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INHIBITION OF MOUTH SKELETAL MUSCLE RELAXATION BY FLAVONOIDS OF *Cistus ladanifer* L.: A PLANT DEFENSE MECHANISM AGAINST HERBIVORES

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Abstract—*Cistus ladanifer* exudate is a potent inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (Ca^{2+}-pump) of rabbit skeletal muscle, a well-established model for active transport that plays a leading role in skeletal muscle relaxation. The low concentration of exudate needed to produce 50% of the maximum inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase activity, 40–60 μg/ml, suggests that eating only a few milligrams of *C. ladanifer* leaves can impair the relaxation of the mouth skeletal muscle of herbivores, as the exudate reaches up to 140 mg/g of dry leaves in summer season. The flavonoid fraction of the exudate accounts fully for the functional impairment of the sarcoplasmic reticulum produced by the exudate (up to a dose of 250–300 μg/ml). The flavonoids present in this exudate impair the skeletal muscle sarcoplasmic reticulum function at two different levels: (i) by inhibition of the Ca^{2+}-ATPase activity, and (ii) by decreasing the steady state ATP-dependent Ca^{2+}-accumulation. Among the exudate flavonoids, apigenin and 3,7-di-*O*-methyl kaempferol are the most potent inhibitors of the skeletal muscle sarcoplasmic reticulum. We conclude that the flavonoids of this exudate can elicit an avoidance reaction of the herbivores eating *C. ladanifer* leaves through impairment of mouth skeletal muscle relaxation.

Key Words—Flavonoids, *Cistus ladanifer*, Ca^{2+}-ATPase, skeletal muscle, plant defense, apigenin, 3,7-di-*O*-methyl kaempferol.

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INTRODUCTION

Secondary metabolites production, accumulation, and release through evolution play a major role in plant survival under stressful conditions (Seigler, 1998; Chaves and Escudero, 1999). The importance of plant chemistry in the evolution of plant–herbivore interactions has been well documented. Among plant secondary metabolites, phenolic compounds have received particular attention because (1) they are present in many plant secretions (Wollenweber and Dietz, 1981; Seigler, 1998), (2) they are enriched in the parts of plants' most vulnerable areas to attack from other organisms (such as leaves and photosynthetic stems), and (3) they show variation in their chemical structure great enough to account for the variety of putative environmental insults from other living organisms.

Arinafril and Suwandi (2001) have pointed out that the presence of compounds in leaves and photosynthetic stems with bioactivity against herbivores eating can also account for plant protection, by acting as dissuasive compounds. Although plant phenolics are believed to play an important role in chemical defense, their specific physiological effects on herbivores are variable and poorly understood (Appel, 1993). It has been reported that they can behave as antifeedants (Zamora et al., 1999), digestibility reducers (Harborne, 1994), and toxins (Rosenthal and Berenbaum, 1991).

Eating plant leaves or tender stems by herbivores involves a significant mouth skeletal muscle exercise, and skeletal muscle sarcoplasmic reticulum plays a central role in the control of contraction/relaxation of the skeletal muscle (Entman and Van Winkle, 1986; Fleischer and Inui, 1989). The lipophilic properties of flavonoids point out that they reach the intracellular space by simple diffusion across the lipid bilayer of the plasma membrane. Therefore, they may reach the skeletal muscle fibers in herbivore mouth by diffusion, without need of previous absorption along the digestive track. Thus, they avoid the herbivore detoxification systems for xenobiotics in the liver (Coulson et al., 1984; Ortiz de Montellano, 1986).

The sarcoplasmic reticulum forms an extensive membrane network that surrounds the skeletal muscle myofibrils. Ca^{2+}-ATPase is by far the major protein component (Andersen, 1989). Ca^{2+}-ATPase couples the hydrolysis of ATP to active Ca^{2+}-transport into the sarcoplasmic reticulum lumen and is responsible for the rise of a high Ca^{2+}-concentration gradients ($\geq$10000) across this membrane in the relaxed state of the skeletal muscle cell (de Meis and Vianna, 1979). This protein plays a leading role in skeletal muscle relaxation in vertebrates, as inhibition of this Ca^{2+}-ATPase or a decrease in the capacity of the sarcoplasmic reticulum membrane to maintain a large Ca^{2+}-gradient results in an impaired relaxation of the skeletal muscle, and eventually leads to tetanic-like myofibril contractions when the cytosolic Ca^{2+} concentration increases to the micromolar range (Ruegg, 1988).

Shoshan and MacLennan (1981) reported that micromolar concentrations of the flavonoid quercetin strongly inhibit the sarcoplasmic reticulum Ca^{2+}-ATPase from rabbit skeletal muscle, and this Ca^{2+}-ATPase has been shown to be a good prototype for other herbivore and insect skeletal muscle Ca^{2+}-ATPases (Martonosi, 1984). Because of the need for proper function of skeletal muscle for eating by herbivorous organisms, leaves or photosynthetic stems containing high flavonoid concentrations may result in impaired mouth muscle relaxation and produce an avoidance reaction.

