

CHAPTER 4

Inhibition of *Candida rugosa* lipase by saponins, flavonoids and alkaloids

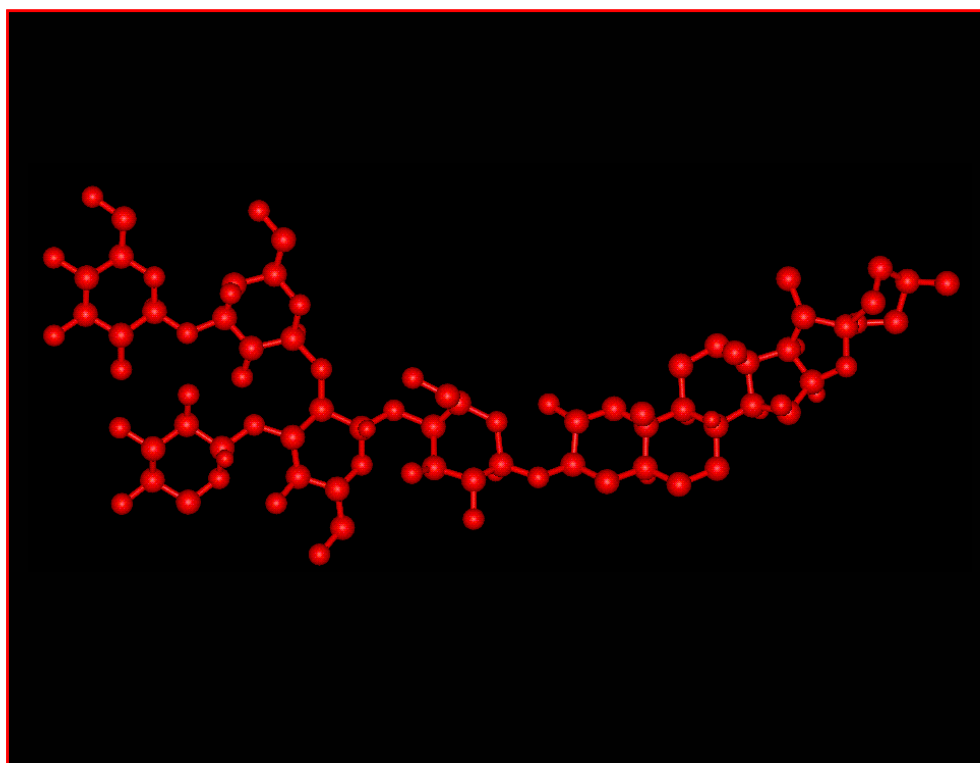


Figure C4.1 Three-dimensional structure of digitonin.

1 INTRODUCTION AND OBJECTIVES

We have mentioned before (see General Introduction 3.4 and 4, and Chapter 3) that lipase inhibitors have a high pharmacological interest because they could help in the therapy of diseases in which lipases play an important role such as obesity or infective diseases produced by lipolytic microorganisms like *P. acnes* and *H. pylori* (Grippa *et al.*, 1999; Higaki, 2003; Gupta *et al.*, 2004). Moreover, inhibition studies on lipases could contribute to better understand the mechanism of action of these enzymes, favoring the design of evolved lipases with improved biochemical properties and more suitable for biotechnological applications (Simons *et al.*, 1999; General Introduction 4).

However, many known lipase inhibitors such as phenylmethylsulfonyl fluoride (PMSF) are not suitable for pharmacological applications due to their elevated toxicity, or due to their lack of activity on some lipases (Gupta *et al.*, 2004). Therefore, there is an increasing interest in finding new lipase inhibitors of natural origin. Plant secondary metabolites present in herbal drugs and food have shown to be very useful in the prevention and treatment of many diseases (Singh *et al.*, 2003). Among these metabolites, saponins, flavonoids and alkaloids are a promising source of lipase inhibitors since they are present in high concentrations in plant extracts capable of inhibiting porcine pancreatic lipase activity (Shimura *et al.*, 1992). Furthermore, these compounds are also present in several plant extracts that have been used for the treatment of lipase-related diseases such as ulcer (Borrelli & Izzo, 2000) and acne (Higaki, 2003). Recent studies have also demonstrated that saponins like platycodin D (Han *et al.*, 2002), flavonoids like quercetin (Gatto *et al.*, 2002) and alkaloids like berberine and sanguinarine (Grippa *et al.*, 1999) are good lipase inhibitors (General Introduction 4.2).

However, further studies are necessary to elucidate the effect of other purified plant metabolites on lipolytic enzymes in order to select the most suitable ones for therapeutic or preventive pharmacological treatments. For this reason, this chapter is focused on the evaluation of the effect of natural substances on the model lipase from *Candida rugosa*. The exact aim of this work was:

1. **To evaluate the inhibitory effect of several saponins, flavonoids and alkaloids (see Figure C4.2 on the model lipase from *Candida rugosa*.**
 - a. Saponins: β -aescin, digitonin, glycyrrhizic acid and *Quillaja* saponin.
 - b. Flavonoids: 3-hydroxyflavone, 5-hydroxyflavone, (\pm)-catechin and kaempferol.
 - c. Alkaloids: aspidospermine, papaverine, physostigmine, pilocarpine, raubasine, rescinnamine, reserpine and trigonelline.

◆ **NOTE:** The work presented in this chapter was mainly performed at the Università degli studi di Roma “*La Sapienza*”, in collaboration with Dr. S. Falcochio, Dr. E. Xoxi and L. Villo, under the supervision of Dr. L. Saso.

2 MATERIALS AND METHODS

Unless otherwise stated, the materials and methods used were those previously described in the General Materials and Methods section.

2.1 EVALUATION OF LIPASE INHIBITION

2.1.1 Sample preparation

Candida rugosa lipase (CRL) was obtained from Sigma (cat. No. L-1754) and was prepared in 50 mM Tris–HCl buffer (pH 7.4) for HPLC assays, or in 50 mM phosphate buffer (pH 7) for the colorimetric microassay.

