Isolation and Identification of Isoquercitrin from Extracts Obtained from Leaves of *Morus alba* (L.) and *Morus nigra* (L.)

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Abstract

The isolation of isoquercitrin from the leaves of *Morus alba* L. and *Morus nigra* L. is part of a greater plan of research. It consists in linking the flavonoids isolated from the leaves and bark of *Morus* sp. with biologically-active metallic ions. The aim is to obtain some semisynthetic compounds with the potential to become new pharmaceutical products.

Isoquercitrin was isolated by column chromatography and purified by recrystallisation using a solvent combination (MeOH/H_2O). The purified compound was subject to analysis by spectroscopic (UV/VIS, IR, ^{1}H-NMR) and electrochemical methods (polarography).

By comparison of chromatographic parameters (R_f=0,55; t_R=8,00 min), spectral data (λ_{max} Band I = 361 nm, Band II = 258, 2666 sh), polarographical parameters ($E_{1/2}$ = - 1,604 V) and melting point (186-187°), the isolated substance was shown to be identical to the standard substance (R_f = 0,55; t_R = 7,80 min, λ_{max} Band I = 359 nm, Band II = 258, 266 sh; $E_{1/2}$ = -1,604 V; pt^o = 188-189). Thus, the isolated flavonglycoside is isoquercitrin, in an advanced grade of purity. IR and ^{1}H-NMR spectra also confirmed this result.

Thus the separation of the flavonoid complex is a laborious method, but suitable choice of solvents/method leads to satisfactory results.

INTRODUCTION

Analysing all aspects of phytotherapy, one can find a rising interest worldwide for natural products, which can offer raw materials for obtaining new pharmaceutical preparations. Taking into account that in Romania there is an ancient tradition in using medicinal herbs and also after establishing that *Morus alba* L. leaves and *Morus nigra* L. bark contain flavonoids, we studied the separation of some flavonoids with important pharmaceutical features. The principal aim of the research work was the isolation of isoquercitrin from *Morus alba* L. leaves and *Morus nigra* L. bark.

METHODS AND MATERIALS

The separation of isoquercitrin was performed by column chromatography and the identification was made from UV, IR and ^{1}H-NMR spectra. The adequately prepared methanolic extracts were subjected to CC with the use of:

Column: 929x70 nm; Adsorbent: Kieselgel 60G; Pump: ProMINENT,
Flow speed: 2ml/min; fractions (20 ml) were collected manually.
Eluent: THF : EtOAc : $CHCl_3$: H_2O = 5 : 4 : 1 : 0,4
Samples: 3,6259 g residues of leaves; 5,4367 g residues of bark;
Compound detection: by TLC

The identification was made with the use of.

- spectrometer JASCO V-530
- NICOLET 210 with Fourier transformation - for recording IR spectra
- VARIAN-GEMINI 300, at ν = 300 MHz for recording ^{1}H-NMR spectra
- polarograph LP-7e with TZ 213S recording device.

RESULTS AND DISCUSSION

Interpretation of the chromatograms obtained after separation by CC of the hydrolysed methanolic extracts of *Morus alba* L. leaves and *Morus nigra* L bark, reveals that the gathered fractions (Table 1, 2), presented in Figures 1 and Figures 2, can be classified as shown below.

The specific chromatographic data obtained are $R_f = 0,55$ and $t_R = 8,00$ min.

The compound identified as isoquercitrin was purified by recrystallization in the solvent mixture MeOH/H_2O and then it was analysed by recording UV/VIS and IR spectra, ^{1}H-NMR spectra and finally by polarography.

The small differences between the experimental spectral data obtained and the ones published come from the purification stage.

