

## Lipolytic effect of a polyphenolic citrus dry extract of red orange, grapefruit, orange (SINETROL) in human body fat adipocytes. Mechanism of action by inhibition of cAMP-phosphodiesterase (PDE)

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### Abstract

The present study investigated the lipolytic (break of fat stored) effect of a citrus-based polyphenolic dietary supplement (SINETROL) at human adipocytes (*ex vivo*), body fat (clinical) and biochemical levels (inhibition of phosphodiesterase). Free fatty acids (FFA) release was used as indicator of human adipocyte lipolysis and SINETROL activity has been compared with known lipolytic products (isoproterenol, theopylline and caffeine). SINETROL stimulated significantly the lipolytic activity in a range of 6 fold greater than the control. Moreover, SINETROL has 2.1 greater activity than guarana 12% caffeine while its content in caffeine is 3 times lower.

Clinically, two groups of 10 volunteers with BMI relevant of overweight were compared during 4 and 12 weeks with 1.4 g/day SINETROL and placebo supplementation. In the SINETROL Group the body fat (%) decreased with a significant difference of 5.53% and 15.6% after 4 and 12 weeks, respectively, while the body weight (kg) decreased with a significant difference of 2.2 and 5.2 kg after 4 and 12 weeks, respectively.

These observed effects are linked to SINETROL polyphenolic composition and its resulting synergistic activity. SINETROL is a potent inhibitor of cAMP-phosphodiesterase (PDE) (97%) compared to other purified compounds (cyanidin-3 glycoside, narangin, caffeine). These results suggest that SINETROL has a strong lipolytic effect mediated by cAMP-PDE inhibition. SINETROL may serve to prevent obesity by decreasing BMI.

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**Keywords:** Lipolysis; Citrus; Adipocytes; Phosphodiesterase; Body fat; Free fatty acids (FFA)

### Introduction

People are becoming fatter in all parts of the world. Recent studies show that excess body fat weight is pandemic, with one-half to two-thirds of the overall study

population (men and women in 65 countries) being overweight or obese in 2006. People who are overweight have a higher risk of heart diseases, type II diabetes and other diseases including some cancers (Balkau et al., 2007).

In this context, it seems interesting to consider a food supplement based on polyphenols that could contribute to the loss of body fat weight without any secondary effect.

SINETROL is a polyphenolic mixture of flavonoids such as anthocyanins and flavanones. It is a citrus-based

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fruits (juice, peels, seeds) extracted by physical treatment (crushing of fruits, cold pressure of juice, extraction, centrifugation, filtration, spray drying) of a specific varieties of red orange (*Citrus sinensis* L. Osbeck (*Blood group*)) sweet orange (*Citrus aurantium* L. var. *sinensis*), bitter orange (*Citrus aurantium* L. var. *amara*), grapefruit (*citrus paradise*) and guarana (*Paulinia cupanna*).

Polyphenols constitutes a widely present organic family of phytochemicals molecules in the vegetal kingdom. They are characterized by the presence of two aromatic rings (A and B) which are linked via an oxygenated heretocycle (ring C). Several phenolic groups are associated in more or less complex structures generally of high molecular weight.

The most important class of polyphenolic compounds is flavonoids. The flavonoids are divided in sub-classes based on the position of the B and C rings as well as the degree of saturation, oxidation and hydroxylation of the C ring. The number of these conjugates contributes to the large number of flavonoids, estimated at more than 5000 compounds.

The flavonoid sub-classes are most commonly known as *anthocyanins* (malvidin, cyanidin, petunidin) red pigments found in the red fruits (red orange, blueberries, red grapes and wine), as *flavanones* (naringin, hesperidin, narirutin, naringenin, etc.) found in citrus fruits (orange, lemons grapefruit), as *flavan-3-ols* (catechins, epigallocatechin, etc.) found in green tea apples, red wine, and as *flavonols* (quercetin, kaempferol) found in onions, apples, broccoli.

Flavonoids take an increasing importance, notably regarding their beneficial effects on health. Indeed, their role of natural antioxidant arouse interest for the prevention and treatment of cancer (Chen et al., 2004), inflammatory diseases (Laughton et al., 1991), cardiovascular diseases (Frankel et al., 1993) and neurodegenerative diseases (Orgogozo et al., 1997). Several studies have shown that flavonoids possess lipolytic activity via inhibition of cAMP-phosphodiesterase and maintaining lipolysis-inducing cAMP levels (Kuppusamy and Das, 1992).

