

CHAPTER II
FLAVONOIDS

INVESTIGATION OF THE FLAVONOIDS

C.dioscoridis

Preparation of the Flavonoids

About 1.5 Kg of the defatted powdered C.dioscoridis were extracted with methanol in Soxhlet. The extract was evaporated in vacuo at about 50°C and the residue was taken with hot water. The aqueous solution was left overnight, filtered and concentrated to about 500ml. The solution was then shaken with methylene chloride (3 X 500 ml), followed by ethyl acetate (5x500 ml). Both the two solvents succeeded in extracting some of the flavonoids. Each of these was separately dehydrated over anhydrous sodium sulphate and evaporated in vacuo. The residues obtained amounted to 2.5 and 6.0 gm respectively.

The ethyl acetate fraction is the subject of the present study.

Thin-layer chromatography

Thin-layer chromatography of the flavonoids was carried out using both silica gel G and polyside/
cellulose

The polyamide powder for TLC is mixed with cellulose powder (for TLC) (8:2), suspended in chloroform-methanol (2:3) and the suspension is then applied to the glass plates (0.25 mm. thick).

The solvents used are:

a- For Silica Gel

Chloroform-methanol-formamide	(80:19:1) (56)
Chloroform-methanol-formamide	(70:29:1)
Benzene-pyridine-formic acid	(36:9 :5)
Ethyl acetate-benzene-methanol-formamide	(50:40:9:1) (56)

b-for polyamide

Butanol-acetic acid-water	(6:2:1) (57)
Ethanol-water	(60:40) (57)
Ethanol-water-acetyl acetone	(20:40:10)

Detection of the flavonoid constituents on TLC was carried out by examining the plates under the Ultra-violet light where fluorescence spots appeared, varying in colour and fluorescence then by spraying with aluminium chloride in ethanol (1%) or by exposing to ammonia vapour and re-examining under the Ultra-violet

Plates coated with polyamide and developed with the solvent butanol-acetic acid-water gave the best separation (Table 5)

Paper chromatography:

Paper chromatography of the flavonoids was carried out using both whatman No 1 and 3MM.

The solvents used are:

- 1 - butanol-acetic acid-water (4:1:5) (58)
- 2 - butanol-acetic acid-water (6:2:1)
- 3 - Acetic acid 30%
- 4 - Acetic acid 20%
- 5 - Ethyl acetate-pyridine-water (2:1:2)(59)

Silica gel column chromatography

About 6 gm of the flavonoid fraction were dissolved in small amount of methanol, mixed with few grams of silica gel, the methanol was evaporated in vacuo and the resulting homogenate was placed on a top of a column (3.5 X 60 CM) packed with silica gel (300 gm) in 1,2 - dichloroethane. Elution was made with 1,2-dichloroethane, then 1,2-dichloroethane-methanol mixtures, collecting

Table (5) : Flavonoid components of *C. dioscoridis* .

Component	R _f [*]	Detection			
		Colour Spot		AlCl ₃	
		D L	U V	D L	U V
I	0.25	—	Y	Y	Y
II	0.57	Y	Br	Y	Br
III	0.47	—	Br	Br	Br
IV	0.60	—	Y	Y	Y
V	0.75	Y	B	Y	B

*

Adsorbent : polyamide

Solvent : butanol-acetic acid-water (6 : 2 : 1)

Y = Yellow

B = Blue

Br = Brown

fractions each 100 ml and the course of the chromatographic fractionation was followed on silica gel plates (table 6).