By adding different substances, fine displacements of the absorption bands appear, as revealed by the values listed in Table 3. So, with sodium methoxide a difference of 52 nm develops, without decomposition, associated with the 4’ free –OH. The additional band at 325 nm indicates the presence of the 7 –OH group. By adding sodium acetate ($NaOOCCH_3$) and boric acid (H_3BO_3), there appears a flavonoid complex by bonding the boric acid to the orthohydroxylic groups, the displacement of the band by 17 nm showing the existence of two ortho positioned hydroxy groups on the B ring. By adding aluminium chloride, stable yellow complexes are formed due to the batochrom displacement by 70 nm. Furthermore, after adding hydrochloric acid there appears an hipsochrom displacement, indicating the presence of the free 5 –OH group, as well as the two ortho positioned –OH groups on ring B.

The displacements of the second band in the presence of sodium methoxide (+15 nm), aluminium chloride (+16 nm) and sodium acetate (+15 nm) indicate a glycoside with a 3 –OH group.

The IR spectra for the isolated isoquercitrin and the pure standard substance, presented in Figures 7 and Figures 8, are identical.

In the spectra one can observe that along with phenolic –OH groups there also appear primary and secondary alcohol groups that can associate themselves. Also, the absorption bands can be distinguished due to the C-H aliphatic bonds (2914 and 2958 cm^{-1}) that are not present in the quercetol spectrum.

In the isoquercitrin spectrum, two absorption bands appear between 1600 – 1700 cm^{-1}, because in this case the two –OH groups bonded in the isolated benzene ring are in positions 3’ and 4’, neither participating in hydrogen bonding to the etheric oxygen.
The values of the vibrational absorption bands for the carbonyl C=O (1664 and 1611 cm^{-1}) are very close to the ones of quercetol (1662 and 1612 cm^{-1}), but those associated with the double bond are identical at 1562 and 1523 cm^{-1}. The isoquercitrin IR spectrum shows, along with the C-O phenolic bond at 1167 cm^{-1}, the corresponding alcohol C-O bond at 1130 cm^{-1}, due to glucose.

From the IR spectra of both the pure standard isoquercitrin and the isolated substance, it could be concluded that the two were identical and that the substance isolated was isoquercitrin, as was supposed.

^{1}H-NMR spectral analysis allows the organic structure to be determined due to the 4 (four) base factors that should be taken into account:

-the number of signals, signal intensities, signal positions in the spectrum and the splitting of the signals (measured by coupling constant *J*).

The values obtained at 300 MHz frequency, 0-15 ppm domain and length of the pulse of $90^o \rightarrow 1,2\ \mu s$, are listed in Table 4 and the spectrum is shown in Figures 9.

The signals appearing in the domain 2-6 ppm are associated with the glucose substituent attached at 3; the aromatic hydrogens are present between 6-8 ppm, and the aliphatic ones between 1-4 ppm.

The fine differences to the published spectra are due to the solvents used, because methanol is transforming hydrogen from –OH groups in deuterium.

The polarography method was used for qualitative purposes in order to identify the separated compound from the mixture extracted from *Morus* sp. leaves and bark, the

carbonyl C=O group present in isoquercitrin being polarographically active.

The procedure involved a basic 50% ethanol solution stabilised at pH = 7.4 with phosphate buffer.

The parameters obtained for the isolated substance are the same as those of the pure standard isoquercitrin, as shown in Tables 5, 6 and 7.

CONCLUSIONS

After extraction using an adequate method/solvent assembly, flavonoid mixtures containing isoquercitrin were isolated, but this involved labour intensive work.

The practical data obtained for the separated compound in comparison with the published and recorded data for isoquercitrin reveals a good correspondence and demonstrate that the separation method used gives a high level of purity of the flavonoid.

By comparison of chromatographic parameters (R_f=0,55; t_R=8,00 min), spectral data (λ_{max} Band I = 361 nm, Band II = 258, 2666 sh), polarographical parameters ($E_{1/2}$ = -1,604 V) and melting point (pt^o = 186-187) of the isolated substance with those of the standard substance (R_f = 0,55; t_R = 7,80 min, λ_{max} Band I = 359 nm, Band II = 258, 266 sh; $E_{1/2}$ = -1,604 V; pt^o = 188-189) it could be concluded that the isolated flavonglycoside is isoquercitrin, with a high grade of purity. IR and ^{1}H-NMR spectra also confirm this result.

