^{13}C NMR spectrum of

TABLE 7. ^1H NMR Spectrum of Ermanin Extracted from *H. calycinus*

Number of proton	Chemical shift δ , ppm	Splitting	Coupling constant J, Hz	Remarks	Integral
C-5 on OH	12.629	S	–	OH Hydrogen Bonding	1
C-7s on OH	8.755	S	–	OH Group	1
H-3',5'	7.112 & 7.134	d	8.8	Aromatic Region	2
H-2',6'	8.051 & 8.073	d	8.8	Aromatic Region	2
H-6	6.899	S	–	Aromatic Region	1
H-8	6.940	S	–	Aromatic Region	1
–	3.938	S	–	4'-OCH ₃	3
–	3.874	S	–	3-OCH ₃	3

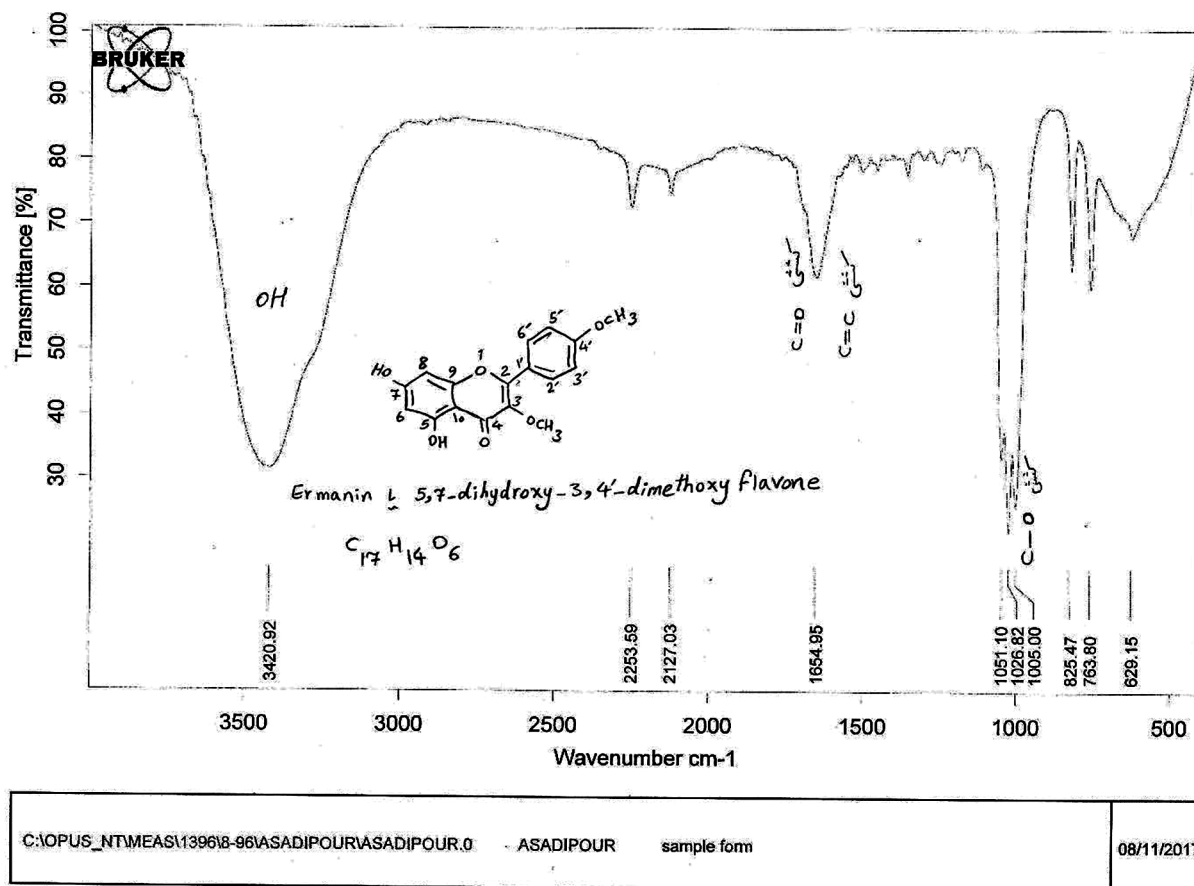


Fig. 8. The IR spectrum of ermanin extracted from *H. calycinus*.