Table(6) Column Chromatographic Fractionation of Flavonoids of C.dioscoridis

Solvent	Fractions No.	R_f^*
1,2- dichloroethane : methanol (95:5)	1 - 32	0.15, 0.67
1,2- dichloroethane : methanol (90:10)	33 - 59	0.43, 0.67
1,2- dichloroethane : methanol (85:15)	60 - 107	0.5, 0.67
1,2- dichloroethane : methanol (80:20)	108-150	0.67

*

: Adsorbent : polyamide/ cellulose

Solvent system: butanol-acetic acid- water (6:2:1)

Identification of Quercetin (Substance 1) :

The combined fractions 1 - 32 (Table 6) was found to contain two flavonoid components, one of which possesses the same R_f as quercetin (R_f 0.25). Separation of the major component was achieved by paper chromatography using Whatman No. 3MM, and the solvent system acetic acid 20%

The obtained flavonoid possesses the same R_f as the authentic quercetin using different solvents and different adsorbents, and gave the same fluorescence under Ultra-violet, both before and after spraying with aluminium chloride (Table 9).

The flavonoid, after crystallization from methanol-water, melted at 309-311°C. both alone and upon admixture with authentic quercetin.

Spectroscopic measurements:

The Ultra-violet absorption spectrum of the isolated flavonoid was measured using Unicam spectrophotometer SP 1800 in the region from 240-400 m μ . A solution of 0.0001 M of the flavonoid, in absolute spectroscopic methanol was prepared and measurements were

carried out as follows :

1- In Methanol:

2 ml of the stock solution (0.0001 M) diluted to 10 ml.

2 - With Sodium Methoxide :

2 ml of the stock solution + 2 ml of 0.01 M sodium methoxide and complete to 10 ml with absolute methanol, spectral measurements were taken after 5 minutes, 1 hour and 18 hours respectively .

3 - With Aluminium Chloride :

2.5 ml of the stock solution + 0.5 ml 0.6% solution of aluminium chloride in absolute methanol and completed to 10 ml.

4 - With Sodium Acetate :

2 ml of the stock solution + excess fused sodium acetate and complete to 10 ml.

5 - With Boric Acid/ Sodium Acetate:

2 ml of the stock solution + 2 ml saturated solution of boric acid in absolute methanol + fused sodium acetate and completed to 10 ml

and then left to stand for 20 minutes before measuring .

The results obtained are shown in table (7) and Fig (14). The spectrum revealed a free-OH at position-3. The unstability of the aglycone, in presence of sodium methoxide and its degradation in few minutes is indicative of free OH at position 3 and 4. The maximum absorbances of the flavonoid are identical with those of quercetin. (60)

(Table 7):

Ultra-violet Absorption Spectra of the isolated quercetin

Compound	Band						
		CH ₃ OH	NaOCH ₃	AlCl ₃	AlCl ₃ +HCl	NaOAc	B/Na
Quercetin	II	256	250	271	265	258	262
(isolated)	I	372	322	458	430	390	390
Quercetin	II	255	247	272	265	257	261
(authentic)	I	371	321	458	428	390	388

