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ISOLATION, CHARACTERIZATION AND PHARMACOLOGICAL ACTIVITY OF *MAGYDARIS PASTINACEA* (LAM) PAOL. GLUCOSIDES

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Summary — The glycoside fraction of fresh rhizomes from *Magydaris pastinacea* (Lam.) Paol. was separated from the alcoholic extract using the method of Kobayashi *et al.*[1] The fraction was found to have six main constituents, the most abundant of which had previously been isolated and identified as 7-O-ß-D-glucopyranosyl-8-(2',3'-dihydroxy-3-methyl-butylcoumarin)[2]. The present paper describes the separation and characterization of the other constituents, all with coumarin or furancoumarin structures. Pharmacological experiments to test these compounds as platelet antiaggregants are also reported.

INTRODUCTION

Magydaris pastinacea (Lam.) Paol. (Umbelliferae) is a very common plant in Sardinia and the Mediterranean area. It has a thick, striated, sulcate stalk, and reaches a height of over two metres (Fig. 1). The leaves are large and white, and velvety underneath; the primordia are undivided or have 3-5 lobes and are crenate, while the leaves that follow have three long oval segments. The umbels are large (40-50 pedicels) with white flowers; the fruit is oblong with slightly flattened sides[3]. Samples of the plant are deposited in the "Sassa" Herbarium, specimen n° 570[4].

It was decided to study the chemical components of *Magydaris pastinacea* after skin reaction was noted, involving intense redness and streaking on the arms of people picking other plants in its vicinity, a reaction which persisted for a few weeks. The chemical components of rhizomes collected in early summer near the intersection to Macomer on Highway 131 in the province of Nuoro (Sardinia - Italy) were initially studied.

Using the method of Kobayashi *et al.*[1], the rhizomes were chopped into pieces and soaked in methanol to release the glycoside fraction. HPLC examination revealed six well-defined peaks, later identified as glucosides of coumarin or furancoumarin derivatives.

MATERIALS AND METHODS

A) CHEMISTRY

The solvents used in HPLC were Licrosolv® from Merck - Darmstadt (D) and RS for HPLC from Carlo Erba, Milan (I); other solvents and chemical products were supplied by the above mentioned companies and by Fluka-Buchs (CH); enzyme hydrolysis was performed with ß-D-glucosidase purchased from Sigma Aldrich, Milan (I), which also supplied the deuterated solvents for HPLC.

HPLC - *Analysis of glycoside fraction*

Apparatus: Hewlett-Packard Liquid Chromatograph mod. 1090 with hp data stations, series 9000/300, hp Diode Array Detector mod. 1040 with 300 nm reading; mobile phase H_2O 64%, MeOH 35%, AcOH 1%; 2ml/min flow; ODS 5μ column, hp 200×4mm; 10 μl injections. Six components with fine resolution were separated with RT between 17.2 min and 95.9 min (Fig. 2a) [System 1]. UV spectra are shown in Fig. 2b.

Semi-preparative analysis of glycoside fraction - Apparatus: Hewlett-Packard Liquid Chromatograph mod.1084 B, supplied with an automatic container for fractions and a wavelength detector positioned at 300 nm. Mobile phase: H_2O 71%, MeOH 28% AcOH 1% until the end, 6 ml/min flow; semi-preparative column Lichrosorb RP8 7μ Merck 250 x 10mm; 100 μl injection [System 2].

The same peaks with RT between 8.7 min and 46.6 min were also separated at this stage.

Sugar analysis - Analysis was performed after chemical or enzyme hydrolysis of the glycoside fraction.

Apparatus: system 1. Detector with refraction index, mobile phase CH_3CN 85%, H_2O 15%; 1 ml/min flow; Lichrosorb NH_2 column 7μ Merck 250×4 mm; 20 μl injection, RT of glucose = 11.4 min.

Examination of enzyme hydrolysis - Apparatus and detector: system 1. Mobile phase CH_3CN 40%; H_2O 60%; 1 ml/min flow; column RP-18 7μ Merck 250×4mm; 10 μl injection; aglycon RT always slightly superior to that of the corresponding glycoside.

MASS SPECTROMETRY - *Characterization of the glycosides*

Finnigan MAT 8222 mass spectrometer with computerized added data; conditions solv. MeOH: source C.I. [NH_3]. Voltage Volt 3000. Risol. 2000. (Dip. Chimico Farmaceutico, University of Pavia).

Magnetic resonance

Carried out at the Spectroscopy Centre of the Dipartimento di Chimica, University of Sassari; apparatus: Varian VCR 300 Mhz.

