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Ferulenol specifically inhibits succinate ubiquinone reductase at the level of the ubiquinone cycle

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Abstract

The natural compound ferulenol, a sesquiterpene prenylated coumarin derivative, was purified from *Ferula vesceritensis* and its mitochondrial effects were studied. Ferulenol caused inhibition of oxidative phoshorylation. At low concentrations, ferulenol inhibited ATP synthesis by inhibition of the adenine nucleotide translocase without limitation of mitochondrial respiration. At higher concentrations, ferulenol inhibited oxygen consumption. Ferulenol caused specific inhibition of succinate ubiquinone reductase without altering succinate dehydrogenase activity of the complex II. This inhibition results from a limitation of electron transfers initiated by the reduction of ubiquinone to ubiquinol in the ubiquinone cycle. This original mechanism of action makes ferulenol a useful tool to study the physiological role and the mechanism of electron transfer in the complex II. In addition, these data provide an additional mechanism by which ferulenol may alter cell function and demonstrate that mitochondrial dysfunction is an important determinant in Ferula plant toxicity.

Keywords: Mitochondria; Ferulenol; Succinate ubiquinone reductase; Adenine nucleotide translocase; Oxidative phosphorylation; Ubiquinone cycle

Plants from the Ferula genus have been used in traditional medicine and as spices in food since the antiquity in the mediterranean regions [1]. These plants contain a large number of sesquiterpene derivatives with biological activity mainly related to the daucane derivative ferutinin [2]. A chemotype of Ferula genus, *Ferula communis*, has been also reported to be toxic inducing severe hemorrhagic disease [3,4]. The toxicity of the plants has been related to the presence of the sesquiterpene prenylated coumarin derivative, ferulenol, which has been shown to induce a lethal hemorrhagic disease known as 'ferulosis' [5]. Several reports also indicate that this compound displays other biological activities such as antibacterial properties and a dose-dependent cytotoxic activity against various human tumor cell lines [6,7].

These latter observations led to the suggestion that mitochondria may be involved in ferulenol cytotoxicity. Indeed, it is now well established that mitochondria are able to induce cell death [8] and are the target of numerous plant-derived natural products [9]. Several mechanisms which may be interrelated by which mitochondria trigger cell death are known. They include (a) disruption of the electron transport through the mitochondrial respiratory chain, (b) inhibition of the oxidative phosphorylation process, (c) increase in the membrane permeability that may lead to the release of apoptogenic factors [10].

In the present study, we hypothesized that mitochondria could be a relevant target by which ferulenol induces its cytotoxicity. We isolated and purified ferulenol from an

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Algerian species of Ferula, *Ferula vesceritensis*, and investigated the effects of this compound on the functions of mitochondria isolated from rat liver.

Materials and methods

Extraction and isolation of ferulenol. Ferulenol was extracted from the roots of *F. vesceritensis* collected from M'zab—ghardaya-Algeria in mars 2003. About 1.5 kg of the air-dried powdered roots was dissolved in a mixing dichloromethane–methanol at room temperature for 48 h and gave 30 g of a viscous oil. This extract was prefractionated over a silica gel column eluted with *n*-hexane containing increasing amounts of dichloromethane. The fraction (*n*-hexane–methylene chloride (1:1) included several compounds which were further separated by repeated column chromatography over a silica gel column (*n*-hexane–methylene chloride–methanol 7:4:1). Ferulenol (70 mg) was then purified by thin layer chromatography developed in *n*-hexane–diethylether (1:2) on a 0.2 mm aluminium sheets silica gel $60F_{254}$.

The structure of ferulenol was confirmed by ^{1}H NMR spectrum (250 MHz, $CDCl_3$) and higher resolution chemical ionization mass spectrum analysis and was in agreement with the molecular formula ($C_{24}H_{30}O_3$) which was previously reported from Ferula species [11–13].

