FLAVONOIDS OF INFLORESCENCES OF Calendula officinalis

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By column chromatography on polyamide sorbent, the inflorescences of pot marigold calendula have yielded eight substances of flavonoid nature: two aglycons – quercetin ($C_{15}H_{10}O_7$, mp 309-311°C) and isorhamnetin ($C_{16}H_{12}O_7$, mp 314-316°C); six glycosides, of which three have been identified as isoquercetin ($C_{21}H_{20}O_{12}$, $[\alpha]_D^{20}$ −36° in methanol, mp 218-220°C), isorhamnetin 3-O-β-D-glucoside ($C_{22}H_{22}O_{12}$, $[\alpha]_D^{20}$ −59° in dimethylformamide, mp 193-195°C), narcissin ($C_{28}H_{32}O_{16}$, $[\alpha]_D^{21}$ −28° in dimethylformamide, mp 180-182°C), and three substances that have proved to be new and have been called calendoflaside ($C_{28}H_{32}O_{15}$, $[\alpha]_D^{21}$ −85° in methanol, mp 192-195°C; calendoflavoside ($C_{28}H_{32}O_{16}$, $[\alpha]_D^{20}$ −106° in methanol, mp 189-192°C), and calendoflavobioside ($c_{27}H_{30}O_{16}$, $[\alpha]_D^{20}$ −105° in methanol, mp 194-197°C).

The isolation from calendula inflorescences of the coumarins umbelliferone, esculetin, and scopoletin has been reported previously [1]. In the present communication we give the results of a study of the flavonoid substances of the inflorescences of the pot marigold Calendula officinialis L., family Asteraceae.

The flavonoids were isolated from the inflorescences by a method described previously [2, 3]. As a result, eight substances (I-VIII) of flavonoid nature were obtained of which two (VII and VIII) were assigned to the aglycons, two (V and VI) to the monosides, and four (I-IV) to the glycosides with two carbohydrate residues. Their flavonoid nature was confirmed by the cyanidin reaction [4] and by characteristic absorption bands in the IR (3400, 3030, 2900, 1670, 1620, 1580, 1560, and 1515 cm^{-1}) and UV ($\lambda_{max}^{C_2H_5OH}$ 350-375, 265-268, 255 nm) regions of the spectrum. The IR spectrum of each of substances (I), (II), (IV), (V), and (VII) had a band at 2975-2980 cm^{-1} indicating the presence of a methoxy group.

When chromatographed on paper, substances (VII) and (VIII) fluoresced in UV light in the form of yellow spots, which indicated the presence in each of them of a free hydroxy group in position 3 of the flavonoid nucleus, while the remaining substances were detected before acid hydrolysis in the form of dark brown spots and after in the form of yellow spots, which showed substitution of a hydroxy group at C-3 [5].

With the aid of UV spectroscopy using ionizing and complex-forming reagents [6], it was established that aglycon (VIII) had free hydroxy groups in positions 3, 3', 4', 5, and 7, and (VII) in positions 3, 4', 5, and 7, of the flavonoid nucleus. A confirmation of this was the formation of their penta- and tetracyl derivatives, respectively. A band in the 2960 cm^{-1} region of substance (VII) showed the possibility of the presence of a methoxy group in the molecule. The demethylation [6] of substance (VII) led to the formation of substance (VIII). On the basis of the spectral characteristics of the substances under investigation and their transformation products and physicochemical properties, they were identified as quercetin (VIII) and isorhamnetin (VII) [7].

On acid and enzymatic hydrolysis, the monosides (V) and (VI) were cleaved to give D-glucose and, respectively, the aglycons quercetin (VIII) and isorhamnetin (VII) (see the scheme of transformations). From their physicochemical properties and their transformation products, they were identified as isorhamnetin 3-O-β-D-glucopyranoside (VI) [3, 8] and quercetin 3-O-β-D-glucopyranoside (isoquercetin) (V) [3, 9].

On acid hydrolysis, substances (II-IV) formed 51.8-53.3% of the aglycon, on which basis it may be considered that these glycosides each contained two sugar residues. It was established chromatographically that these residues were those of D-glucose and L-rhamnose,