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Tables

Table 1. The obtained fractions to the component separation from *Morus alba* L. leaves

No. crt.	**Fractions (20 ml)**	**Residues (g)**
1	1 – 42	-
2	43 – 78	-
3	79 – 91	0,078
4	92 – 121	0,123
5	122 – 203	0,967
6	204 – 280	1,258

Table 2. The obtained fractions to the component separation from *Morus nigra* L. bark

No. crt.	Fractions (20 ml)	Residues (g)
1	1 – 35	-
2	36 – 74	-
3	75 – 1013	0,041
4	104 – 144	0,023
5	145 – 189	0,382
6	190 – 236	0,324
7	237 – 274	0,537
8	275 – 298	0,626
9	299 – 345	0,986

Table 3. Spectral data experimentally obtained in comparison with the published ones

Studied compound	λ_{max} (nm)					
	MeOH		+ $NaOCH_3$		+ $AlCl_3$	
	Band I	Band II	Band I	Band II	Band I	Band II
Pure isoquercitrin	359	259 266sh	327; 410	272	303sh 433	275
Separated compound	361	258 266sh	312; 413	273	300sh 431	274

Studied compound	λ_{max} (nm)					
	$AlCl_3$/HCl		+ $NaOOCCH_3$		+$NaOOCCH_3$/H_3BO_3	
	Band I	Band II	Band I	Band II	Band I	Band II
Pure isoquercitrin	364; 402	271 300sh	325sh 383	271	387	262 298sh
Separated compound	359; 398	265 299sh	326sh 380	273	378	261 299sh

Table 4. The ^{1}H-NMR spectral characteristics of isoquercitrin

POSITION	δ (ppm)	INTENSITY	OBSERVATION
1" - 6"	0,86-3,38	1,38 (1p)	other –OH appear between 9 - 11 ppm
6	7,53	2,11 (2p)	superimposed with 1' or 2'
8	6,37	0,71 (1p)	superimposed with the proton from 6
1' 2'	} 7,53 (d)		
	6,85 6,82	0,67 (1p)	$J_{1'-2'}$ = 8,37 Hz
5'	6,10	0,71 (1p)	

Table 5. Semiwave potential values of the pure standard and separated compound

No. crt.	Studied substance	$E_{1/2} \pm S_M$ (V)
1	Pure Standard Isoquercetrin	1,606 ± 0,018
2	Separated Compound	1,598

Table 6. Variation of the limit current with the unhydrolysed concentration extract from the leaves

C (mM)		1,00	1,25	1,50	1,75	2,00	The Regression Equation	r
I	i_l (μA)	13,38	14,30	15,28	16,33	17,09	(7,66±0,13)C + (4,75±0,08)	0,998
	$E_{1/2}$ (V)	-1,62	-1,58	-1,60	-1,62	-1,60	$E_{1/2}$ = (-1,604 ± 0,018)	n = 5
II	i_l (μA)	3,30	4,50	5,25	6,25	7,50	(7,74±0,33)C - (0,10±0,08)	0,996
	$E_{1/2}$ (V)	-1,62	-1,58	-1,60	-1,60	-1,61	$E_{1/2}$ = (-1,598 ± 0,005)	n = 5

Table 7. Current limit dependence with the Hg column height for the unhydrolysed extract 1,0 nM

	$h_{Hg\ (cm)}$	90	80	70	60	50	The Regression Equation	r
	$\lg h_{Hg}$	1,95	1,90	1,84	1,78	1,70		
I	$\lg i_l$	1,10	1,01	0,96	0,90	0,86	$(0{,}83 \pm 0{,}12)\lg h_{Hg} - (0{,}63 \pm 0{,}22)$	0,995
	$E_{1/2}$(V)	-1,60	-1,60	-1,63	-1,58	-1,57	$E_{1/2} = (-1{,}596 \pm 0{,}010)$	n = 5
II	$\lg i_l$	0,29	0,24	0,20	0,17	0,14	$(0{,}60 \pm 0{,}16)\lg h_{Hg} - (0{,}87 \pm 0{,}123)$	0,981
	$E_{1/2}$(V)	-1,62	-1,60	-1,60	-1,58	-1,61	$E_{1/2} = (-1{,}602 \pm 0{,}005)$	n = 5

