



## Assessment of *in vitro* inhibitory effect of khaya tea infusion on porcine pancreatic lipase activity

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### Abstract

The inhibition of digestive lipases is a widely studied mechanism for the determination of the potential efficacy of natural products as anti-obesity agents. In this regard, the use of natural products might be an excellent alternative strategy for the development of safe and effective anti-obesity drugs that lack the unpleasant side effects of existing chemical drugs. The aim of this study was to assess the potential effect of Khaya tea in the treatment of obesity through the evaluation of its *in vitro* inhibitory effects on porcine pancreatic lipase activity. Extract was obtained by infusion of powder in water 4g/150 ml at 95°C for 10 min and was subjected to chemical analysis and kinetics studies. The *in vitro* inhibitory effect of Khaya tea on lipase activity was studied by assessing porcine pancreatic lipase activities at varying concentrations of khaya tea extract (0, 1, 2, 3, 4, 5 mg/ml). Results showed that Khaya tea induced a competitive inhibition (85±2%) of the porcine pancreatic lipase activity in the hydrolysis of substrate emulsified with gum arabic : I.C (inhibition Concentration)<sub>50</sub> = 0.96 ± 0.03 mg of Khaya tea /ml. These *in vitro* inhibition results suggest that Khaya tea could be a potential therapeutic alternative in the treatment of obesity caused by fat-rich diets.

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## Introduction

Human obesity is one of the most serious health problems associated with an increase risk of several diseases like hypertension, coronary heart disease, type II diabetes, stroke, osteoarthritis and cancer. One of the most important strategies in the treatment of obesity is the development of nutrient digestion and absorption inhibitors, as an attempt to reduce the energy intake through gastrointestinal mechanism, without altering any central mechanism (Hi *et al.*, 2004). This relies on the fact that the consumption of dietary fat is an important contribution factor to human obesity (Kumar *et al.*, 2010).

The hydrolysis of dietary triglycerides to monoglycerides and free fatty acids in gastro intestinal tract is catalyzed by gastric and pancreatic lipase. The enzyme suspected to be the main responsible of in gastrointestinal lipid digestion is pancreatic lipase with its cofactor co-lipase (Berit *et al.*, 2002). Pancreatic lipase or triacylglycerol acyl hydrolase (*EC* 3.1.1.3) inhibition is a widely studied mechanism for the determination of the potential efficacy of natural products as anti-obesity agents (Birari *et al.*, 2007). Pancreatic lipase is responsible for the hydrolysis of 50–70% of the total dietary fats. It removes fatty acids from the  $\alpha$  and  $\alpha'$  positions of dietary triglycerides, yielding  $\beta$ -monoglycerides and long chain saturated and polyunsaturated fatty acids as lipolytic products (Mukherjee *et al.*, 2003). Orlistat, one of the two clinically approved drugs for obesity treatment, has been shown to act by inhibiting pancreatic lipase. It has been used in several *in vitro* lipase inhibition studies, and found to inhibit gastric lipase, pancreatic lipase and carboxyl ester lipase, but not phospholipase A2 (Krebs *et al.*, 2000). Porcine and human pancreatic lipases have both a similar mechanism of inhibition with Matea tea (Fernanda *et al.*, 2009).

The mechanism by which Orlistat inhibits the action of lipases is well known (Cudrey *et al.*, 1993). It also has certain unpleasant gastrointestinal side effects such as oily stools, oily spotting and flatulence, among others. The success of Orlistat has prompted

research for the identification of new pancreatic lipase inhibitors that lack some of these unpleasant side effects.

