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Radical scavenging and xanthine oxidase inhibitory activity of phenolic compounds from *Bridelia ferruginea* stem bark

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Abstract

Bridelia ferruginea Benth. (Euphorbiaceae) is a subtropical medicinal plant widely used in traditional African medicine against various diseases, including rheumatic pains. Seven of its constituents (3-*O*-methylquercetin (**1**), 3,7,3′,4′-tetra-*O*-methylquercetin (rutisin, **2**), myricetin (**3**), 3′,4′,5′-tri-*O*-methylmyricetin (ferrugin, **4**), 3,3′,4′,5′-tetra-*O*-methylmyricetin (**5**), quercetin 3-*O*-glucoside (**6**), and a biflavanol gallocatechin-[4′-*O*-7]-epigallocatechin (**7**)) have been evaluated in-vitro in the xanthine-xanthine oxidase enzymatic system for inhibition of xanthine oxidase and radical scavenging activity. Results indicated that compounds **1**, **3**, **4** and **6** exhibited, at different levels, xanthine oxidase inhibiting and superoxide scavenging activity at micromolar concentrations, whereas compound **7** showed scavenging activity only. Compounds **2** and **5** were inactive in both cases. Study of the structure–activity relationship demonstrated that for flavonoids the xanthine oxidase inhibitory activity was reduced by methylation of the hydroxyl functionality at C-3 and in rings A and B. These results may partly explain and support the use of *B. ferruginea* stem bark for the treatment of rheumatic pains in traditional medicine.

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Introduction

The oxygen involved in the respiratory process can be transformed under some conditions into superoxide anion, hydroxyl radical, singlet oxygen and hydrogen peroxide (Pignol et al 1988). These reactive oxygen species are implicated in some diseases such as inflammation, cancer, ageing, anaemia and degenerative diseases (Halliwell & Gutteridge 1989).

Superoxide anions are produced by several oxidative systems, especially the xanthine–xanthine oxidase system. Xanthine oxidase is an important enzyme which catalyses the oxidation of hypoxanthine to xanthine and then to uric acid in man. The accumulation of uric acid can not only lead to hyperuricaemia and gout but can also provoke inflammation by various mechanisms such as the stimulation of a respiratory burst in neutrophils and production of leukotriene B_4 (Kerher & Smith 1994). Inhibition of superoxide anion production should lead to a decrease of H_2O_2, and also of HOCl and, possibly, OH radicals implicated in inflammation (Halliwell & Gutteridge 1989). This suggests that a compound which inhibits superoxide anion production could have a beneficial effect not only in the treatment of hyperuricaemia and gout, but also in the alleviation of inflammation.

Bridelia ferruginea Benth. (Euphorbiaceae) is a subtropical medicinal plant widely used in traditional African medicine. In Congo-Kinshasa, an aqueous decoction of the stem bark is used to treat diarrhoea, dysentery, intestinal disorders, female sterility, and rheumatic pains. It is also used as a purgative and a vermifugal agent (Kambu 1990). The antimicrobial (Irobi et al 1994; Muanza et al 1994; Tona et al 1999), antispasmodic (Onuruvwe et al 1994; Tona et al 1999) and anti-HIV (Muanza et al 1995) activities of crude extracts from the stem bark are described without isolation of the active constituents. An aqueous decoction of the leaves is used for the treatment of diabetes (Iwu 1983). Some flavonoids were isolated from this part of the plant (Iwu 1980; Addae-Mensah & Achenbach 1985), among which rutin (rutoside) was claimed to be responsible for the hypoglycaemic effect (Addae-Mensah & Munenge 1989). This study reports the inhibitory effect on xanthine oxidase, as well as the superoxide scavenging activity of some phenolic compounds from *B. ferruginea* stem bark, isolated as reported previously (De Bruyne et al 1997; Cimanga et al 1999a).

Materials and Methods

Plant material

The stem bark of *B. ferruginea* was collected in Congo-Kinshasa in December 1989. The plant was identified by Mr Breyne of the Institut National d'Etudes et de Recherches en Agronomie (INERA) of the University of Kinshasa, where a voucher specimen was deposited (INERA 891204). The stem bark was dried under 40°C and reduced to powder.

Isolation, purification and structure elucidation

General experimental procedures detailed for the extraction, isolation, purification and structure elucidation of polyphenolic compounds from *B. ferruginea* stem bark are described in previous reports (De Bruyne et al 1997; Cimanga et al 1999a). Allopurinol, xanthine and xanthine oxidase were purchased from Sigma (USA).