I - pure standard isoquercitrin
II – separated compound from the *Morus* sp. extract

Table 8. Comparison of the experimental and published data for pure and isolated Isoquercitrin

Studied Compounds	R_f	t_R	λ_{max} (nm)		pt C^0	$E_{1/2}$ (V)
			Band I	Band II		
Pure Standard Isoquercitrin	0,55	7,80	359	258, 266 sh	188-189	1,606
Separated Compound	0,55	8,00	361	258, 266 sh	186-187	-1,604

Figures

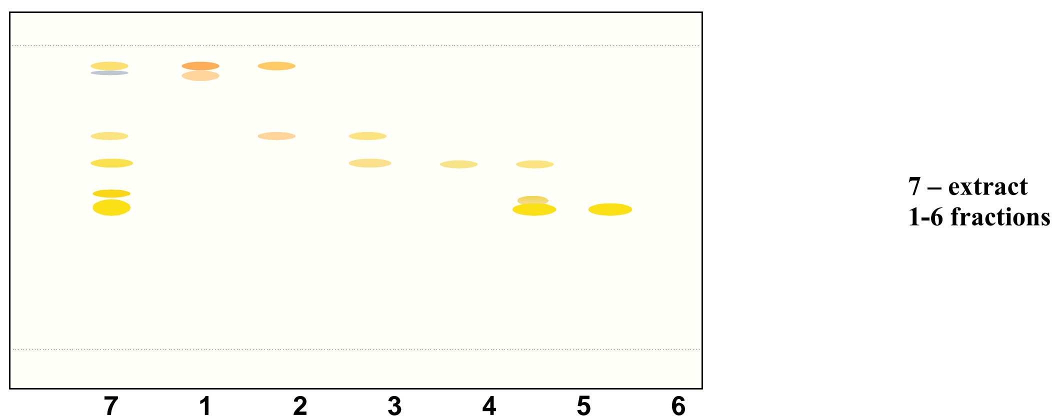

Fig. 1. The chromatogram obtained for the fraction separated from hydrolysed *Morus alba* L. leaves extract

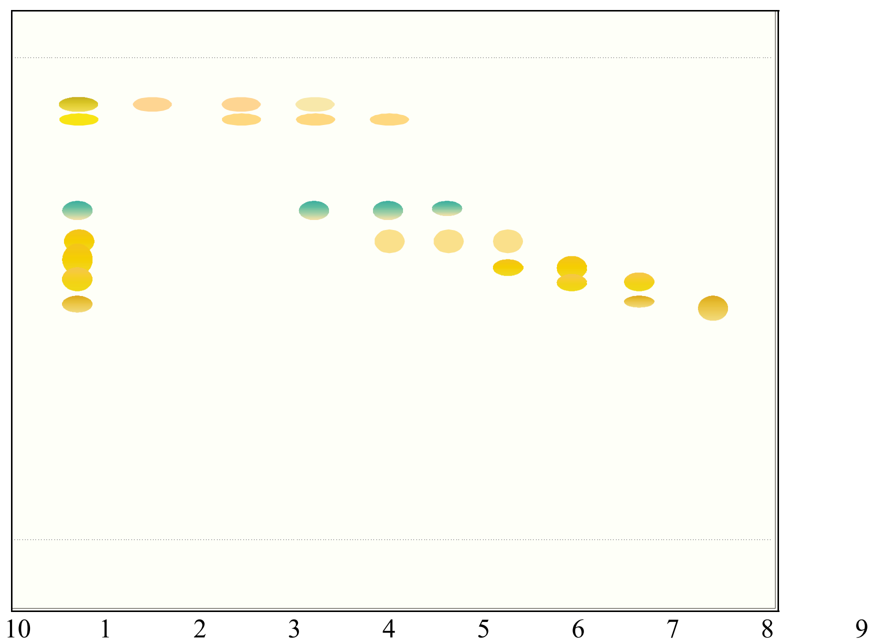

Fig. 2. The chromatogram obtained for the fractions separated from *Morus nigra* L. extract

