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IDENTIFICATION OF PHENOLIC CONSTITUENTS ISOLATED FROM MACEDONIAN PROPOLIS

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Phenolic constituents isolated from Macedonian propolis, were investigated by ultraviolet and infrared spectroscopy. Three flavonoids (chrysin, tectochrysin, galangin) and two phenylpropanoids (caffeic and hydrocaffeic acid) were identified in propolis samples. Identification of the present hydroxyl groups in the flavonoid nucleus from the isolated compounds was carried out using shift reagents and observing the resultant shifts in the UV spectrum. The identification of isolated substances was proved by recording their IR spectra and authentic standards.

Key words: propolis; phenolic constituents; UV and IR spectroscopy; flavonoids; chrysin; tectochrysin; galangin; caffeic acid; hydrocaffeic acid

INTRODUCTION

Propolis is a resinous hive product, collected by bees from various plant sources. It has been used for a long time in folk medicine, because propolis possess such versatile biological properties as antibacterial, antiviral, fungicidal, local anaesthetic, antiulcer, immunostimulating, antiinflamatory and cytostatic [1–4].

These valuable properties of propolis created an increasing interest in its chemical composition. The last appears to be very complex (the bees collect propolis from resinous buds of various trees) and till now more than a hundred propolis constituents have been identified [5–8].

Over the last years, the number of investigations involving isolation and identification of the biologically active constituents on propolis has increased. A substantial part of the physiological activity of propolis is connected with its phenolic compounds (flavonoids, phenolic acids and phenyl propanoids), which constitute more than 50% of its composition [9, 10].

The main phenolic constituents of propolis are flavonoids: flavanones, flavones, flavonols and dihydroflavonols. The interest in propolis flavonoids is due to their significant physiological activities, which can be responsible for a large part of propolis activity. Pinocembrin and galangin determine the antibacterial activity of propolis; pinocembrin has also fungicidal and local anesthetic activities. Quercetin, kaempferide and pectolinarigenin have spasmolytic activity; acacetin has antiinflamatory activity and luteolin and apigenin possess antiulcer activity [1, 12, 13].

The aim of this paper is identification of isolated phenolic compounds from Macedonian propolis, with ultraviolet and infrared spectroscopy.

EXPERIMENTAL

The propolis sample was collected in the Skopje region, Macedonia.

The propolis was ground and extracted with boiling methanol for 1 h. The obtained methanolic extract was reextracted with petrol ether and ethyl ether. The diethyl ether extract was evaporated to dryness.

The obtained DEEP extract was subjected to column chromatography (CC). CC was done with silica gel (Kieselgel 60 F_{254} 0.063–0.200 mm Merck) and elution was with dichloroethane and increasing quantities of methanol and methyl-ethyl ketone. The obtained fractions were investigated by thinlayer chromatography (TLC) on silica gel plates (Silica gel F_{254} 20 × 20 cm 0.25 mm Merck) using benzene – ethylacetate – formic acid (40 : 10 : 5 v/v) as a mobile phase. The fractions with identical compounds were combined and rechromatographed on silica gel column with different mixtures of eluents: benzene – chloroform (1 : 1), chloroform, ethyl acetate, ethyl acetate – acetone (1 : 1), ethyl acetate – methanol (1 : 1), acetone – methanol (9 : 1) and methanol.

Isolated flavonoids were purifed by recrystallization and also by preparative TLC on silica gel plates.

The identification of the isolated flavonoids was accomplished with TLC, ultraviolet (UV) and infrared (IR) spectroscopy.

The ultraviolet spectral data of isolated substances were obtained in methanol solution and after adding the shift reagents: sodium hydroxide, sodium acetate, boric acid, aluminium chloride and hydrochloric acid (11). Measurements were performed on the Hewlett Packard Diode Array 8452A UV spectrophotometer.

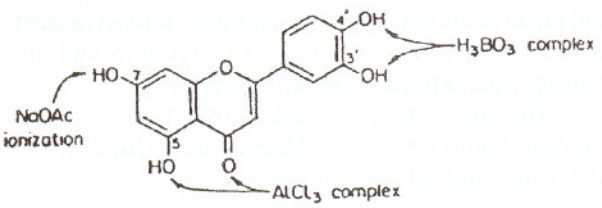
The infrared spectra of isolated phenolic constituents were recorded in KBr on the Perkin Elmer 580 IR spectrophotometer.

Spectra of isolated compounds were compared with those of authentic standards: chrysin, galangin, caffeic and hydrocaffeic acids, products of Aldrich.

RESULTS AND DISCUSSION

From the investigated sample of propolis isolated were five compounds: A, B, C, D and E. The obtained spectral data from UV and IR spectroscopy, provided evidence for the presence of different groups of phenolic compounds: flavonoids and phenyl propanoids. The composition od these different groups will be discussed separately.