Isolation of rat liver mitochondria and measurement of mitochondrial respiration. Rat liver mitochondria were isolated from male Wistar rats as described previously [14].

Oxygen consumption was measured by a Clark type oxygen microelectrode in a thermostat controlled chamber fitted to an oxygen monitoring system (Eurosep Instruments). Mitochondria (1 mg) were added to 1 ml of phosphate buffer (250 mM sucrose, 5 mM KH_2PO_4, pH 7.2 at 25 °C). Mitochondrial respiration was initiated by addition of 10 mM glutamate/malate or 6 mM succinate in the presence of 2 μM rotenone, and oxidative phosphorylation was initiated by addition of ADP to a final concentration of 0.2 mM. The following parameters were evaluated: substrate-dependent respiration rate (State 2 or State 4, oxygen consumption in the absence of exogenous ADP); ADP-stimulated respiration rate (State 3, oxygen consumption during ADP phosphorylation), and respiratory control ratio (RCR) as State 3/State 4 ratio.

Measurement of mitochondrial respiratory complex activities. Mitochondrial complex I activity (NADH decylubiquinone oxidoreductase, NQR) was measured at 30 °C by monitoring the decrease in fluorescence resulting from the oxidation of NADH. The incubation medium contained 25 mM KH_2PO_4, 5 mM $MgCl_2$, 10 μM decylubiquinone, 250 μM KCN, 1 mg/ml bovine serum albumin and 0.4 mg/ml of freeze-thawed liver mitochondria. The reaction was started by the addition of 10 μM NADH. The rotenone-sensitive NQR activity was measured in the presence of 5 μM rotenone.

Others respiratory chain enzyme activities were measured according to Barrientos [15]. Briefly, mitochondrial complex II activity (succinate ubiquinone reductase, SQR) was measured by monitoring the absorbance changes of 2,6-dichloroindophenol (DCIP) at 600 nm. The assay mixture contained 10 mM KH_2PO_4, 2 mM EDTA, 2 μM rotenone, 6 mM succinate, 1 mg/ml bovine serum albumin and 0.05 mg/ml of freeze-thawed liver mitochondria. After a preincubation period of 10 min, the activity of the complex was measured in the presence of 80 μM DCIP. To measure succinate dehydrogenase (SDH) activity alone, 1 mM of the SQR inhibitor thenoyltrifluoroacetone (TTFA) and 0.8 mM of the water soluble electron acceptor phenazine were added in the medium. Succinate cytochrome *c* reductase activity (complex II + III) was measured as the rate of cytochrome *c* reduction at 550 mm. The reaction mixture contained 10 mM KH_2PO_4, 2 mM EDTA, 2 μM rotenone, 6 mM succinate, 1 mg/ml bovine serum albumin, 0.05 mg/ml of freeze-thawed liver mitochondria and was preincubated for 10 min. The reaction was started by the addition of 40 μM oxidized cytochrome *c*.

Mitochondrial complex IV activity (cytochrome *c* oxidase) was performed at 550 mm following the decrease in absorbance resulting from the oxidation of reduced cytochrome *c*.

Assay of adenine nucleotide translocase (ANT) activity. ANT activity was assessed by measuring the transport of ^{14}C-ADP into mitochondria. Freshly prepared liver mitochondria (0.4 mg/ml) were suspended in the phosphate buffer in a total volume of 200 μl. These samples were preincubated for 5 min at 4 °C in the absence or in the presence of increasing concentrations of ferulenol and the transport was initiated by the addition of 8 μM ^{14}C-ADP (Perkin-Elmer Life Sciences, 1.85 MBq/μmol). The initial rate of ^{14}C-ADP transport was performed in exchange for mitochondrial endogenous ATP. After incubation of the suspension for 1 min at 4 °C, the reaction was terminated by the addition of 100 μM atractyloside and the suspension was immediately filtered through Millipore glass fiber filters. The bound radioactivity was determined by liquid scintillation counting.