Lipolysis is a catabolic process leading to the breakdown of triglycerides (TG) stored in fat cells (adipocytes) and the release of free fatty acids (FFA) and glycerol (Renold and Cahill, 1965). Fatty acids are important oxidative fuel for liver, kidney, skeletal muscle and myocardium. Adipose tissue lipolysis is the major regulator of the body supply of lipid energy because it controls the release of fatty acids into plasma, where they circulate as FFA complexed to albumin (Spector, 1975).

The first step of this lipolytic process in adipocytes is regulated by a variety of hormones such as epinephrine, norepinephrine, glucagons and adrenocorticotrophic hormone (ACTH) (Robidoux et al., 2006). The mechanisms of action of these lipolytic hormones are believed to

be mediated by the cAMP cascade. Lipolytic hormones activate adenylate cyclase, resulting in increased synthesis of cAMP, leading to activation of cAMP-dependant protein kinase and activation of hormone-sensitive lipase (HSL), so named because of its responsiveness to insulin and catecholamines (Steinberg and Khoo, 1977). Activation of hormone-sensitive lipase results in the hydrolysis of stored triglycerides into FFA and glycerol.

The lipolytic process is stimulated by beta adrenergic agonists (Mochizuki and Hasegawa, 2004a, b) with high sympathomimetic activity, but also by the inhibition of 2 enzymes: (i) catechol-*O*-methyl transferase, which degrades norepinephrine (Shixian et al., 2006), and (ii) c-AMP-dependent phosphodiesterase (PDE) (Girotti et al., 2005), which degrades cyclic cAMP and consequently inhibits the activation of HSL.

In the present study we firstly investigated the lipolytic effect of SINETROL in human adipocytes by measuring free fatty acid (FFA) release and secondly the potential of a daily intake of 1.4 g/SINETROL in decreasing the body weight fat and the body mass index (BMI) in human healthy subjects. In a third step, SINETROL was tested for its ability to inhibit cAMP-PDE.

## Materials and methods

### Reagents

SINETROL and the extract of guarana 12% caffeine were supplied by Fytexia (Beziers, France). SINETROL composition of active ingredients was total polyphenols (expressed as catechin): 60%; total flavanones (expressed as naringin): 16.7%; total anthocyanins (expressed as cyanidin-3-glycoside): 2%; and caffeine: 3.6%.

Guarana (*Paulinia cupana*) 12% is a fruit extract standardised naturally in caffeine (12%).

Purified cyanidin-3-glucoside (96% by HPLC) and naringin (70% by HPLC) were supplied by Extrasynthèse (Lyon, France).

Purified theopylline (99%), isoproterenol (99%) and caffeine (99%) were purchased from Sigma-Aldrich (St Quentin Fallavier, France).