TABLE 8. ^{13}C NMR Spectrum of Ermanin Extracted from *H. calycinus*

Number of carbon	δ , ppm
1'	123.45
2	143.64
3	130.44
4	182.69
4'	162.73
5	145.87
& 5' 3'	115.02
6	103.62
2' & 6'	128.68
7	163.76
8	91.66
9	150.23
10	105.56
4'-OCH ₃	56.03
3-OCH ₃	56.77

ermanin are given in Table 8. In the ^{13}C NMR spectrum, 17 peaks of 17 carbons are also present according to the molecular formula. As can be seen from Fig. 11, there are 9 cases of the fourth type of carbons with numbers 7, 5, 9, 10, 2, 3, 4, 1' and 4', which do not appear in the DEPT spectrum. In addition, the lack of observation of the downstream peak in the DEPT spectrum is the reason for the lack of CH_2 in the ermanin structure. The most deshielded carbons in this compound are first carbonyl group, then olefinic carbon, and then the one attached to the electronegative oxygen atom. The peak of the septet of solvent dimethyl sulfoxide (DMSO) appeared in the range of δ from 39.36 to 40.61 ppm. Alkane carbons belonging to two different Methoxy groups are also deshielded due to the binding of methyl carbon to oxygen and can be seen separately at $\delta = 6.03$ and 56.77 ppm. As can be seen in Fig. 11, this is due to the symmetry and equivalence of carbons 6' and 2' as well as carbons 5' and 3' with each other, so that peaks of two carbons appear in the same place. The high electronegativity of oxygen in the carbonyl group causes the carbon 4 signal, which is related to the carbonyl group, to appear in a higher chemical shift, in the region of 182.69 ppm. Oxygen-bonded carbons such as 7, 5, 9, 2, 3, and 4' are also deshielded due to