Boric acid/ NaOAc . = B/Na

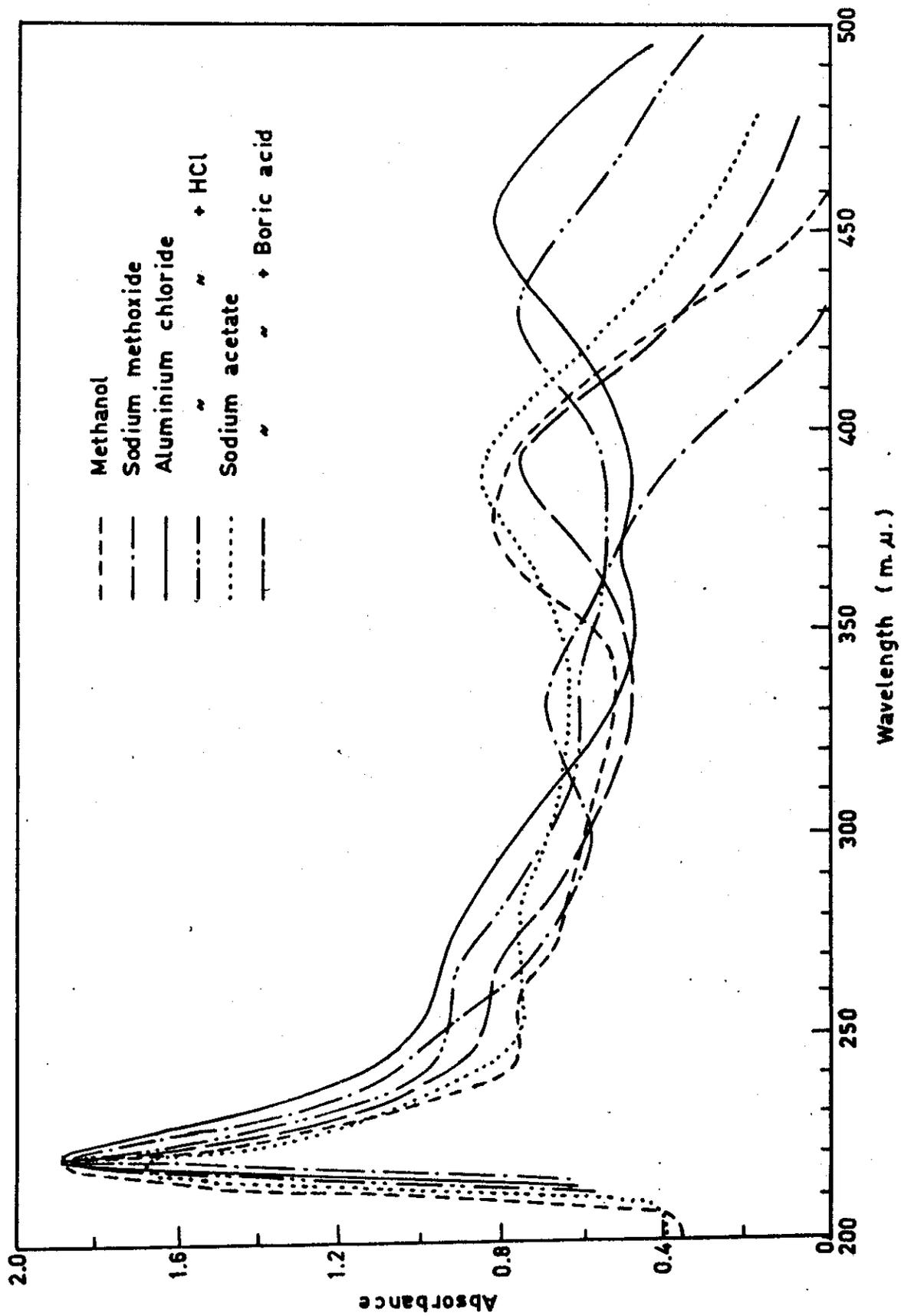


Fig. (14): Ultra violet spectra of quercetin isolated.

The mass spectrum of the aglycone showed a molecular peak at 302 which represents the molecular ion of quercetin ($C_{15} H_{10} O_7$). The fragmentation of the mass spectrum agreed with those expected for quercetin .

The infra-red absorption spectrum of the aglycone showed exactly the same bands of the spectrum given by the authentic quercetin .

The NMR spectrum of the aglycone was found to be identical with that of quercetin (60) .

Identification of Quercetin - 7 - arabinoside :

Fractions 33-59 (Table 6) were found to contain two flavonoids, one of which was isolated by preparative paper chromatography (Whatman No 1,3 MM). (Table 9).

Hydrolysis of the glycoside :

To about 10mg of the isolated flavonoid (in methanol) were added 10 ml of H_2SO_4 20% and heated on a water bath for 6 hours. The solution was then diluted with about 10 ml cold water. The precipitated aglycone was extracted with ethyl acetate, washed thoroughly with distilled water

till free from acidity then dried over anhydrous sodium sulphate and evaporated in vacuo.