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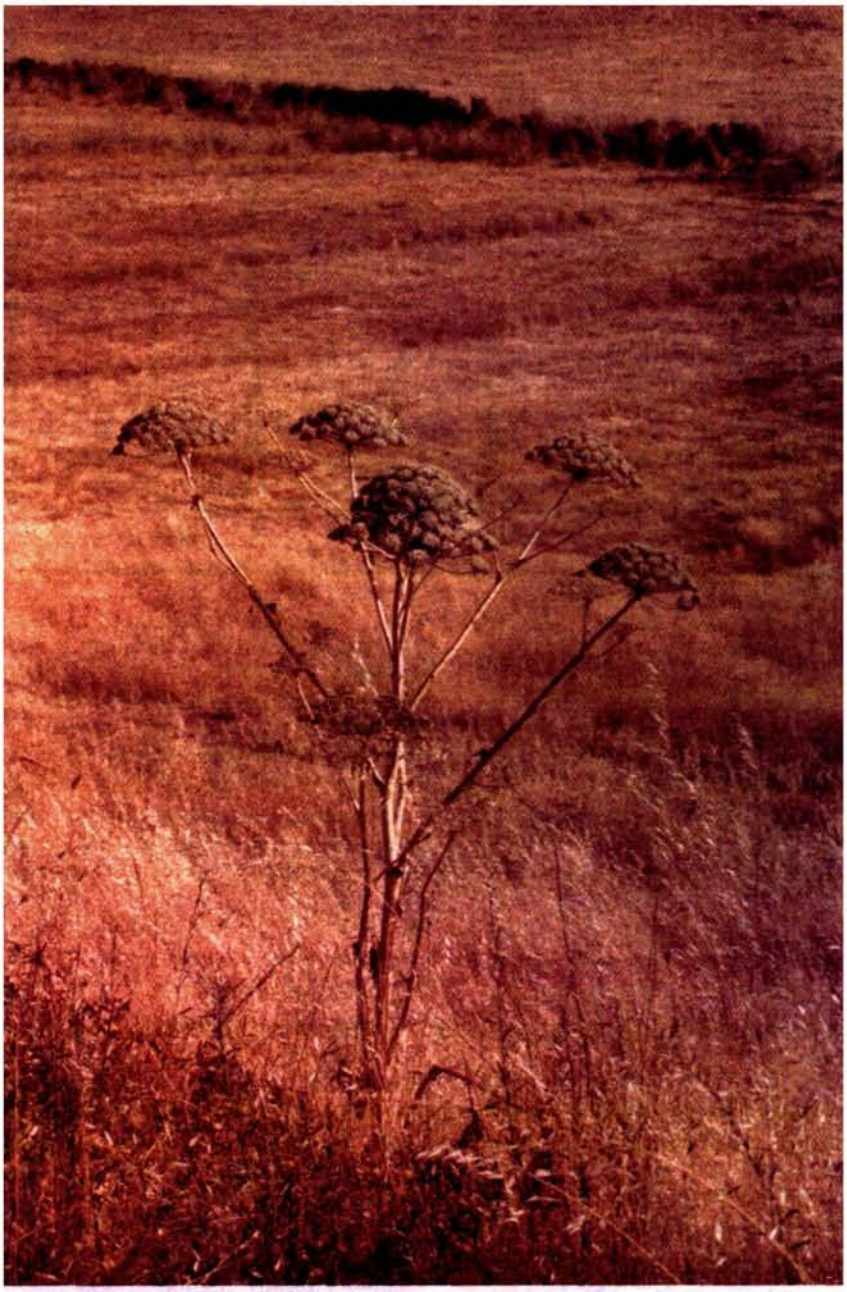


Fig. 1

^{1}H-NMR

Spectral width 0-15 ppm, integral, exchange with D_2O, parameter and frequency list, integral spectrum expansion.

^{13}C-NMR

Spectral width O÷400 ppm., decoupling, off/resonance DM = 1, NB = 0; Dec offset arom., Dec offset Aliph.

HYDROLYSIS OF GLUCOSIDES

Chemical hydrolysis - The mixture of glucosides was treated with 2N HCl at room temperature for 60 min; the solution was evaporated under vacuum, then water was added, followed by re-evaporation. This treatment was repeated until the pH was nearly neutral. Subsequently, the solution was analysed by HPLC as described above: RT 11.4 min, corresponding to the D-glucose as the only sugar present.

Enzyme hydrolysis - 200 µl of the single glucoside solution (2 mg/ml) was added with 40 ml of acetate buffer solution (410 mg NaOAc, 0.45 ml acetic acid 1M in 5 ml of H_2O) and 100 µl of a solution containing ß-D-glucosidase (20 mg of enzyme/ml of H_2O). The mixture was left at 37°C for a few hours; a 10µl sample was drawn approximately every 60 min in order to follow the evolution of hydrolysis by HPLC.

B) PHARMOCOLOGY

PREPARATION OF BLOOD SAMPLES FOR ANALYSIS

Materials: blood samples were taken from 20 healthy males (voluntary donors), drawn from a vein on the volar surface of the forearm in recumbent subjects. Donors had not taken any medication during the previous ten days. Samples were drawn with 19'' dia. needles in vacuum-sealed Vacutainer® test tubes, with 3.8% sodium citrate with a ratio of 1:10