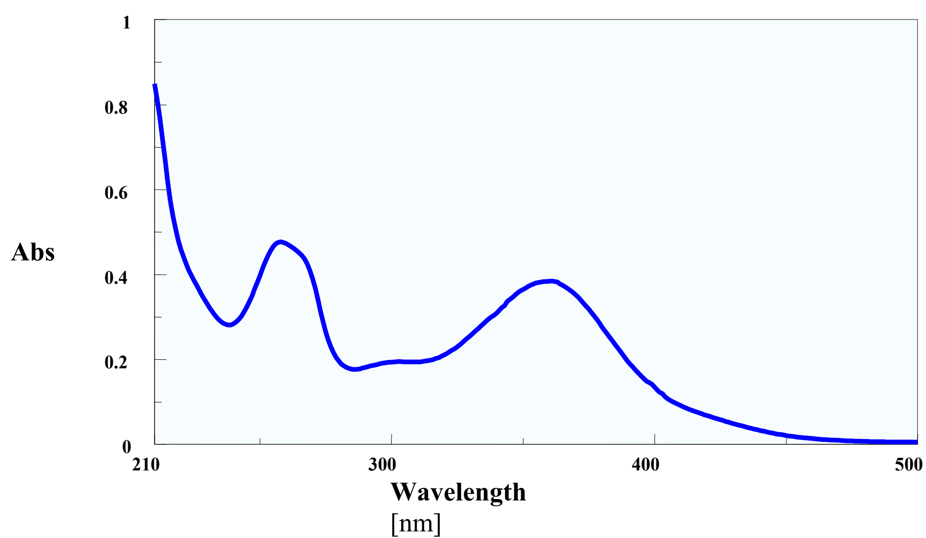

Fig. 3. The UV absorption spectrum in MeOH recorded for the separated compound

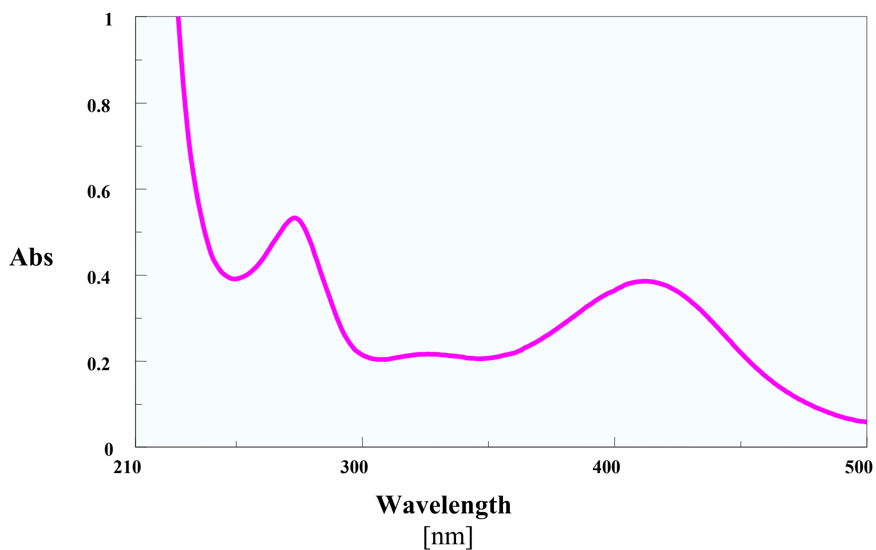

Fig. 4. The UV absorption spectrum in $NaOCH_3$ recorded for the separated compound