In previous studies, we have shown the high concentration of flavonoids in the exudate of *Cistus ladanifer* L. and their possible function as allelopathic agents (Chaves et al., 1993, 1998, 2001; Chaves and Escudero, 1999). The exudate is enriched in flavonoids during summer, particularly in flavonols (kaempferols) over flavones (apigenins) (Chaves et al., 1993, 1997). In the summertime, *C. ladanifer* is often exposed simultaneously to different types of stress (high levels of UV, drought, and high temperatures), and protection of the growing plant from herbivory should play a critical role in plant survival.

Only low doses of the *C. ladanifer* exudate are needed to impair the skeletal muscle sarcoplasmic reticulum capacity to sequester Ca^{2+}, and this effect can be accounted for by the flavonoids present in the exudate.

METHODS AND MATERIALS

Extraction, Purification, and Quantification of Flavonoids. C. ladanifer exudate was prepared as described in detail in a previous paper (Chaves et al., 1997). Apigenin was obtained from Aldrich Chemical (Madrid, Spain). Methylated flavonoids (3-*O*-methylkaempferol, 3,4′-di-*O*-methylkaempferol, 3,7-di-*O*-methylkaempferol, 4′-*O*-methylapigenin, and 7-*O*-methylapigenin) were prepared by HPLC from the *C. ladanifer* exudate as indicated in Chaves et al. (1998). Briefly, the exudate was dissolved in hot methanol and chilled at -20°C for 12 hr. Precipitated waxes were removed by centrifugation at 4°C (4500 $\times g$ for 10 min). The extract was loaded onto a 25 $\times$ 1.5 cm hydrated Sephadex LH-20 column (equilibrated with methanol for 24 hr), and eluted with methanol. Three fractions were obtained and subsequently analyzed by HPLC to determine which fraction contained the flavonoids. Flavonoids were separated on a semipreparative Nucleosil 5μ C-18 (250 $\times$ 10 mm) column using water–methanol–acetonitrile–tetrahydrofuran (56:16:6:22) as elution solvent. The flow rate was maintained at 1.75 ml/min. The flavonoids were detected with a diode array detector (350 nm). As each flavonoid was detected, it was collected into a separate tube. To eliminate any possible contamination from other compounds eluting close to the flavonoid of interest, the fraction was reseparated by HPLC with a methanol:water (80:20) at a flow rate of 2.5 ml/min.

TABLE 1. CONTENT OF *Cistus ladanifer* LEAF EXUDATE FLAVONOIDS

	Spring		Summer	
	Exudate (mg/g)	Dry mass (mg/g)	Exudate (mg/g)	Dry mass (mg/g)
Ap	1.43	0.17	1.68	0.23
K-3	8.58	0.99	11.80	2.31
Ap-4′	19.17	2.18	13.15	1.76
Ap-7	22.51	2.54	26.61	3.63
K-3,4′	17.35	1.97	33.71	4.47
K-3,7	32.34	5.95	161.96	21.98

Note. Ap: apigenin; K-3: 3-*O*-methylkaempferol; Ap-4′: 4′-*O*-methylapigenin; Ap-7: 7-*O*-methylapigenin; K-3,4′: 3,4′-di-*O*-methylkaempferol; K-3,7: 3,7-di-*O*-methylkaempferol. $N = 12$.

Quantitative analysis of leaf flavonoids was performed as indicated in previous publications (Chaves et al., 1993, 1997). Leaves (2–3 g; $N = 12$) were collected in spring and summer. They were dipped several times into chloroform (2 ml), and the chloroform was removed by evaporation. The exudate was dissolved in methanol (2 ml), and analyzed by HPLC as follows: extract (20 μl) was injected onto a Nucleosil 5μ C-18 (150 $\times$ 4 mm) column and eluted with water–methanol–acetonitrile–tetrahydrofuran (56:16:6:22) at a flow rate of 0.7 ml/min (Chaves et al., 1993, 1997). After extraction, leaves were weighed, oven-dried at 60°C for 12 hr, and reweighed again to determine dry biomass. The content of exudate flavonoids is indicated in the Table 1.

Preparation of Sarcoplasmic Reticulum Vesicles. Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as indicated in Cuenda et al. (1994). Protein concentrations were measured following the method of Lowry et al. (1951), using bovine serum albumin as a standard. The Ca^{2+}-ATPase accounted for 75–80% of the total protein in sarcoplasmic reticulum membranes, as determined by densitometry of Coomasie blue stained SDS/gels (7–10% acrylamide) using a BioRad Molecular Imager® FX.