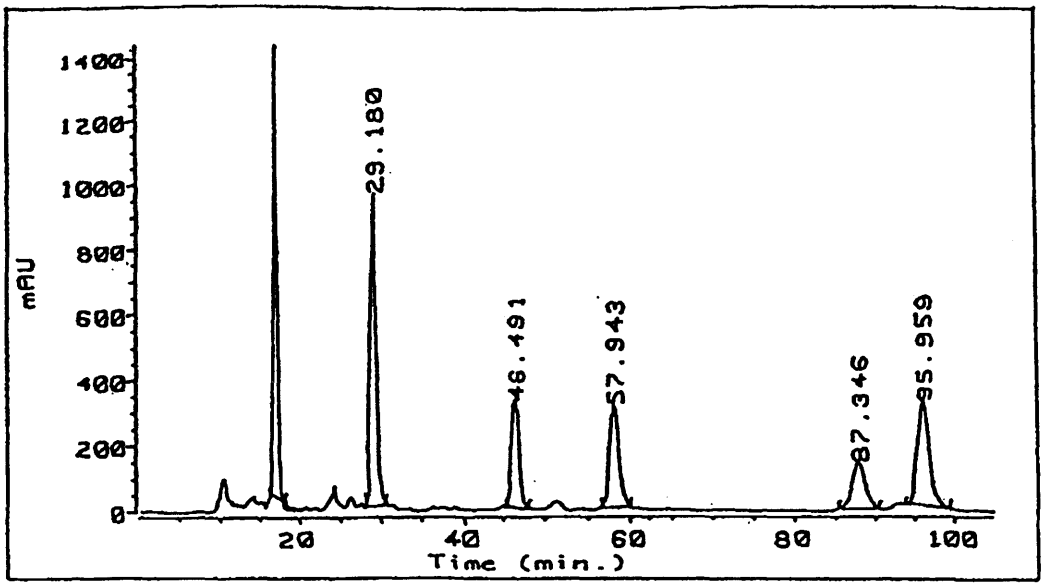


Fig. 2a

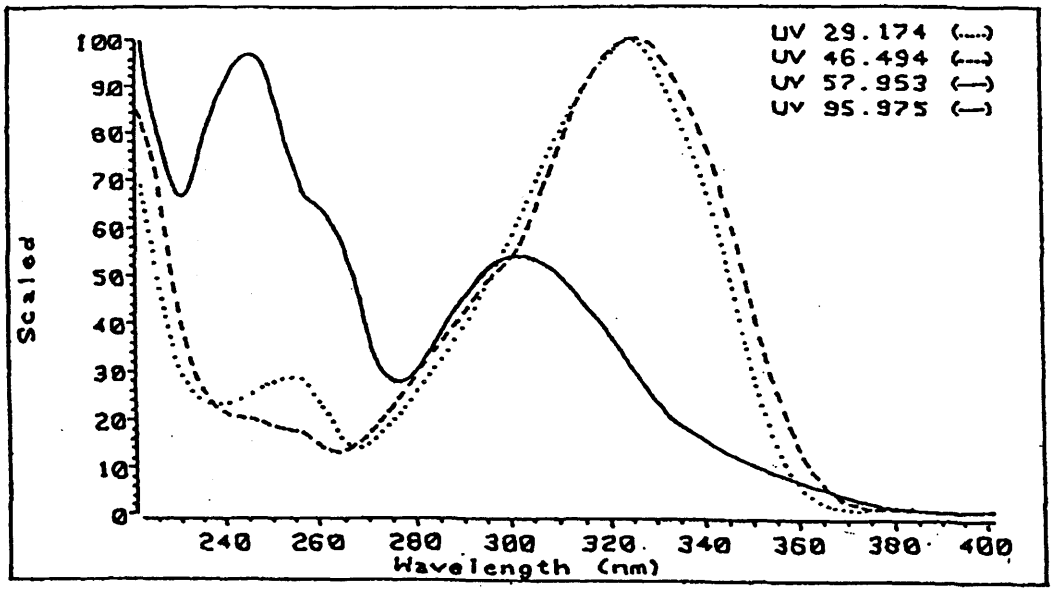


Fig. 2b

anticoagulant/blood.

Platelet Rich Plasma (PRP) was obtained by centrifugation at 800 rpm (200 × g) at room temperature. Platelet Poor Plasma (PPP) was obtained by centrifugation at 3000 rpm (700 × g) of the same samples after separation of the PRP. The PRP and PPP of all samples were collected in two separate pools. Samples were stored at room temperature in sealed PVC test tubes.

Reagents

All reagents were supplied by Menarini Diagnostici (I). Working solution: a) ADP: 0.5 μM/l and 3.0 μM/l; b) Arachidonic acid 1.3 μg/l; c) Collagen: 2.0 μg/l; d) Ionophore A23187:20 μM/l; e) Epinephrine: 2.5 μg/l.

Operating conditions

Apparatus: DAIICHI-MENARINI Aggrecorder mod. II-PA 3220.

Each substance to be tested was dissolved in bi-distilled water (1 mg/ml); 200 μl of the solution was used, always at the same concentration.

During aggregation, the samples were constantly shaken at 1000 rpm in a magnetic stirrer covered with teflon. The reaction of the solutions to each inductor was observed for 10 minutes. Basic tracings were made using PRP samples prepared by adding 200 μl of Michaelis buffer at pH 7.35 to 250 μl of PRP. In order to assess the action of the isolated substances on platelet aggregation, 200 μl of the solution of each substance was mixed with 250 μl of PRP (so as to maintain a platelet concentration

of not less than 200,000/ml), and 50 µl of the inductors.

Analysis of aggregation curves

After tracing the profiles of aggregation curves produced by epinephrine, collagen and arachidonic acid in Michaelis buffer without inhibitor, the peacks of maximum amplitude of the curve were obtained at 5.0, 9.4 and 7.2 minutes respectively. The maximum amplitude times were taken into consideration when assessing the antiaggregation effect of the tested substances (Fig. 3).

For solutions containing collagen and arachidonic acid, the Slope and Latent Period between the addition of the inductor and the beginning of the aggregation wave were recorded as well as Max. Amp. (Fig. 4).

In cases in which aggregation had been induced by ADP, the maximum height of the curve in the first phase induced by 0.5µM ADP was taken as the value of the primary wave, whereas that of the second phase of aggregation was expressed as the slope of the aggregation curve induced by 3.0mM ADP (Fig. 5).