Assay of xanthine oxidase inhibition and superoxide scavenging activity

The inhibition of xanthine oxidase and the superoxide anion scavenging activity were assessed in-vitro in one assay. The inhibition of xanthine oxidase activity was evaluated by measuring the UV absorbance at 290 nm, while the superoxide anion scavenging activity was detected spectrophotometrically by the nitrite method as by Cimanga et al (1999b). Briefly, the assay mixture consisted of 100-μL test solution of compound, 200 μL xanthine solution (final concentration 50 μM) and hydroxylamine (final concentration 0.2 mM), 200 μL EDTA (0.1 mM), 300 μL distilled water. The reaction was initiated by adding 200 μL xanthine oxidase (final concentration 1.25 mU mL^{-1}) dissolved in phosphate buffer solution (0.2 M, pH 7.5). The assay mixture was incubated at 37°C for 30 min. Before measuring the uric acid production at 290 nm, the reaction was stopped by adding 0.1 mL 0.58 M HCl. The absorbance was measured spectrophotometrically against a blank solution prepared in the same way as described above, replacing xanthine oxidase by buffer solution (no production of uric acid). A control solution without test compound was prepared in the same manner as the assay mixture to measure the total uric acid production. Allopurinol was used as a positive control. The uric acid production was calculated from the differential absorbance. To detect the superoxide scavenging activity, 2 mL of the colouring reagent consisting of sulfanilic acid solution (final concentration 300 μg mL^{-1}), *N*-(1-naphthyl)ethylene diamine dihydrochloride (final concentration 5 μg mL^{-1}) and acetic acid (16.7%, v/v) was added after incubation of the test mixture. After 30 min at room temperature, the absorbance was measured at 550 nm. The IC50 values were calculated by regression analysis.

Statistical analysis

Student's *t*-test was used to compare results of different samples. Statistical significance was set at $P = 0.05$.

Results

The interaction of methylated and glycosylated quercetin derivatives, myricetin and its methylated derivatives, and a biflavanol isolated from *B. ferruginea* stem bark with the xanthine–xanthine oxidase enzyme system have been tested. Results indicated that 3-*O*-methylquercetin (**1**), myricetin (**3**), 3′,4′,5′-tri-*O*-methylmyricetin (ferrugin, **4**) and quercetin 3-*O*-glucoside (**6**) were inhibitors of xanthine oxidase (IC50 = 27.3 ± 3.8, 2.41 ± 0.21, 9.2 ± 2.3 and 20.3 ± 1.7 μM, respectively) and

Table 1 Activity of compounds (**1**–**7**) from *B. ferruginea* and (**8**–**11**) from *M. morindoides* as inhibitors of xanthine oxidase and scavengers of superoxide anion.

Compounds	Inhibition of xanthine oxidase activity (IC50, μM)	Scavenging activity (IC50, μM)
3-*O*-Methylquercetin (**1**)	27.3 ± 3.8	15.7 ± 2.2
3,7,3′,4′-Tetra-*O*-methylquercetin (**2**)	> 100	> 100
Myricetin (**3**)	2.41 ± 0.21	0.43 ± 0.02
3′,4′,5′-Tri-*O*-methylmyricetin (**4**)	9.2 ± 2.3	5.8 ± 1.4
3,3′,4′,5′-Tetra-*O*-methylmyricetin (**5**)	> 100	> 100
Quercetin 3-*O*-glucoside (**6**)	20.3 ± 1.7	7.5 ± 2.6
Gallocatechin-[4′-*O*-7]-epigallocatechin (**7**)	> 100	2.01 ± 0.03
Quercetin (**8**)	2.60 ± 0.05	1.63 ± 0.02
7,4′-Di-*O*-methylquercetin (**9**)	7.7 ± 0.8	2.60 ± 0.10
Quercetin 3-*O*-rhamnoside (**10**)	> 100	8.1 ± 0.3
Quercetin 3-*O*-rutinoside (**11**)	52.2 ± 0.4	10.6 ± 1.2
Allopurinol	0.24 ± 0.06	0.23 ± 0.06

