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Comparative inhibitory effect of prenylated coumarins, ferulenol and ferprenin, contained in the 'poisonous chemotype' of *Ferula communis* on mammal liver microsomal VKORC1 activity

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ABSTRACT

Two distinguishable chemotypes of *Ferula communis* have been described: the 'nonpoisonous' chemotype, containing as main constituents the daucane esters; and the 'poisonous' chemotype containing prenylated coumarins, such as ferulenol and ferprenin. Ferulenol and ferprenin are 4-oxygenated molecules such as dicoumarol and warfarin, the first developed antivitamin K molecules. Antivitamin K molecules specifically inhibit VKORC1, an enzyme essential for recycling vitamin K. This latest is involved in the activation of clotting factors II, VII, IX, X. The inhibiting effect of ferulenol on VKORC1 was shown in rat, but not for species exposed to *F. communis* while in vivo studies suggest differences between animal susceptibility to ferulenol. The inhibiting effect of ferprenin on VKORC1 was never demonstrated. The aim of this study was to compare the inhibiting effect of both compounds on VKORC1 of different species exposed to *F. communis*. Vitamin K epoxide activity was evaluated for each species from liver microsomes and inhibiting effect of ferulenol and ferprenin was characterized. Ferulenol and ferprenin were shown to be able to inhibit VKORC1 from all analyzed species. Nevertheless, susceptibility to ferulenol and ferprenin presented differences between species, suggesting a different susceptibility to 'poisonous' chemotypes of *F. communis*.

1. Introduction

Ferula communis L. (Apiaceae) is a perennial and robust weed, native to the Mediterranean basin. Its presence was reported in Syria, Turkey, North Africa, Italy, Sardinia, Portugal, Greece, Croatia, Albania and Palestine (Cauwet-Marc, 1981; Infante, 1965). Consumption of *F. communis* L. has been reported to be associated to an hemorrhagic syndrome, also called ferulosis, often fatal in the absence of treatment (Cauwet-Marc, 1990). This intoxication affected almost all mammals and the National Center of Veterinary Toxicology of Lyon (France) diagnosed 28 clinical cases of Ferula communis intoxication in cattle, sheep, pig and horse from 1990 to 2013 (Gault et al., 2015). Even man may be affected after an uncontrolled therapeutic use of Ferula extract (Cornevin, 1887; Lannehoa et al., 1998).

Two distinguishable chemotypes of *F. communis* L. were reported: the 'non-poisonous' chemotype and the 'poisonous' chemotype associated to the hemorrhagic syndrome (Benkhalti and Lamnaouer, 1994; Sacchetti et al., 2003). Different chemical investigations have reported the presence of daucane esters or drimane ethers, as main constituents in the 'non-poisonous' chemotype, according to the geographic area and the presence of prenylated coumarins as main constituents in the 'poisonous' chemotype (Appendino et al., 1987, 2001; Fraigui et al., 2001; Miski and Mabry, 1985; Valle et al., 1987; Rubiolo et al., 2006). Among the prenylated coumarins of the toxic variety, both ferulenol, a 4-hydroxycoumarin derivative, and ferprenin, a pyrane (3,2-c) coumarin derivative (Appendino et al., 1988; Carboni et al., 1964) (Fig. 1) could affect blood clotting, as described for other 4-oxygenated coumarin derivatives such as dicoumarol or warfarin, the first developed antivitamin K molecules used in human medicine and as rodenticide for pest control.

Abbreviations: VKOR, vitamin K epoxide reductase; VKORC1, vitamin K epoxide reductase complex subunit 1; Vitamin K > O, vitamin K epoxide.

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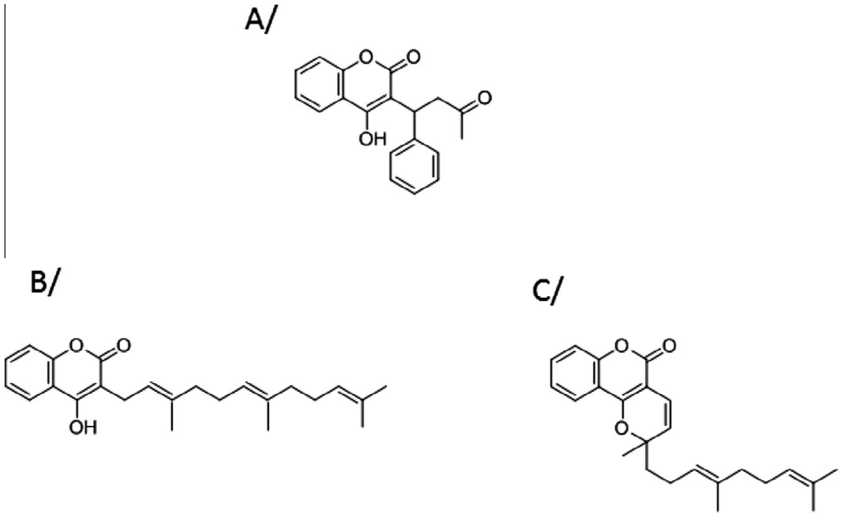

Fig. 1. Chemical structures of warfarin (A), ferulenol (B) and ferprenin (C).