The aglycone possesses the same R_f and fluorescence as quercetin. (Table 9)

Identification of the sugar Residue :

The acidic solution (after separation of the aglycone) was neutralized with barium carbonate. The filtrate, after removal of the precipitated barium sulphate was evaporated in vacuo and the residue was extracted with redistilled pyridine and filtered. Pyridine was distilled off and the residue was dissolved in isopropanol (10%) and subjected to paper chromatographic analysis, using Whatman No. 1 filter paper and applying two different solvents viz n-butanol - acetic - acid - water (4 : 1 : 5) (58) and ethyl acetate - pyridine - water (2 : 1 : 2) (59) .

The chromatogram was revealed by spraying with aniline phthalate (6I) then dried in hot air oven at 105°C for few minutes. Arabinose was the only sugar detected in the hydrolysate.

The ultraviolet spectra of the glycoside and its aglycone (table 8) (Fig 15) showed that the sugar is attached to the aglycone in position-7 (60).

A bathochromic shift of band I of the glycoside from 372 to 457 m μ with sodium methoxide is indicative of free 4'-OH. Also the alkali-unstability of the glycoside in presence of sodium methoxide or sodium acetate is indicative of free 3, 4' OH. On the other hand a bathochromic shift of band I of the glycoside was produced with AlCl₃ from 372 to 458 m μ indicating the presence of 3 and 5 free OH. Moreover, a hypsochromic shift of band I of the AlCl₃ spectrum on the addition of acid from 358 to 426 m μ indicating the presence of ortho-dihydroxy groups at 3', 4' which form acid-labile complex. On addition of sodium acetate no shift of band II was detected, which indicates the absence of free 7-OH group.

The results of ultra-violet spectrum of the aglycone revealed a free OH at position 7 and the bathochromic shift of band II of the aglycone from 255 to 274 m μ is another confirmation that the sugar is attached to position 7. The maximum absorbances of the aglycone, before and after the addition of the different reagents, are in agreement

with those of quercetin (Table 8).

The identity of quercetin was further proved by NMR, infra-red, and mass spectrum.

Table (8): Ultra-violet Absorption Spectra of Quercetin 7- arabinoside and its Aglycone

Compound	Band	λ_{max}					
		CH ₃ OH	NaOCH ₃	AlCl ₃	AlCl ₃ /HCl	NaOAc	Na/B
Quercetin 7	II	257	293	272	270	286	261
arabinoside	I	372	457	458	426	378	384
(isolated)							
Quercetin 7	II	256	291	273	268	286	261
arabinoside	I	372	457	458	426	378	386
(authentic)							
Quercetin	II	256	247	273	265	257	264
(isolated)	I	372	321	458	428	390	388
Quercetin	II	255	247	272	265	254	261
(authentic)	I	371	321	458	428	390	388