The substances under evaluation (Figure C4.2) were dissolved at their maximum solubility in a proper solvent such as water (*Quillaja* saponin, trigonelline, papaverine and pilocarpine), acetone (3-hydroxyflavone and 5-hydroxyflavone), dimethyl sulfoxide (glycyrrhizic acid and kaempferol) or methanol (the rest of substances evaluated).

2.1.2 Inhibition assays by HPLC

Lipase inhibition assays by HPLC were performed as previously described (General Materials and Methods 5.5). Essentially, 2020 μl reaction mixtures containing varying inhibitor concentrations (from 0 to their maximum solubility), 3.75% inhibitor solvent, 0.46 mM β -NL, 1.25 % acetone, 1 mM sodium taurocholate, 3.5 mM NaCl, 1.5 mM CaCl_2 , 50 mM Tris–HCl buffer (pH 7.4) and 10 $\mu\text{g ml}^{-1}$ CRL, were incubated for 30 minutes at 37 °C under gentle mixing. Then, β -naphthol was extracted with 2 ml of ethyl acetate, and 500 μl of the organic phase were withdrawn, evaporated at room temperature under a nitrogen stream and redissolved in 1 ml methanol. Aliquots of 50 μl

were then analyzed at room temperature by HPLC and the enzymatic activity was determined as previously described (General Materials and Methods 5.5).

2.1.3 Inhibition assays by colorimetric microassay

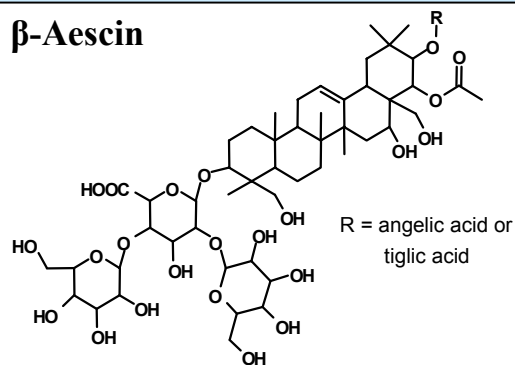
The inhibition produced by saponins was also determined by using the colorimetric microassay developed in Chapter 3. The assay was performed as is described (General Materials and Methods 5.3.2) with the following modifications: after a preincubation of the enzyme solution and the reaction premix for 15 min at 37 °C, the absorbance at $\lambda = 405$ nm was measured from each reaction mixture incubated for 30 min at 37 °C and containing the following final concentrations: inhibitor (from 0 to their maximum solubility, prepared as explained for the HPLC assay), 2.5% inhibitor solvent, 1 mM *p*-NPL, 2.5% isopropanol, 0.6% Triton X-100, 50 mM phosphate buffer (pH 7) and 10 $\mu\text{g ml}^{-1}$ of CRL. This method was only performed for saponins due to the fact that some of the flavonoids and alkaloids evaluated in this work produced yellow or not clear solutions that interfered with the measurement of *p*-NP absorbance.

2.1.4 Determination of lipase inhibition

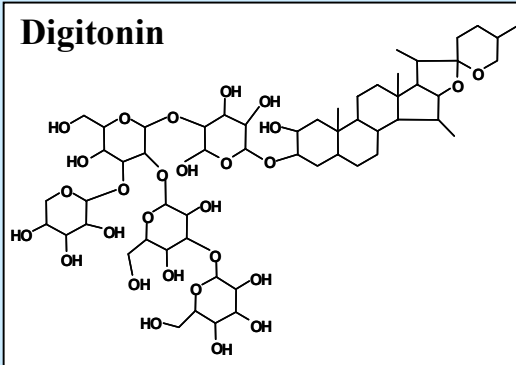
Inhibition experiments were performed at least in duplicate (HPLC) or at least in triplicate (colorimetric microassay), being each replicate the result of an independent assay performed in duplicate. Lipase inhibition was calculated from the residual activity detected in the presence of the compound under assay with respect to that of untreated samples (without inhibitor but including the inhibitor solvent). The concentrations yielding a lipase inhibition of 16% (IC_{16}) and 50% (IC_{50}) were calculated by regression analysis with Rsquare coefficients higher than 0.99, as previously described (General Materials and Methods 5.7).