4-Hydroxycoumarin derivatives, also designed as antivitamin K molecules were reported to specifically inhibit VKORC1, an enzyme encoded by the recent discovered Vkorc1 gene (Li et al., 2004; Rost et al., 2004). This enzyme catalyzes the vitamin K 2,3-epoxide reductase activity. This enzymatic activity is essential for recycling vitamin K. The function of VKORC1 is to regenerate vitamin K and vitamin K hydroquinone (K and KH2) from vitamin K 2,3-epoxide (K > O), a byproduct of the vitamin K-dependent gamma carboxylation reaction (Oldenburg et al., 2008). Inhibition of VKORC1 by 4-hydroxycoumarin derivatives limits the amount of KH2 available for the carboxylation reaction and results in partially carboxylated vitamin K-dependent blood clotting factors II, VII, IX, X. The specific inhibition of VKORC1 results in a stop of the clotting factors activation leading to a delayed death by hemorrhage. The inhibiting effect of ferulenol on VKORC1 was shown in rat (Gebauer, 2007), but not on VKORC1 of species exposed to *F. communis* L.

Previous studies in sheep treated with ferulenol indicated a sharp decreased in activity for several coagulation factors (Tligui et al., 1994). This observation seems to be coherent with the VKORC1-mediated antivitamin K properties of 4-oxygenated coumarins. Nevertheless, another mechanism leading to decrease in coagulation factors was proposed. This decrease was proposed to be due to an impairment of coagulation factors biosynthesis induced by a cytotoxic effect of ferulenol (Monti et al., 2007). Moreover, the toxicity of *F. communis* L. was reported to be not correlated with its contents in ferulenol (Appendino et al., 1988) and another prenylated coumarin, the pyranoferprenin, isolated from *F. communis* L., showed in vivo haemorrhagic activity (Appendino et al., 1988). Nevertheless, the inhibiting effect of ferprenin on VKORC1 was never demonstrated. This study aims to compare the inhibiting effect of ferulenol and ferprenin on VKORC1 from different species in order to demonstrate the mode of action of phytochemicals presumed to be responsible for the toxicity of *F. communis*, plant associated to intoxication that has considerable importance in plant/animal interactions in the Mediterranean countries.

2. Results

2.1. Determination of vitamin K epoxide reductase activity in liver of animal species susceptible to be exposed to F. communis L

In order to evaluate the vitamin K recycling ability of animal species exposed to *F. communis* L., liver microsomes were prepared from cow, calf, goat, lamb, pig, boar and horse. VKOR activity was thus measured at saturating concentration of vitamin K epoxide substrate (i.e., 200 μM) from the various microsomal fractions. Fig. 2 presents the results obtained. All the liver microsomal fractions tested in this study were able to reduce vitamin K epoxide in vitamin K. VKOR activities were found statistically different between species (VKOR activities determined from horse and boar were excluded of the one way analysis of variance). VKOR activity measured in cow liver microsomes was 1.5–2-fold higher than that measured in goat, lamb and pig liver.

2.2. Determination of the inhibiting effect of ferulenol and ferprenin on vitamin K epoxide reductase activity

In order to analyze the efficiency of ferulenol and ferprenin as inhibitor of the VKOR activity, susceptibilities to ferulenol and ferprenin were first determined in the presence of calf liver microsomes. The plots of the velocity of the VKOR activity catalyzed by calf liver microsomes, versus the substrate concentration in the presence of different concentrations of ferulenol or ferprenin are presented in Fig. 3A and B, respectively. The inhibiting effects of ferulenol and ferprenin on VKOR activity catalyzed by calf liver microsomes were compared with that obtained in the same conditions with warfarin (Fig. 3C).

Ferulenol was able to inhibit VKOR activity. The addition of ferulenol did not modify the apparent K_m (40.3 ± 5 μM), while it decreased the apparent V_{max} (Fig. 3A). Ferulenol is thus able to inhibit VKOR activity catalyzed by calf liver microsomes in a non-competitive manner, as observed with warfarin (Fig. 3C). Data were fitted to the Michaelis–Menten model, which takes into account the presence of either competitive, non-competitive, or uncompetitive inhibitor by non-linear regression. A fit was possible when the model that takes into account a non-competitive inhibitor was used only. Finally, K_i towards ferulenol for calf liver microsomes was 0.076 ± 0.007 μM. K_i towards warfarin obtained in the same conditions was 0.51 ± 0.04 μM.

Ferprenin was also able to inhibit the VKOR activity catalyzed by calf liver microsomes, but with concentration much higher than those used for inhibition of VKOR activity by ferulenol (Fig. 3B). Ferprenin inhibited the VKOR activity in a non-competitive manner, as described for ferulenol. K_i towards ferprenin for calf liver microsomes was 8.2 ± 0.05 μM. VKOR activity in calf liver microsomes was thus 100-fold more resistant to the action of ferprenin than ferulenol.

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