RESULTS

The constituents of the glycoside mixture were separated by semi-preparative HPLC. After repeated tests, suitable quantities of four out of six components corresponding to the peaks were obtained: Tr = 29.2 min (1); Tr = 46.5 min (2); Tr = 57.9 min (3); and Tr = 95.9 min (4). (Table 1).

Details of the first constituent of the series (Tr = 17.2 min) have already been described by us.[2] The sixth component had been isolated in sufficient quantity to allow characterization and pharmacological testing.

The UV-Vis. absorption curves which were recorded at the same time as analytical elution showed spectral behaviour for 1 (nm = 325 max., 297 fl., 255) and 2 (nm = 327 max., 295 fl., 255) comparable to that of coumarin 7-hydroxy substituent, the chromophore of the umbelliferone. The curves of 3 and 4 were perfectly identical and also matched the xanthotoxin chromophore.

These preliminary results were confirmed by the shift tests with variable pH described in the literature for these types of compounds[5].

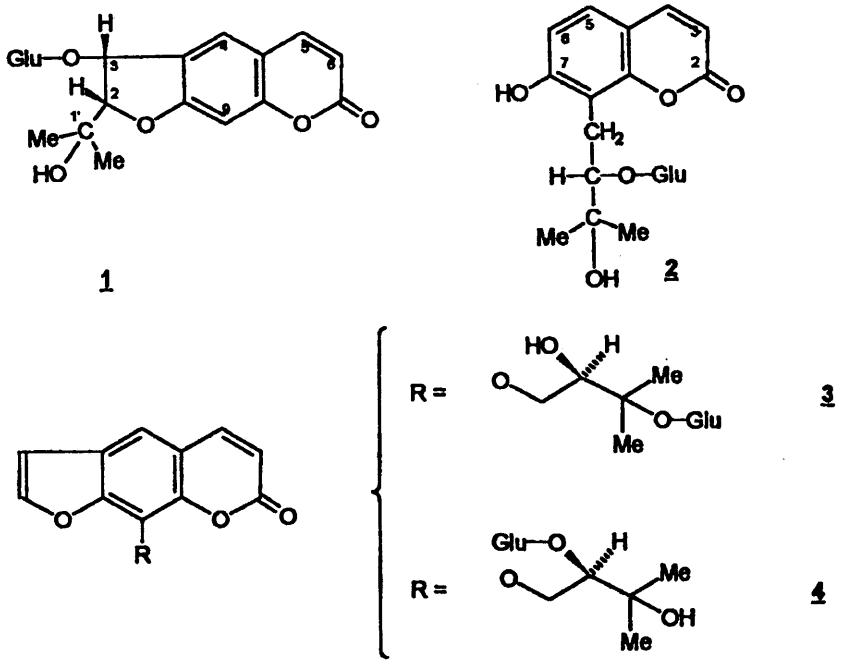
Chemical hydrolysis, performed on the mixture of glycosides as well as on the single products which were later separated, in all cases showed the presence of glucose only. This result was supported by enzyme hydrolysis with ß-D-glucosidase, which confirmed D-glucose as the only component of the sugar fractions. Besides D-glucose, hydrolysis of 3 and 4 produced an identical aglycon with the same structure as komaline, identified by spectroscopic data relating to the corresponding glucosides.

On the basis of the data obtained by ^{1}H-NMR, ^{13}C-NMR and MS, the following structures were assigned to the separated products:

Compound 1 (Table I), as a derivative of 2,3-dihydro-furancoumarin, has only four signals of H aromatics: H4 and H9 as singlets, typical of hydrogen atoms in para between each other (δ = 7.85 and 6.88 ppm), and H5 and H6 as doublets with J = 10Hz (δ = 7.95 and 6.27 ppm) (ortho coupling).

The signal of H-3 (δ = 5.73), which has a shift value of about 0.5 δ to lower fields due to the effect of the glucoside bond, and H2 (δ = 4.55) are mutually influenced, resulting in two doublets, as revealed not only by the NOE technique but also by double

Table I



resonance radiation on H3. This reduces the multiplicity to a singlet on H2. The value of J = 6 Hz allows a cis conformation of the two hydrogen atoms H2 and H-3[7]. The two singlets of geminal methyl fall at $\delta = 1.53$ and 1.46 ppm respectively, in fields significantly different from those described for the same compound isolated by Lemmich[7], in which the geminal-dimethyl system is bound to the same carbon OH atom that supports glucose.

The ^{13}C-NMR spectrum was fully consistent with the structure hypothesized. The mass spectrometry results (C.I. + NH_3) show the molecular ion 442 u.m.a. corresponding to the m.w. of 1 which is (424 + NH_4^+), and a considerable base peak $M^{++}/z = 221$ (442/2). The fragmentation found was also consistent with the structure proposed.

Compound 1 differs from 1'-O-ß-glucopyranosyl-(2s,3r)-3-hydroxycoumarin, described in the literature[7], on account of the different position of D-glucose which, as previously stated, is bound to OH on C3 of the nucleus 2,3-dihydro-furancoumarin instead of C'-1. However, it is important to note that, although the absolute configuration of this molecule was not resolved, only the two enantiomers (for which H2 and H3 are in cis, from the NMR data) can be proposed, rather than the four theoretically possible ones.