Saponins

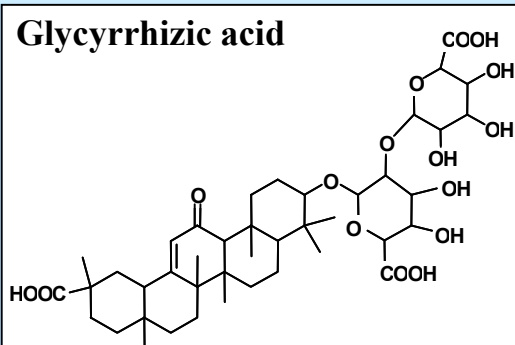
β -Aescin



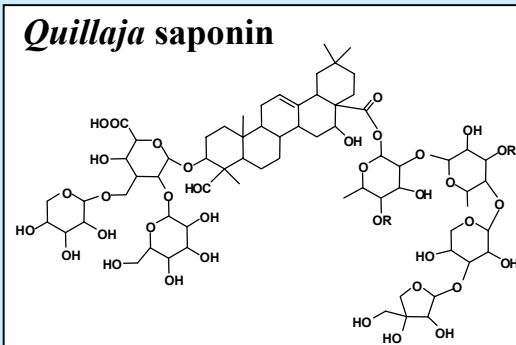
Digitonin



Glycyrrhizic acid

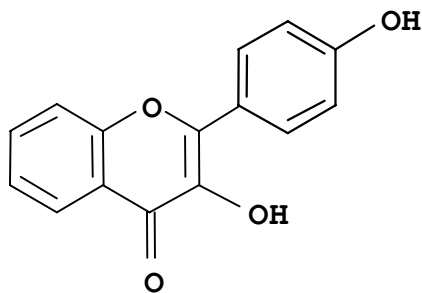


Quillaja saponin

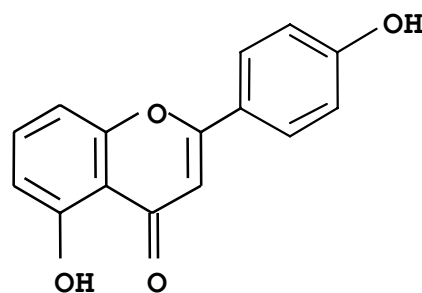


Flavonoids

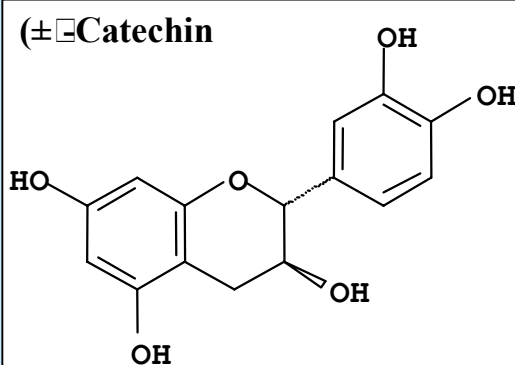
3-hydroxyflavone



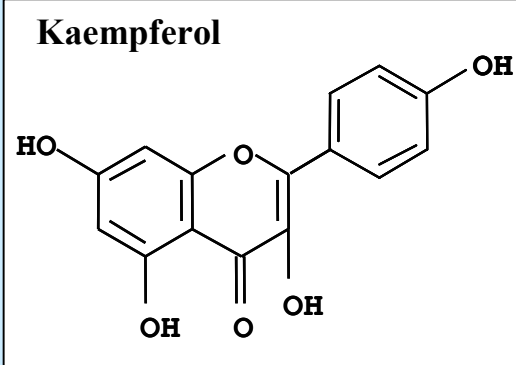
5-hydroxyflavone



(\pm) Catechin



Kaempferol



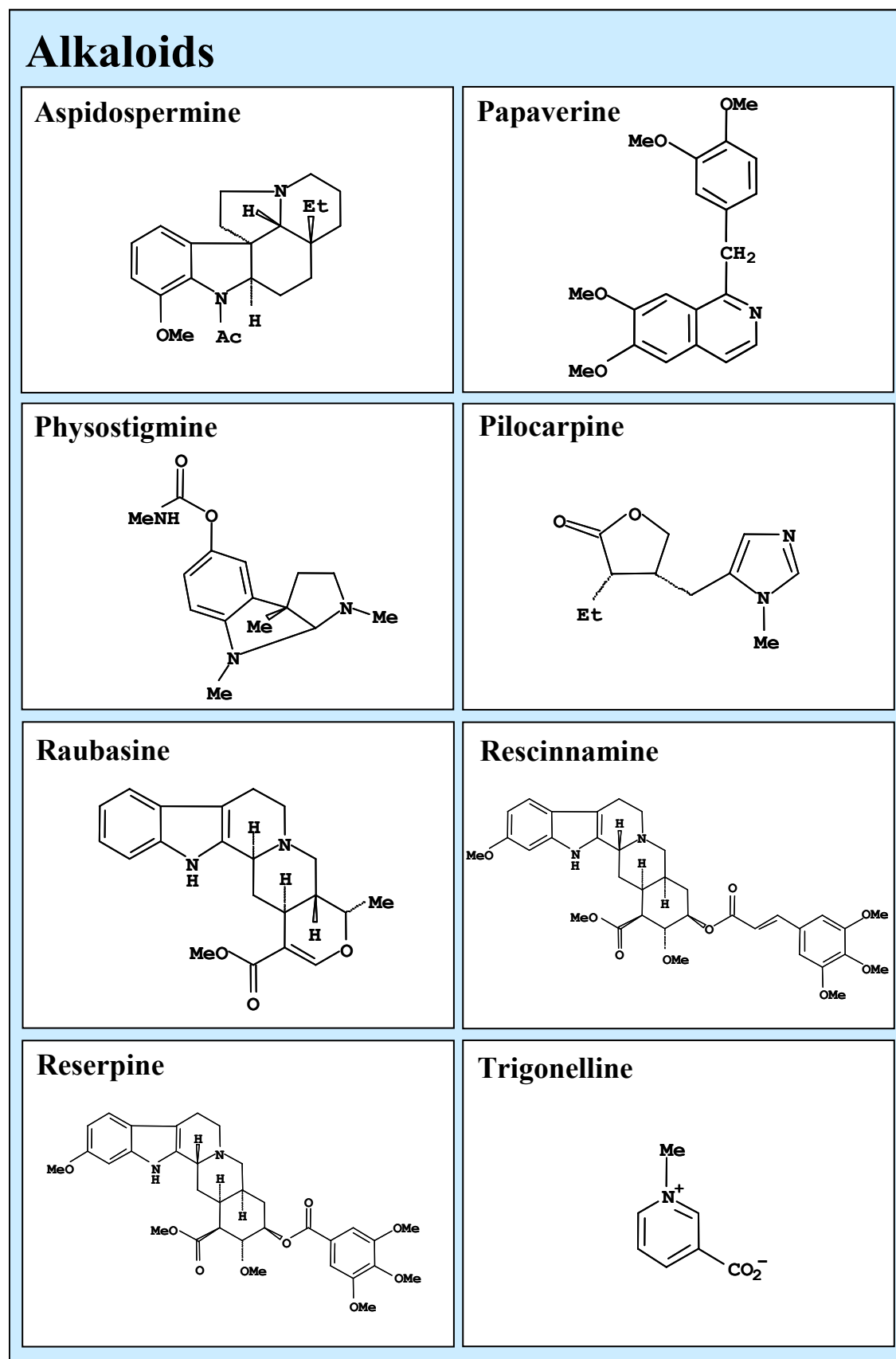


Figure C4.2 Structure of the saponins, flavonoids (previous page) and alkaloids analyzed.

3 RESULTS

The results obtained by HPLC with respect to the effect of several saponins, flavonoids and alkaloids on CRL are shown in Table C4.1 and Figure C4.3.

Table C4.1 Effect of natural substances on CRL evaluated by HPLC.