To date, the potential of natural products for the treatment of obesity is still largely unexplored and might be an excellent alternative strategy for the development of safe and effective anti-obesity drugs (Birari *et al.*, 2007). Many polyphenolics components such as flavones, flavonols, tannins, and chalcones are active against pancreatic lipase. Several reports have described the inhibition of human pancreatic lipase in the presence of unfermented (green tea) (Juhel *et al.*, 2000) and semi-fermented (oolong tea) (Nakai *et al.*, 2005) extracts of *Camellia sinensis* L. Tea polyphenols have been reported to have various biological and pharmacological functions, such as an anti-HIV effect, antioxidant, antimutagenic, anticarcinogenic, antitopoisomerase, antiobesity, and hypocholesterolemic activities (Masaaki *et al.*, 1991). Khaya is a tree of the family Meliaceae consisting of seven species which originated from tropical Africa. *Khaya senegalensis* is present in Mauritania, Senegal and right up to Northern Uganda. Its bark is often used to treat certain illnesses such as malaria, headaches, fever, smallpox, diarrhea, lumbago (back pain), rheumatism, wounds, etc... In addition to polyphenols such as alkaloids, saponins, flavonoids, tannins and DPPH anti-radical activity, Khaya bark is also rich in phenols, and some phragmalin limonoids such as khayanolides, khayanosides, 2,6-dihydrofissinolide and two mexicanolides named khayanone and 2-hydroxyseneganolide ( Khalid *et al.*, 2002). Khaya Tea, a beverage produced from extracts of the bark of khaya tree is even said to be effective in weight loss therapies. Though the effect of some polyphenols in the inhibition of digestive enzymes has been demonstrated (Griffiths *et al.*, 1986) no study on the inhibitory activity of Khaya tea against pancreatic lipases has been reported to date.

Therefore, the aim of this study was to assess the potential effect of Khaya tea in the treatment of obesity through the evaluation of its *in vitro* inhibitory effects on porcine pancreatic lipase activity.

Emphasis will be laid on the use of a pH-stat titrator for the enzyme kinetic study.

## Materials and methods

### Reagents

Porcine pancreatic lipase (type II, 100– 400 U/mg protein using olive oil), and gum arabic (10 % v/v) were purchased from Sigma Chemicals (St Louis, MO, USA).

### Khaya Tea preparation and determination of $IC_{50}$ (Inhibition Concentration)

Fresh barks were harvested in September 2010 early in the morning (6 A.M.) from 3 mature trees located at Meskine, a locality close to Maroua, in the far north-region of Cameroon. The barks were coarsely crushed in a mortar with a pestle, dried in a ventilated-convention oven at 35°C for 24h, then ground in a hammer mill (Culatti Polymix, Germany) endowed with a sieve of pore size 250  $\mu$ m. The powder obtained was used to prepare the beverage or Khaya tea obtained by infusion of 4 g of bark's powder of *Khaya senegalensis* in 150 ml of hot water at 95°C for 10 min, followed by filtration through a 250  $\mu$ m pore size diameter sieve. The resultant filtrate served as Khaya tea extract and was used for further experiments throughout our study. The inhibitory action of Khaya tea on porcine pancreatic lipase was assessed using various concentrations (0.5-5 mg/ml) of this Khaya tea extract.

### Effect of khaya tea on the activity of porcine pancreatic lipase

Porcine pancreatic lipase activity was determined by measuring, through back-titration on a pH-stat titrator equipment (842-Titrando, Metrohm, swiss made), the rate of release of free fatty acids using an olive oil emulsion as substrate. The substrate emulsion (5 ml) was prepared by ultrasonification of olive oil (10 ml) in a solution containing 10% W/V gum arabic, 225 mM NaCl, 3 mM tris-HCl (pH 8.0), and 15 mM  $CaCl_2$  according to Fernanda *et al.* (2009). In the principle of the pH-stat functioning, the pure enzyme solution is injected under constant agitation in a thermostated vessel (maintained at 37 °C)

containing the emulsified substrate and in which the reaction of lipolysis proceeds. During hydrolysis, the pH which normally decreases due to  $H^+$  release is maintained constant by an automatic addition of a soda solution (NaOH) contained in a glass jar. In these conditions, the mole number of NaOH added automatically to maintain the constant pH with the set point during reaction is equal to the quantity in mole of free fatty acid released. Lipase activity was calculated knowing the volume of 0.1N Sodium hydroxide solution (NaOH) added automatically by the pH-stat up to the set point (Sarda *et al.*, 1958).

After addition of 50  $\mu$ l of Khaya tea extract (final concentration: 0, 0.5, 1, 2, 3, 4, 5 mg /ml respectively), or 50  $\mu$ l tetrahydrolipstatin standard inhibitor (final concentration: 0, 0.01, 0.1, 1, 10, 100, 200, 300, 400, 500  $\mu$ g.ml<sup>-1</sup> respectively) dissolved in 20 ml Tris-HCl (pH 8.0), the assay tube was then centrifuged at 1 000g for 5 min and pre-incubated for 10 min at 37 °C. The enzyme reaction was started by the addition of 50  $\mu$ l porcine pancreatic lipase solution, containing 3 mmol/l Tris-HCl (pH 8.0). After incubation for 30 min at 37 °C, the concentration of free fatty acids in the reaction mixture was measured knowing the volume of 0.1 sodium hydroxide solution (NaOH) added automatically by the titrator to maintain the pH constant with the set point during reaction (Sarda *et al.*, 1958).The inhibitory activity of each sample was reported as the relative percentage compared with the control value.