Compound 2 (Table I) has four aromatic signals, corresponding to H3, H4, H5 and H6. All are doublets, because of the evident influence in ortho (J = 10Hz), which indicates that positions 7 and 8 of the coumarin ring are occupied by substituents. The ^{1}H-NMR and ^{13}C-NMR spectra were very similar to those obtained for 7-O-ß-D-glucopyranosyl-1-(2',3'-dihydroxy-3'-methyl-)-butylcoumarin[2]. The most marked difference is the different position of D-glucose in the isobutyl-diol chain. In Compound 2, two singlets relating to two geminal-dimethyl groups ($\delta = 1.32$ and 1.24 ppm) are visible, while in the case reported by Cerri et al.[2] only one $\delta = 1.33$ signal appeared, as six hydrogen atoms.

Mass spectroscopy data (C.I. + NH_3) confirmed the expected molecular ion 444 u.m.a. (426 + NH_4^+) and fragmentation consistent with the structure hypothesized.

Compound 3 (Table I) yielded an aglycon by hydrolysis, with identical ^{1}H-NMR and ^{13}C-NMR spectra and MS data consistent with the structure of tert-O-ß-D-glucopyranosyl-R-heraclenol, as described by Thastrup O. et al.[8]. However, in the absence of polarimetric data which could confirm both identification and also absolute configuration, the komalin identified as the racemic structure of the aglycon was taken as evidence.

Like compound 3, compound 4 produces the same aglycon by acid or enzyme hydrolysis (Table I). However, the ^{1}H-NMR spectra of the two glucosides 3 and 4 differ on account of the double singlet of the two geminal- methyls ($\delta = 1.12$ and 1.17) found in 4, compared with the single signal on the same trace in compound 3 ($\delta = 1.33$). Other differences are shifts in the area between $\delta = 3.0$ and 4.5 ppm, related to hydrogen atoms in the prenyl chain. Since D-glucose binds to the OH of this chain, compounds 3 and 4 differ from each other both because of the different position of the glucoside bond on the genin (which explains the difference in the multiplicity of geminal methyl signals) and on account of the different alignment of the bond between genin and glucose, on the same chiral carbon atom.

However, in view of the marked difference in retention times between the two glucosides observed during HPLC separation, it may reasonably be assumed that there are two separate molecules rather than two enantiomers. This is supported by the well-known difficulty in separating two enantiomers of the same racemic mixture, even when appropriate chiral phases are used. The frequency which characterizes the stereo-specificity of biogenetic synthesis in natural compounds is also well known.

In the context of our research, we naturally did not take into account the many times compound 3 had been identified as tert-O-ß-D-glucopyranosyl-(R)-heraclenol by Tharstrup *et al.*[8].

3-O-β-D-glucopyranosyl-2-(-2'-hydroxy-2'-methyl-ethane-)-2,3(cis)-dihydrofuran-coumarin (1)

UV (MeOH) λ_{max} 324, 296 (sh), 258, 246.

^{1}HNMR (δ) (330 Mhz, MeOH-D_4) 7.95 (1H, d, J = 10.0 Hz, H-5), 7.85 (1H, s, H-4) 6.94 (1H, s, H-9), 6.27 (1H, d, J = 10.0 Hz, H-6), 5.73 (1H, d, J = 6.3 Hz,H-3) 4.55 (1H, d, J = 6.3 Hz, H-2); Double Res. 1719 Hz (5.73) ⇒ 4.54 (1H, s, H-2), 1.53 (3H, s, *gem-dimethyl*), 1.46 (3H, s, *gem-dimethyl*).

^{13}CNMR (δ) (MeOH-D_4); 165.0 (C-9a); 162.9 (C-7), 158.2 (C-8a), 145.9 (C-5), 127.9 (C-3a), 125.2 (C-4), 114.3 (C-4a), 112.8 (C-6), 100.1 (C-9), 99.3 (C-1''), 93.5 (C-2), 78.3 (C-3), 77.9 (C-5''), 75.9 (C-3''), 74.6 (C-2''), 72.3 (C-4''), 71.6 (C-1), 62.8 (C-6''), 26.4 e 26.7 (2x3H, s, *gem-dimethyl*).

MS-CI [NH_3](rel. abun.)m/z = 442 [426 + NH_4^+] (15), 390 (48), 262 (18), 221 [442/2] (100), 204 (80).

8-(-2'-O-β-D-glucopyranosyl-3'-methyl-3'-hydroxybutyl-)-Umbelliferone (2)

UV MeOH λ_{max} 324, 296 (sh), 260, 254, 214; + NaOH = shift bathocromic from 324 to 376.

^{1}HNMR (δ) (330 Mhz, MeOH-D_4): 7.84 (1H, d, J = 10.0 Hz, H-4), 7.32 (1H, d, J = 10.0 Hz, H-5), 6.80 (1H, d, J = 10.0 Hz, H-6), 6.18 (1H, d, J = 10.0 Hz, H-3) 2.95 ÷ 3.15 (3H, m, H1' and H2''), 1.34 (6H, s, *gem-dimethyl*),

^{13}CNMR (δ) (MeOH-D_4); 161.2 (C-2), 155.8 (C-7), 146.6 (C-4), 146.0 (C8a),128.0 (C-5), 116.2 (C-8), 113.5 (C-4a), 113.2 (C-6), 111.9 (C-5), 26.4 e 25.6 (2x3H, s, *gem-dimethyl*).

MS-CI [NH_3] (rel. abun.) m/z = 444 [426 + NH_4^+] (90), 426 (12), 282 (90), 264 (80), 246 (40)

3'-O-β-D-glucopyranosyl-Komaline (3)

UV MeOH λ_{max} 296, 262 (sh), 248.