SUBSTANCE	S _{max} * (M□)	IC ₁₆ (M□)	IC ₅₀ (M□)
<i>I. Saponins</i>			
β-Aescin	4.0·10 ⁻⁴	7.5·10 ⁻⁷	8.0·10 ⁻⁶
Digitonin	1.5·10 ⁻⁴	5.9·10 ⁻⁶	2.4·10 ⁻⁵
Glycyrrhizic acid	2.0·10 ⁻³	8.5·10 ⁻⁴	> S _{max}
<i>Quillaja</i> saponin	1.4·10 ⁻³	1.0·10 ⁻⁴	5.5·10 ⁻⁴
<i>II. Flavonoids</i>			
3-hydroxyflavone	1.5·10 ⁻³	1.1·10 ⁻³	> S _{max}
5-hydroxyflavone	2.5·10 ⁻³	4.5·10 ⁻⁴	> S _{max}
(±)-Catechin	3.0·10 ⁻²	8.3·10 ⁻⁴	6.3·10 ⁻³
Kaempferol	1.4·10 ⁻²	2.8·10 ⁻⁴	7.5·10 ⁻³
<i>III. Alkaloids</i>			
Aspidospermine	2.5·10 ⁻³	6.3·10 ⁻⁴	1.0·10 ⁻³
Papaverine	2.0·10 ⁻³		Inactive
Physostigmine	3.8·10 ⁻²		Inactive
Pilocarpine	1.0·10 ⁻²		Inactive
Raubasine	1.2·10 ⁻³		Inactive
Rescinnamine	8.0·10 ⁻⁴	2.3·10 ⁻⁶	1.6·10 ⁻⁴
Reserpine	4.5·10 ⁻⁴	1.1·10 ⁻⁴	4.0·10 ⁻⁴
Trigonelline	5.0·10 ⁻²	6.8·10 ⁻³	2.0·10 ⁻²

S_{max}*: Highest concentration at which each substance was tested.

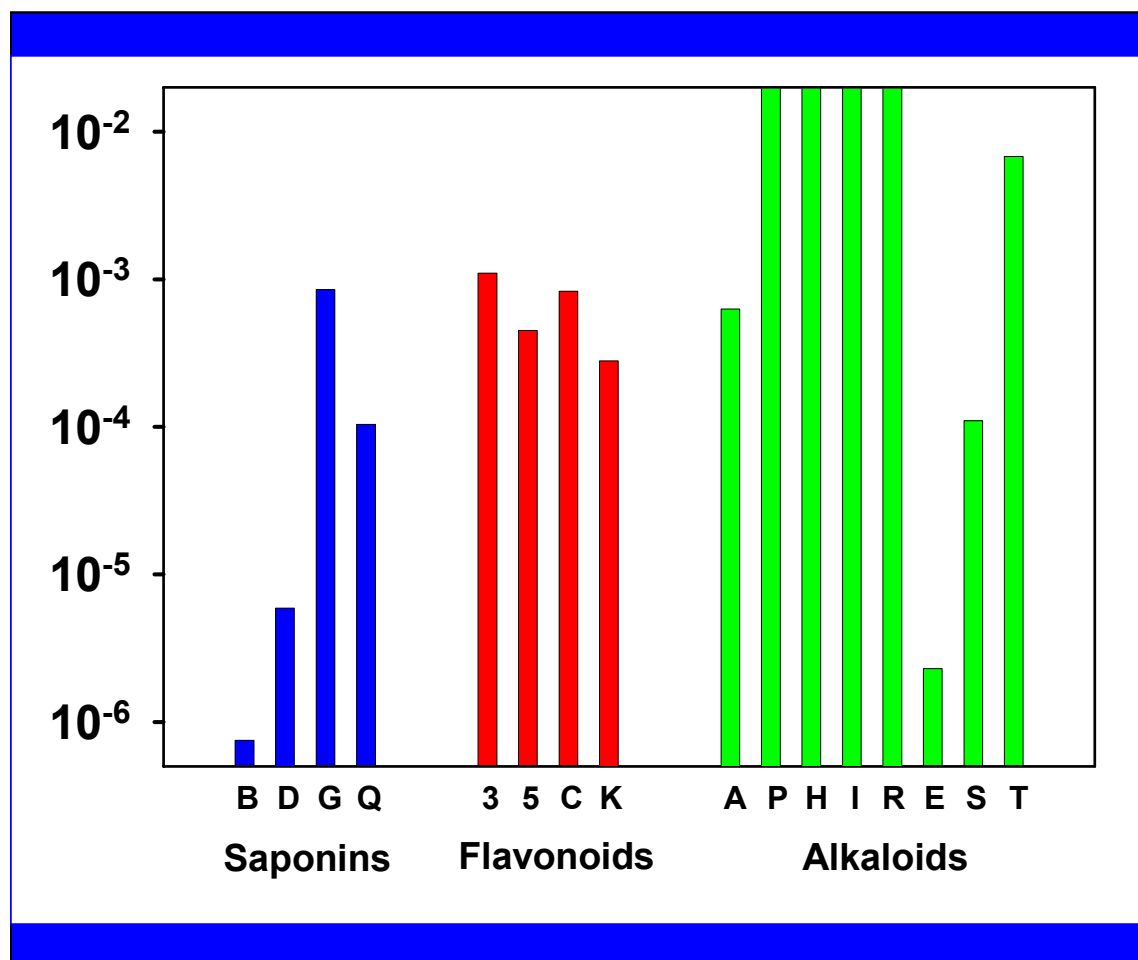


Figure C4.3 Comparison of the IC_{16} of natural substances evaluated on CRL by HPLC.

The concentrations producing a 16% inhibition (IC_{16}) on CRL activity are compared for the 16 compounds evaluated, since the IC_{50} was not reached in most of them. These substances are plotted in the following order (from left to right): **saponins**: β -aescin (B), digitonin (D), glycyrrhizic acid (G) and *Quillaja* saponin (\square); **flavonoids**: 3-hydroxyflavone (3), 5-hydroxyflavone (5), (\pm)-catechin (C) and kaempferol (K); **alkaloids**: aspidospermine (A), papaverine (P), physostigmine (H), pilocarpine (I), raubasine (R), rescinnamine (E), reserpine (S) and trigonelline (T). The compounds being inactive are plotted as bars reaching the top of the graphic.