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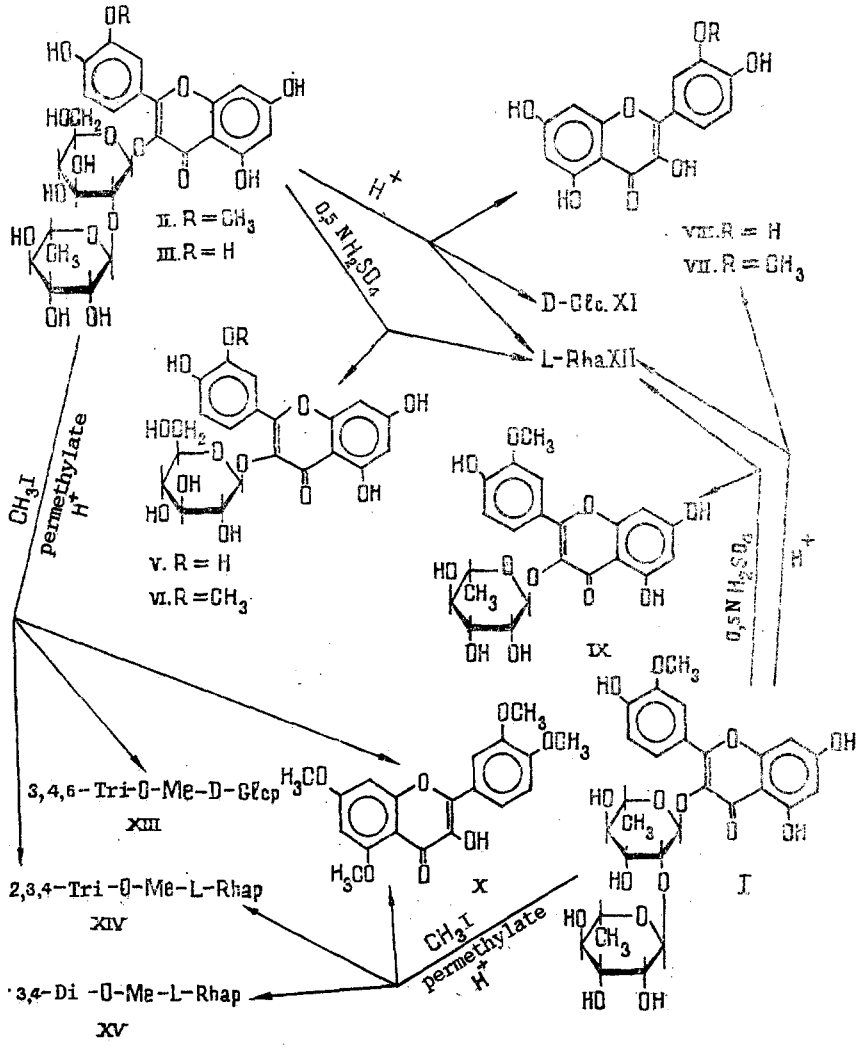

Scheme of the transformations of flavonoid glycosides isolated from calendula inflorescences.

while substance (I) contained a L-rhamnose residue. The aglycon of glycoside (I), (II), and (IV) was isorhamnetin (VII), and that of glycoside (III) was quercetin (VIII).

It was established by UV spectroscopy with ionizing and complex-forming additives that their carbohydrate residues were attached at position 3 of the flavone nucleus.

Stepwise acid hydrolysis led to the formation of monosides: substance (I) formed isorhamnetin 3-O-α-L-rhamnopyranoside (IX), substances (II) and (IV) formed isorhamnetin 3-O-β-D-glucopyranoside (VI), and (III) formed quercetin 3-O-β-D-glucopyranoside (isoquercitrin (V)). The results obtained showed that the terminal sugar in each of the biosides was L-rhamnose.

To establish the position of attachment of the L-rhamnose residue to the D-glucose residue in each of glycosides (II-IV), we used enzymatic hydrolysis with rhamnodiastase [7]. Only substance (IV) was cleaved, to give isorhamnetin (VII) and rutinose, which enabled it to be identified as narcissin: 4',5,7-trihydroxy-3'-methoxy-3-[O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy]flavone. The resistance to rhamnodiastase of glycosides (II) and (III) enabled a 1 → 6 bond between the carbonhydrate residues in them to be excluded.

No free D-glucose of L-rhamnose was detected in the reaction mixtures after the periodate oxidation [10] of glycosides (I-III) followed by their acid hydrolysis which showed the absence of 1 → 3 bonds between the sugar residues. For an unambiguous determination of the structure of the carbohydrate moieties we carried out exhaustive methylation of the glycosides (I-III) by Kuhn's method [11]. Paper chromatography [12] showed the presence of 3,4,6-tri-O-methyl-D-glucose and 2,3,4-tri-O-methyl-L-rhamnose in the products of the hydrolysis of the methyl derivatives while in the hydrolysate of (I) 3,4-di-O-methyl-L-rhamnose and 2,3,4-tri-

O-methyl-L-rhamnose were detected. 3-Hydroxy-3',4',5,7-tetramethoxy flavone (X) was isolated from the hydrolysate of all three methylated glycosides. On analyzing the methylation results, it may be concluded that the sugar residues in the glycosides investigated were linked with one another by 1 → 2 bonds.