Ca^{2+}-ATPase Activity. Ca^{2+}-ATPase activity was measured spectrophotometrically at 25°C using the coupled enzyme system pyruvate kinase/lactate dehydrogenase as in Cuenda et al. (1990, 1994). The following reaction mixture was used: 0.1 M TES/KOH (pH 7.45), 0.1 M KCl, 0.1 mM $CaCl_2$, 3 mM $MgCl_2$, 2.5 mM ATP, 0.42 mM phosphoenolpyruvate, 0.25 mM NADH, 7.5 IU pyruvate kinase, and 18 IU lactate dehydrogenase. On average, sarcoplasmic reticulum membranes had less than 5% of Ca^{2+}-independent ATPase activity, measured in the presence of 5 mM EGTA. The specific Ca^{2+}-ATPase activity of sarcoplasmic reticulum membrane preparations was stimulated between 8 and 10-fold following addition of 0.04 μg of the Ca^{2+}-ionophore calcimycin per microgram of sarcoplasmic reticulum protein.

Ca^{2+} Accumulation by Sarcoplasmic Reticulum Vesicles. Steady state ATP-dependent Ca^{2+} accumulation by sarcoplasmic reticulum was measured at 25°C using arsenazo III as the metallochromic indicator from the difference of absorbance between 650 and 700 nm, as in Fernandez-Salguero et al. (1990). The following assay mixture was used: 0.1 M TES/KOH (pH 7.45), 0.1 M KCl, 0.1 mM $CaCl_2$, 3 mM $MgCl_2$, 2.5 mM ATP, and 100 μM arsenazo III. The conversion of absorbance change into Ca^{2+} concentration was carried out by calibration with EGTA Ca^{2+} solutions.

For titration studies, flavonoids were dissolved in DMSO at a concentration at least 100-fold the maximum concentration to be used in activity measurements. Control experiments confirmed that up to 1% DMSO did not produce any significant effect on the sarcoplasmic reticulum Ca^{2+}-ATPase activity nor on steady state ATP-dependent Ca^{2+} accumulation by sarcoplasmic reticulum vesicles.

Fluorescence. Steady-state fluorescence measurements were performed in a continuously stirred cuvette with the following spectrofluorimeters: Hitachi-Perkin–Elmer (model 650-40) and SLM-4800C, with 280 and 335 nm as excitation and emission wavelengths, respectively. Inner filter effects, due to the absorbance of flavonoids at 280 and 335 nm, were corrected using the following equation: $F_{corr} = C \times F_{obs}$, where F_{corr} and F_{obs} are the values of the corrected and observed fluorescence, and $C = \text{antilog}((A_{280} + A_{335})/2)$ (Lackowicz, 1983). Flavonoid extinction coefficients at 280 nm (ε_{280}) and 335 nm (ε_{335}) were determined in 0.1 M TES/0.1 M KCl and 3 mM $MgCl_2$ (pH 7), the buffer used for fluorescence measurements. The sum of the absorbance at 280 and 335 nm at the highest flavonoid concentrations used in the titration of intrinsic sarcoplasmic reticulum fluorescence (25–30 μM) was close to 0.3, and only for concentrations of the flavonoid higher than 10 μM was the value of the correction factor C higher than 1.2.

Egg lecithin liposomes containing 10% octyl-tryptophan were prepared by the ethanol injection method of Fung and Stryer (1978), and characterized as in a Antollini et al. (1996).

The experimental data obtained for inhibition of Ca^{2+}-ATPase activity and for fluorescence quenching by the flavonoids were analyzed by nonlinear least-squares two-parameters fit to the equations indicated in the text using the program Origin 5.0™.

RESULTS

Sarcoplasmic reticulum Ca^{2+}-ATPase was inhibited by the exudate of *C. ladanifer*. The summer exudate was several-fold more potent than the spring exudate (Figure 1A). Since the content of leaf flavonoids increased several-fold from spring to summer (Table 1 and Chaves et al., 1993, 1997), this suggested that *C. ladanifer* flavonoids are inhibitors of the Ca^{2+}-ATPase. This was confirmed using the flavonoid fraction, which showed that only 13 μg flavonoids/ml are needed to produce 50% inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase

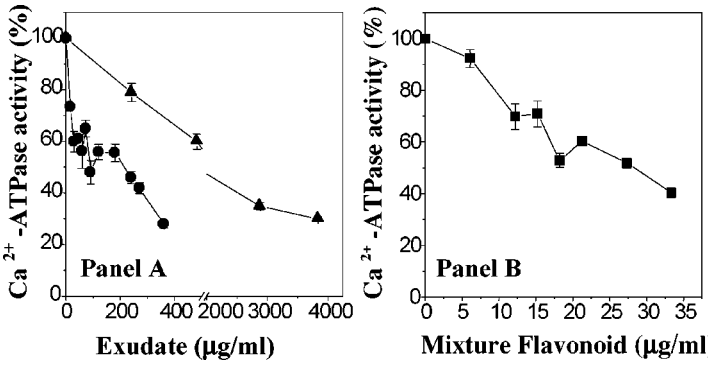

FIG. 1. Inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase activity by the spring (triangles) and summer (circles) *C. ladanifer* exudate (Panel A) and by the flavonoid mixture of the summer exudate (Panel B). Ca^{2+}-ATPase activity was assayed in the presence of 4% calcimycin.

