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IN VITRO ANTICOMPLEMENTARY ACTIVITY OF CONSTITUENTS FROM *MORINDA MORINDOIDES*

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ABSTRACT.—In a screening program for complement classical pathway modulation, an 80% MeOH extract of the leaves of *Morinda morindoides* showed potent dose-dependent anticomplementary activity. Bioassay-guided chromatographic separation of the active constituents led to the isolation of ten flavonoids of which two were aglycones. The compounds were tested in vitro for their putative complement-inhibiting properties on the classical (CP) and the alternative (AP) pathways of the complement system. The results indicated that quercetin [**1**], quercetin 3-*O*-rhamnoside (quercitrin) [**5**], and quercetin 3-*O*-rutinoside (rutin) [**7**] showed similar anticomplementary activities (inhibition) on the CP of complement. A mixture of two kaempferol triglycosides isolated and denoted as M_{015}, also had a good inhibitory effect. The effects of these compounds were dose-dependent for this pathway. On the AP of complement, quercetin [**1**] and M_{015} had, respectively, more pronounced inhibitory and activatory effects than the other tested flavonoids, but their effects were not dose-dependent for this pathway. The other isolated flavonoids showed weak effects or were inactive for both pathways.

The human complement system plays an important role in the host defense system against foreign invasive organisms such as bacteria, fungi, and viruses. Activation of the system may contribute to or evoke pathologic reactions in a variety of inflammatory and degenerative diseases, for example, various hemolytic anemias, dermatological diseases, rheumatoid arthritis, gout, and microbial infections (1–3). Moreover, its effects are normally beneficial for the host, but they can also cause adverse effects depending on the site, extent, and duration of complement activation. Therefore, the modulation of complement activity can be important (3).

Medicinal plants with anti-inflammatory properties are considered as good sources for some kinds of regulators of the complement system (4–7). As part of a systematic study in our laboratory to detect medicinal plants and/or natural products with anticomplementary activity, the complement-inhibiting properties of the leaves of *Morinda morindoides* (Baker) Milne Redh. (Rubiaceae) and of the isolated active compounds have been investigated.

A decoction of the leaves of *M. morindoides* is used in Zairese traditional medicine against various diseases such as malaria, amoebiasis, scabies, hemorrhoids, worms, and gonorrhea. Similarly, decoctions of this same plant part are also used for the treatment of patients (particulary children) recovering from surgery or suffering from chronic disease to promote improvement of their debilitated general condition (8,9).

RESULTS AND DISCUSSION

An initial 80% MeOH extract of the leaves of *M. morindoides* (MMS-1), as well as the EtOAc (MMS-2) and the *n*-BuOH (MMS-3) fractions from this MeOH extract, inhibited the classical pathway (CP) of the complement (C) system in a dose-dependent way. Fraction MMS-2 showed a more pronounced complement inhibition than fraction MMS-3 (Figure 1), as measured by the diminished hemolysis of sensitized erythrocytes in the presence of complement factors (Figure 2). At high dilutions (1/640 to 1/1280),

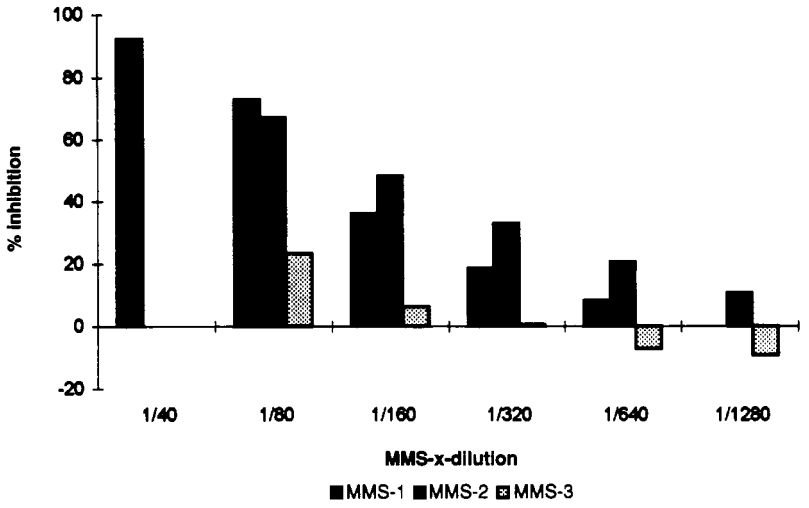


FIGURE 1. Inhibitory effect of extracts of *M. morindoides* on complement activity.