### SINETROL polyphenols analysis

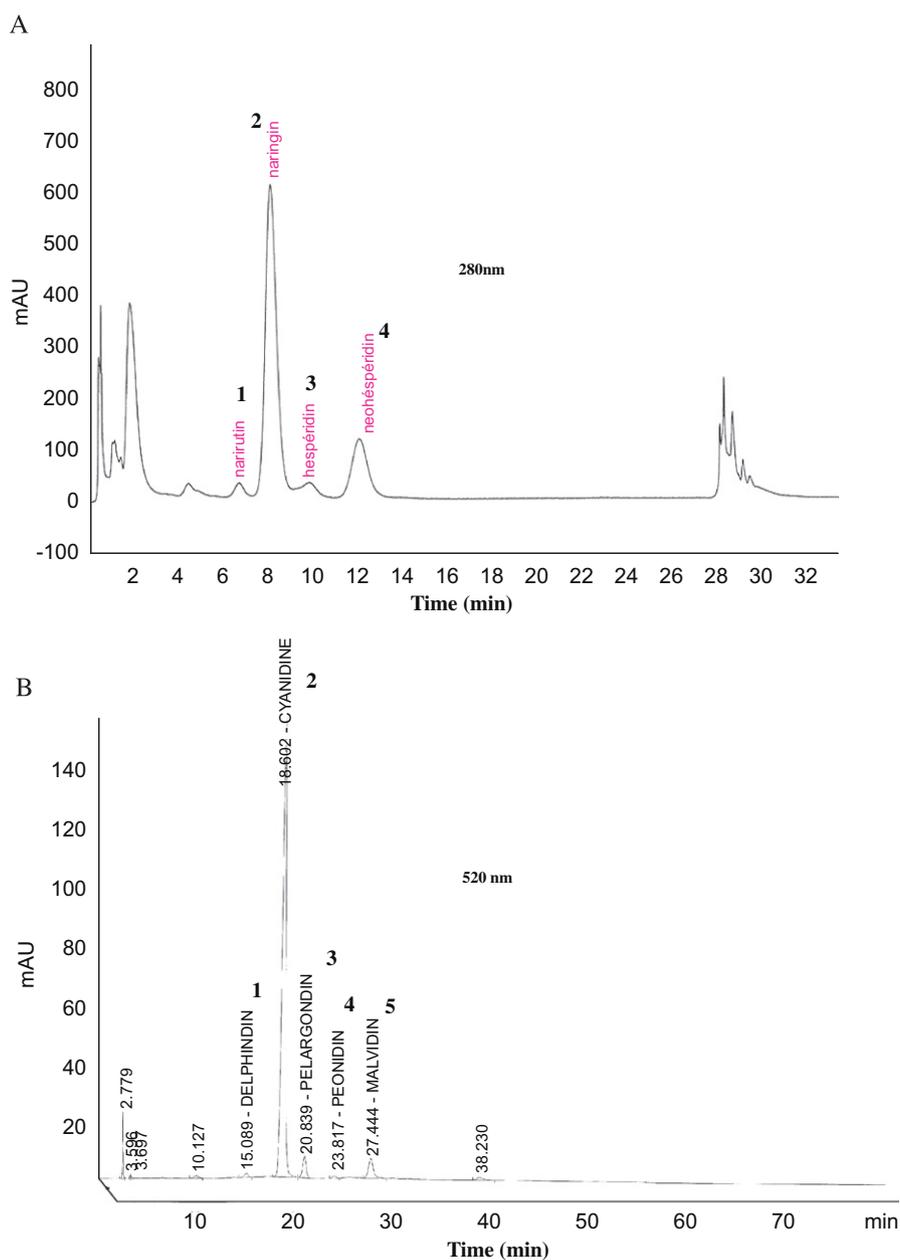
Total polyphenols analysis was performed by the UV-method using a spectrophotometer SHIMADSU 1601 with a detection at 280 nm wavelength (as described by Dallas and Laureano, 1994a, b). SINETROL sample for analysis was obtained by dissolution of 20 mg in 50 ml of distilled water. A volume of 1 ml of this solution was

removed and completed to 50 ml with distilled water and absorbance was measured at 280 nm. An external standard (catechin) (Extrasynthese, France) was used to quantify the total polyphenols. A standard curve was prepared by using catechin from 4 to 16 mg/l and related absorbance was measured ( $A_{280}$ ).

### SINETROL flavanones analysis

Flavanones HPLC-UV analysis was performed using a using Thermo Electron (UV 600) system, equipped

with an analytical column (PLRP-S; 1000 Å; 8 µm). The mobile phase was composed of acetonitrile (A)/water (B)/acetic acid 0.5% (C). A linear gradient was run from 10%(A)/80%(B)/10%(C) to 30%(A)/50%(B)/20%(C) during 40 min. Flow rate was 1 ml/min and detection was made at 280 nm (Fig. 1A). Flavanones were identified by using previously Narirutin, Naringin, Hesperidin and Neohesperidin as external standard obtained from (Extrasynthese, France) and quantified by using Naringin (Extrasynthese, France) as external standard.



**Fig. 1.** (A) A typical flavanones HPLC Chromatogram of SINETROL recorded at 280 nm. (1) Narirutin; (2) naringin; (3) hesperidin; (4) neohesperidin. (B) A typical Anthocyanin HPLC Chromatogram of SINETROL recorded at 520 nm. (1) Delphinidin-3-glucoside; (2) cyanidin-3-glucoside; (3) pelargonidin-3-glycoside; (4) peonidin-3-glucoside; (5) malvidin-3-glucoside.