Measurement of ATP synthesis and F1F0-ATPase activity. Mitochondrial ATP synthesis and ATP synthase (F1F0-ATPase) activity were evaluated as described recently [16]. Briefly, ATP synthesis was evaluated by measuring ATP produced in liver mitochondria by means of a well-established bioluminescence technique using the ATP dependency of the light emitting luciferase catalyzed oxidation of luciferin. This was made by means of a commercial kit (ATP bioluminescence assay kit HS II, Boehringer Mannheim).

Mitochondrial F1F0-ATPase activity was measured in the direction of ATP hydrolysis [17] and was monitered by measuring the concentration of Pi released. Mitochondria (0.05 mg/ml) were incubated at 37 °C for 3 min in 0.5 ml of a medium containing 50 mM Tris, 5 mM $MgCl_2$ (pH 7.4 at 37 °C) in the absence or in the presence of ferulenol. ATPase activity was started by the addition of 100 μM ATP and the reaction was stopped after 10 min of incubation by the addition of 1 μM oligomycin. The solution was centrifuged at 10,000*g* for 3 min and Pi concentration was determined in the supernatant.

Quantum mechanical calculations. All calculations were performed on a Silicon Graphics Origin 2000 workstation. Following the strategy already validated for antioxidant drugs [18,19], all compounds and intermediates were minimized at the semi-empirical level. Semi-empirical calculations were performed using the AM1 Hamiltonian [20,21] as implemented in SPARTAN 5.0 [22]. Closed-shell species were optimized using the restricted Hartree–Fock (RHF) approximation, while the open-shell species were calculated using the unrestricted Hartree–Fock (UHF) approximation.

Results and discussion

Interaction of ferulenol with the mitochondrial respiratory chain

Addition of increasing concentrations of ferulenol to a mitochondrial suspension energized with glutamate/malate decreased RCR value. The same result was observed when mitochondria were energized with succinate in the presence of rotenone, i.e. when complex I was inhibited and electron provided to complex II (Table 1). This effect was due to the decrease in both State 3 and State 4 respiration, suggesting that both mitochondrial oxygen consumption and ATP synthesis promoted by ADP were altered by ferulenol. This was confirmed with bioluminescence experiments measuring the quantity of ATP produced: at 10 μM, ferulenol inhibited ATP synthesis (Fig. 1). This effect involved two different mechanisms.

Below 1 μM, ferulenol decreased State 3 respiration without altering State 4. This tends to indicate that the inhibition of ATP synthesis was not related to the inhibition of mitochondrial oxygen consumption. This hypothesis was confirmed by uncoupled respiration experiments: ferulenol

Table 1
Effect of ferulenol on mitochondrial respiration, ATP synthesis, and FCCP induced oxygen consumption

Ferulenol (M)	Glutamate/malate			Succinate			Glutamate/malate	
	State 3	State 4	RCR	State 3	State 4	RCR	State 2	+FCCP
0	179 ± 12.1	34.8 ± 3.00	5.14 ± 0.15	122 ± 5.00	39.9 ± 1.46	3.06 ± 0.08	40.9 ± 2.3	169 ± 3.90
10^{-8}	195 ± 21.6	38.3 ± 8.90	5.09 ± 0.34	118 ± 1.53	41.0 ± 0.72	2.88 ± 0.03	43.3 ± 1.80	168 ± 4.50
10^{-7}	154 ± 7.20*	37.6 ± 3.40	4.10 ± 0.14*	112 ± 2.65*	42.0 ± 1.34	2.67 ± 0.06*	39.7 ± 1.40	173 ± 2.00
2×10^{-7}	137 ± 10.6*	36.3 ± 4.25	3.77 ± 0.19*	97.7 ± 4.88*	39.1 ± 4.59	2.50 ± 0.17*	40.2 ± 2.45	170 ± 3.85
4×10^{-7}	115 ± 12.6*	35.5 ± 5.10	3.24 ± 0.25*	80.5 ± 1.90*	40.8 ± 1.00	1.97 ± 0.05*	41.4 ± 1.40	164 ± 4.40
10^{-6}	61.4 ± 6.30*	38.8 ± 7.40	1.58 ± 0.29*	41.6 ± 0.62*	39.4 ± 0.47	1.06 ± 0.03*	42.2 ± 3.92	92.2 ± 3.50*
10^{-5}	26.8 ± 3.90*	23.7 ± 4.90*	1.13 ± 0.35*	24.2 ± 0.44*	24.2 ± 0.44*	1.00 ± 0.04*	29.6 ± 2.71	72.5 ± 3.20*
10^{-4}	11.0 ± 0.90*	11.0 ± 0.90*	1.00 ± 0.16*	11.4 ± 0.30*	11.4 ± 0.30*	1.00 ± 0.05*	12.8 ± 1.35	22.3 ± 1.82*