(Figure 1B). Taking into account the molecular weight of the flavonoids present in the flavonoid fraction (Table 1 and Chaves et al., 1993, 1997), this implies that one or several of the components inhibit the sarcoplasmic reticulum Ca^{2+}-ATPase with an inhibitory dissociation constant in the micromolar range.

Inhibition of the Ca^{2+}-ATPase activity is not due to solubilization of the sarcoplasmic reticulum membranes by the exudate nor by the flavonoid fraction, because the light scattering of the membranes decreases less than 10% in the presence of exudate and flavonoid fractions producing more than 50% inhibition of the Ca^{2+}-ATPase activity (data not shown). Furthermore, in the absence of the Ca^{2+} ionophore calcimycin, the Ca^{2+}-ATPase activity is largely inhibited in well-sealed sarcoplasmic reticulum vesicles by the Ca^{2+} gradient established by the operation of the Ca^{2+}-pump (de Meis and Vianna, 1979; Entman and Van Winkle, 1986; Andersen, 1989). This is shown experimentally by a large stimulation of the Ca^{2+}-ATPase by the Ca^{2+} ionophore, calcimycin (Table 2). Table 2 also shows that, in presence of exudate and flavonoid fraction concentrations that produced a large inhibition of the Ca^{2+}-ATPase activity, the ratio between Ca^{2+}-ATPase activity in the absence and presence of calcimycin is much lower than 1, and closer to that found for control (well sealed) sarcoplasmic vesicles. Therefore, inhibition of Ca^{2+}-ATPase activity cannot be rationalized in terms of a large perturbation of the sarcoplasmic reticulum membrane structure, which should be monitored by a loss of the large Ca^{2+} gradient generated across this membrane by Ca^{2+}-ATPase activity. In fact, impairment of the sarcoplasmic reticulum vesicles to accumulate Ca^{2+} required concentrations of the summer exudate (LD_{50} about 500 μg/ml, Table 3) much higher than the IC_{50} value obtained for the inhibition of Ca^{2+}-ATPase activity.

TABLE 2. EFFECTS OF *Cistus ladanifer* SUMMER EXUDATE AND THE EXUDATE FLAVONOID FRACTION ON CA^{2+}-GRADIENT INHIBITION OF THE SARCOPLASMIC RETICULUM CA^{2+}-ATPase

Sarcoplasmic reticulum	Ca^{2+}-ATPase activity ratio (−calcimycin/+calcimycin)
Control	0.10 ± 0.04
+30 μg exudate/ml	0.17 ± 0.04
+120 μg exudate/ml	0.32 ± 0.04
+15 μg flavonoids/ml	0.14 ± 0.04
+33 μg flavonoids/ml	0.25 ± 0.04

Note. $N = 5$.

Flavonoids are lipophilic compounds, and should be expected to strongly adsorb to biological membranes, such as the sarcoplasmic reticulum membrane, thereby promoting specific functional perturbations that lead to altered physiological responses. Thus, we have measured the adsorption of the flavonoids used in this work onto the sarcoplasmic reticulum membranes.

As flavonoids quench the intrinsic fluorescence of the sarcoplasmic reticulum membranes (Figure 2), and more than 80% of the intrinsic fluorescence of sarcoplasmic reticulum (largely Trp fluorescence) is accessible to quenching through the lipid bilayer (London and Feigenson, 1981), we made use of this property to monitor the incorporation of flavonoids into sarcoplasmic reticulum membranes

TABLE 3. EFFECTS OF *Cistus ladanifer* SUMMER EXUDATE ON STEADY STATE ATP-DEPENDENT CA^{2+} ACCUMULATION BY THE SARCOPLASMIC RETICULUM

Exudate (μg/ml)	Ca^{2+} uptake (% of control)[a]
0 (control)	100
30	98
180	100
360	85.5
450	74.6
540	35
720	28
2160	30

Note. $N = 3$.

[a] The control value of measured steady state ATP-dependent Ca^{2+} uptake was 99.3 nmoles Ca^{2+}/mg sarcoplasmic reticulum protein.