The configurations of the glycosidic bonds were determined by a comparison of molecular rotations with those of the corresponding phenyl and methyl glycosides [16].

On the basis of the facts obtained, the structures of flavonoids (I-III) can be represented as 4',5,7-trihydroxy-3'-methoxy-3-(O-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyloxy)flavone (substance (I)), 4',5,7-trihydroxy-3'-methoxy-3-[O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy)flavone (substance (II)), and 3',4',5,7-tetrahydroxy-3-[O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]flavone (substance (III)).

Thus, eight flavonoid substances have been isolated from the inflorescences of pot marigold calendula, of which five have been identified as quercetin, isorhamnetic, quercitrin, isorhamnetin 3-glucoside, and narcissin. Three substances are new glycosides and we have called them calendoflaside (I), calendoflavoside (II), and calendoflavobioside (III).

EXPERIMENTAL

General Observations. Pot marigold calendula inflorescences of pharmacopeal quality were investigated. The following solvent systems were used: 1) 2% acetic acid; 2) butan-2-ol–acetic acid–water (4:1:2); 3) benzene–ethyl acetate–acetic acid (24.5:73.5:2)/formamide; 4) pyridine–ethyl acetate–water (1:2:2); and 5) water-saturated methyl ethyl ketone.

UV spectra were obtained on a Hitachi spectrophotometer, and IR spectra on a UR-10 instrument (tablets with KBr). Optical rotations were measured on a ÉPL-1A instrument. The Kapton (polycaprolactan) sorbent was prepared by the procedure described in [13].

A solution of aniline phthalate was used to reveal sugars on paper chromatograms [14].

Isolation of the Flavonoids. The comminuted pot marigold inflorescences (6 kg) were extracted three times with 80% ethanol, and the resulting 60 liters of extract was evaporated in vacuum to a volume of 8 liters. The aqueous residue so obtained was left in the refrigerator for a day, and the precipitate that had deposited was separated off by centrifugation and then the filtrate was additionally purified with chloroform (3 × 3 liters). From the residue purified in this way the flavonoids were extracted successively with ethyl acetate (12 liters) and n-butanol (12 liters). The extracts were evaporated until the solvents had been eliminated completely, giving 31 g and 44 g, respectively, of dry residues.

The residue from the butanolic fraction (40 g) was dissolved in 250 ml of 50% ethanol, and the solution was mixed with 140 g of polyamide sorbent and, after careful stirring, the mixture was dried at 50°C and was deposited on a column of the same sorbent (10 × 75 cm). The column was washed first with water and then with aqueous ethanol in which the concentration of ethanol was gradually increased to 50%. The eluates were collected in 0.5-liter portions, and the flavonoid composition of each of them was determined by paper chromatography in systems 1 and 2. Fractions having the same composition were combined and evaporated, and the residues were crystallized from ethanol. As a result substances (I) (1.25 g), (II) (1.42 g), (III) (1.07 g), and (IV) (0.98 g) were obtained.

The flavonoids of the ethyl acetate fraction (30 g) were separated on a column of Kapron sorbent (8 × 75 cm) by the method described above, the column being eluted with aqueous alcohols containing from 5 to 80% of ethanol. As a result, flavonoids (V) (1.14 g), (VI) (1.03 g), (VII) (0.11 g), and (VIII) (0.09 g) were obtained.

The fractions containing mixtures or uncrystallizable compounds were passed through columns of Kapron sorbent again.

Calendoflaside (I). This crystallizes from ethanol in the form of acicular light yellow crystals with the composition $C_{28}H_{32}O_{15}$, mp 192-195°C, $[\alpha]_D^{21}$ −85° (c 0.2; methanol). On paper chromatography in solvent systems 1 and 2, the substance had R_f 0.74 and 0.39, respectively. After the chromatogram had been sprayed with a solution of zirconyl chloride followed by treatment with ammonia vapor, substance (I) fluoresced light yellow in UV light.

Quantitative Acid Hydrolysis of (I). The substance (150 mg, accurately weighed) was dissolved in 15 ml of 50% aqueous ethanolic solution containing 4% of hydrochloric acid, and the

resulting solution was boiled in a flask with a reflux condenser for 2 h. When the hydrolysate was chromatographed on paper in system 3, only the aglycon was detected. Then the alcohol was distilled off from the reaction mixture and the aqueous residue was left in the refrigerator for 3 h. The crystals that had deposited were filtered and dried. This gave 77.8 mg of the aglycon isorhamnetin [7], making up 51.8% of the weight of the initial glycoside.