it was also observed that fraction MMS-3 lost its inhibitory effect and became a slight activator of complement activity. This activity is not significant because its influence on complement activity was very low (Figure 2).

Thus, it was concluded that these crude materials contained active anticomplementary components of greatest potency in the EtOAc fraction. Activity-guided fractionation of MMS-2 and MMS-3 was performed based on the effects on the CP and the AP pathways of the complement system. From them, ten flavonoids were isolated (Figure 3) and their anticomplementary activity was assessed in vitro.

As shown in Table 1, quercetin [**1**], quercetin 3-*O*-rhamnoside [**5**], and quercetin 3-*O*-rutinoside [**7**], followed by the mixture M_{015}, showed a higher inhibitory effect towards the CP of C than the other flavonoids at the same concentration. Quercetin 7,4′-dimethyl ether [**2**] and luteolin 7-*O*-glucoside [**3**] were moderately active and kaempferol 3-*O*-rutinoside [**8**] was weakly active, while the other compounds were all inactive. On

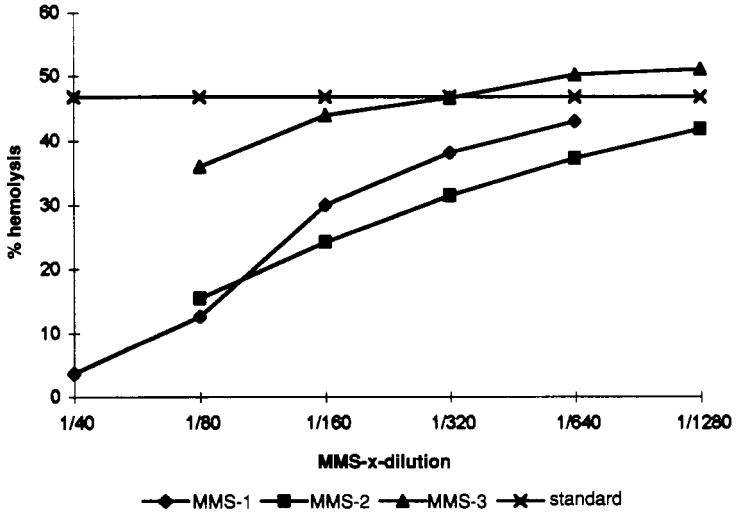


FIGURE 2. Inhibitory effect of extracts of *M. morindoides* on complement hemolytic activity.

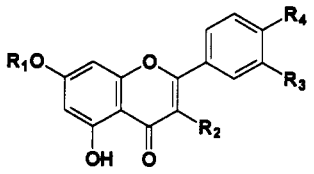
TABLE 1. Anticomplementary Activity of Flavonoids from *M. morindoides*.[a]