^{1}HNMR (δ) (330 Mhz, MeOH-D_4): 8.15 (1H, d, J = 9.4 Hz, H-5), 7.90 (1H, d, J = 2.0 Hz, H-2), 7.58 (1H, s, H-4), 6.98 (1H, d, J = 2.0 Hz, H-3), 6.38 (1H, d, J = 9.4 Hz, H-6), 4.65 (1H, m, H-1'a), 4.40 (1H, m, H-1'b), 4.02 (1H, m, H-2'), 1.25 (6H, s, *gem-dimethyl*),

^{13}CNMR (δ) (MeOH-D_4); 160.0 (C-7); 148.5 (C-2), 147.5 (C-9a), 145.2 (C-5), 143 (C-8a), 133.5 (C-9), 127.8 (C-3a), 116.8 (C-4a), 113.6 (C-4), 114.0 (C-6), 107.0 (C-3), 77.0.(C1' o C2''), 77.1 (C1' o C2''), 23.8 e 21,6 (2x3H, s, *gem-dimethyl*).

MS-CI [NH_3] (rel. abun.) m/z = 484 [466 + NH_4^+] (100), 322 (15), 282 (18), 220 (15), 180 (35).

2'-O-b-D-glucopyranosyl-Komaline (4)

UV MeOH λ_{max} 296, 262 (sh), 248.

^{1}HNMR (δ) (330 Mhz, MeOH-D_4): 8.15 (1H, d, J = 9.4 Hz, H-5), 7.90 (1H, d, J = 2. Hz, H-2), 7.58 (1H, s, H-4), 6.98 (1H, d, J = 2.0 Hz, H-3), 6.38 (1H, d, J = 9.4, (H-6), 4.40 (1H, m, H-1'b) 3.96 (1H, m, H-2'), 1.25 (6H, s, *gem-dimethyl*),

^{13}CNMR (δ) (MeOH-D_4); 160.0 (C-7); 148.5 (C-2), 147.5 (C-9a), 145.2 (C-5), 143 (C-8a), 133.5 (C-9), 127.8 (C-3a), 116.8 (C-4a), 113.6 (C-4), 114.0 (C-6), 107.0 (C-3), 77.0. (C1' o C2''), 77.1 (C1' o C2''), 23.8 e 21,6 (2 × 3H, s, *gem-dimethyl*).

MS-CI [NH_3] (rel. abun.) m/z = 484 [466 + NH_4^+] (65), 466 (12), 322 (100), 180 (10).

Biological activity

The curve of platelet aggregation induced by ADP 0.5M, 3.0M, collagen and arachidonic acid showed that all the substances tested, in particular the latter two, had some inhibitory action on platelet aggregation. No inhibitory action was observed when aggregation was induced by Ionophore A23187.

When compound **1** was used, the Maximum Amplitude (M.A.) value was slightly reduced with all inductors, while the slope of the curves showed a significant reduction, indicating a slowing down of the process of aggregation induction (Figs. 3 and 4). This result was confirmed by an increase in the Latent Period.

Inhibitory action was observed particularly in compounds **2** and **3**. All parameters were reduced, especially M. A., Slope and Latent Period when platelet aggregation was induced by arachidonic acid and collagen. However, some aggregation produced by collagen remained, possibly due to the flow of calcium from the outside to the inside of the cell which, in this case, may be the major cause of aggregation.