For lipase activity calculations, it is assumed through the chemical reaction of triglyceride hydrolysis that the number of moles of free fatty acids released is equal to the number of moles of sodium hydroxide (0.1 N) injected in the reaction medium, as the latter can be calculated knowing the volume.

The concentration of inhibitor,  $IC_{50}$ , which reduced fifty percent of pancreatic lipase activity, was deduced by extrapolation from the curve activity against Khaya concentration axis (Figure 1).

### Determination of the mechanism of inhibition

In order to evaluate the mechanism of inhibition, the

effects of substrate and khaya extracts were assessed following a factorial design 3x3 with 3 oleic acid concentrations (33; 50 and 100 mmol/l) and 3 khaya tea concentrations (0; 1 and 3 mg/ml). From the derived curves the initial velocity,  $V_i$ , were determined as the tangent of the curve during the first min of reaction. Following these curves of Michaelis-Menten ( $V_i$  versus  $S$ ), Lineavewear-Burk graph ( $1/V_i$  versus  $1/S$ ) were plotted in order to determine the mechanism of inhibition using the sigmaplot software<sup>R</sup> (Aspire software International, Ashburn, VA). The inhibition constant,  $K_i$  was determined based on the competitive-type inhibition equation as followed and confirmed by figure 4:

$$K_{m,app} = K_m \left( 1 + \frac{[I]}{K_i} \right);$$

Where  $K_{m,app}$  and  $K_m$  represent the Michaelis-Menten constant in the presence and absence of Khaya tea, respectively,  $[I]$  represents the concentration of inhibitor Khaya tea.

### Statistical Analysis

All of numerical data were expressed as means  $\pm$  SD (Standard Deviation). Significance of differences was examined by variance analysis, followed by Dunnett' test. The statistical significance for the expression of the analysis was also assessed by ANOVA. Results with  $P < 0.05$  were considered to be significant.

### Results

#### *Inhibition Concentration ( $IC_{50}$ ) of khaya tea and orlistat standard*

The curve representing the variation of percent activity of Lipase versus khaya tea concentration is presented in Figure 1. The curve showed that as the level of Khaya tea was increasing, the lipase activity decreased as an exponential function up to an inhibitory percentage of  $85 \pm 2\%$  corresponding to 3 mg/ml khaya tea. The positive standard Orlistat also revealed a significant decrease of the lipase activity, but the concentrations used were much lower than for khaya tea.

**Table 1.** Phytochemical analysis of extracts from Khaya tea.

Chemical compounds	Hot Water Extract
Saponins	+
Cardiac Glycosides	+

- = Absent; + = present.

**Table 2.** Quantitative results of phytochemical analysis of Khaya tea.

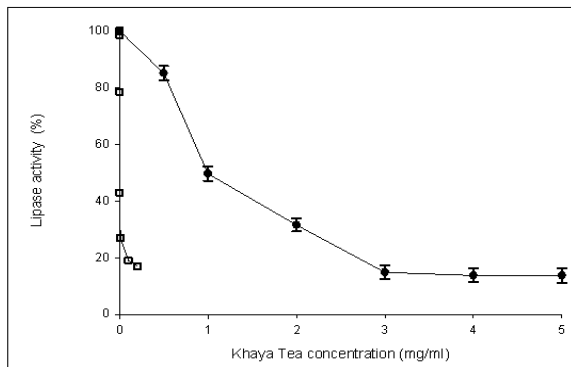
Nature	Quantity( /100g DM)	Percentage(%)
Total polyphenols (mg)	1400 $\pm$ 0.05	64,218
Flavonoids(mg)	0.078 $\pm$ 0.004	0,004
Tannins(mg)	69 $\pm$ 3.2	3,165
DPPH (%)	77 $\pm$ 2.1	3,532
Saponins(mg)	470 $\pm$ 20	21,559
Alkaloids(mg)	164 $\pm$ 8	7,523
Total	2180,078	100,000

$IC_{50}$  representing the concentration of khaya tea which inhibited 50% of pancreatic lipase activity was  $0.96 \pm 0.03$  mg.ml<sup>-1</sup> for khaya tea and  $0.85 \pm 0.02$   $\mu$ g.ml<sup>-1</sup> for tetrahydrolipstatin.