3.1 EFFECT OF SAPONINS ON *CANDIDA rugosa* LIPASE

The effect of β -aescin, digitonin, glycyrrhizic acid (GA) and *Quillaja* saponin (\square S) on CRL was evaluated by HPLC (Table C4.1; Figure C4.3 and C4.4). All saponins

showed a high inhibition on CRL, being the most active group of inhibitors in general terms.

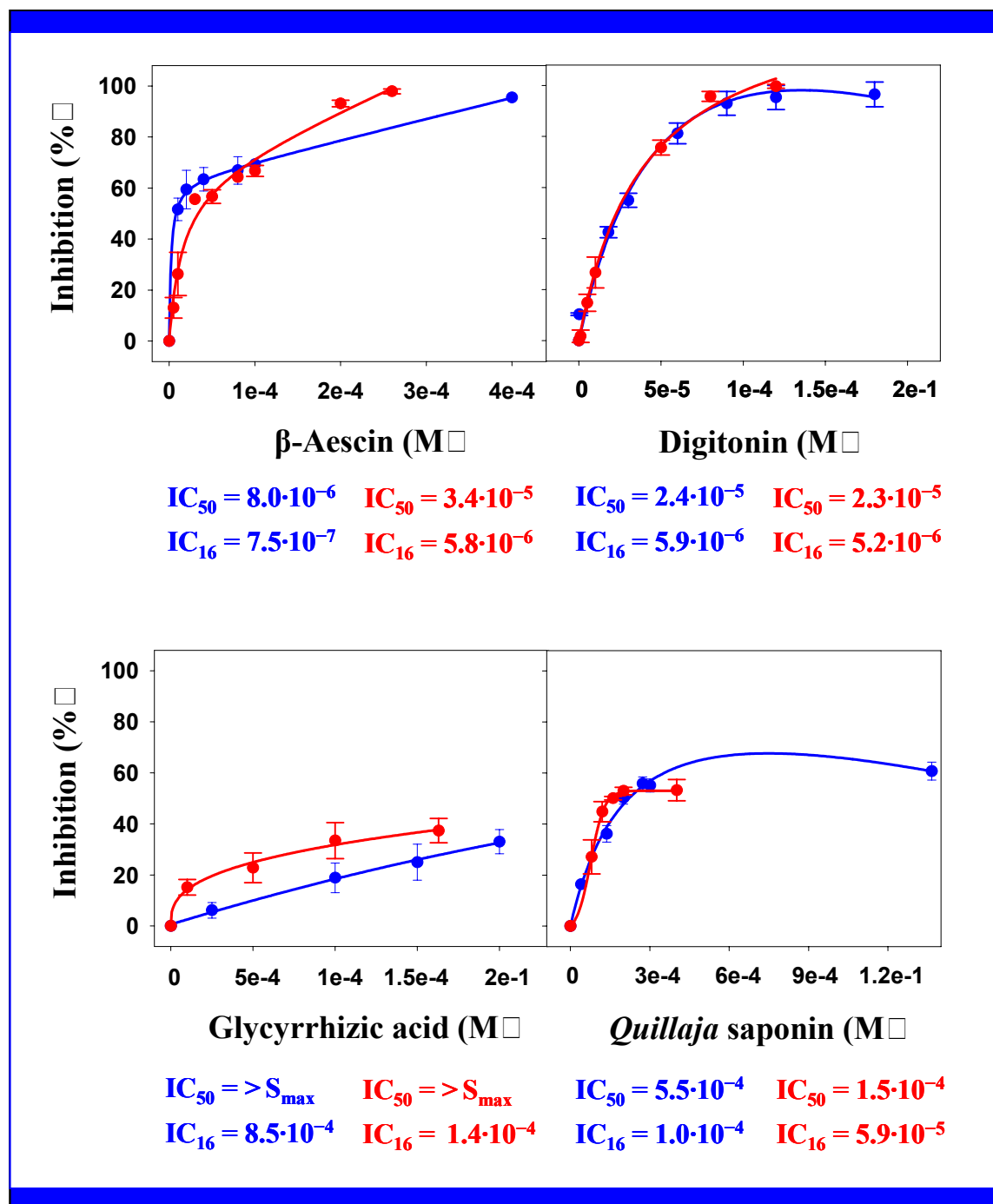


Figure C4.4 Comparison of the inhibition produced by saponins on CRL evaluated by HPLC (blue) and colorimetric microassay (red)

Both digitonin and β -aescin were the most active compounds ($IC_{50} = 0.8\text{--}2.4 \cdot 10^{-5}$ M). β -Aescin was the most active inhibitor at concentrations below $2\text{--}5 \cdot 10^{-5}$ M, and digitonin displayed the highest inhibition above this concentration. On the other hand, GA produced the lower inhibition among the saponins evaluated ($IC_{16} = 8.5 \cdot 10^{-4}$ M). \square S showed an increasing inhibition at concentrations up to $1.5\text{--}2.2 \cdot 10^{-4}$ M (about the IC_{50}). However, above these concentrations the inhibition by \square S remained stable between 50–60 %, in contrast to the other saponins which always produced a higher inhibition on CRL when increasing concentrations were assayed (Table C4.1, Figure C4.3).

The inhibition produced by saponins on CRL activity was also analyzed by using the colorimetric microassay developed in Chapter 3 in order to confirm the results obtained by HPLC. Saponins displayed almost the same profile than in the HPLC experiments when they were assayed by colorimetric microassay, and only β -aescin and GA showed relevant but small differences between the two methods when they were assayed at low concentrations (Figure C4.4).

3.2 EFFECT OF FLAVONOIDS ON *CANDIDA rugosa* LIPASE

Flavonoids produced a lower inhibition than saponins on CRL (except for GA) when they were assayed by HPLC. All flavonoids displayed a similar IC_{16} (from $2.8 \cdot 10^{-4}$ M to $1.1 \cdot 10^{-3}$ M), whereas the IC_{50} was only achieved for (\pm)-catechin and kaempferol (IC_{50} of $6.3 \cdot 10^{-3}$ M and $7.5 \cdot 10^{-3}$ M, respectively). At low concentrations kaempferol was the most active flavonoid, although (\pm)-catechin was the most efficient at high concentrations. 5-hydroxyflavone (5-HF) showed an intermediate inhibition at low concentrations, halfway between (\pm)-catechin and kaempferol. However, 3-hydroxyflavone (3-HF) caused an inhibition more than two-fold lower than its isomer 5-HF (Table C4.1, Figure C4.3 and C4.4).