The flavonoids spectra typically consist of two absorption maxima in the ranges 240–285 nm (band I) and 300–550 nm (band II). The precise position and relative intensities of these maxima give valuable information on the nature of the flavonoid and its oxygenation pattern. The position of the unsubstituted phenolic hydroxyl groups on the flavonoid nucleus may be established by adding shift reagents (sodium hydroxide, sodium acetate, boric acid, aluminium chloride and hydrochloric acid) to the sample solution and observing the resultant shifts in the absorption peaks. These reagents cause ionization of flavonoid hydroxyl groups or forme complexes between the hydroxyl groups and neighbouring ketones inducing significant changes in the spectrum.

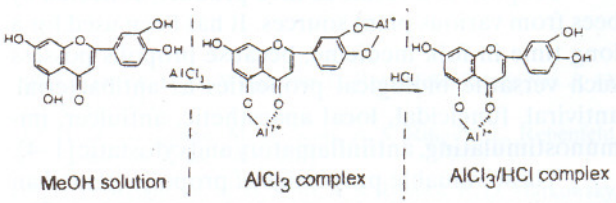


The obtained resultant shifts may be useful in determining the number and location of hydroxyl groups or methyl groups attached to one of the phenolic hydroxyls.

Sodium acetate causes significant ionization of only the most acidic of the flavonoid hydroxyl groups. Thus it is used primarily to detect the presence of a free 7-hydroxyl group. After measuring the spectrum of the isolated substance A (Rf = 0.70) in methanol and adding sodium acetate, it induces bathochromic shift to longer wavelengths of band I from 276 to 282 nm. The obtained change is characteristic for the presence of a free 7-OH group.

In the same solution after adding boric acid, the $NaOAc/H_3BO_3$ spectrum is recorded. $NaOAc/H_3BO_3$ bridges the two hydroxyls in an ortho-dihydroxy group and it is used to detect their presence. In the obtained spectrum there are no changes showing that in the structure of isolated flavonoid A ortho-hydroxyl groups in A and B ring are absent.

Aluminium chloride formes acid-stable complexes between hydroxyls and neighbouring ketones and acid-labile complexes with ortho-dihydroxyl groups, so this reagent is used to detect both groupings. The $AlCl_3$ spectrum thus represents the sum effect of all complexes in the spectrum, whilst the same spectrum recorded in presence of hydrochloric acid represents the effect only of the hydroxy-keto complexes, which are stable in acid.



On adding aluminium chloride in the methanolic solution of isolated substance B, it caused a bathochromic shift of band II for 70 nm. The $AlCl_3/HCl$ spectrum was recorded after adding HCl, but no changes were induced. From the obtained spectra we conclude that isolated flavonoid B (Rf = 0.84) formed only the acid-stable complexes between hydroxyl group on C-5 and neighbouring ketone, so the presence of 5-hydroxyl group was established. The absence of band I bathochromic shift in the UV spectra, after the addition of NaOAc showed an absence of free 7-OH group and no changes in $NaOAc/H_3BO_3$ spectra, was an indication for absence of *o*-diOH groups in A and B ring.

The NaOH spectrum represents the flavonoid with all phenolic hydroxyl groups ionized to some extent. It is therefore generally a good "fingerprint" indicator of the hydroxylation pattern as well as being useful for the detection of the more acidic hydroxyl groups in unsubstituted form. In the NaOH spectrum of isolated flavonoid C (Rf = 0.78) a new band at 324 nm with low intensity appeared, that indicated the presence of free 7-OH group. Also in the spectrum a 52 nm bathochromic shift of band II typical of 3-OH group was noticed.

Table I

UV spectral shifts of isolated flavonoid A

Methanol solution	Spectral maxima /nm		Spectral effect	Structural diagnosis
	Band I	Band II		
Isolated flavonoid A	270	314		Flavon
Plus powdered NaOAc	276	360	6 nm bathochromic shift (band I)	7-OH free
Plus NaOAc and H_3BO_3	270	316	No changes in spectrum	Absence of o-diOH in A and B ring
Plus $AlCl_3$	282	380	66 nm bathochromic shift (band II)	5-OH free
Plus $AlCl_3$ and HCl	282	380	No changes in spectrum	Absence of 3', 4'*o*-diOH

Table II

UV spectral shifts of isolated flavonoid B

Methanol solution	Spectral maxima /nm		Spectral effect	Structural diagnosis
	Band I	Band II		
Isolated flavonoid B	270	310		Flavon
Plus powdered NaOAc	270	310	No changes in spectrum	7-OH free substituted
Plus NaOAc and H_3BO_3	270	310	No changes in spectrum	Absence of *o*-diOH in A and B ring
Plus $AlCl_3$	282	380	70 nm bathochromic shift (band II)	5-OH free
Plus $AlCl_3$ and HCl	282	380	No changes in spectrum	Absence of 3', 4'*o*-diOH