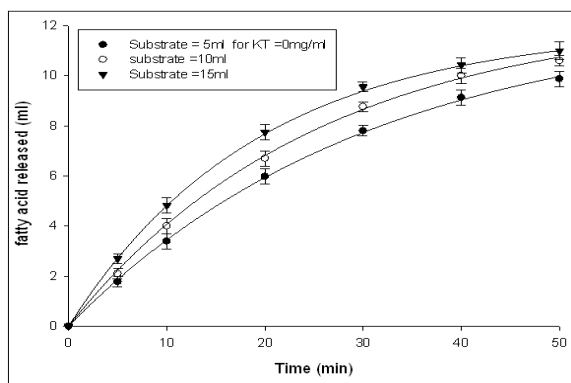
#### *Active compounds in khaya tea*

The content of some active compounds in khaya tea is presented in Table 1. Generally polyphenols were the most represented with an average of  $1.4 \pm 0.05$  g/100g DM from which flavonoids represented  $78 \pm 3.9$   $\mu$ g/100g DM and tannins  $69 \pm 3.2$  mg/100g DM. Total saponins were also quite represented with a

content of  $470 \pm 20$  mg/100g DM and alkaloids  $164 \pm 8$  mg/100g DM. Khaya tea extract also exhibited radical scavenging activity, with a percentage inhibition of the DPPH radical  $77 \pm 2.1\%$ .



**Fig. 1.** Inhibitory effects of Khaya Tea (filled circle) and Orlistat (open square) on porcine pancreatic lipase activities. The reaction mixture contained olive oil emulsified with gum Arabic (10% W/V).



**Fig. 2 a.** Influence of substrate, and Khaya Tea (KT) concentration (0mg/ml) on olive oil lipolysis. Data represent means  $\pm$  SD,  $n=3$ .

#### Kinetics of inhibition of porcine pancreatic lipase by Khaya tea

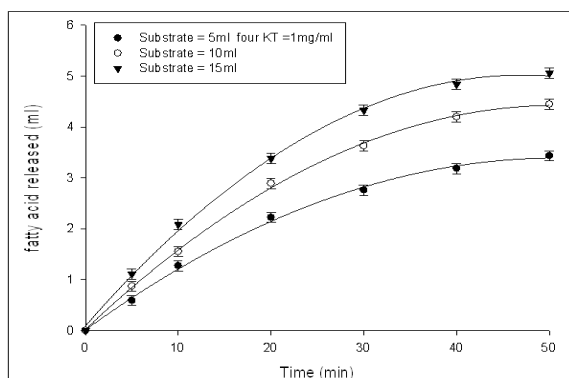
Figure 2 shows the kinetic change of fatty acid liberation at varying substrate (olive oil) and inhibitor (khaya tea) concentrations. Irrespective of the kinetics conditions, the amount of fatty acids liberated increased linearly during the first stage, and then slowed down progressively to become constant at the end. In addition, as the substrate concentration was increasing, the amount of fatty acids liberated also increased; while it decreased with the increase in inhibitor concentration. The initial rate of free fatty acids formation, usually called initial velocity, ( $V_i$ ), was determined as a function of substrate

concentration for different inhibitor concentrations as shown in Figure 2. The resultant curves were of hyperbolic form. Irrespective of the inhibitor's concentration, the rate of fatty acid liberation increased linearly as the substrate concentration increased, and then slowed down progressively to a constant rate called maximum velocity,  $V_{max}$ . The hyperbolic curve is known as Michaelis-Menten curve, from which  $V_{max}$  represents the maximum velocity and the concentration corresponding to the half  $V_{max}$  is the Michaelis constant,  $K_m$ . These constants are best determined with the reverse representation of Lineweaver-Burk as shown in Figure 3. From the Lineweaver-Burk curves, the  $y$ -intercept represented the  $1/V_{max}$  while the  $X$ -intercept represented the  $1/K_m$ . In this respect the value for the  $y$ -intercept in the equation,  $V_{max} = 52.6 \pm 0.5$   $\mu\text{mol}/\text{min}/\text{ml}$  which for each curve remained at a fixed point, revealing that the maximal velocity did not change following variation of inhibitor concentration. However  $1/K_m$  changed as the concentration of inhibitor varied. The value for  $K_m$  without khaya tea was  $29.1 \pm 0.3$  mmol/l, and with the addition of 1.0 and 3.0 mg/ml of Khaya tea respectively, the values shifted to  $43.7 \pm 0.8$  and  $97.1 \pm 0.6$  mmol/l. Results from figure 3 showed that  $K_m$  which represents the affinity of the enzyme for the substrate significantly increased with the increase in Khaya tea concentrations, while the maximum velocity did not change. The mechanism that describes such variation is the competitive inhibition. In such mechanism, the apparent Michaelis-Menten constant vary linearly with the Inhibitor's concentration. The constant of inhibition,  $K_i$ , was then graphically evaluated to be  $1 \pm 0.3$  mg/ml according to Figure 4.