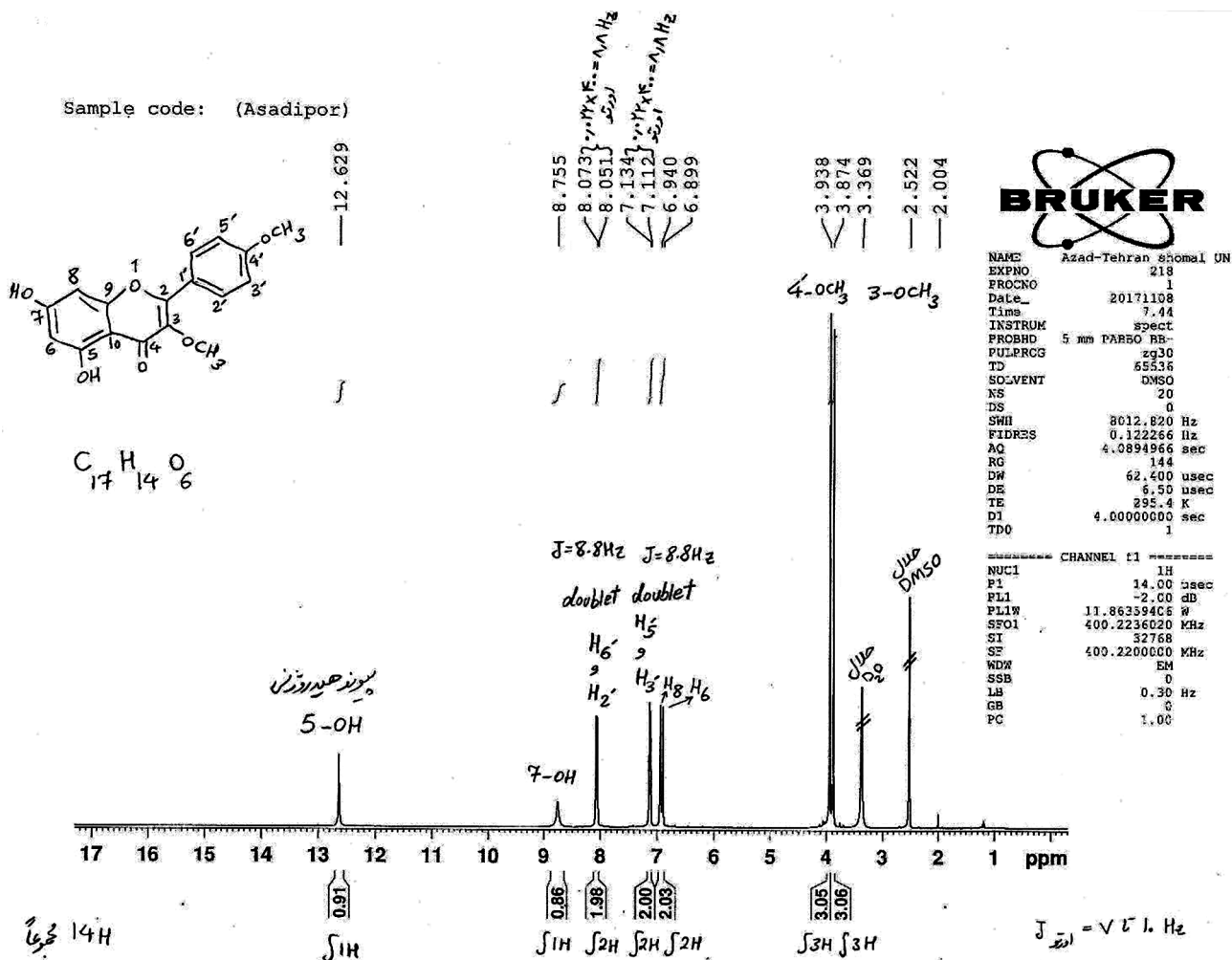


Fig. 9. ¹H NMR spectrum of ermanin extracted from *H. calycinus*.

their binding to the electronegative oxygen atom and appear at higher δ values. As can be seen from Table 8, the peaks of aromatic carbons in the ¹³C NMR spectrum range from 91.66 to 163.76 ppm and can be slightly shifted by the corresponding substituents.

In this study, it has been found out that there is a large amount of flavonoid compounds in the extract of *H. calycinus* (Boiss) Benth. Other compounds that have been confirmed to exist were glycosides, saponins, and tannins. Flavonoid compounds, in addition to antioxidant properties, are effective antimicrobial agents against various microorganisms [12]. The health effects of flavonoids, which are abundant in the plant studied, are mostly due to their antioxidant properties and chelating ability. Numerous studies have been performed to prove the effectiveness of flavonoids as antifungal, antibacterial, antiviral, anti-inflammatory, antioxidant agents, etc. Flavonoids can also attract insects and help pollinate plants by creating attractive colors [13]. Due to the deposition of proteins, tannins are considered as blood-blocking agents in traditional medicine, and their oral administra-

tion is used to protect inflamed surfaces of the mouth and throat. Biological tests prove the antiviral, antibacterial and especially the anti-tumor effects of tannins. In the treatment of burns, tannins form a thin layer due to the deposition of proteins on the surface of tissues, which causes the production of new cells under this layer and the healing of burns [14].

In [5], the antioxidant and antibacterial activity of various extracts of *H. calycinus* was reported. These results showed that phenolic and flavonoid compounds play a major and direct role in the antioxidant and antibacterial properties of this plant, which requires further studies to isolate and purify these compounds from this plant. As was previously mentioned in the present study, two flavonols called santin and ermanin were isolated from *H. calycinus* essential oil and their molecular structures were confirmed. In our research on analysis of the essential oil of *H. calycinus* (Boiss) Benth, compounds like 1,8-cineole (12.54%), δ -cadinene (11.27%), α -cadinol (7.08%), β -pinene (6.59%), α -pinene (6.56%) and α -thujene (4.50%) had the highest content. Although the essential oil compounds of this species have simi-