## SINETROL anthocyanins analysis

Anthocyanins HPLC-UV analysis was performed using a PERKIN ELMER system, equipped with a reversed phase column Superpher 100, C18 (Merck, Germany) (as described by Dallas and Laureano, 1994a, b; Dallas et al., 1995, 1996a, b). The solvent was 40% formic acid (A)/acetonitrile (B)/water (C). The initial conditions were 25%(A)/6%(B)/69%(C) for 15 min followed by a linear gradient to 25%(A)/25,5%(B)/49,5%(C) during 70 min. Flow rate was 0.7 ml/min and detector wavelength at 520 nm (Fig. 1B). Anthocyanins in SINETROL were identified by using previously external standard obtained from (Extrasynthese, France) and concentration of monomeric anthocyanins was quantified by using cyanidin-3-glucoside chloride (Extrasynthese, France) as external standard.

## SINETROL caffeine analysis

Caffeine HPLC-UV analysis was performed using a Thermo Electron (UV 600) system, equipped with a reversed column C18. A preliminary extraction with aqueous acidified solution was realized. The mobile phase was composed of water/acetic acid/acetonitrile. Flow rate was 1 ml/min and detection was made at 270 nm. Concentration of caffeine in SINETROL was quantified by using caffeine obtained from Extrasynthese, France, as external standard.

## Normal human adipocyte isolation and treatments

Normal human adipocytes were freshly isolated from surgical samples of healthy abdominal skin (35-year-old woman) as described (Rodbell, 1964). Briefly, pieces of human adipose tissue were incubated for 30 min at 37 °C with 12,500 CDU/ml of collagenase solution (EG/EC 2325829 Sigma-Aldrich, St Quentin Fallavier, France). Adipocyte suspensions were washed and diluted in minimum essential medium supplemented with 1.87 mg/ml sodium bicarbonate, 50 UI/ml penicillin/streptomycin, 2 mM L-glutamine, 0.5% fatty acid-free bovine albumin. Normal human adipocytes were incubated under gentle shaking for 2 h at 37 °C with or without 20 mg/ml guarana 12% caffeine or 20 mg/ml SINETROL; theophylline (1 mM), isoproterenol (1 μM) and caffeine (0.5 mM) were used as positive controls.

## Lipolysis assay

Free fatty acid release was used as the indicator of adipocyte lipolysis and was measured using FFA-C kit (OXOID, Dardilly, France). Results were expressed as micromoles of FFA or percentage of control. The

absence of interference of the test substances on the FFA assay was checked (data not shown).

## Statistical analysis

The raw data were analysed with PRISM<sup>®</sup> software (Graph Pad Software, Sigma-Aldrich, St Quentin Fallavier, France). The inter-group comparisons were performed by variance analysis (ANOVA) using the Dunnett's multiple comparison test.

## Human clinical study

### Subjects- enrolled criteria

A total of 20 volunteers participated in a randomized, placebo, doubled blinded trial protocol. The pre-inclusion of volunteers was made based on

- *inclusion criteria*: to be between 25 and 55 years old, to have a body mass index (BMI) between 27 and 33, to be in full health, not taking any drugs or dietary food supplements.
- *excluding criteria*: pregnancy, smokers, persons with hepatic, cardiovascular, renal dysfunctions, having pathologies on going or active during the last month, having received medical treatment (allopathic or homeopathic) during the previous months, having taken a dietary food supplement or drugs during the last month.

After pre-inclusion, volunteers were screened using our evaluation test and after screening 20 volunteers were used as the subjects for our clinical trial. Participation in the study was based on informed consent.

### Treatment protocol

The subjects were assigned by randomisation into two groups of 10 peoples. The *treatment group* received a dietary supplement of 4 pieces hard capsules per day containing 350 mg of SINETROL and maltodextrin (1.4 g/day) supplied by Fytexia, France, while the *placebo group* received 4 pieces hard capsules per day containing 350 mg of maltodextrin alone. The two tested products (placebo and SINETROL) were administrated twice daily, in the morning and during the main meal.

Hard capsules (red color) were indistinguishable and were administrated in a double blind approach. The subjects were tested 5 times during a visit to the doctor and dietician. The first time was before the supplementation (T0). A test was planned at 1 week (W1) after taking the dietary supplement, at 4 weeks (W4), at 8 weeks (W8) and finally at the end of the trial, 12 weeks (W12). The evaluation tests were filled by the doctor. During the clinical trial, participants maintain their

previous daily physical exercise and eating habits (1500–2000 cal/day) without any particular dietetic program.