Oxygen consumption is expressed in nmol O_2/mg protein/min. Each value represents the means ± SD of three experiments performed in duplicate.
* $p < 0.05$ versus the value observed in the absence of ferulenol.

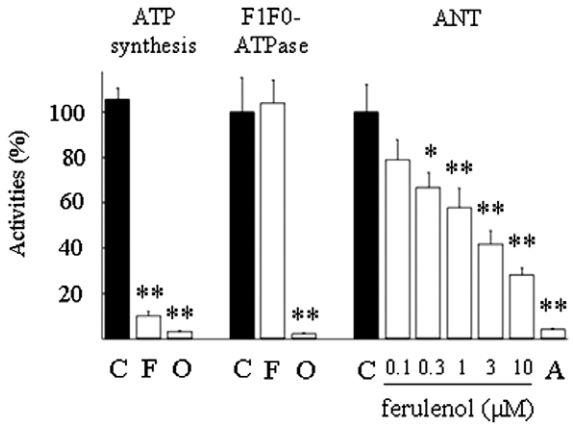

Fig. 1. Effect of ferulenol on ATP synthesis. ATP synthesis was estimated in the absence (C) or in the presence of either 10 μM ferulenol (F) or 1 μM oligomycin (O), a specific inhibitor of F1F0-ATPase. The effect of ferulenol (F, 10 μM) on F1F0-ATPase activity was monitored by measuring the apparition of Pi released by ATP hydrolysis. ANT activity was assessed by measuring the incorporation of ^{14}C-ADP into mitochondria. In these experiments, ferulenol was compared to atractyloside (A, 10 μM), a specific inhibitor of the exchanger. Data are expressed as percentage of their respective control values and represent means ± SD of three experiments performed in triplicate. Control values were 1.25 nmol ATP/mg protein, 5.17 nmol Pi/mg protein/min and 1.4 nmol ^{14}C-ADP/mg protein/min for ATP synthesis, F1F0-ATPase and ANT activities, respectively. *$p < 0.05$; **$p < 0.01$.

did not inhibit oxygen consumption when the demand of oxygen by the respiratory chain was increased by the presence of 1 μM of the uncoupling agent carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) (Table 1). Moreover, the decrease in State 3 respiration was not due to a direct inhibition of F1F0-ATPase as ferulenol did not alter the activity of the enzyme when it was measured in the direction of ATP hydrolysis [17]. ATP synthesis inhibition was rather caused by the inhibition of ANT which was inhibited by ferulenol in a concentration-dependent manner (Fig. 1). It should be noted that the concentrations of ferulenol required to block ADP transport were a bit higher than those inhibiting ADP-stimulated respiration (complete effect at 1 μM; Table 1). This is probably ascribable to the temperature used to measure these parameters: 25 °C for respiration and 4 °C for ANT.