Compound/Fraction	Percent Inhibition[b]	
	CP[c]	AP[c]
Quercetin [**1**]	52.5 ± 1.5	24.0 ± 6.4
Quercetin 7,4′-dimethyl ether [**2**]	24.3 ± 5.3	13.7 ± 5.5[e]
Luteolin 7-*O*-glucoside [**3**]	25.2 ± 8.9	2.0 ± 7.0
Apigenin 7-*O*-glucoside [**4**]	1.9 ± 2.34[e]	2.4 ± 4.8[e]
Quercetin 3-*O*-rhamnoside [**5**]	56.2 ± 2.3	13.5 ± 4.2
Kaempferol 3-*O*-rhamnoside [**6**]	5.6 ± 2.2	6.2 ± 3.7
Quercetin 3-*O*-rutinoside [**7**]	54.4 ± 8.1	18.0 ± 0.1
Kaempferol 3-*O*-rutinoside [**8**]	19.2 ± 8.3	18.4 ± 0.1[e]
Chrysoeriol 7-*O*-neohesperidoside [**9**]	3.9 ± 4.6[e]	11.2 ± 4.0[e]
Chrysoeriol[d] [**10**]	9.0 ± 3.0[e]	59.0 ± 2.0[e]
M_{015}	40.8 ± 8.6	56.1 ± 4.5[e]
MFL-22 (**1–6**)	77.1 ± 6.4	38.6 ± 16.3
MFL-23 (**7–9**+M_{015})	43.1 ± 4.7	23.3 ± 15.2[e]
Rosmarinic acid (control)	93.3 ± 0.1	67.8 ± 3.8

[a]Concentration 10^{-3} M.
[b]Data are expressed as mean ±S.E. of three experiments.
[c]For definition of terms see text.
[d]Chrysoeriol [**10**] was obtained by acid hydrolysis of **9**.
[e]Denotes activation.

the alternative pathway (AP) of complement (C) system, compound **1** showed moderate inhibition and compounds **5** and **7** showed weak inhibition of this pathway at 10^{-3} M. Compounds **3**, **4**, and **6** were considered inactive, while the other flavonoids showed some activation of the complement activity at this tested concentration. Concerning this last observation, M_{015} and chrysoeriol [**10**] were found to have relatively higher effects on activation, followed by **8**, **2**, and **9**, in decreasing order.

Table 1 also shows that the anticomplementary activity of each compound obtained is lower than the activity of the respective fraction from which it was isolated. MFL-22 (a mixture of flavonoids **1–6** prepared in the proportions in which they were isolated in



	R_1	R_2	R_3	R_4
1	H	OH	OH	OH
2	CH_3	OH	OH	OCH_3
3	Glc	H	OH	OH
4	Glc	H	H	OH
5	H	O-Rha	OH	OH
6	H	O-Rha	H	OH
7	H	O-[Rha-(1→6)-glc]	OH	OH
8	H	O-[Rha-(1→6)-glc]	H	OH
9	Rha-(1→2)-glc	H	OCH_3	OH
10	H	H	OCH_3	OH

FIGURE 3. Structural relations of the test compounds listed in Table 1 (rha=rhamnose, glc=glucose).

the plant and were present in fraction MMS-2) exhibited a strong inhibitory effect (77.1±6.4%) on the CP of C system and a moderate inhibitory effect (38.6±16.3%) on the the AP of C system. This observation suggests possible synergistic effects between these flavonoids. On the other hand, MFL-23, prepared as described above (a mixture of flavonoids **7–9**+M_{015} isolated from MMS-3) showed an inhibitory effect (43.1±4.7%) on the CP of C and an activation effect on the AP of C (23.3±15.2%). This last effect was lower than that of M_{015}, and higher than those of **8** and **9**.

In order to determine whether or not the anticomplementary activity was dose-dependent, compounds **1**, **5**, **7**, M_{015}, and rosmarinic acid, MFL-22, and MFL-23 were diluted 2,4,...16 times with the corresponding buffer solution. Figures 4 and 6 illustrate the dose-dependent profile of the effects on the CP of C and Figures 5 and 7 show clearly that the inhibiting effect is not dose-dependent towards the AP of C. These figures also show that the anticomplementary activity of the tested flavonoids is in general lower than that of rosmarinic acid.