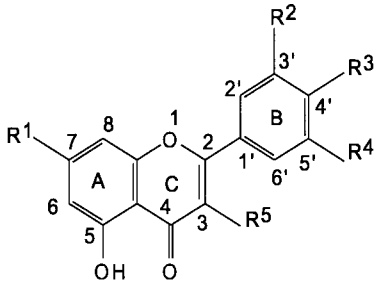
Values (mean ± s.d., n = 3) are IC50 (μM), the concentration resulting in 50 % inhibition.

scavengers of superoxide anion (IC50 = 15.7 ± 2.2, 0.43 ± 0.02, 5.8 ± 1.4 and 7.5 ± 2.6 μM, respectively) (Table 1). The biflavanol gallocatechin-[4′-*O*-7]-epigallocatechin (**7**) showed only a scavenging activity (IC50 = 2.01 ± 0.03 μM). 3,7,3′,4′-Tetra-*O*-methylquercetin (rutisin, **2**) and 3,3′,4′,5′-tetra-*O*-methylmyricetin (**5**) were inactive in both cases at a higher tested concentration of 100 μM. Table 2 shows the structural relationship of the flavonoids listed in Table 1. A detailed structure–activity relationship demonstrated the influence of the position and the number of methoxyl groups, and the nature and the position of glycosyl residue on the manifestation of both activities.

Discussion

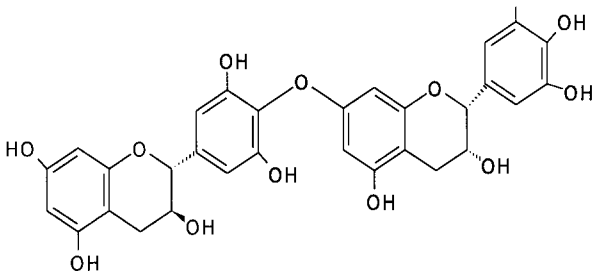
A previous bioassay-guided fractionation of an 80 % acetone extract from *B. ferruginea* stem bark, exhibiting a dose-dependent inhibitory effect towards the classical and the alternative pathways of the complement system, resulted in the isolation and identification of 3-*O*-methylquercetin (**1**), 3,7,3′,4′-tetra-*O*-methylquercetin (rutisin, **2**), myricetin (**3**), 3′,4′,5′-tri-*O*-methylmyricetin (ferrugin, **4**), 3,3′,4′,5′-tetra-*O*-methylmyricetin (**5**), quercetin 3-*O*-glucoside (**6**), a biflavanol gallocatechin-[4′-*O*-7]-epigallocatechin (**7**) and 3,5-dicaffeoylquinic acid and 3,4,5-tricaffeoylquinic acid as the active constituents (Cimanga et al 1999a). The effect of these compounds on xanthine oxidase activity in an enzymatic oxidation system of hypoxanthine to xanthine and to uric acid as the final product, and their influence on the production of superoxide anions ($O_2^{\cdot -}$) generated by this system were assessed in-vitro as described above. Quercetin (**8**), 7,4′-di-*O*-methylquercetin (**9**), quercetin 3-*O*-rhamnoside (**10**) and quercetin 3-*O*-rutinoside (**11**) isolated from the leaves of *Morinda morindoides* (Cimanga et al 1995) were included for a structure–activity relationship study.The IC50 values as inhibitors of xanthine oxidase and scavengers of superoxide anion are given in Table 1. Myricetin (**3**) and 3′,4′,5′-tri-*O*-methylmyricetin (ferrugin, **4**) exhibited an inhibitory activity on xanthine oxidase with an IC50 $\leqslant$ 10 μM. Compounds **1** and **6** showed the same effect with 20 < IC50 < 30 μM. Quercetin (**8**) and myricetin (**3**) were equally active ($P = 0.05$), and were the most active inhibitors of xanthine oxidase. Methylation, as in **4** and **9**, led to a decrease in activity ($P < 0.001$). All four compounds still had a free 3-OH group. When the IC50 value on xanthine oxidase of quercetin (**8**) (IC50 = 2.60 ± 0.05 μM), 3-*O*-methylquercetin (**1**) (IC50 = 27.3 ± 3.8 μM), or ferrugin (**4**) (IC50 = 9.2 ± 2.3 μM) was compared with **5** (IC50 > 100 μM), it was evident that this free 3-OH functionality was important for a high xanthine oxidase inhibiting activity. All compounds where the 3-OH group was methylated or glycosylated were less active. The nature of the glycosyl residue also influenced the xanthine oxidase inhibiting activity, quercetin 3-*O*-glucoside (**6**) (IC50 = 20.3 ± 1.7 μM) and quercetin 3-*O*-rutinoside (**11**) (IC50 = 52.2 ± 0.4 μM) being more active than quercetin 3-*O*-rhamnoside (**10**) (IC50 > 100 μM). Also rutisin (**2**) and the biflavanol (**7**) were completely inactive as inhibitors of xanthine oxi-

Table 2 Structural relationship of flavonoids listed in Table 1.