3.3 EFFECT OF ALKALOIDS ON *CANDIDA rugosa* LIPASE

The inhibition obtained by HPLC assays on CRL by alkaloids was very different among the compounds analyzed. On the one hand, rescinnamine was the most active alkaloid, displaying an IC_{16} of $2.3 \cdot 10^{-6}$ M, similar to those of digitonin and β -aescin. However, this alkaloid was less active than the mentioned saponins at high concentrations ($IC_{50} = 1.6 \cdot 10^{-4}$ M). Reserpine, the second most active alkaloid, showed an inhibition rate higher than flavonoids, and similar to \square S. Aspidospermine displayed an inhibition lower than saponins (except GA) and similar to most of the flavonoids at low concentrations (IC_{16}), but it was more active than flavonoids at high concentrations ($IC_{50} = 1.0 \cdot 10^{-3}$ M). Trigonelline produced the lowest inhibition among the active compounds, while papaverine, physostigmine, pilocarpine and raubasine produced almost no inhibition (Table C4.1, Figure C4.3).

4 DISCUSSION

4.1 INHIBITION OF *Candida rugosa* LIPASE BY NATURAL SUBSTANCES

The effect of several saponins, flavonoids and alkaloids on *C. rugosa* lipase (CRL) was analyzed to evaluate their potential as antilipase drugs. CRL, one of the best-known lipases, widely used in biotechnology, and being commercially available (Benjamin & Pandey, 1998; General Introduction 2.4), was used as a model lipase to evaluate these substances. Moreover, CRL inhibition could be of interest in the treatment against *C. rugosa* strains refractory to antifungal therapy involved in veterinary mycology and in emerging pathogenesis on immunocompromised patients (Colombo *et al.*, 2003), and could serve as a model for other fungal pathogenic lipases. In addition, analysis of CRL activity and inhibition could help in controlling and increasing its effectiveness in a wide range of biotechnological processes.

The HPLC method was chosen to evaluate these substances due to several reasons. First of all, the analysis of natural substances reported here forms part of a more extensive screening carried out using this method. Thus, the HPLC method was maintained for the assays reported here in order to obtain results directly comparable to those previously reported (Grippa *et al.*, 1999; Gatto *et al.*, 2000), since there are some differences in the inhibition degree obtained when using different methods, as previously explained (Chapter 3). Another reason was the fact that the colorimetric microassay was not completely developed at the moment these experiments started. Moreover, some of the flavonoids and alkaloids evaluated produced yellow or not clear solutions that interfered with the measurement of absorbance. However, the colorimetric microassay was used to confirm the inhibition results obtained for saponins, which were the most active natural substances, and producing clear and non-colored solutions.

With respect to the compounds evaluated, they were selected for different reasons. Some of them had demonstrated a high antilipase activity in a preliminary screening performed previously in the research group of Dr. L. Saso from the Università degli studi di Roma “*La Sapienza*”, whereas others displayed an elevated antilipase potential in computational analyses performed by Dr. G. Nicolosi from the CNR (*Centro Nazionale di Ricerca*, Italy) or were structurally related to previously reported lipase inhibitors (Grippa *et al.*, 1999; Gatto *et al.*, 2000)

Among the compounds tested, saponins were very active, flavonoids displayed lower inhibition, and alkaloids, the most heterogeneous group, showed a wide inhibition range (Table C4.1, Figure C4.3). The different inhibition produced by the latter substances is probably related to their different structure (Figure C4.2) and physicochemical properties, as explained below.

4.1.1 Saponins

As it has been mentioned before (General Introduction 4.2.1), saponins are glycosidic compounds containing a steroid or triterpenoid sapogenin nucleus. They are characterized by antimicrobial, hypocholesterolemic, anti-inflammatory and other activities and by a low toxicity (Milgate & Roberts, 1995; Sirtori, 2001; Singh *et al.*, 2003). They have also a certain activity against ulcer (Borrelli & Izzo 2000) and human pathogenic *Candida* spp. (Bader *et al.*, 2000). Furthermore, *in vitro* inhibition by saponin-containing formulations on *P. acnes* lipase (Higaki *et al.*, 2000) and by purified saponins (platycodin D and dioscin) on pancreatic lipases (Han *et al.*, 2002; Kwon *et al.*, 2003) has been also reported recently. However, saponins from *Medicago sativa* activated porcine pancreatic lipase (Sroka *et al.*, 1997).

Here, we have analyzed the effect of β -aescin, digitonin, glycyrrhizic acid (GA) and *Quillaja* saponin (\square S) on CRL by HPLC and by colorimetric microassay (Table C4.1; Figure C4.3 and C4.4). Saponins displayed almost the same profile when they were assayed by both methods, in agreement with the similar results previously obtained by using these two methods when inhibition by saturated fatty acids was evaluated (Chapter 3). Only β -aescin and GA showed relevant but small differences

between the two methods when they were assayed at low concentrations. The differences found could be explained by the different composition of the reaction mixture of each method with respect to the type and amount of substrate used and the ionic strength of the medium (see Chapter 3). In fact, a different type and amount of substrate used can affect the competition of the inhibitor and the substrate for the active site of the enzyme, and the ionic strength of the medium can affect the interaction between charged substances, as well as the properties of the emulsion by altering the critical micellar concentration (CMC) of detergents like saponins (Edwards *et al.*, 1994; Chapter 3).

Direct inhibition of a lipase by these saponins is described here for the first time. Only inhibition of the hydrolysis of acetyl fluorescein (substrate for lipases and esterases) by β -aescin on several bacterial strains (Zablotowicz *et al.*, 1996), inhibition of a cholesterol esterase by digitonin (Karmanskii, 1983), inhibition of pancreatic lipase by GA-containing plant extracts (Shimura *et al.*, 1992), and the presence of GA and \square S in plant formulations that inhibited *P. acnes* lipase (Higaki *et al.*, 2000) have been reported before.