For concentrations upper than 1 μM, ferulenol inhibited State 4 respiration rate in a concentration-dependent manner. Interestingly, the inhibiting effect of ferulenol was reversed by 10 mM of L-glycerol 3-phosphate (Fig. 2), a substrate which provides electrons directly to the complex III. This indicates that ferulenol acts upstream to the complex III. In addition, ferulenol did not inhibit oxygen consumption when it was triggered at the level of complex IV (not shown). Taken together, these data led us to investigate whether the mitochondrial respiratory complexes I and II could be involved in the inhibiting effect of ferulenol.

Interaction of ferulenol with the mitochondrial respiratory complexes

Ferulenol inhibited the activity of the rotenone-sensitive NADH-decylubiquinone oxidoreductase (complex I) evaluated by the oxidation of NADH (Table 2). The inhibiting

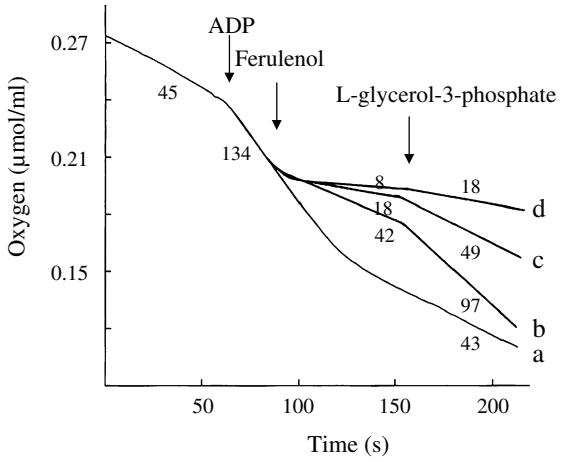

Fig. 2. Typical experiment showing the restoration by L-glycerol-3-phosphate of the oxygen consumption rate inhibited by ferulenol. Mitochondria were incubated with 6 mM succinate, 2 μM rotenone and then 0.2 mM ADP was added (a). Increasing concentrations of ferulenol (b, 1 μM; c, 10 μM; d, 100 μM) inhibited the oxygen consumption which was restored by adding L-glycerol-3-phosphate (10 mM). A similar profile was observed in the presence of malonate (not shown). Rates of oxygen consumption (nmol/min/mg protein) are given alongside each curve.

Table 2
Effect of ferulenol on NQR and SDH activities

NQR activity		SDH activity	
NADH	18.0 ± 1.08	Succinate − TTFA	2.37 ± 0.14
NADH + rotenone (5 μM)	3.47 ± 0.27*	Succinate + TTFA (1 mM)	88.5 ± 5.32
NADH + ferulenol (100 μM)	3.25 ± 0.23*	Succinate + TTFA (1 mM) + malonate (10 mM)	1.03 ± 0.15
NADH + ferulenol (100 μM) + DUQ (100 μM)	21.1 ± 0.85#	Succinate − TTFA + ferulenol (100 μM)	111 ± 7.70
NADH + rotenone (5 μM) + DUQ (100 μM)	4.92 ± 0.39*	Succinate + TTFA (1 mM) + ferulenol (100 μM)	137 ± 11.5

The activity observed in the presence of rotenone (a specific inhibitor of NQR) results from the contaminating enzyme NADH cytochrome b5 reductase. Values are expressed as nmol/min/mg protein and represent the means ($n = 3$) ± SD. DUQ, decylubiquinone.
* $p < 0.05$ versus NADH alone.
# $p < 0.05$ versus NADH + ferulenol.

effect of ferulenol was reversed by 100 μM decylubiquinone which provides electrons directly to the ubiquinone cycle, indicating that the drug acts downstream to the complex I.