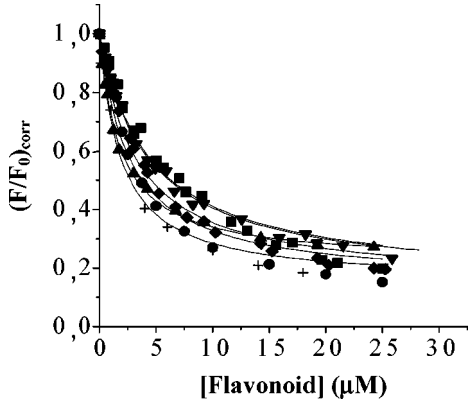

FIG. 2. Quenching of intrinsic fluorescence of sarcoplasmic reticulum membranes by *C. ladanifer* exudate flavonoids. Symbols correspond to apigenin (squares), 4′-*O*-methylapigenin (up-triangles), 7-*O*-methylapigenin (down-triangles), 3-*O*-methylkaempferol (diamonds), 3,4′-di-*O*-methylkaempferol (+), and 3,7-di-*O*-methylkaempferol (circles). The lines are the best nonlinear least squares fit of the data to the equation indicated in the text, which yielded the following IC_{50} values: 5 μM ($\chi^2 = 0.0011$) for apigenin; 3.2 μM ($\chi^2 = 0.0034$) for 4′-*O*-methylapigenin; 1.8 μM ($\chi^2 = 0.0001$) for 7−*O*−methylapigenin; 4.5 μM ($\chi^2 = 0.0003$) for 3-*O*-methylkaempferol; 3.6 μM ($\chi^2 = 0.0011$) for 3,4′-di-*O*-methylkaempferol; 1.9 μM ($\chi^2 = 0.0012$) for 3,7-di-*O*-methylkaempferol.

by using a methodological approach similar to that outlined in Gutiérrez-Merino et al. (1989). Briefly, we measured: (1) the intrinsic fluorescence of sarcoplasmic reticulum vesicles (Figure 2), and (2) the fluorescence of Trp incorporated as *n*-octyl Trp into egg lecithin liposomes (Figure 3) in the presence of different concentrations (up to 50 μM) of each flavonoid in a pH 7 buffered solution. The data for each flavonoid were fitted (using the iterative nonlinear squares two parameters fit algorithm of Origin 5.0^{TM} software) to the following equation:

$$\frac{F}{F_0} = 1 - \left\{ \frac{F_{\min}(\text{Flavonoid})}{IC_{50} + [\text{Flavonoid}]} \right\},$$

where F_0 and $F_{\min}$ are the fluorescence intensity in the absence and at saturation of the flavonoid, respectively; IC_{50} is the concentration of flavonoid that produces 50% of the maximum fluorescence quenching attained at saturation by the flavonoid, and F is the fluorescence in the presence of each concentration of flavonoid. The two parameters that were fit by iteration were $F_{\min}$ and IC_{50}. The flavonoids studied are more potent as quenchers of Trp fluorescence in a lipid phase than in an aqueous environment (compare Figures 3 and 4), reflecting the large partition of these flavonoids into the lipid bilayer. To obtain the IC_{50} value for the

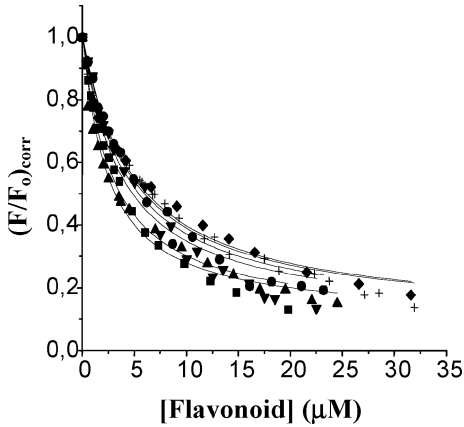

FIG. 3. Quenching of octyl-Trp incorporated in to egg lecithin liposomes by *C. ladanifer* exudate flavonoids. Octyl-Trp was incorporated into egg lecithin liposomes at a molar ratio of 1% octyl-Trp/lipid. Different symbols correspond to the different flavonoids present in the exudate (as in Figure 2). The lines are the best nonlinear least squares fit of the data to the equation indicated in the text, which yielded the following IC_{50} values: 3 μM ($\chi^2 =$ 0.0013) for apigenin; 4.5 μM ($\chi^2 = 0.0017$) for 4′-*O*-methylapigenin; 2.7 μM ($\chi^2 =$ 0.001) for 7-*O*-methylapigenin; 4.2 μM ($\chi^2 = 0.0033$) for 3-*O*-methylkaempferol; 5.3 μM ($\chi^2 = 0.001$) for 3.4′-di-*O*-methylkaempferol; 5.1 μM ($\chi^2 = 0.001$) for 3.7-di-*O*-methylkaempferol.

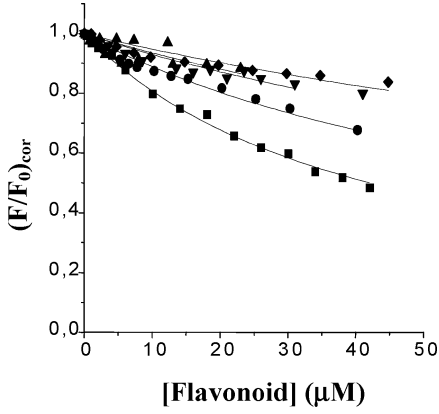

FIG. 4. Quenching of Trp fluorescence in aqueous solution by flavonoids in *C. ladanifer* exudate. The symbols correspond to apigenin (squares); 4′-*O*-methylapigenin (circles); 7-*O*-methylapigenin (up-triangles); 3-*O*-methylkaempferol (down-triangles); and 3,4′-di-*O*-methylkaempferol (diamonds).

sarcoplasmic reticulum by iteration, F_{min}/F_0 was allowed to vary between 0.15 and 0.25, taking into account previous studies on the quenching of intrinsic sarcoplasmic reticulum fluorescence by lipid quenchers (London and Feigenson, 1981).