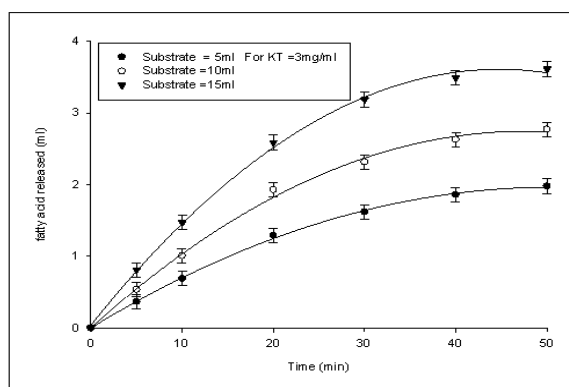
#### Discussion

The inhibition of pancreatic lipase is one of the most studied mechanisms for the determination of the efficacy of the anti-obesity potential of natural products such as hypolipidemic agents (Carriere *et al.*, 2001). The kinetic results attested the activity of porcine pancreatic lipase at a half-maximal inhibitory concentration of  $0.96 \pm 0.03$  mg.ml<sup>-1</sup> of Khaya tea

extract, which was justified by a drastic drop of the lipolytic activity of this enzyme (Figure1), corresponding to 85% (> 80 %) inhibition in the presence of 3 mg/ml of Khaya tea with respect to that of the blank (0 mg/ml). This result was higher than the 75% and 55% inhibition (Han *et al.*, 2001) with green and black teas respectively at a concentration of 2.0 g/l on porcine pancreatic lipase activity. It was also higher than 66.5% inhibition with 6.0 mg/g of green tea on the same enzyme (Juhel *et al.*, 2000).



**Fig. 2 b.** Influence of substrate, and Khaya Tea (KT) concentration (1 mg/ml) on olive oil lipolysis.

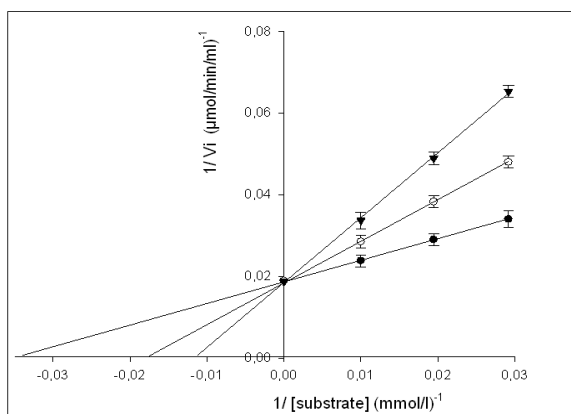


**Fig. 2 c.** Influence of substrate, and Khaya Tea (KT) concentration (3mg/ml) on olive oil lipolysis.

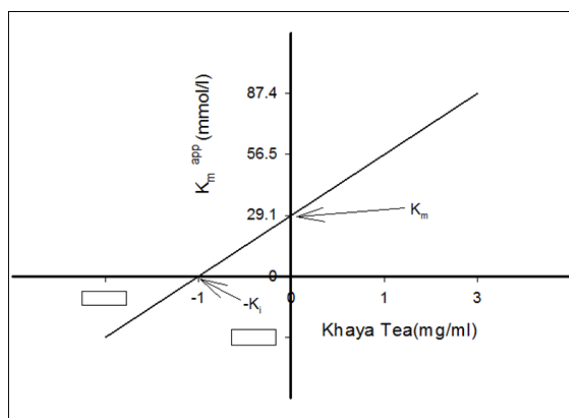
Inhibitors might prevent the enzyme activity in various ways, including specific, non-specific, competitive and non-competitive inhibitory processes. Experimental data clearly shows that, the inhibition process induced by Khaya tea extract was of a competitive type. However, it was not so easy as for the specific or non-specific aspect of this inhibition. Specific inhibition of lipases is generally obtained by “active site-directed” compounds which interact with molecules of the active site of the enzyme (catalytic serine, free cysteine residue, C-