Mitochondrial complex II (SQR) catalyzes the oxidation of succinate to fumarate (SDH activity of the complex) and transfers the electrons released during this reaction to the ubiquinone cycle [23]. Thus, in a first step, we measured the effect of ferulenol on SDH activity. It was made in the presence of both TTFA which inhibits the transport of electrons at complex II and the water-soluble electron acceptor, phenazine. As shown in Table 2, ferulenol did not inhibit but rather tended to increase SDH activity. This could be due to the fact that ferulenol increased the inhibitory effect of SQR, increasing the electron transfer from succinate to phenazine. In the same experiment, the well-known SDH inhibitor malonate was effective. Moreover, when TTFA was omitted from the medium, ferulenol served as a substitute for TTFA and allowed the measurement of SDH activity (Table 2). This indicates that the two compounds might act at the same level. Thus, we further characterized the effects of ferulenol on SQR activity measured with 2,6-dichloroindophenol as an electron acceptor. Decylubiquinone stimulated the basal activity of the enzyme with an EC_{50} value of 12 μM, the maximal effect being obtained at 100 μM (not shown). Ferulenol inhibited SQR activity in a concentration-dependent manner (Fig. 3A) and was as effective as TTFA (Fig. 3B). A similar inhibitory effect was observed when we analysed the transfer of electrons from complex II to complex III by monitoring the reduction of cytochrome *c* (Fig. 3D) and this suggested that ferulenol might interact with the ubiquinone cycle. Thus, we tested whether decylubiquinone was able to counteract the effect of ferulenol. Fig. 3B shows that a low concentration of decylubiquinone antagonized the effect of ferulenol. In the presence of 10 μM decylubiquinone, IC_{50} of ferulenol was about 10-fold higher than that observed in the absence of decylubiquinone indicating that the two molecules act in a competitive way. Similarly, increasing low concentrations of decylubiquinone restored the activity of complex II previously inhibited by a high ferulenol concentration (250 μM; Fig. 3C). This result rules out a possible covalent interaction of ferulenol with the SQR complex and suggests that ferulenol could impair the rate of electron transfer from complex II to complex III via the ubiquinone cycle.

Possible mechanism by which ferulenol inhibits complex II activity

In order to get information on the ability of this 4-hydroxy-coumarin derivative to interfere with the electron transfer reaction in the complex II, a quantum mechanical study reaction was performed centred on its behaviour in redox reaction in comparison with ubiquinone. The reaction schemes (Fig. 4) were explored at AM1 level using the unrestricted Hartree–Fock (UHF) Hamiltonian for open-shell species (see Experimental procedures). In order to save computation time, only simple model reactive fragments for ubiquinone or ferulenol were used, the long alkyl side-chains removed being not involved in redox reactions. It appears immediately from these two reaction schemes that the overall processes are not identical for these two compounds, ubiquinone being involved in two reduction steps for a global exchange of two electrons and two protons, while only one reduction step is possible for the reduction of ferulenol since subsequent reaction intermediates cannot be reduced. Moreover, the two compounds differ also according to their reactivity towards the first electron transfer reaction, namely their reduction. Indeed, the reduction of ubiquinone is easier than the reduction of ferulenol by an amount of 15.0 kcal/mol ($\Delta H_{reac} = -54.7$ kcal/mol for ubiquinone and −39.7 kcal/mol for ferulenol; Fig. 4). Thus, the structural properties of the 4-hydroxy-coumarin nucleus of ferulenol are compatible with a potential interaction with complex II in the mitochondrial respiratory chain but the fate of the reduced species involved in this electron trap step appears largely different from the fate of reduced species of ubiquinone. These observations may be at the origin of the inhibition of mitochondrial complex II observed with ferulenol and may explain the counteraction of ubiquinone in the biological assays described above. This is an original mechanism which makes ferulenol a useful tool to further study the biological roles of the complex. Indeed, numerous evidence suggest that complex II dysfunction is coupled to a wide variety of clinical disorders [24–26] but the lack of specific inhibitors has hampered the detailed