The aqueous acid solution after the separation of the aglycon was passed through AV-17 anion-exchange resin (in the OH form), and the neutral eluate obtained was evaporated to 1-1.5 ml and was chromatographed in systems 2 and 4. After the chromatogram had been treated with aniline phthalate, L-rhamnose was detected.

Enzymatic Hydrolysis of (I). A solution of 10 mg of the substance in 3 ml of water, prepared with heating, was treated with 8 mg of a dry enzyme preparation from the pancreatic juice of the grape snail and the mixture was left for 2 days, with the degree of hydrolysis of glycoside (I) being monitored periodically by paper chromatography in solvent system 1. After the complete cleavage of the initial substance, the enzyme preparation was precipitated with 4 volumes of ethanol, the precipitate was separated off, and the filtrate was evaporated to 2.9-3 ml. The crystals of the aglycon isorhamnetin (VII) that deposited under these conditions was separated off, and the aqueous residue was chromatographed in solvent system 2. A single sugar, L-rhamnose, was found, as in the case of acid hydrolysis.

Stepwise Hydrolysis of (I). A solution of 30 mg of the substance in 3 ml of 0.5 N sulfuric acid solution was treated with 1.5 ml of 95% ethanol, and the mixture was left at room temperature for 15 days with periodic checking of the reaction mixture by paper chromatography. The hydrolysis products were separated on a column of polyamide sorbent (1 × 12 cm) with elution first by water and then with aqueous ethanol. The concentration of ethanol was increased from 2 to 50%. As a result, 6 mg of the initial glycoside (I), 12 mg of isorhamnetin 3-α-L-rhamnopyranoside – $C_{22}H_{22}O_{11}$, mp 118-119°C, $[\alpha]_D^{20}$ −120.5° (in methanol) – and 9 mg of the aglycon (VII) – $C_{16}H_{10}O_7$, mp 314-316°C – were obtained. No L-rhamnose was detected in the aqueous eluates after their treatment with AV-17 anion-exchange resin and evaporation to 1.5-2 ml (systems 1 and 2).

Periodate Oxidation of (I). Substance (1) (40 mg) was oxidized with a 1% solution of sodium metaperiodate at 5-7°C for 48 h. The excess of periodate was decomposed with ethylene glycol. The subsequent treatment of the reaction mixture was carried out as described in [10]. After acid hydrolysis of the products of the oxidation of substance (I), no L-rhamnose was detected in the reaction mixture.

Methylation of (I) [11]. The substance under investigation (80 mg) was dissolved in 3 ml of dimethylformamide that had been redistilled over phosphorus pentoxide and had previously been heated to 40°C, and, after the addition of 3 ml of methyl iodide, 0.8 g of silver oxide was added in small portions with stirring over 30 min. Then the reaction mixture was worked up as shown in [10]. The methylated glycoside was hydrolyzed with 2% H_2SO_4. The hydrolysate deposited crystals of the methyl derivative of an aglycon with mp 196-198°C which we identified as 3',4',5,7,tetra-O-methylquercetin. The carbohydrate fraction of the hydrolysate was chromatographed on paper in system 5 in parallel with 2,3,4,6-tetra-O-methyl-D-glucose to determine its R_g value (g = 2,3,4,6-tetra-O-methyl-D-glucose) [12]. Two methylated sugar were detected that corresponded to 2,3,4-tri-O-methyl-L-rhamnose and 3,4-di-O-methyl-L-rhamnose.

Calendoflavoside (II). This crystallized from aqueous alcohol (on evaporation) in the form of yellow acicular crystals with the composition $C_{28}H_{32}O_{16}$, mp 189-192°C, $[\alpha]_C^{20}$ −106° (c 0.2, methanol). When chromatographed in solvent systems 1 and 2 it had R_f values of 0.61 and 0.57, respectively.

Enzymatic Hydrolysis of (II). A solution of 11 mg of the glycoside under investigation in 3.5 ml of water was treated with 10 mg of rhamnodiastase. Then the experiment was continued by the procedure described above. Paper chromatography in solvent systems 1 and 2 showed the presence of the initial substance (II).

Quantitative Acid Hydrolysis of (II). The substance (100 mg, accurately weighed) was hydrolyzed in the same way as substance (I). This gave 51.07 mg of the aglycon isorhamnetin, which corresponds to the presence of two sugar residues in glycoside (II).

In the aqueous part of the hydrolysate, D-glucose (XI) and L-rhamnose (XII) were detected by paper chromatography in solvent systems 2 and 4.