Table III

UV spectral shifts of isolated flavonoid C

Methanol solution	Spectral maxima /nm		Spectral effect	Structural diagnosis
	Band I	Band II		
Isolated flavonoid C	266	360		Flavonol
Plus NaOH	282	412	52 nm bathochromic shift (band II)	3-OH free
			New band at 324 nm	7-OH free
Plus $AlCl_3$	272	414	54 nm bathochromic shift (band II)	5-OH free
Plus $AlCl_3$ and HCl	272	414	No changes in spectrum	Absence of 3', 4'*o*-diOH

The absorption bands in the ultraviolet spectra and spectral shifts obtained by adding reagents for isolated flavonoids A, B and C are presented in Table I, II, III.

According to the obtained spectral data we deduced the structures of three flavonoids: chrysin (5,7-dihydroxy-flavone), tectochrysin (5-hydroxy-7-methoxy-flavone) and galangin (3,5,7-trihydroxy-flavone).

Together with the flavonoids we isolated and identified another two compounds D and E. According to their obtained spectral data (UV and IR) and physical properties, we deduced that these compounds belong to phenyl-propanoids.

The compound D (Rf = 0.45) was brownish-yellow substance and its aqueous solution has acid pH (pH = 3.96). Its melting point was 195–196 °C. The aqueous or alcoholic solution of the isolated substance gave these characteristic chemical reactions: discoloured a solution of $KMnO_4$ and a solution of Br_2. With a solution of alkalies it gave an intensely yellow colour, with $FeCl_3$ a green colour.

The UV spectrum of this substance has absorption bands at 220, 244 and 326 nm, with a distinctive shoulder at 304 nm. These absorption bands are characteristic for caffeic acid and its derivatives. According to the obtained same Rf values, melting points and UV spectral data between the standard and the isolated acid, we deduced the presence of 3,4-dihydroxy-cinnamic acid (caffeic acid).

The UV spectrum of the second isolated phenyl-propanoid E (Rf = 0.40), consists of two absorption maxima at 210 and 288 nm. The absorption maximum at 210 nm indicated the presence of hydroxyl groups (from COOH or substituents) and absorption band on 288 nm indicate carbonyl group. The UV spectrum of isolated substance E was compared to spectra of many substituted phenolic acids and according to spectral data we deduced the presence of hydrocaffeic acid.

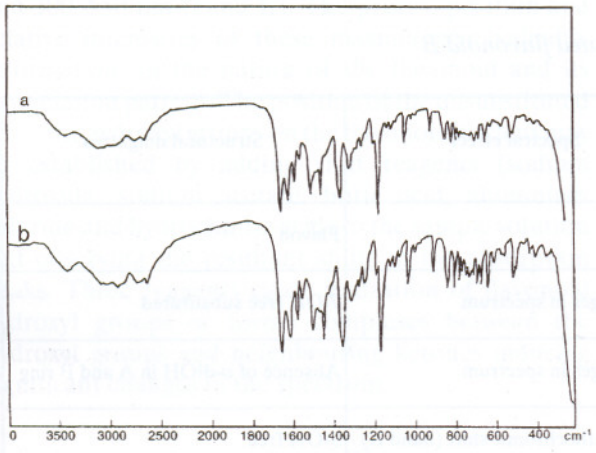


Fig. 1. IR spectrum of isolated flavonoid A (a) and of standard substance chrysin (b)

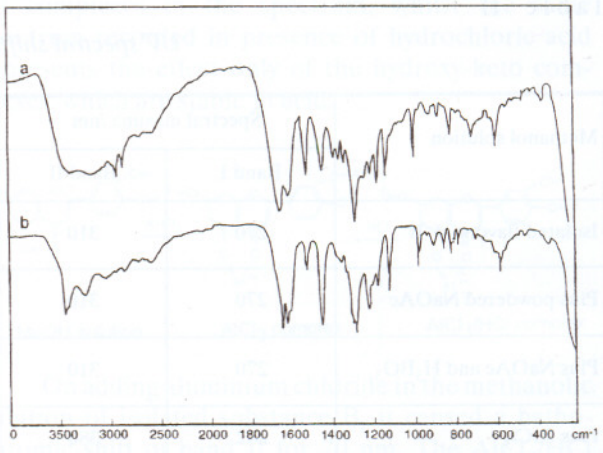


Fig. 3. IR spectrum of isolated phenyl-propanoid D (a) and of standard substance caffeic acid (b)