Saponins are known detergents due to their amphiphilic nature and surface activity (Milgate & Roberts 1995). Detergents are considered unspecific reversible lipase inhibitors that possibly interfere with the enzyme, the substrate or the interface (Patkar & Björkling, 1994). The higher contents in glycosidic groups and their branching could be responsible for the higher inhibition found for β -aescin, digitonin and \square S with respect to GA, which bears only a disaccharide. In fact, CRL catalytic triad is in the first part of a deep and narrow tunnel (diameter of 4 Å) (Pleiss *et al.*, 1998; Cygler & Schrag, 1997 and 1999) in which the aglicone nucleus of saponins is too large to enter. We think that the aglicone nucleus remains outside of the tunnel, in part at the hydrophobic patch located just under the tunnel entrance (Cygler & Schrag, 1997 and 1999), and only the carbohydrate chains enter in the first part of the tunnel interfering with the catalytic triad of the enzyme. Thus, longer glycosidic chains would be more able to reach the catalytic triad inside the tunnel than the short ones, which would explain the lower inhibition produced by GA, the compound with the shorter carbohydrate chains.

4.1.2 Flavonoids

As previously explained (General Introduction 4.2.2), flavonoids are benzo- γ -pyrone derivatives that can be grouped according to the presence of different substituents and the degree of benzo- γ -pyrone ring saturation. They can also be glycosidated or methylated. Flavonoids are capable of modulating the activity of many enzymes and cell systems producing, among others, antitumoral, cardioprotective or anti-inflammatory effects (Di Carlo *et al.*, 1999). They are also components of antiacne formulations, and some of them, like kaempferol, have shown antiulcer activity (Izzo *et al.*, 1994). Furthermore, some flavonoids like quercetin (Gatto *et al.*, 2002) and hesperidin or neohesperidin (Kawaguchi *et al.*, 1997) are known lipase inhibitors.

We have evaluated the inhibition produced by (\pm)-catechin, kaempferol, 5-hydroxyflavone (5-HF) and 3-hydroxyflavone (3-HF) on CRL (Table C4.1, Figure C4.3). All of them displayed a similar IC_{16} and a lower inhibition than saponins (except GA). It has been previously reported that catechin-rich extracts or catechin-related compounds like (–)-epicatechin inhibit pancreatic or gastric lipases and rat adipose tissue-derived lipoprotein lipase, although they do not inhibit the hormone-sensitive lipase (Shimura *et al.*, 1992; Juhel *et al.*, 2000; Yoshikawa *et al.*, 2002). Inhibition of pancreatic lipase by kaempferol, a compound similar to quercetin, or by extracts of *Tea sinensis* (rich in kaempferol) has also been reported (Shimura *et al.*, 1992; Shimura *et al.*, 1994). However, microbial lipase inhibition by (\pm)-catechin and kaempferol, as well as lipase inhibition by 5-HF and 3-HF, are described here for the first time.

The mechanism of enzyme inhibition by flavonoids is unclear. The flavonoids analyzed are small molecules able to enter the tunnel of CRL and able to interact with the hydrophobic and hydrophilic residues of this tunnel (Pleiss *et al.*, 1998; Cygler & Schrag, 1997 and 1999; see General Discussion) because of being aromatic molecules with hydrophilic groups (mainly hydroxyl groups). The similar inhibition observed in this work for (\pm)-catechin and kaempferol, in comparison with the lower effect produced by 3-HF and 5-HF, mainly at high concentrations, suggests that a larger number of hydroxyl groups is necessary to obtain a higher solubility of these compounds, which would allow to reach a higher lipase inhibition in the assay. With respect to the structure of these molecules, the non-planar conformation of catechin

seems to favour inhibition of prostaglandin endoperoxide synthase with respect to planar compounds like quercetin and kaempferol (Kalkbrenner *et al.*, 1992). However, the similar inhibition produced by (\pm)-catechin and kaempferol discards this hypothesis and suggests that a pivotal role could be associated to the presence of the hydroxyl group found at position 7 (ring A) of both compounds. This group, which is missing in the less active flavonoids 3-HF and 5-HF, could interact with the catalytic amino acids of CRL, favouring the interaction between these flavonoids and the active site of this lipase (see General Discussion). On the contrary, the occurrence of a carbonyl function on the benzopyran moiety seems to be no essential, considering the efficiency observed for (\pm)-catechin. Moreover, it must be considered that two enantioforms of (\pm)-catechin are operative, perhaps with different activity grade. Anyhow, the requirement for a polyoxydrylated compound for the inhibition of CRL by the above considered polyphenols is also in agreement with the model proposed for the inhibition of the H^+ , K^+ -ATPase by polyphenols (Murakami *et al.*, 1999).

4.1.3 Alkaloids

As it has been mentioned before (General Introduction 4.2.3), alkaloids are very diverse natural substances (and their related synthetic compounds) that contain nitrogen, usually as a part of a cyclic system. They are active on many enzymes and biological systems, such as the central nervous system, producing a wide range of effects (Evans, 1996). Moreover, berberine and other alkaloids such as cheliritrine, chelidonine and sanguinarine, but not palmatine, have demonstrated antilipase activity on CRL (Grippa *et al.*, 1999).

We have analyzed here the effect on CRL of trigonelline and several heterocyclic alkaloids. Inhibition was very different among the compounds assayed, even for those belonging to the same structural group. Among the indole-benzopyrrole alkaloids, rescinnamine was the most active, displaying an IC_{16} similar (but a higher IC_{50}) to those of digitonin and β -aescin. Reserpine was the second most active alkaloid, showing an inhibition higher than flavonoids and similar to $\square S$. Aspidospermine displayed an inhibition rate similar to most flavonoids, and physostigmine and

raubasine caused almost no inhibition of CRL. Among the non-indole-benzopyrrole alkaloids, only trigonelline (pyridine and piperidine group) was active, but it caused a low inhibition, whereas papaverine (isoquinoline group) and pilocarpine (imidazole or glyoxaline group) did not inhibit CRL (Table C4.1, Figure C4.3). Lipase inhibition by the mentioned alkaloids is reported here for the first time, except for reserpine and physostigmine. Among other effects, reserpine increased lipoprotein lipase activity of heart tissue (Heinroth & Forster, 1980) and inhibited epididymal hormone-sensitive lipase but not that of other tissues (Matsuura, 1970). Physostigmine, a known esterase inhibitor, inhibited also lipases like bile-salt stimulated lipase (Ellis & Hamosh, 1992) or pancreatic lipase (Hall, 1961), but not lipolytic liver extracts (Katz, 1957). Moreover, some isoquinoline-group alkaloids similar to papaverine like palmatine were inactive on CRL, although berberine and others inhibited CRL (Grippa *et al.*, 1999).