terminal domain). In this category, we can name components such as Diethyl p-nitrophenyl phosphate ( $E_{600}$ ), a phosphorylating agent that reacts specifically and irreversibly with the active serine site and is known to be a strong inhibitor of both pancreatic and gastric lipases (Masaaki *et al.*, 1991). Tetrahydrolipstatin (THL) used as a positive control in our study is also a specific inhibitor of gastrointestinal lipases (Zhi *et al.*, 1999). Specific inhibition of human pancreatic lipase by monoclonal antibodies was also reported (Aoubala *et al.*, 1995). Khaya tea may have act as a specific inhibitor of lipase activity through some components present in the extract. It may be reasonable to consider that, the components of Khaya tea efficiently inhibited the activity of porcine pancreatic lipase by interacting with olive oil micelles emulsified with gum arabic (10 %,v/v), by adsorbing at the surface of the substrate and thus slowing down the lipolytic reaction. It is known that higher molecular weight polyphenols (for example condensed tannins) have a high affinity for proteins, and produce insoluble complexes with proteins and other macromolecules (Sarda *et al.*, 1958). The process is established by the interaction of at least two groups of polyphenol hydroxides with proteins (Sarda *et al.*, 1958). The activities of human pancreatic and gastric lipases were significantly reduced (96.8% and 66.5 % respectively) in the presence of green tea extract containing 25% catechins, especially in the form of epigallocatechin-3-gallate. On the other hand, epicatechin gallate and epigallocatechin gallate had no effect on the activity of human pancreatic lipase, but saponins (a mixture of theasaponins  $E_1$  and  $E_2$ ) isolates from Oolong tea inhibited the activity of human pancreatic lipase *in-vitro* (Han *et al.*, 2001). The dimeric components of flavanol-3-ol gallate, esters of Oolong such as proanthocyanidins, oolonghomobisflavans and theasinensins were more activated in the inhibition of pancreatic lipase than epigallocatechingallate, and the presence of galloyl particles within the structure was necessary to increase the inhibition of pancreatic lipase (Nakai *et al.*, 2005). This does not exclude the fact that, other components found in Khaya tea could be involved in certain inhibitory effects. According to

our previous analyses, Khaya tea has antioxidant activities and the main polyphenols present are tannins, saponins, flavonoids, alkaloids, cardiac glycosid, phenols. In previous studies, certain components were said to be very good inhibitors of pancreatic lipase activity such as saponins, flavonoids and tannins, but for other components, no relationship was established between them and the inhibition potential of pancreatic lipase.



**Fig. 3.** Lineweaver-Burk graph of free fatty acid (product) released from gum arabic and olive oil emulsion. Various concentrations of khaya tea were used: 0 mg/ml (closed), 1 mg/ml (open circles), and 3 mg/ml (Inverted triangle).



**Fig. 4.** Variations of the affinity constant in absence (0 mg/ml) and presence (1 and 3 mg/ml) of Khaya tea in order to determine the inhibition constant of Khaya tea.

A non-specific inhibition process can also be observed on lipases with proteins and tension-active agents, which usually do not interact specifically with lipases but generally adsorb at the lipid/water interface, thus affecting the “quality” of this interface and hence lipase activity (Pieroni *et al.*, 1990). It may also be

reasonable to consider that the active components of Khaya tea efficiently inhibited the activity of pancreatic lipase by interacting non-specifically at the lipid/water interface, the adsorption at the surface of this interface affecting the “quality” of the interface and thus slowing down the lipolytic reaction.

### Conclusion

Results from our study showed that Khaya tea competitively and efficiently (> 80%) inhibited porcine pancreatic lipase activity *in vitro* and could play a major role in a therapeutic strategy for the treatment of obesity caused by fat-rich diets. However, the specific nature of this inhibition was clearly established using more accurate techniques such as monomolecular lipid films, continuous monitoring of the reaction allowing the simultaneous measurement of enzyme activity and the partitioning process (using the pH-stat technique) and tridimensional enzyme-inhibitor interaction studies are required. Also, determination of the large chemical composition of Khaya tea extract may contribute to this goal and also to clearly identify the active component.

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