The lipid concentrations used in the titration of Trp fluorescence shown in the Figures 2 and 3 were similar (the molar lipid/Ca^{2+}-ATPase ratio in sarcoplasmic reticulum membranes is 90 ± 10 (Entman and Van Winkle, 1986). Therefore, the ratio between the IC_{50} values obtained for egg lecithin liposomes with 10% octyl-tryptophan, $(IC_{50})_{PC}$, and for sarcoplasmic reticulum vesicles, $(IC_{50})_{SR}$, is proportional to the partition coefficient of the flavonoid in the sarcoplasmic reticulum membrane relative to that into egg lecithin lipid bilayer, $K_{SR/PC}$.

The values obtained for $(IC_{50})_{PC}$, $(IC_{50})_{SR}$, and $K_{SR/PC}$ for each flavonoid are listed in Table 4. These flavonoids produced 50% quenching of the intrinsic fluorescence of sarcoplasmic reticulum vesicles at molar ratios ranging between 0.04 and 0.1 moles of flavonoid per mole of sarcoplasmic reticulum lipid. In addition, 3,7-di-*O*-methylkaempferol is adsorbed to the sarcoplasmic reticulum membrane more strongly than all of the other flavonoids, i.e., more than a twofold higher incorporation than in egg lecithin liposomes.

In summer, the season of the year in which the secretion of exudate by *C. ladanifer* reaches its maximum value, the predominant flavonoids are (percentage of total flavonoids given in parenthesis): 3,7-di-*O*-methylkaempferol (65.07%) > 3,4′-di-*O*-methylkaempferol (13.54%) > 7-*O*-methylapigenin (10.69%) > 4′-*O*-methylapigenin (5.28%) > 3-*O*-methylkaempferol (4.74%) > apigenin (0.67%) (Table 1; see also Chaves et al., 1993, 1997).

The compounds 3,7-di-*O*-methylkaempferol, 3,4′-di-*O*-methylkaempferol, 3-*O*-methylkaempferol, 7-*O*-methylapigenin, and apigenin inhibited Ca^{2+}-ATPase activity (Figure 5). Only 4′-*O*-methylapigenin (up to 100 μM) had less than 20% effect on sarcoplasmic reticulum Ca^{2+}-ATPase activity in the presence of calcimycin (to relieve it from inhibition by the Ca^{2+}-gradient across the sarcoplasmic reticulum membrane). Inhibition produced by these flavonoids is rapid,

TABLE 4. CONCENTRATIONS OF *Cistus ladanifer* EXUDATE FLAVONOIDS THAT PRODUCED HALF-QUENCHING OF INTRINSIC FLUORESCENCE OF SARCOPLASMIC RETICULUM MEMBRANES AND OF THE FLUORESCENCE OF OCTYL-TRYPTOPHAN IN EGG LECITHIN LIPOSOMES

Flavonoid	$(IC_{50})_{PC}$ (μM)	$(IC_{50})_{SR}$ (μM)	$K_{SR/PC}$
Apigenin	3.0 ± 0.3	5.0 ± 0.5	0.6 ± 0.15
4′-*O*-Methylapigenin	4.5 ± 0.4	3.2 ± 0.3	1.4 ± 0.25
7-*O*-Methylapigenin	2.7 ± 0.3	1.8 ± 0.2	1.5 ± 0.3
3-*O*-Methylkaempferol	4.2 ± 0.4	4.5 ± 0.5	0.9 ± 0.2
3,4′-Di-*O*-Methylkaempferol	5.3 ± 0.4	3.6 ± 0.4	1.5 ± 0.25
3,7-Di-*O*-Methylkaempferol	5.1 ± 0.4	1.9 ± 0.3	2.7 ± 0.5

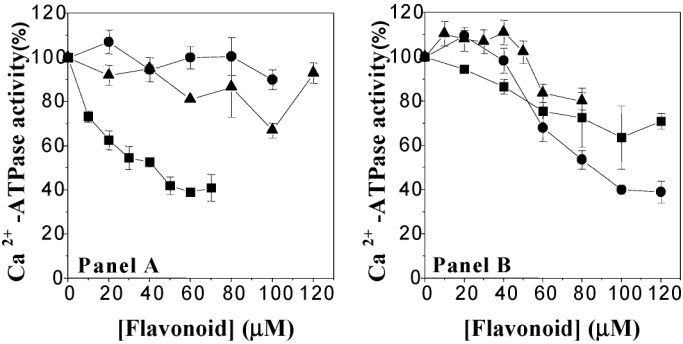

FIG. 5. Effects of different *C. ladanifer* flavonoids on the Ca^{2+}-ATPase activity of sarcoplasmic reticulum membranes in the presence of calcimycin. Panel A: Exudate apigenins: apigenin (squares); 4′-*O*-methylapigenin (circles); and 7-*O*-methylapigenin (up-triangles). Panel B: Exudate kaempferols: 3-*O*-methylkaempferol (squares); 3,4′-di-*O*-methylkaempferol (circles); and 3,7-di-*O*-methylkaempferol (up-triangles).