Na/B = NaOAc/ Boric acid

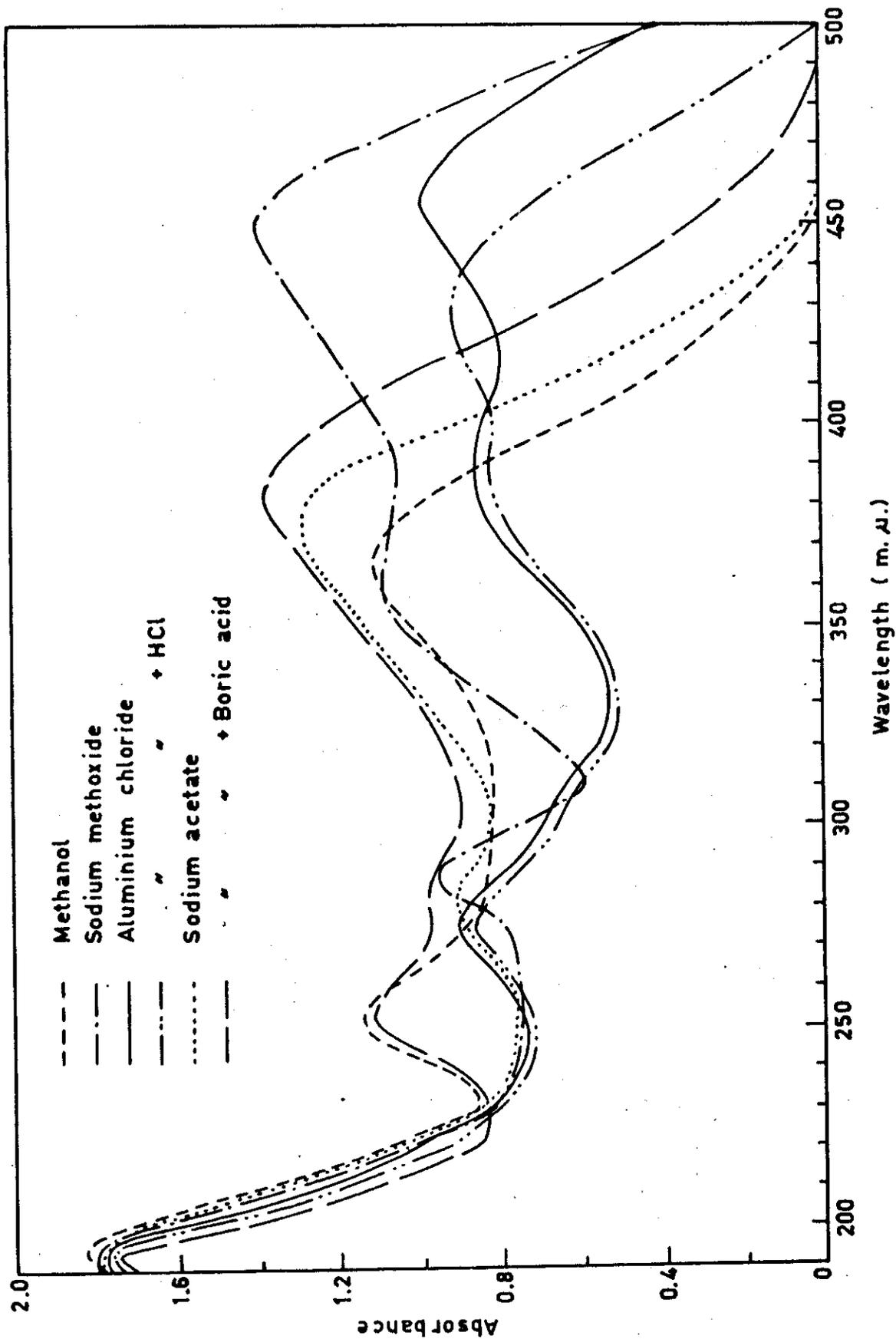


Fig. (15) : Ultra-violet spectra of quercetin-7-arabinoside.

Identification of Quercetin-3-rhamnoside:

The methylene chloride extract (A) was found to contain three flavonoids one of which possesses the same R_f as the authentic quercitrin (Quercetin-3-rhamnoside) (Table 9)

The glycoside was isolated by preparative paper chromatography (Whatman No. 1, 3MM) and the solvent system acetic acid 20% applying the two development technique.

Hydrolysis was carried out using the same condition mentioned above (cf page 60). The aglycone was found to possess the same R_f as authentic quercetin in different solvents using different adsorbents (silica gel and polyamide/cellulose) and the same fluorescence under ultra-violet both before and after spraying with aluminum chloride.

The results of ultra-violet spectra of the flavonoid (Table 10) and (Fig 16) are in agreement with those reported for quercitrin.

Table (9) : The R_f and Colour of the Isolated Flavonoids and Available Authentics.