Stepwise Hydrolysis of (II). Substance (II) (35 mg) was hydrolyzed by the procedure described above. After the mixture had been allowed to stand for 15 days and had been separated on polyamide sorbent, 15 mg of isorhamnetin 3-O-β-D-glucopyranoside (VI) ($C_{22}H_{22}O_{12}$, mp 193-195°C, $[\alpha]_D^{20}$ −59° (in dimethylformamide) [8] and 7 mg of the aglycon isorhamnetin (VII) were obtained.

In the aqueous eluates L-rhamnose (XII) was detected as the main product, with a small amount of D-glucose (XI).

Periodate Oxidation of (II). On the periodate oxidation of 35 mg of substance (II) (compare substance (I)) and hydrolysis of the reaction products, no D-glucose was detected.

Methylation of (II) [11]. In the methylation products (compare substance (I)), 2,3,4-tri-O-methyl-L-rhamnose (XIV), and 3,4,6-tri-O-methyl-D-glucose were detected. The separated methylated aglycon was identified as 3-hydroxy-3',4',5,7-tetramethoxyflavone (X).

Calendoflavobioside (III). This crystallized like substance (II) from aqueous ethanol in the form of acicular crystals with the composition $C_{27}H_{30}O_{16}$, mp 194-197°C, $[\alpha]_D^{20}$ −105° (c 0.2 in methanol); R_f 0.63, 0.54, (solvent systems 1 and 2).

Enzymatic Hydrolysis of (III). Substance (III) (15 mg) was treated with 12 mg of rhamnodiatase as described for glycosides (I) and (II). The initial substance was recovered.

Quantitative Acid Hydrolysis of (III). Substance (III) (15 mg, accurately weighed) was hydrolyzed by the procedure described above. As a result of hydrolysis, 50.3% of the aglycon quercetin (VIII) was obtained, on the basis of which it was possible to assign (III) to the biosides.

In the aqueous fraction of the hydrolysate, D-glucose (XI) and L-rhamnose (XII) were detected in solvent systems 2 and 4.

Stepwise Hydrolysis of (III). Glycoside (III) (40 mg) was hydrolyzed with 4 ml of a 0.5 N solution of sulfuric acid as described for substance (I). As a result, quercetin 3-O-β-D-glucopyranoside (V), $C_{21}H_{20}O_{12}$, mp 218-220°C, $[\alpha]_D^{21}$ −36° (in methanol) [3, 9], quercetin (VIII), $C_{15}H_{10}O_7$, mp 309-311°C [7], and the initial substance (III) were isolated.

The periodate oxidation of (III) was carried out as for glycosides (I) and (II). No D-glucose was found in the oxidation products.

Methylation of (III). Substance (III) was methylated in the same way as substances (I) and (II). After hydrolysis of the methylation product, 3-hydroxy-3',4',5,7-tetramethoxyflavone (X) was obtained; when the hydrolysis products were chromatographed in solvent system 5, 2,3,4-tri-O-methyl-L-rhamnose (XIV), and 3,4,6-tri-O-methyl-D-glucose (XIII) were detected.

Substance (IV) ($C_{28}H_{32}O_{16}$, $[\alpha]_D^{21}$ −28°, in dimethylformamide, mp 180-182°C; substance (V) ($C_{21}H_{20}O_{12}$, $[\alpha]_D^{20}$ −36°, in methanol, mp 218-220°C), substance (VI) ($C_{22}H_{22}O_{12}$, $[\alpha]_D^{20}$ −59° in dimethylformamide, mp 193-195°C; substance (VII) ($C_{16}H_{12}O_7$, mp 314-316°C), and substance (VIII) ($C_{15}H_{10}O_7$, mp 309-311°C) were identified with substances which we have isolated previously from other plants: narcissin [15], isoquercitrin [3. 9], isorhamnetin 3-glucoside [3, 8], isorhamnetin [7], and quercetin [7], respectively.

CONCLUSIONS

Eight new substances of flavonoid nature have been isolated from the inflorescences of Calendula officinialis L.

It has been established that substances (I-III) are new compounds, and their structures can be represented as 4',5,7-trihydroxy-3'-methoxy-3-[O-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyloxy]flavone (calendoflaside (I)), 4',5,7-trihydroxy-3'-methoxy-3-[O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]flavone (calendoflavoside (II)); and 3',4',5,7-tetrahydroxy-3-[O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyloxy]flavone (calendoflavobioside (III)).

Substances (IV-VIII) have been identified with flavonols known previously: narcissin (IV), isoquercetrin (V), isorhamnetin 3-O-β-D-glucopyranoside (VI), isorhamnetin (VII), and quercetin (VIII).

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