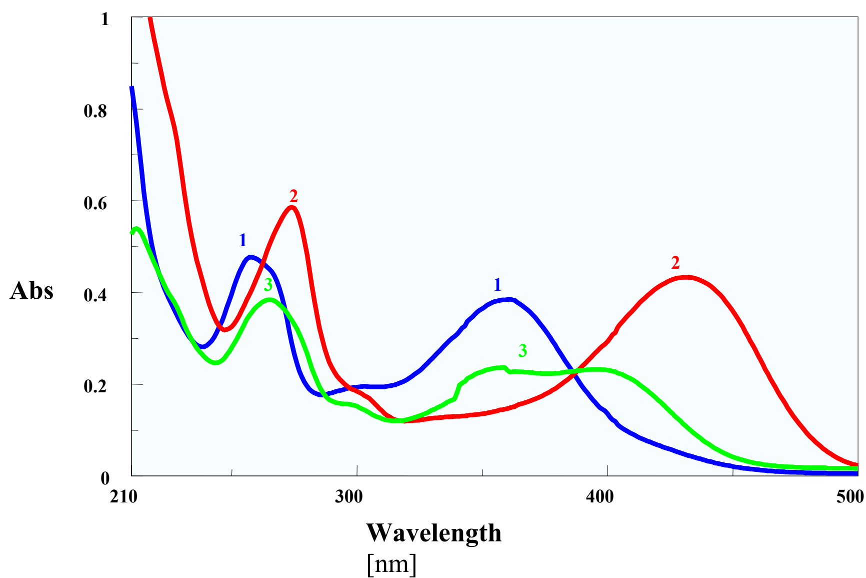

Fig. 5. The UV absorption spectrum recorded for the separated compound
in MeOH 1
+ $AlCl_3$ 2
+ $AlCl_3$/HCl 3

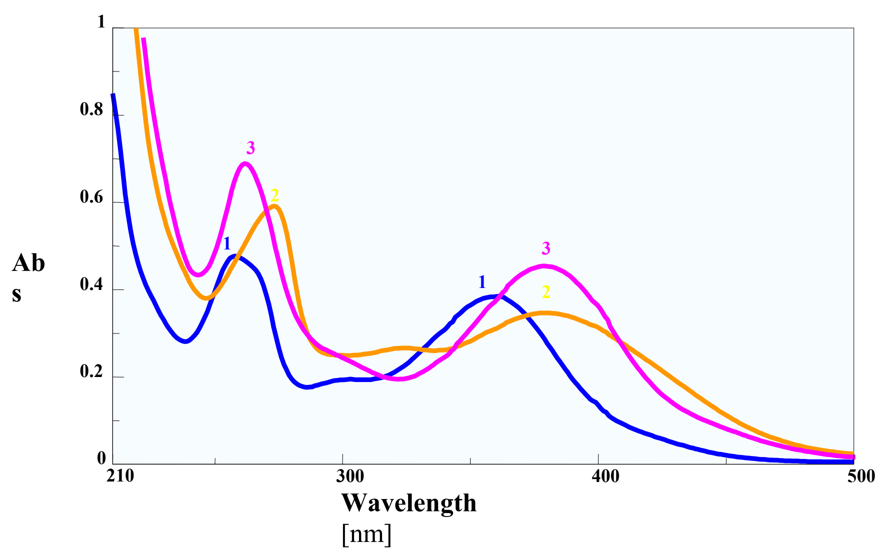

Fig. 6. The UV absorption spectrum recorded for the separated compound
in MeOH 1
+ $NaOOCCH_3$ 2
+ $NaOAc/H_3BO_3$ 3