Compound	R_f			Detection				
				Colour Spot		$AlCl_3$		
	1	2	3	4	DL	UV	DL	UV
Quercetin-7 arabinoside (isolated)	0.47	0.58	0.15	0.40	Y	Br	Y	Br
Quercetin-3 rhamnoside (isolated)	0.57	0.38	0.27	0.80	Y	Br	Y	Br
Quercetin-3 rhamnoside (authentic)	0.57	0.38	0.27	0.80	Y	Br	Y	Br
Quercetin (isolated)	0.25	0.08	0.06	0.73	Y	Y	Y	Y
Quercetin (authentic)	0.25	0.08	0.06	0.73	Y	Y	Y	Y
Apigenin-7 glycoside	0.62	0.08	0.57	0.63	Y	Br	Y	Br
Luteolin-7 glycoside	0.50	0.42	0.90	0.38	---	Br	Y	Br
Kaempferol-3,7 diglycoside	0.62	0.09	0.56	0.63	---	Br	Y	Br
Naringin-5 glycoside	0.69	0.40	0.92	0.66	---	Br	Y	Br
Vitexin	0.56	0.58	0.15	0.40	Y	Br	Y	Br
Casticin	0.72	0.40	0.33	0.90	Y	Br	Y	Br

Table "9" (Cont.) :

Compound	R _f				Detection			
					Colour Spot		AlCl ₃	
	1	2	3	4	DL	UV	DL	UV
Kaempferol	0.22	0.12	0.07	0.54	Y	Br	Y	Br
Apigenin	0.23	0.13	0.07	0.55	Y	Br	Y	Br

1 = Butanol:Acetic acid:Water (6:2:1)

(polyamide/cellulose)

2 = Ethanol:Water (60:40) (polyamide/cellulose)

3 = Ethanol:Water:Acetyl acetone (20:40:10)

(polyamide/cellulose)

4 = Butanol:Acetic acid:Water (4:1:5) (PC Whatman No.1)

Y = yellow

Br= brown

Table 10: Ultra-violet Absorption Spectra
of Quercitrin.

Compound	Band	λ_{max}					
		CH ₃ OH	NaOCH ₃	AlCl ₃	AlCl ₃ /HCL	NaOAC	Na/B
Quercitrin	II	256	270	272	270	274	260
(isolated)	I	350	391	430	401	372	365
Quercitrin	II	256	270	276	272	272	260
(authentic)	I	352	393	430	401	372	367