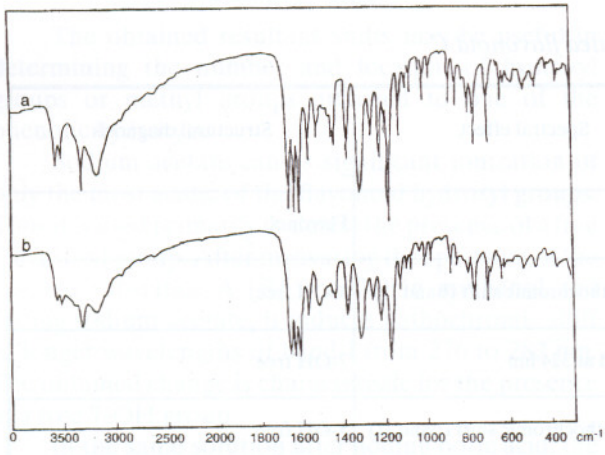


Fig. 2. IR spectrum of isolated flavonoid C (a) and of standard substance galangin (b)

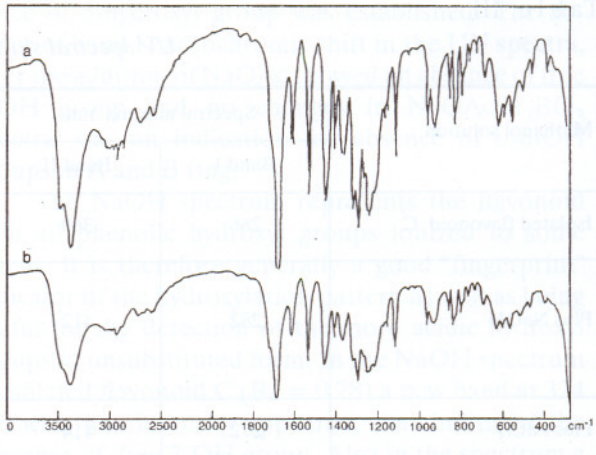


Fig. 4. IR spectrum of isolated phenyl-propanoid E (a) and of standard substance hydrocaffeic acid (b)

IR spectroscopy is most frequently used in phytochemical studies as a "fingerprinting" device, for comparing a natural with a synthetic sample. The complexity of the IR spectrum lends itself particularly well to this purpose and such comparisons are very important in the complete identification of natural compounds. The correctness of all our conclusions about identification of five isolated substances were proved by recording their IR spectra and authentic standards (Fig. 1–4). The IR spectra of isolated substances were strikingly similar to those of standard substances: chrysin, galangin, caffeic acid and hydrocaffeic acid. The IR spectrum of isolated flavonoid tectochrysin (Fig. 5) was compared with its IR spectrum from the Aldrich catalogue.

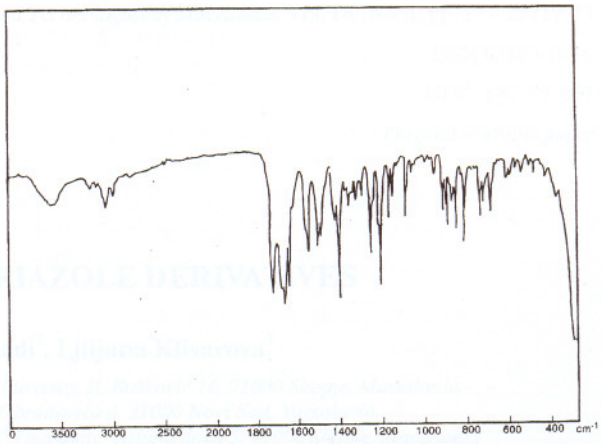


Fig. 5. IR spectrum of isolated flavonoid B

CONCLUSION

Five compounds were isolated and identified from Macedonian propolis. The obtained UV and IR spectral data provided evidence for the presence of different groups of phenolic constituents: flavonoids and phenyl-propanoids. The presence of three flavonoids: chrysin, tectochrysin and galangin, and two phenyl-propanoids: caffeic and hydrocaffeic acid was determined.

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Резиме

ИДЕНТИФИКАЦИЈА НА ФЕНОЛНИ КОНСТИТУЕНТИ ИЗОЛИРАНИ ОД МАКЕДОНСКИ ПРОПОЛИС

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Клучни зборови: прополис; фенолни конституенти; UV и IR спектроскопија; флавоноиди; хризин; тектохризин; галангин; кафеична киселина; хидрокафеична киселина

Испитувани се фенолни конституенти, изолирани од македонски прополис, со примена на UV и IR спектроскопија. Во примероците прополис се идентификувани три флавоноиди (хризин, тектохризин и галангин) и два фенилпропаноиди (кафеинска и хидрокафеинска киселина) се. Бројот и положбата на присутните хидроксилни групи во флавоноидното јадро на изолираните супстанци беа определени со примена на shift реагенси. Идентификацијата на изолираните конституенти беше потврдена со споредување на нивните IR спектри со стандардни супстанци.