The present results of our screening system for complement on classical and alternative pathway modulation have indicated a correlation between the activity and the structures of quercetin derivatives. On the CP of C, the glycosylation of the OH

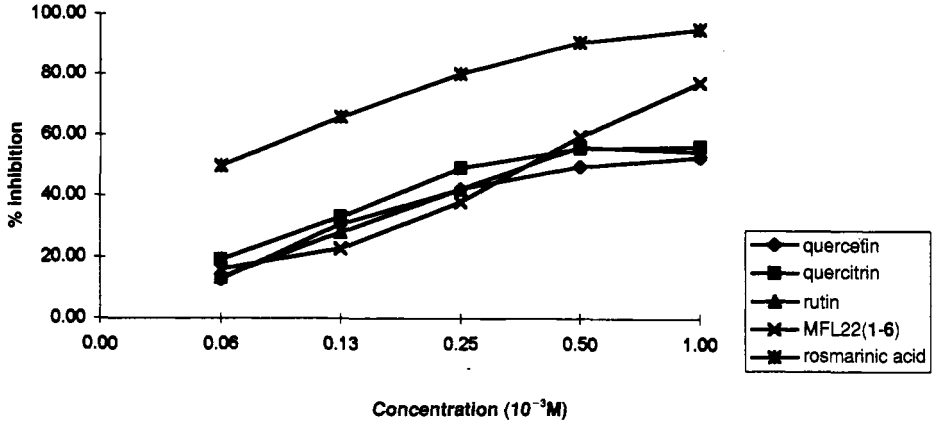


FIGURE 4. Inhibitory effect of flavonoids from MMS-2 and rosmarinic acid on the CP of C.

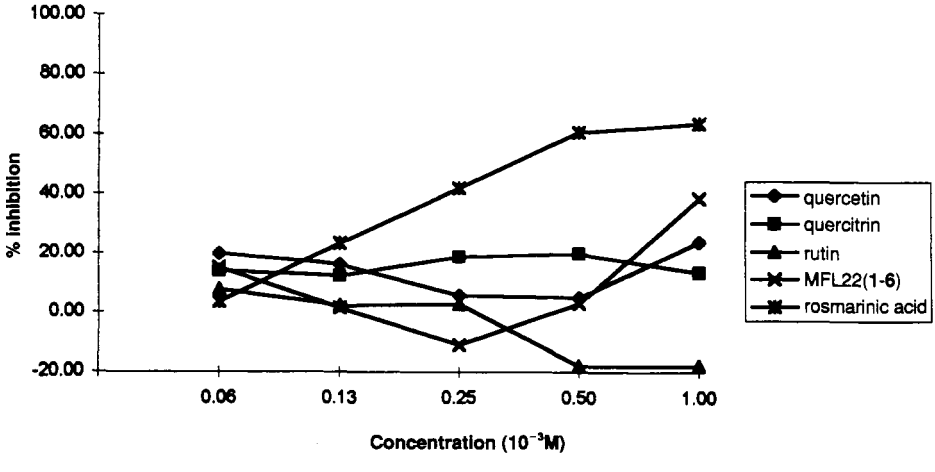


FIGURE 5. Anticomplementary activity of flavonoids from MMS-2 and rosmarinic acid on the AP of C.

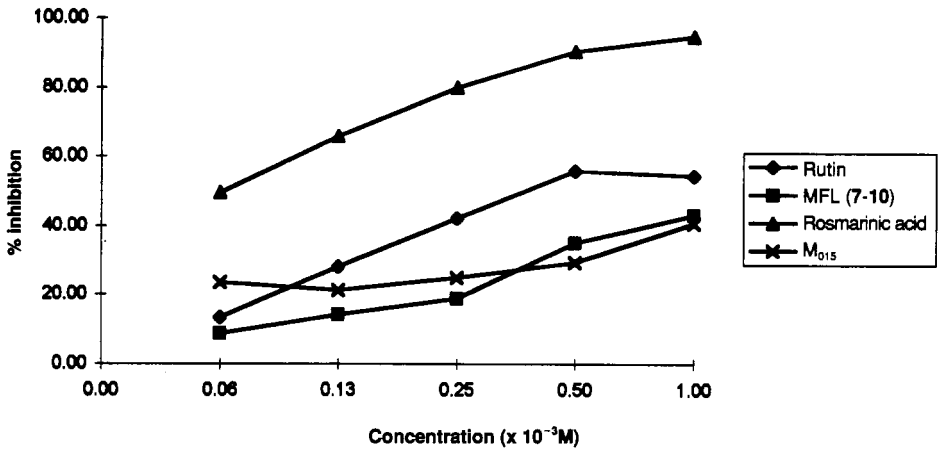


FIGURE 6. Inhibitory effect of flavonoids from MMS-3 and rosmarinic acid on the CP of C.