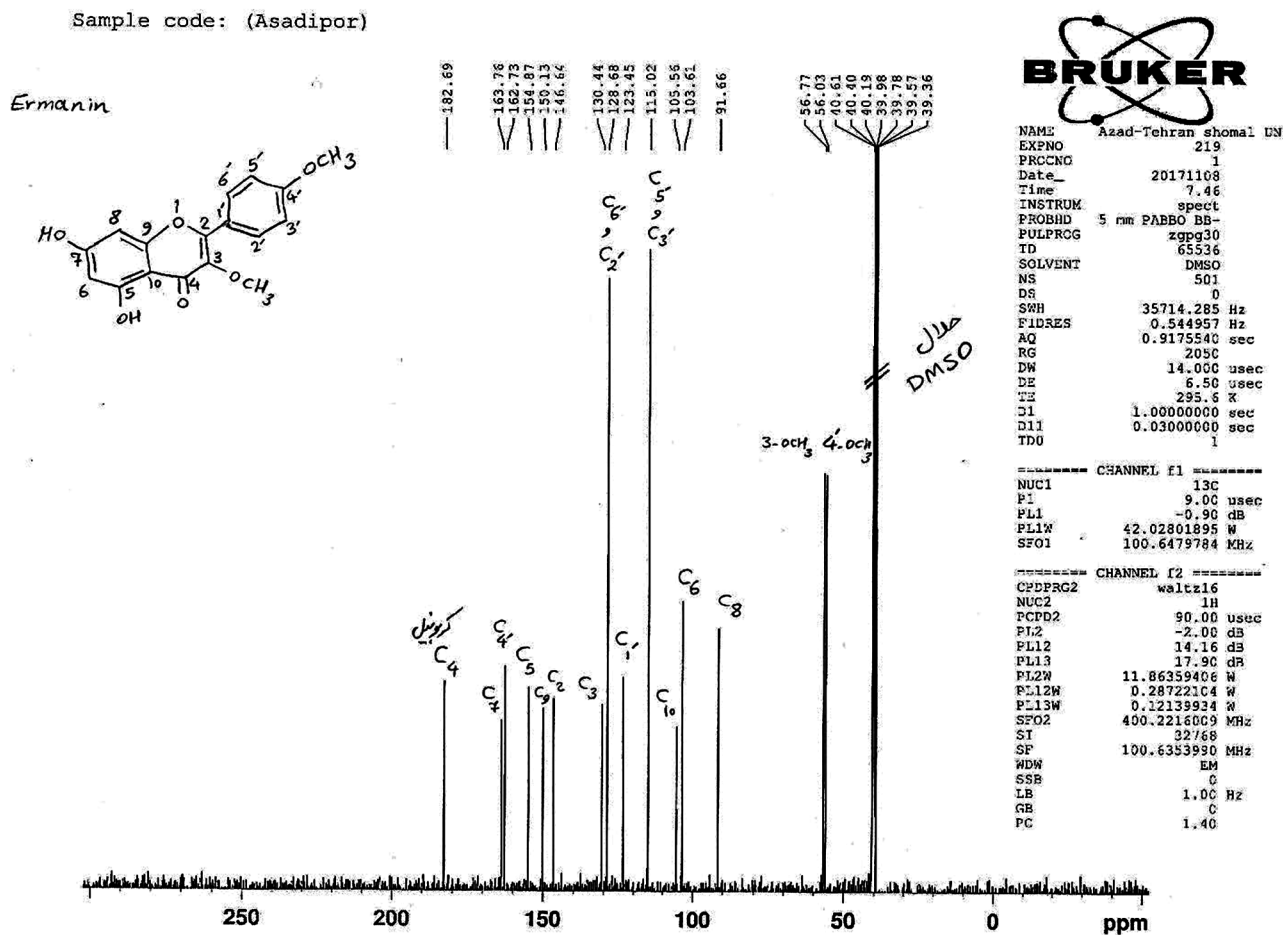


Fig. 10. The ^{13}C NMR spectrum of ermanin extracted from *H. calycinus*.

larities, it is clear that the type of species, place of growth and time of plant collection affect the type of terpene compounds as well as the quality and quantity of constituents of the essential oils.