i.e., it develops in less than 30 sec after mixing of the solutions and remains constant for at least 1 hr. The maximum inhibition attained for each flavonoid (I_{max}) and the concentration of the flavonoid that produced 50% of the maximum inhibition (IC_{50}) are listed in Table 5. However, for methylated kaempferols, these values should be interpreted as apparent IC_{50} values, because of the biphasic pattern shown by the plots of the Ca^{2+}-ATPase activity versus the concentration of these flavonoids in the presence of calcimycin (apparent lag phase at the lower flavonoid concentrations tested, Figure 5).

TABLE 5. KINETIC PARAMETERS OF THE MODULATION OF THE CA^{2+}-ATPase ACTIVITY OF SARCOPLASMIC RETICULUM MEMBRANES BY THE *Cistus ladanifer* EXUDATE FLAVONOIDS

	−Calcimycin		+Calcimycin	
Flavonoid	Stimulation	IC_{50} (μM)	I_{max} (%)	IC_{50} (μM)
Apigenin	−	—	65 ± 5	12 ± 2
4′-*O*-Methylapigenin	+	> 100	< 20	—
7-*O*-Methylapigenin	+	70–80	30 ± 5	40 ± 5
3-*O*-Methylkaempferol	−	—	35 ± 5	45 ± 5
3,4′-Di-*O*-Methylkaempferol	−	—	65 ± 5	55 ± 5
3,7-Di-*O*-Methylkaempferol	+ + +	25–30	30 ± 5	60 ± 5

The Ca^{2+}-ATPase activity was measured in the absence/presence of 4% calcimycin (to relieve the inhibition by Ca^{2+} gradient across the sarcoplasmic reticulum membrane). $N = 5$.

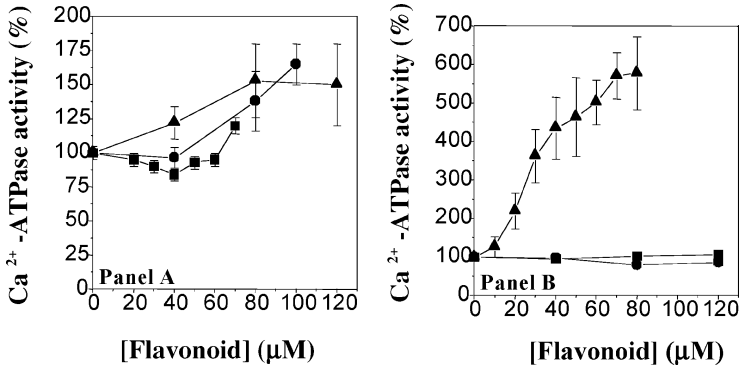

FIG. 6. Effects of the different *C. ladanifer* exudate flavonoids on Ca^{2+}-ATPase activity of sarcoplasmic reticulum membranes in the absence of calcimycin. Panel A: Exudate apigenins: apigenin (squares); 4′-*O*-methylapigenin (circles); and 7-*O*-methylapigenin (up-triangles). Panel B: Exudate kaempferols: 3-*O*-methylkaempferol (squares); 3,4′-di-*O*-methylkaempferol (circles); and 3,7-di-*O*-methylkaempferol (up-triangles).

In contrast, the flavonoids that are less potent as inhibitors of the Ca^{2+}-ATPase activity in presence of calcimycin—4′-*O*-methylapigenin, 7-*O*-methylapigenin, and 3,7-di-*O*-methylkaempferol—were found to stimulate Ca^{2+}-ATPase activity of sealed sarcoplasmic reticulum vesicles when assayed in the absence of calcimycin (Figure 6). Among these flavonoids, 3,7-di-*O*-methylkaempferol stimulated a higher Ca^{2+}-ATPase activity in the absence of calcimycin (Figure 6 and Table 5). All the other flavonoid (up to approximately 100 μM) produced less than 20% stimulation of the Ca^{2+}-ATPase activity in the absence of calcimycin, and no significant decrease (less than 10%) of the steady state ATP-dependent Ca^{2+} accumulation by sarcoplasmic reticulum vesicles. Stimulation of the Ca^{2+}-ATPase activity by methylated apigenins and 3,7-di-*O*-methylkaempferol correlated with a decrease in (close to 30%) steady state ATP-dependent Ca^{2+}-accumulation by sarcoplasmic reticulum vesicles. The flavonoid 3,7-di-*O*-methylkaempferol was the more potent of them, as 60 μM of this flavonoid produced the same effect as 100 μM of the methylated apigenins.