group at the C-3 position enhanced the inhibitory effect according to the nature of the sugar. Methoxylation at the C-7 and C-4′ positions appears to decrease this effect. However, on the AP of C, the presence of a free OH at the C-3 position is necessary for the observation of an inhibitory effect. Glycosylation at C-3 decreases this effect, depending also on the nature of the sugar, whereas methoxylation at the C-7 and C-4′ positions favors an activation effect. Both kaempferol and quercetin derivatives belong to the flavonol group, for which the inhibitory effect increased with the number of hydroxylated groups in ring B, with glycosylation at the C-3 position playing a secondary role, as the anticomplementary activity depends on the nature of the aglycone. Free hydroxyl groups in ring A at C-5 and C-7 and/or at the C-3 position are involved in mediating an inhibitory effect on the complement system. For the flavone group, samples were limited, but it was observed that a hydroxyl group in the C-5′ position (ring B) is necessary for anticomplement activity (inhibitory) on the CP of C (e.g., activity of luteolin 7-*O*-glucoside compared to that of apigenin-7-*O*-glucoside). The presence of a methoxyl group in the C-5 position caused activation of both pathways (e.g., chrysoeriol). This activity was decreased with glycosylation at the C-7 position (e.g., chrysoeriol 7-

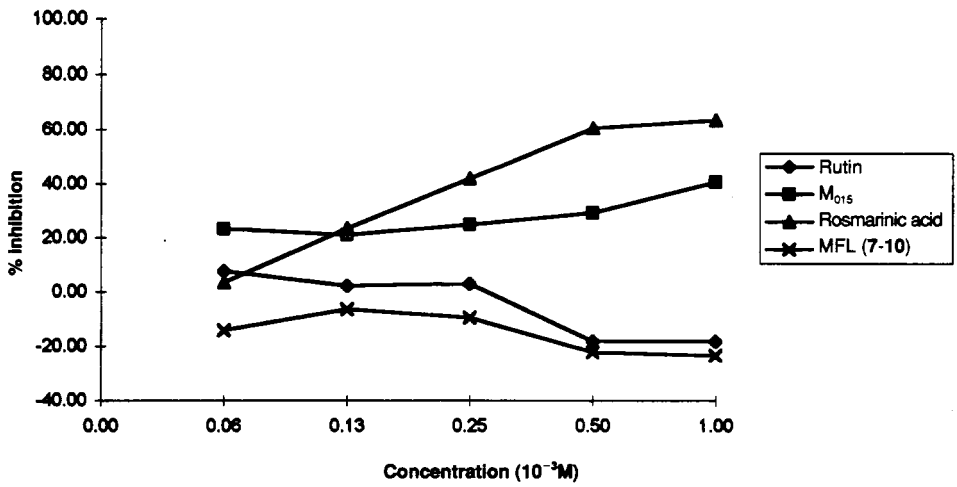


FIGURE 7. Anticomplementary activity of flavonoids from MMS-3 and rosmarinic acid on the AP of C.

O-neohesperidoside). Some of our observations on the structure-activity relationship of flavonoids are in agreement with those made by Lasure *et al.* (10).

In conclusion, flavonoids isolated from the leaves of *M. morindoides* were found to contribute to its anticomplementary activity. However, the presence of other putative active compounds in this plant cannot be excluded.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Chromatographic methods including cc, tlc, and prep. tlc, the solvent systems used for the isolation and purification, and the spectroscopic techniques used for the identification of these flavonoids are detailed by Cimanga *et al.* (12).

PLANT MATERIAL.—Leaves of *M. morindoides* (Baker) Milne Redh. (Rubiaceae) were collected in Kinshasa, Zaire, and the plant was identified by Mr. Breyne of the Institut National d'Etudes et de Recherches Agronomiques (INERA) of the University of Kinshasa, where a voucher specimen has been deposited.