Na/B = NaOAC/ Boric acid

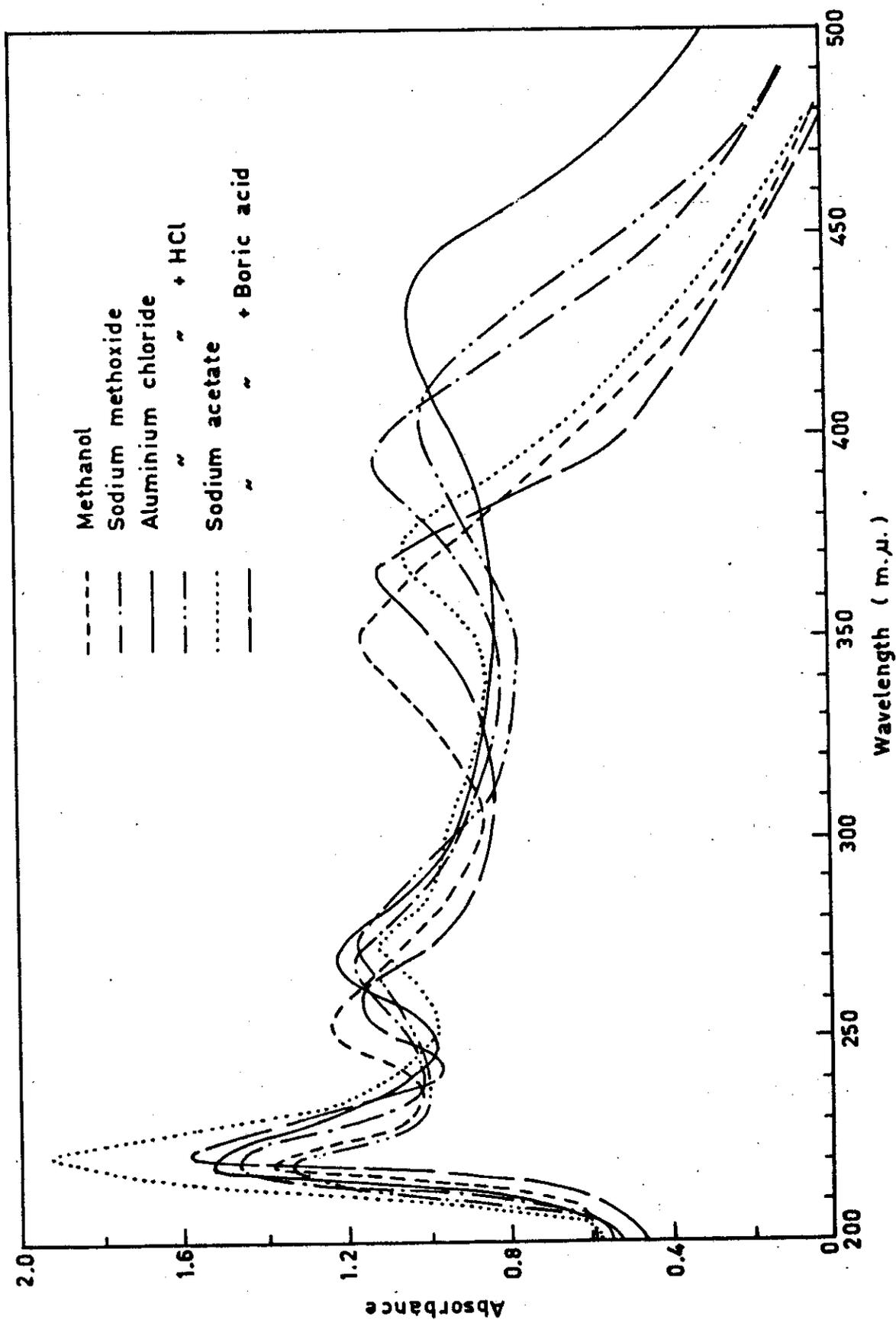


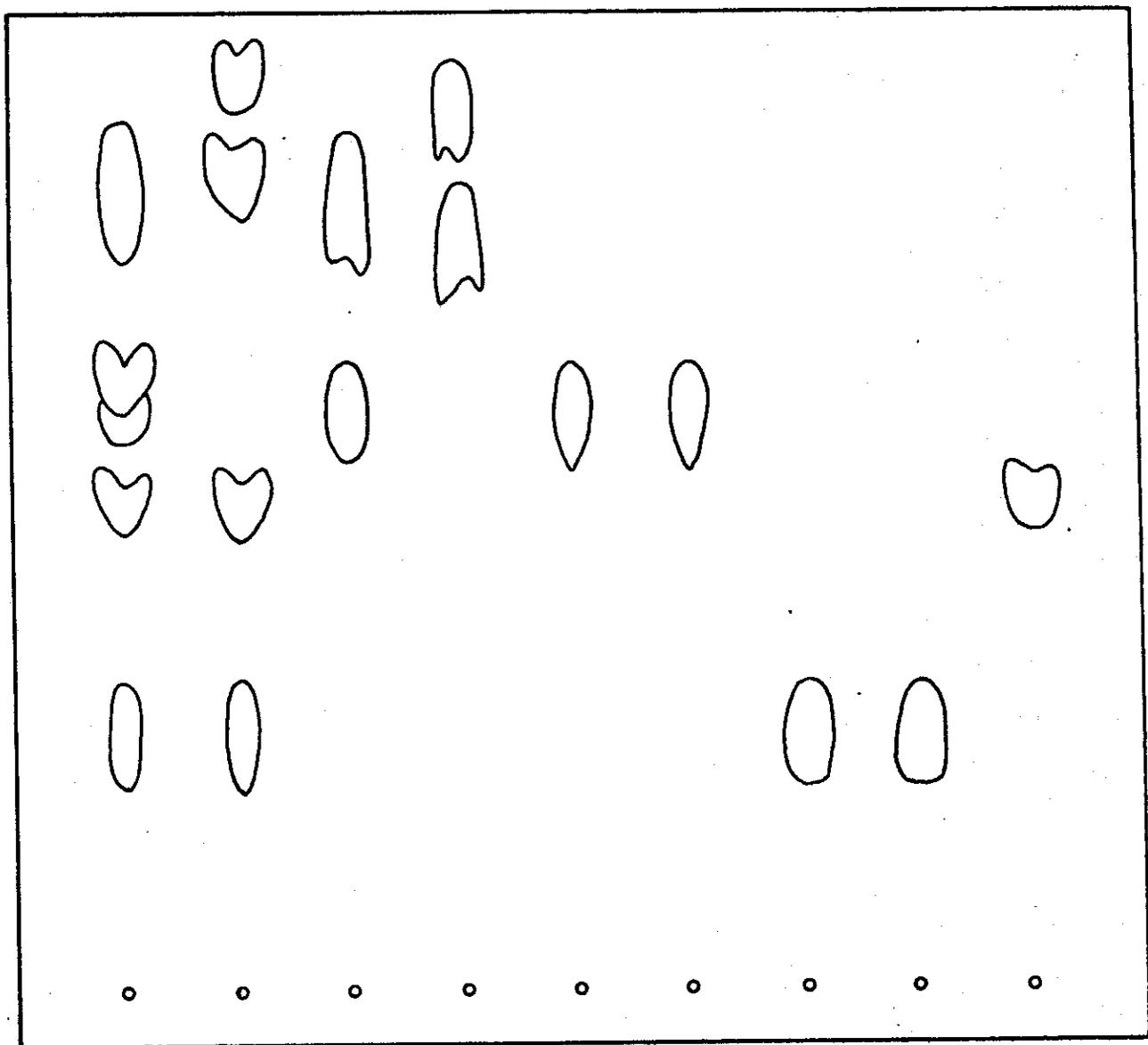
Fig. (16): Ultra violet spectra of quercitrin isolated.

II C.aegyptiaca:

The flavonoids of C.aegyptiaca were prepared in the usual manner as previously mentioned (cf. page 51).

TLC of the total flavonoid mixture (extracted with methylene chloride and ethyl acetate) revealed qualitative differences from that of C.dioscoridis.

The absence of quercitrin, detected in C. dioscoridis, was proved using different solvent systems and adsorbents. On the other hand, quercetin and quercetin-7-arabinoside were detected in C.aegyptiaca, and their presence was proved by fractionation of the flavonoids using preparative paper chromatography. The identity of these two flavonoids was confirmed by R_f , ultra-violet as well as hydrolysis of the glycoside and the detection of both quercetin and arabinose. Two more flavonoid components were found to be present, in trace amounts, in only C.aegyptiaca using TLC technique and applying different adsorbents (silica gel G and polyamide/ cellulose) and different solvent systems (butanol-acetic acid-water (6:2:1), chloroform-methanol-formamide(70:29:1).



Ethyl acetate extract of C. dioscoridis
 Ethyl acetate extract of C. aegyptiaca
 Methylene chloride extract of C. dioscoridis
 Methylene chloride extract of C. aegyptiaca
 Quercitrin (authentic)
 Quercitrin (isolated)
 Quercetin (authentic)
 Quercetin (isolated)
 Quercetin 7-arabinosid. (isolated.)

Fig. (17) : Total flavonoid of C. dioscoridis and C. aegyptiaca with authentic and isolated flavonoids.

Adsorbent : Polyamide/ cellulose
 Solvent : Butanol - Acetic acid - Water (6 : 2 : 1)