According to previously reported investigations, 49 compounds with a total concentration of 84.764% were identified in the essential oil of *Hymenocrater bituminosus*, of which the highest concentration was related to (E)-caryophyllene (10.5%), α -pinene (15.1%), β -farnesene (6%), germacrene D (5%) and β -pinene (4%) [15, 16]. In [15], *H. calycinus* Boiss plant was collected from three areas of Yekeh Shakh near Bojnourd town and in the Nodeh and Golestan forests in northeastern Iran and their essential oil compounds were identified. The main compounds included α -pinene (10.5%) and sabinene (10.5%) in the sample collected in Bojnourd, spathulenol (35.4%) and abietadiene (13.4%) in the Nodeh sample, and β -caryophyllene (32.8%) and caryophyllene oxide (16.1%) in the Golestan sample. From analysis of the essential oil of *Hymenocrater platystegius*, 59 compounds with a total concentration of 84.72% were identified, the highest amount of which was related to 1,8-cineole (14.27%), (-pinene (4.89%), terpinolene (4.83%) and sabinene (4.59%).

The essential oil of this plant reduced PC12 cell death caused by the oxidative stress [17].

In another study, the essential oil of dried aerial parts of *H. calycinus* (Boiss) was studied by GC and GC-MS and 67 compounds were identified, the most important of which were 1,8-cineole (12.2%), (-pinene (6.3%), and (-pinene (5.7%). The antimicrobial properties of the essential oil were studied by the disk diffusion, and the MIC values were determined against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Candida albicans* microorganisms. Results showed that the essential oil of this plant exhibited antibacterial properties against *B. subtilis*, *S. aureus*, *E. coli* and *S. typhi*, while no antifungal properties were observed [18]. Four compounds were isolated and identified from ethyl acetate and methanol extracts of *H. calycinus* using chromatography and spectroscopy (MS, ^1H and ^{13}C NMR, HMQC, HMBC, and ^1H - ^1H COSY) techniques. These compounds included (-sitosterol, ursolic acid, rosmarinic acid and quercetin-3-O-rutinoside. Results of this study against bacteria and fungi showed that rosmarinic acid as the main component of *H. calycinus* extract exhibited antifungal properties