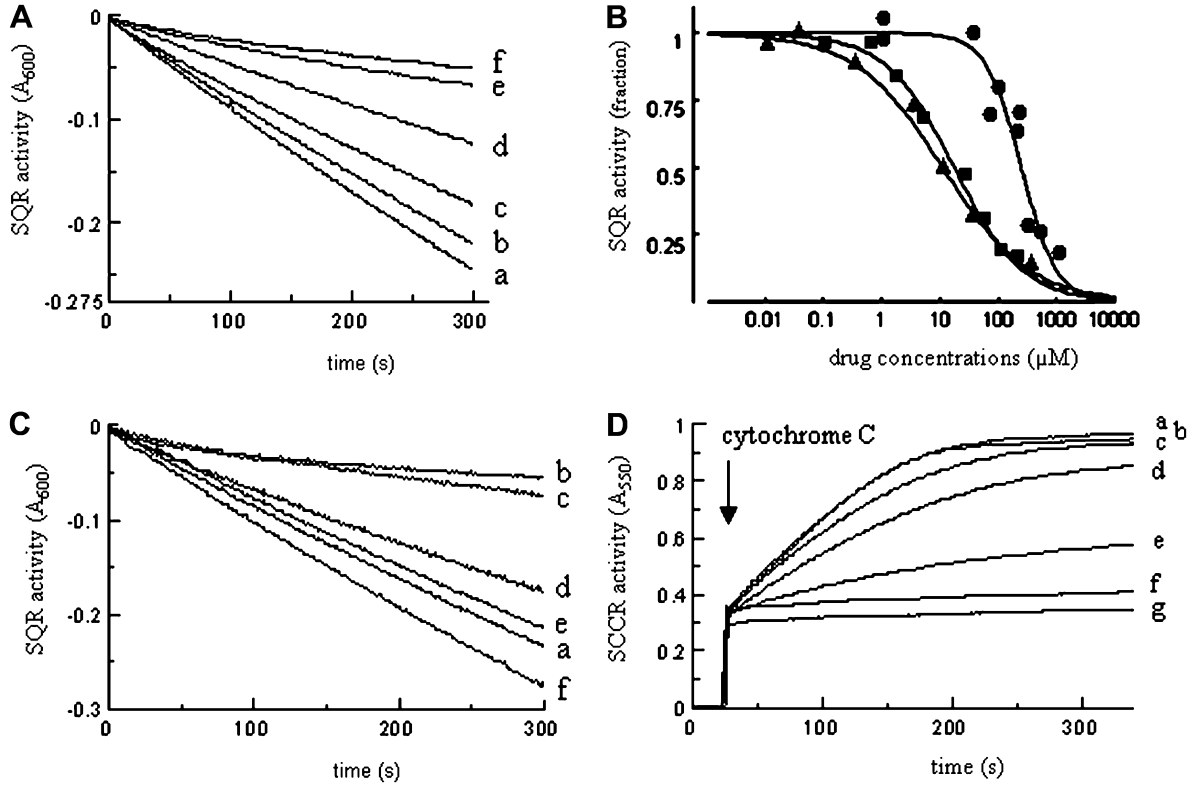

Fig. 3. Effect of ferulenol on SQR and SCCR activity. (A) Typical experiment showing the inhibiting effect of increasing concentrations of ferulenol 1.5 μM (b), 5 μM (c), 25 μM (d), and 100 μM (e)) or of 100 μM TTFA (f) on SQR activity evaluated by measuring the decrease in absorption of 2,6-dichloroindophenol at 600 nm. SQR activity was determined by using $\varepsilon = 21$ l/mmol/cm for 2,6-dichloroindophenol . Control activity (a) was 0.65 μmol/min/mg proteins. (B) Concentration-dependent inhibition of SQR activity by ferulenol (■), TTFA (▲) and ferulenol in the presence of 6 μM decylubiquinone (●). The results were expressed as the percentage of the maximal activity of the enzyme and IC_{50} values are as follows: ferulenol, 17 μM; TTFA, 11 μM; ferulenol in the presence of decylubiquinone, 245 μM. (C) Decylubiquinone reversed the inhibiting effect of ferulenol. Control SQR activity (a) was inhibited by 250 μM ferulenol (b). This effect was reversed in the presence of increasing concentrations of decylubiquinone (c, 1 μM; d, 5 μM; e, 10 μM; f, 15 μM). (D) Ferulenol inhibits succinate cytochrome C reductase (SCCR) activity. The reaction was started by the addition of 40 μM oxidized cytochrome C (a: control) which initially induced a sudden increase in absorption unrelated to the activity of the enzyme. Ferulenol inhibited SCCR activity in a concentration-dependent manner: b, 1 μM; c, 5 μM; d, 25 μM; e, 50 μM, and f, 100 μM. Succinate oxidation was also inhibited by the addition of 10 mM malonate (g).