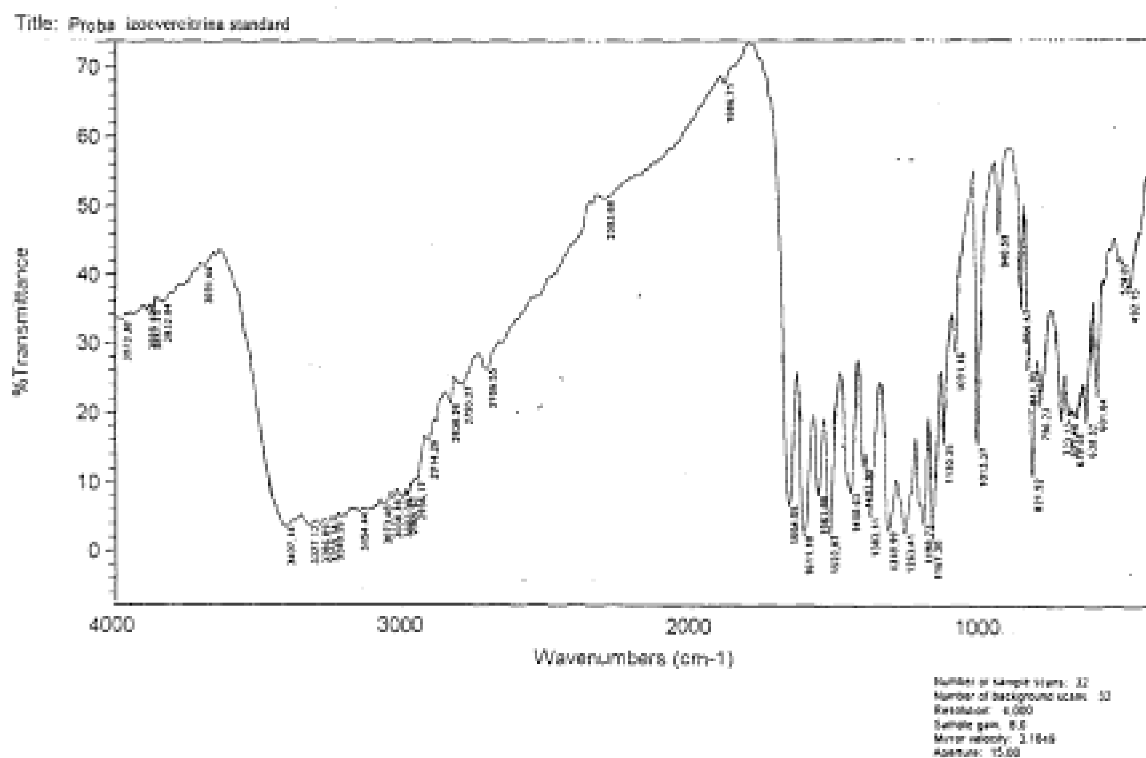

Fig. 7. The IR spectrum of pure standard isoquercitrin

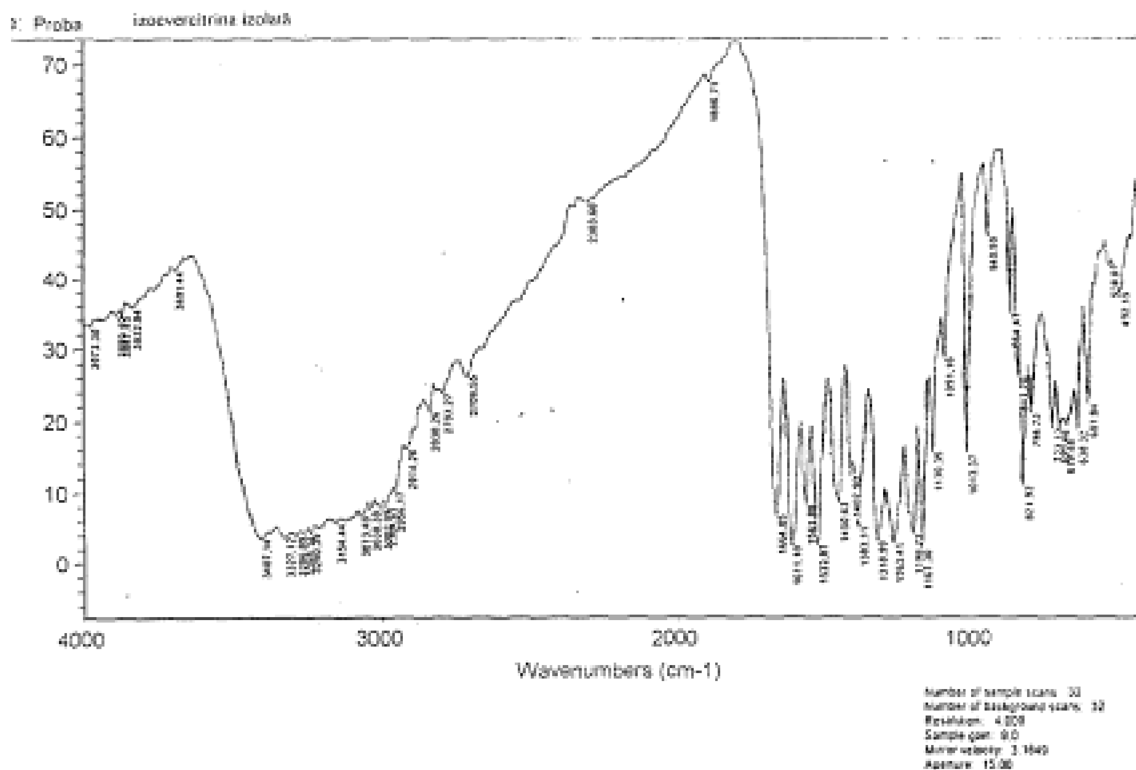