Inhibition by alkaloids is difficult to explain since they have very different structures. Except trigonelline (the only monocyclic alkaloid evaluated), most of them have a large structure with several rings, which makes difficult their entering into the narrow tunnel of CRL, and thus, their access to the active site of this lipase (Pleiss *et al.*, 1998; Cygler & Schrag, 1997 and 1999). This fact could explain the lack of inhibition of some of them. However, further considerations are possible with respect to the indole-benzopyrrole alkaloids. Raubasine (inactive) and rescinnamine or reserpine (the most active) share the same indole nucleus (Figure C4.2). Thus, it is clear that the inhibition produced by rescinnamine and reserpine is, respectively, due to the [(2E)-1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]oxy or the [(3,4,5-trimethoxybenzoyl)oxy] groups bound to C18 in the indole nucleus of the alkaloid. As we suggested before for the carbohydrate chains of saponins, we think that these two groups can enter the tunnel of CRL and interfere with the active site of the enzyme, while the indole nucleus remains outside the tunnel, probably associated with the hydrophobic patch located under the tunnel entrance or with other hydrophobic structures of CRL surface (Cygler & Schrag, 1997 and 1999). This hypothesis is supported by the fact that rescinnamine, whose longer group could enter more deeply into the tunnel of CRL than that of reserpine, is itself more active than reserpine.

Inhibition by alkaloids seems to be also related, at least partially, to their logP, a known measure of the hydrophobicity of the molecule. The logP is 3.8–5.4 (more

hydrophobic) for the alkaloids found to be active (except trigonelline whose structure is very different because it is monocyclic), and 0.0–3.4 (less hydrophobic) for the inactive alkaloids. The relationship between logP and inhibition is not directly proportional, but suggests that a more hydrophobic nature could facilitate the interaction between the inhibitor and the enzyme, the micelle or the micelle-enzyme complex.

4.2 FUTURE PERSPECTIVES

The results obtained in this chapter are interesting with respect to their application in pharmacology, in the design of new lipase inhibitors, or in the evolution of the active site of the CRL in order to obtain an enzyme more suitable for performing new biotechnological processes.

Saponins, (±)-catechin and kaempferol produced a high CRL inhibition, and some of them have been described to be active against acne, ulcer or pancreatic lipase, as explained before. Moreover, almost all of them have a low toxicity. For example, β-aescin is currently used in the treatment of peripheral vascular diseases as it displays more effectiveness and tolerability than the conventional therapy (Sirtori, 2001). However, at high concentrations, (±)-catechin produces toxic effects like haemolysis, and kaempferol is genotoxic, although they are some of the most occurring compounds among the approximately 1 g of flavonoids that contains the daily human diet (Di carlo *et al.*, 1999). Therefore, these inhibitors are promising candidates for the prevention (creams, shampoos, etc) and the therapy of diseases in which lipases play an important role like acne, *H. pylori*-associated ulcers, obesity, or fungal diseases. For example, β-aescin, which has also a fungistatic activity (Gruiz, 1996), could be very suitable for candidiasis. However, further studies are necessary to confirm their pharmacological potential. On the contrary, the potential use of rescinnamine and reserpine to treat lipase-related diseases is more limited by their other marked pharmacological activities (La Barre, 1960).

5 CONCLUSIONS

- I. The effect of several saponins: β -aescin, digitonin, glycyrrhizic acid (GA) and *Quillaja* saponin; flavonoids: 3-hydroxyflavone, 5-hydroxyflavone, (\pm)-catechin and kaempferol; and alkaloids: aspidospermine, papaverine, physostigmine, pilocarpine, raubasine, rescinnamine, reserpine and trigonelline on the model lipase from *C. rugosa* has been analyzed by HPLC to evaluate their potential as antilipase drugs.
- II. The inhibition produced by most of these compounds is described here for the first time, and the differences found among them could be related to their structure and physicochemical properties.
- III. Saponins appeared very active, being β -aescin and digitonin the most active compounds among all the inhibitors assayed. *Quillaja* saponin produced a lower inhibition and glycyrrhizic acid was the less active saponin. The degree of inhibition produced by these saponins seems to be related to larger and more branched carbohydrate side chains.
- IV. The inhibition produced by saponins in the HPLC experiments has been confirmed by the colorimetric microassay, reinforcing the reliability of the results obtained.
- V. The inhibitory activity of flavonoids was lower than that of saponins (except for glycyrrhizic acid). (\pm)-Catechin and kaempferol were the most active whereas 3-hydroxyflavone and 5-hydroxyflavone were less active. The number and disposition of the hydroxyl groups of these flavonoids seems to be related to the inhibition they produce.
- VI. Alkaloids was the most heterogeneous group assayed. Rescinnamine and reserpine were the most active, and produced an inhibition similar to some saponins, whereas aspidospermine and trigonelline produced lower inhibition. The other

alkaloids evaluated showed almost no inhibition. The inhibition produced by these alkaloids seems to be related to their structure and hydrophobicity.

- VII. β -Aescin, digitonin, kaempferol or (\pm)-catechin, strong lipase inhibitors with a low toxicity and present also in antiacne or antiulcer herbal drugs, are promising candidates for the treatment or prevention of lipase-related diseases.

6 PUBLICATIONS

The work corresponding to this chapter has been submitted for publication in the following scientific article (see Annex I for the full text):

- **Ruiz, C., Falcocchio, S., Xoxi, E., Villo, L., Nicolosi, G., Pastor, F.I.J., Díaz, P. and Saso, L.** (2005) Inhibition of *Candida rugosa* lipase by saponins, flavonoids and alkaloids. *Biosci. Biotechnol. Biochem.* Submitted.