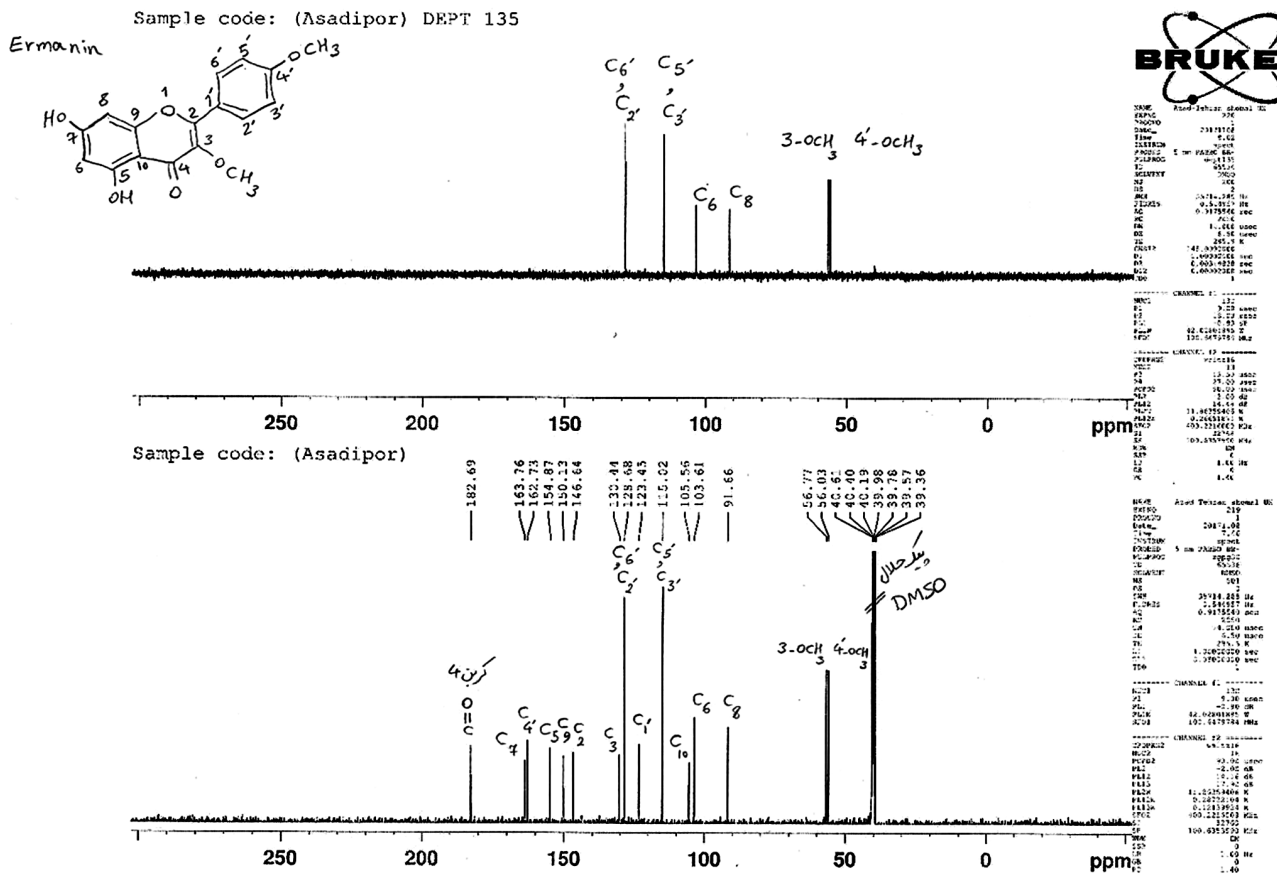


Fig. 11. The DEPT spectrum along with ¹³C NMR spectrum of ermanin extracted from *H. calycinus*.

against *C. albicans* [4]. In 2010, Gohari, et al. [19] extracted a derivative of quinic acid called 3-(3,4-dihydroxyphenyl) lactic acid 2-O-quinic acid from *H. calycinus* plant collected from Firoozkooh region and determined the molecular structure of this compound. Methanol extract of the aerial parts of *H. calycinus* showed significant activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Morganella morganii*, *Klebsiella pneumoniae* and *Staphylococcus aureus* [20]. Previous reports confirmed the potential of Hymenocraete plants in development of new drugs. These plants contain antibiotic, anti-fungal, anti-parasitic, anti-cancer, anti-diabetic and anti-oxidant compounds that can be effective in treating diseases [21]. Previously, two anti-inflammatory flavonoids, santin (5,7-dihydroxy-3,6,4'-trimethoxyflavone) and ermanin (5,7-dihydroxy-3,4'-dimethoxyflavone) were isolated from CH₂Cl₂ extract of *Tanacetum microphyllum* [22, 23]. Ermanin was reported to have several effects including anti-inflammatory, cytotoxic, antiviral, and antibacterial activity [24]. According to previous studies, plant flavonoids such as santin and ermanin exhibited anti-inflammatory activity both *in vitro* and *in vivo* and might be potential agents for the treatment of inflammatory diseases [25]. The extract of *H. calycinus* has high content of flavonoids, especially of two anti-inflammatory flavonols named santin and ermanin.

As a result, the extract of this plant has high antioxidant properties and can play a potential role in modulating inflammatory processes.

In conclusion, results obtained in this study and previous research of *H. calycinus* indicate the importance of this medicinal plant as a source of antioxidants and antibacterial components. Therefore, in the future, more research can be done on the pharmacological properties of this plant so that it would be used to make a wide range of drugs.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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