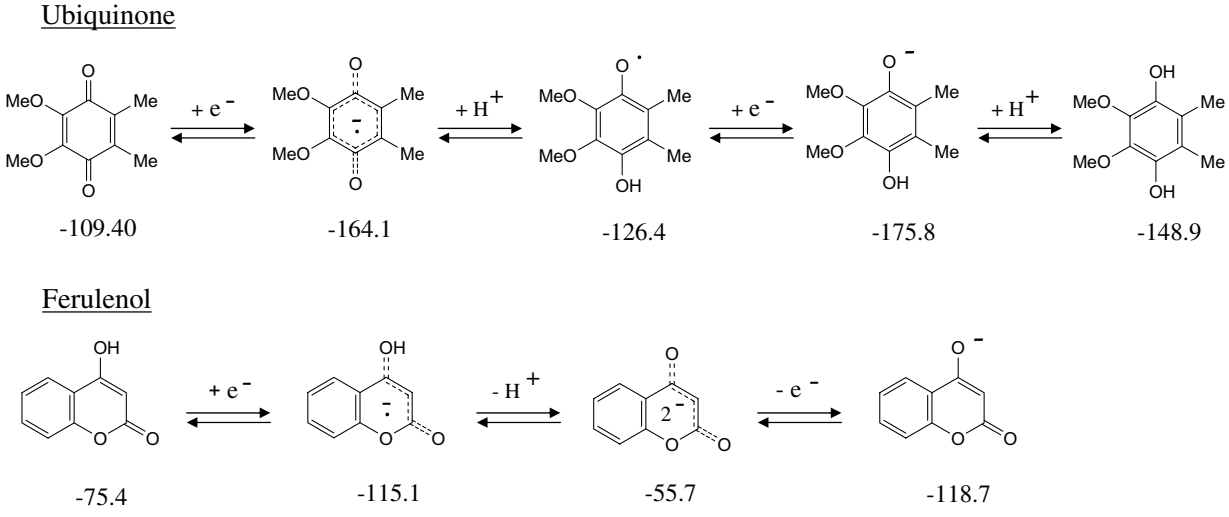

Fig. 4. Redox and proton exchange pathways explored for model compounds of ubiquinone and ferulenol using AM1 semiempirical calculations. The heat of formation ΔH_f is given in kcal/mol. Stabilization of the intermediate obtained by the first reduction of ubiquinone was compared with the correspondent intermediate for ferulenol using the isodesmic reaction: ubiquinone + reduced ferulenol [⇄] reduced ubiquinone + ferulenol. Thus, a stabilization of 15.0 kcal/mol was determined for the reduced ubiquinone ($\Delta H_{reac} = -164.1 - 75.4 + 115.1 + 109.4 = -15.0$ kcal/mol).

analysis of its functions. In this context, ferulenol could represent an interesting agent.

In conclusion, our data demonstrate that ferulenol inhibits the oxidative phosphorylation process by interacting with ANT and the complex II of the respiratory chain. These data provide an additional mechanism by which ferulenol may alter cell function and support the idea that mitochondrial dysfunction is an important determinant in Ferula plant toxicity. In addition, ferulenol appears to be a useful tool to study the biochemical properties, the physiological roles of complex II and the mechanism of electron transfer in this complex.

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