Compound	R_1	R_2	R_3	R_4	R_5
3-*O*-Methylquercetin (**1**)	OH	OH	OH	H	OCH_3
3,7,3',4'-Tetra-*O*-methylquercetin(**2**)	OCH_3	OCH_3	OCH_3	H	OCH_3
Myricetin (**3**)	OH	OH	OH	OH	OH
3',4',5'-Tri-*O*-methylmyricetin (**4**)	OH	OCH_3	OCH_3	OCH_3	OH
3,3',4',5'-Tetra-*O*-methylmyricetin (**5**)	OH	OCH_3	OCH_3	OCH_3	OCH_3
Quercetin 3-*O*-glucoside (**6**)	OH	OH	OH	H	O-Glucosyl
Quercetin (**8**)	OH	OH	OH	H	OH
7,4'-Di-*O*-methylquercetin (**9**)	OCH_3	OH	OCH_3	H	OH
Quercetin 3-*O*-rhamnoside (**10**)	OH	OH	OH	H	O-Rhamnosyl
Quercetin 3-*O*-rutinoside (**11**)	OH	OH	OH	H	O-Rutinosyl

Gallocatechin-[4'-*O*-7]-epigallocatechin (7)



dase (IC50 > 100 μM being the highest concentration tested).

In our test system, the inhibition of xanthine oxidase led to a decreased production of superoxide radicals. Only those compounds where the IC50 as radical scavenger was lower than the IC50 as inhibitor of xanthine oxidase had an additional scavenging effect and can be considered as true superoxide anion scavengers. Inhibitors of xanthine oxidase with an additional scavenging effect included 3-*O*-methylquercetin (**1**), myricetin (**3**), ferrugin (**4**), and quercetin 3-*O*-glucoside (**6**). The biflavanol (**7**) was a radical scavenger without any xanthine oxidase inhibitory effect. Methylation or glycosylation at C-3 significantly decreased the activity. Compound **3** was more active than **1**, **2**, **5** or **6**. The presence of methoxyl groups in ring B and a free hydroxyl group at C-3 also significantly diminished the activity ($P < 0.01$) (i.e. compound **3** compared with **9** and **4**). In an investigation of flavonoids from medicinal plants and commercial sources as inhibitors of xanthine oxidase and scavengers of superoxide anion, Cos et al (1998) classified the tested compounds into five categories noted A to F. According to the criteria of Cos et al (1998), our flavonoid samples, isolated from *B. ferruginea*, could be classified into: category A (compound **7**) of flavonoids as scavengers of superoxide anions without inhibitory activity on xanthine oxidase; category C (compounds **1**, **3**, **4** and **6**) including flavonoids showing an inhibitory

effect on xanthine oxidase and having an additional superoxide scavenging activity; and category F (compounds **2** and **5**) of flavonoids devoid of any effect as inhibitors of xanthine oxidase and scavengers of superoxide anion. Although 3,4-dicaffeoylquinic acid and 3,4,5-tricaffeoylquinic acid isolated from *B. ferruginea* stem bark were not tested in our enzymatic oxidation system, these quinic acid derivatives isolated from *Tetaria integrifolia* R.et P. and *Mikania cordifolia* Wild. (Asteraceae) produced significant decreases of superoxide anion production in another enzymatic model (Peluso et al 1995).

In relation to the use of the stem bark of *B. ferruginea* in the treatment of rheumatic pains, Olajide et al (1999) reported the anti-inflammatory activity of an aqueous extract. This extract was found to inhibit significantly the carrageenan-induced rat paw oedema at oral doses of 40–80 mg kg^{-1}, while in the mouse paw oedema test, the extract did not show a significant inhibitory effect.

Those results, together with the anti-complementary effect of a series of phenolic compounds we reported previously (Cimanga et al 1999a), and the results reported here, explain and support, at least partly, the traditional use of *B. ferruginea* stem bark extract for the treatment of rheumatic pains.

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