Fig. 8. The IR spectrum of the separated compound from the Morus sp. extract

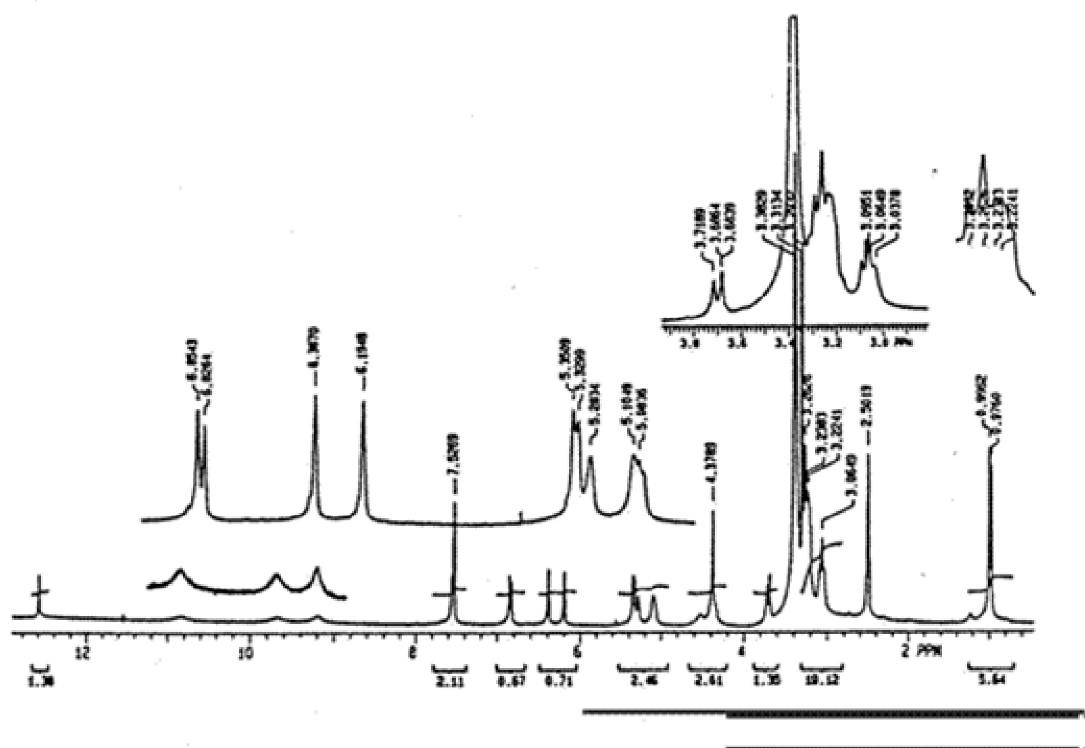

Fig. 9. The ^{1}H-NMR spectrum of isoquercitrin