It is known that a break in the process of

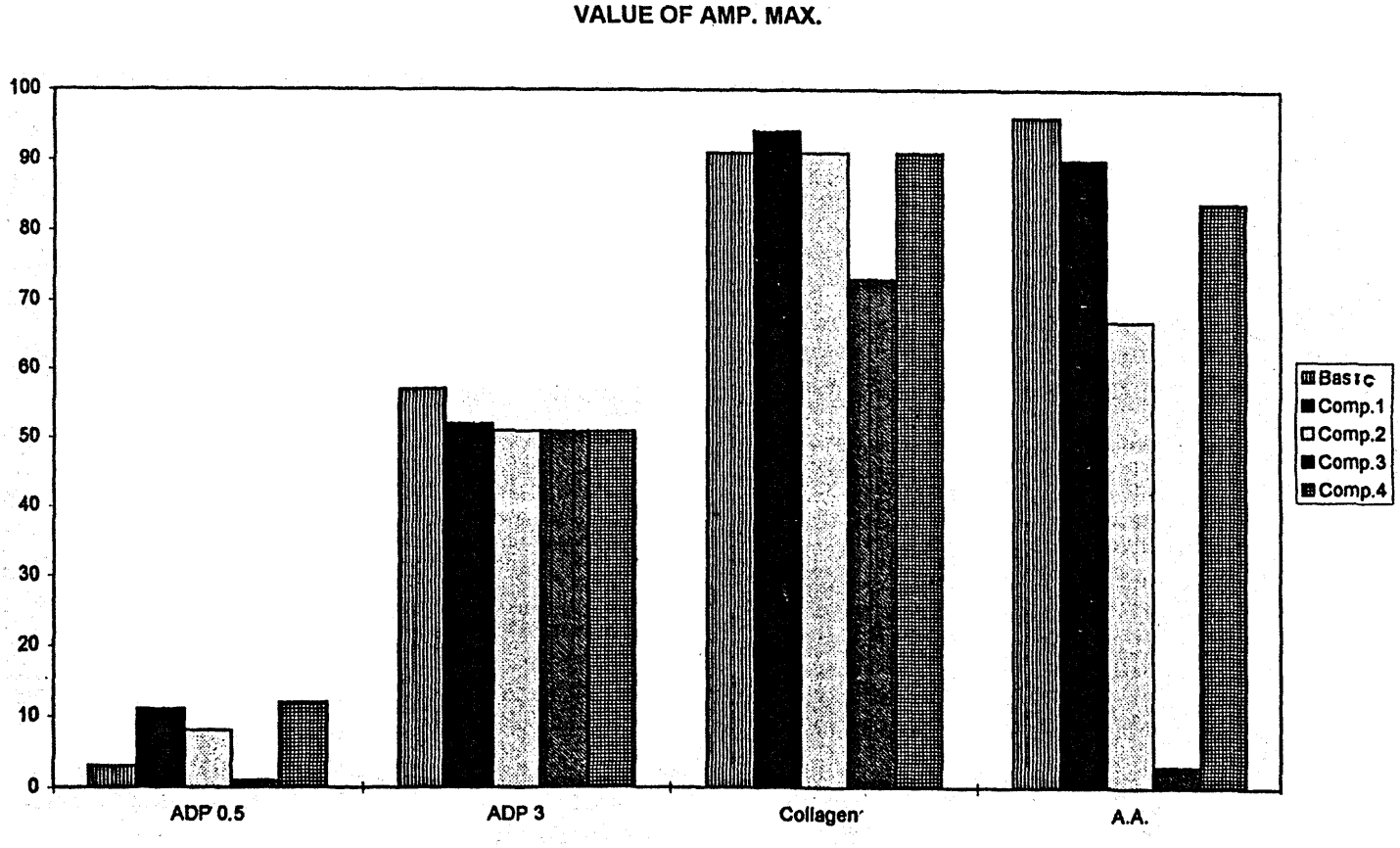


Fig. 3

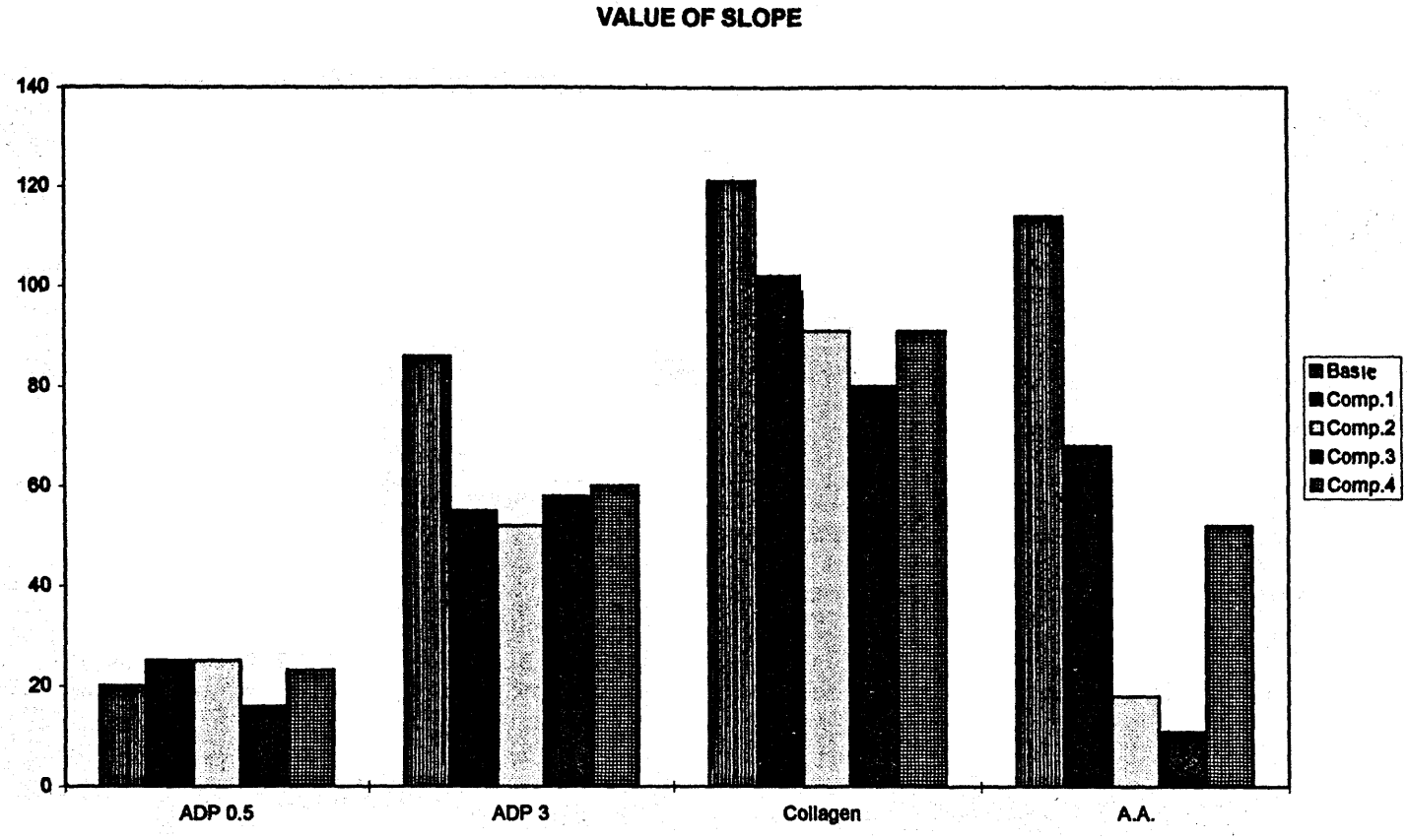

VALUE OF SLOPE
140
120
100
80
60
40
20
0
ADP 0.5
ADP 3
Collagen
A.A.
Basic
Comp.1
Comp.2
Comp.3
Comp.4