EXTRACTION AND ISOLATION.—Dried and powdered leaves (500 g) were defatted by Soxhlet extraction with *n*-hexane. The dried residue was macerated and percolated exhaustively with 80% aqueous MeOH. The percolate was concentrated to dryness under reduced pressure and denoted MMS-1. The residue was dissolved in hot H_2O and filtered after 24 h. The filtrate was extracted successively with $CHCl_3$, EtOAc, and *n*-BuOH, and these fractions were designated MMS-10, MMS-2, and MMS-3, respectively, after evaporation of solvent. All flavonoids except chrysoeriol [**10**] were isolated from fractions MMS-2 and MMS-3 according to the procedure described elsewhere (11,12). Rosmarinic acid was isolated from *Apeiba tibourbou* by the Antwerp group (13) and was used as an anticomplementary reference product.

IN VITRO ANTICOMPLEMENTARY ACTIVITY.—*Preparation of test samples.*—Each extract (50 mg) was dissolved in MeOH and diluted 20, 40, 80,...1280 times with barbital buffer to test the classical pathway. The highest compound concentration obtained in each tested solution was 10^{-3} M.

Complement tests.—Classical (CP) and alternative (AP) pathway of complement (C) activities were assessed based on the procedures described by Mayer (14) and Platts-Mills *et al.* (15), respectively. For CP, 125 μl of 1% sheep erythrocytes (Bio-Mérieux No. 7 214 1) were sensitized by pre-incubation (37°, 10 min) with 125 μl 1/400 anti-sheep haemolysin (Bio-Mérieux No. 7 220 2). After adding 250 μl of barbital buffer solution containing the test compound, a second pre-incubation was carried out (37°, 15 min). Afterwards, 125 μl of guinea pig complement (Bio-Mérieux No. 7 212 1) (dilution C/50-C/1000) were added and the reaction mixture was incubated (37°, 60 min).

On the other hand, for the AP, 200 μl of a 1% suspension of non-sensitized rabbit erythrocytes (RaRBC) (Bio-Mérieux No. 7 229 1) and 250 μl of AP-CFTD buffer containing test compound were pre-incubated (37°, 60 min), together with 200 μl of plasma diluted 1/5–1/125 (fresh human plasma obtained from healthy volunteers).

Both reactions were stopped by adding 4 ml of an EDTA solution. The reaction mixture was then centrifuged (1600 g, 20 min). Absorbances in the hemolytic assay were measured on a Uvikon 931 spectrophotometer at 412 nm. Each absorption value determination was the mean of triplicate experiments. Buffer solutions were composed as follows: barbital buffer (pH 7.3): 0.145 M NaCl; 4 mM diethylbarbituric acid; 0.25 mM Ca^{2+}, 0.83 mM Mg^{2+}, 0.1% gelatin and 0.02% NaN_3, EDTA buffer (pH 7.3): 10 mM EDTA, 4 mM diethylbarbituric acid, 0.154 mM NaCl, 0.1% gelatin, 0.02% NaN_3; AP-CFTD buffer (pH 7.3): 0.145 mM NaCl, 4 mM diethylbarbituric acid, 7 mM Mg^{2+}, 10 mM EGTA, 0.1% gelatin, and 0.02% NaN_3.

The standard was constituted by erythrocytes with complement (dilution C/300) in buffer solution. This dilution of complement was used in order to obtain ±50% hemolysis in our experimental conditions. A first control consisted of erythrocytes incubated with H_2O (100% hemolysis), and a second control consisted of erythrocytes sensitized (for CP) or erythrocytes non-sensitized (for AP) incubated in corresponding buffer.

The anti-complementary activity was expressed as the influence of the test compound upon the CH_{50} or AP_{50}-value respectively for CP or AP of complement, which means the amount of complement necessary to lyse half of a given quantity of erythrocytes under optimal conditions (16).

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