Most of the flavonoids of *C. ladanifer* strongly impair the ability of skeletal muscle sarcoplasmic reticulum to sequester Ca^{2+} either through inhibition of the Ca^{2+}-ATPase activity or through a decrease of the steady state ATP-dependent accumulation of Ca^{2+}.

DISCUSSION

C. ladanifer exudate impaired the ability to sequester and/or to accumulate Ca^{2+} by the skeletal muscle sarcoplasmic reticulum. The flavonoids within this

exudate strongly adsorb onto the sarcoplasmic reticulum membrane and can account for the functional perturbations of the sarcoplasmic reticulum produced at low summer leaf exudate doses (i.e., up to near 450 μg/ml). Because of the well-established leading role of Ca^{2+} uptake by the sarcoplasmic reticulum to promote skeletal muscle relaxation (Ruegg, 1988), this suggests that *C. ladanifer* exudate flavonoids are potent bioactive agents that impair skeletal muscle relaxation. The most potent flavonoid in the exudate is 3,7-di-*O*-methylkaempferol, the flavonoid that becomes most enriched in the *C. ladanifer* exudate from spring to summertime (Chaves et al., 1997). The decrease of the ATP-dependent steady state Ca^{2+} accumulation in sarcoplasmic reticulum, such as that produced by 3,7-di-*O*-methylkaempferol and to a minor extent by 4′-*O*-methyl and 7-*O*-methylapigenin, produces a typical phenotype of flaccid muscle paralysis associated with a long lasting rise of cytosolic Ca^{2+} (Ruegg, 1988).

Flavonoid impairment of sarcoplasmic reticulum function is a rapid effect. The results obtained with egg lecithin liposomes (Figure 3) showed that *C. ladanifer* exudate flavonoids strongly partition into the lipid bilayer, indicating that they can rapidly reach the intracellular spaces, and into exposed tissues by lipid-mediated diffusion. Owing to the anatomical disposition of mouth skeletal muscles in herbivores, these flavonoids could be expected to impair mastication of leaves above a threshold dose and induce an avoidance reaction of the herbivores for eating *C. ladanifer* leaves. Thus, within the conceptual framework of Arinafril and Suwandi (2001), *C. ladanifer* exudate flavonoids can be categorized as dissuasive compounds for herbivores. The exudate includes *C. ladanifer* leaves and photosynthetic stems and should afford an efficient protection against herbivores to the most vulnerable parts of the plant. An approximate threshold dose can be estimated from the overall volume of tissue exposed to flavonoids in the mouth of the herbivores (approximately 10 ml for an adult rabbit) and from the flavonoid composition of the exudate. Since *C. ladanifer* exudate shows a strong seasonal variation (Chaves et al., 1993, 1997; see also Table 1), the threshold dose should be expected to vary as well.

In summer, the exudate secretion reaches a peak of 140 mg/g of leaf dry weight (Chaves et al., 1997). Figure 1 suggests that only a few milligrams of *C. ladanifer* leaves (dry weight) are needed to produce an impaired relaxation of the mouth skeletal muscles of a rabbit ($>25\%$ functional decrease of the sarcoplasmic reticulum ability for Ca^{2+} withdrawal, either by inhibition of the Ca^{2+}-ATPase or by decrease of the ATP-dependent steady state accumulation of Ca^{2+}). For smaller herbivores, such as insects, the amount of *C. ladanifer* leaves needed will be lower, roughly proportional to their relative mouth sizes, as their skeletal muscle Ca^{2+}-ATPases show large functional and structural homology with rabbit skeletal muscle Ca^{2+}-ATPase (Entman and Van Winkle, 1986; Andersen, 1989). During the spring season, the amount of exudate per gram of dry leaves is only about one third of that in summertime (Chaves et al., 1993, 1997), with significant

quantitative variation in the flavonoid composition in the summer season (see Table 1). The potency of the summer exudate as an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase was found to be between four to sixfold higher than the spring exudate (Figure 1A). This closely correlates with the fourfold increase of 3,7-di-*O*-methylkaempferol plus 3,4′-di-*O*-methylkaempferol content in the exudate. However, impairment of sarcoplasmic reticulum function by summer exudate should be longer lasting than that afforded by the spring exudate, due to the enrichment in 3,7-di-*O*-methylkaempferol (see above). Seasonal variation of exudate flavonoids is by far the most relevant ecological variable, because other variables, i.e., average rainfall and UV irradiation, produce at most a twofold change in the exudate secretion, with only minor quantitative variations of the flavonoid composition (<20% variation in the content of total apigenins and kaempferols) (Chaves et al., 1997).

Thus, only 0.5–1 fresh *C. ladanifer* leaf (e.g., 20–40 mg dry-weight) should be needed to produce a long-lasting functional impairment of the mouth skeletal muscle of insect herbivores, and should elicit an avoidance reaction.

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