Fig. 4

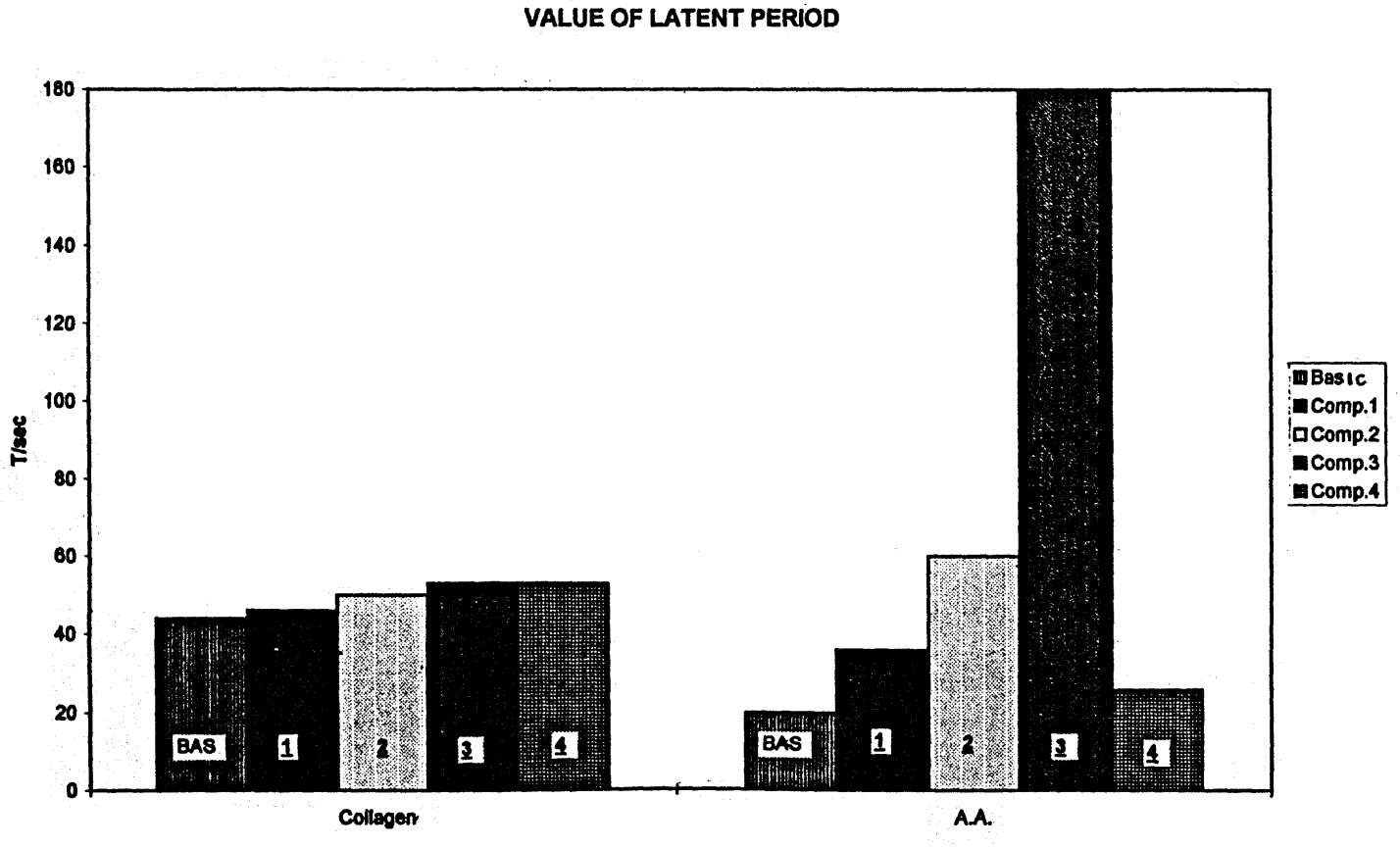

VALUE OF LATENT PERIOD
T/sec
180
160
140
120
100
80
60
40
20
0
BAS
1
2
3
4
Collagen
A.A.
Basic
Comp.1
Comp.2
Comp.3
Comp.4

Fig. 5

metabolizing arachidonate does not usually cause complete inhibition of aggregation induced by collagen. This supports the assumption that the substances tested act specifically on the intraplatelet metabolism of arachidonic acid.

CONCLUSIONS

The compounds isolated and characterized (three of which have not yet been described in the literature, particularly **1, 2** and **4**) showed some antiaggregation activity. Compound **3** was particularly active, as shown in the test to assess the Latent Period (Fig. 5). We intend to carry out further experiments to determine activity on the part of the 7-O-ß-D-glucopyranosyl-8-(-2,3 dihydroxy-3-methyl-butylcoumarin)[2] molecule, already isolated by us, and if we had had greater quantities of the isolated compounds, we would have performed dose response curves. Other studies will be carried out to characterize the compound (RT = 87.84 min), not yet available in sufficient quantities to perform recognition tests and determine pharmacological activity.

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