### Measurement

The International Day for the Evaluation of Obesity (IDEA) study looked at 2 measures of fatness: waist circumference and body mass index (BMI). A BMI (weight in kg divided by square of height in meters) of 18.5–25 is considered healthy. A BMI over 25 is deemed overweight and greater than 30 is obese.

Subjects for our study were monitored for body composition (body fat/body lean) by impedance bioelectrical balance (TANITA) analysis and by anthropometric measures (BMI, body weight, waist circumferences). A global satisfaction test (silhouette, acceptability, efficacy, secondary effects) was monitored at the end of the clinical trial (W12).

The placebo group was 10 overweight persons (9 women, 1 man) with BMI between 27 and 30, age between 22 and 55 years old and mean weight: 73 kg.

The treatment (SINETROL) group was 10 persons (7 women, 3 men), 4 obese women with a BMI between 29 and 33 and a overweight group (3 women, 3 men) with a BMI between 27 and 30, age between 25 and 55 years old and mean weight 70.50 kg.

### Statistical analysis

Results are expressed as mean  $\pm$  SD. A Kolmogorov–Smirnov test for normality and a Bartlett test for homogeneous variance were performed for each group. All the data were analyzed using a nonparametric Kruskal–Wallis test, and differences between groups were tested using the Mann–Whitney U test ( $p < 0.05$  was considered significant). All analyses were done using the Statview software version 4.51.1 (Abacus Concepts, Berkley, CA, USA).

### Phosphodiesterase activity assay

Phosphodiesterase activity was measured by a scintillation proximity assay (SPA)-based method (Amersham Biosciences, Orsay, France). The tested substances

guarana 12% caffeine, SINETROL, cyanidin-3 glucoside and naringin were diluted to 0.01% in DMSO. Caffeine diluted to 0.01% in DMSO was used as a positive control and DMSO diluted to 1% (the maximal amount of DMSO in the assay) was used as a negative control. Phosphodiesterase 3'-5'-cyclic nucleotide 5'-nucleotidohydrolase (EC: 3.1.4.17 Sigma-Aldrich, St Quentin Fallavier, France) was incubated for 10 min at +4 °C with or without the tested substances. The reaction was initiated by the addition of 3'-5'-[3 H]cAMP at 0.5  $\mu$ Ci/ml and incubated for 15 min at +30 °C. Yttrium SPA PDE beads (Amersham Biosciences, Orsay, France) were added to the reaction and incubated for 20 min at +30 °C. The 5'-[3 H]AMP produced by the phosphodiesterase activity specifically binds to SPA yttrium silicate beads and excites the scintillation liquid finally added to tubes. The relative amount of the reaction product was measured by scintillation counting.

### Statistical analysis

The raw data were analysed with PRISM<sup>®</sup> software (Graph Pad Software, Sigma-Aldrich, St Quentin Fallavier, France). The inter-group comparisons were performed by variance analysis (ANOVA) using the Dunnett's multiple comparison test.

## Results and discussion

### Lipolytic activity on human adipocyte

The lipolytic effect of SINETROL, three purified substances (theophylline, isoproterenol and caffeine) and guarana 12% on human adipocytes is presented in Table 1 and Fig. 2. FFA release was used as indicator of adipocyte lipolysis as described in Material and methods. Isoproterenol stimulated lipolysis via beta adrenergic receptor activation and cAMP-dependent signalling (Robidoux et al., 2006), while caffeine (Jiang et al., 1998) and theophylline (Beavo et al., 1971) induced lipolysis by inhibition of PDE. Moreover, as described in our PDE experiments, caffeine also act with weak

**Table 1.** FFA assay after treatment of human adipocytes (fat cells) by various lipolytic products

Tested products	Concentrations	FFA ( $\mu$ M)	FFA/control (%)
Control	–	36 $\pm$ 10 <sup>a</sup>	100 $\pm$ 28 <sup>a</sup>
Theophylline	1 mM	381 $\pm$ 9 <sup>b</sup>	1057 $\pm$ 26 <sup>b</sup>
Isoproterenol	1 $\mu$ M	377 $\pm$ 11 <sup>b</sup>	1048 $\pm$ 31 <sup>b</sup>
Caffein	0.5 mM	339 $\pm$ 4 <sup>b</sup>	941 $\pm$ 12 <sup>b</sup>
Guarana 12% caffeine	20 mg/ml	101 $\pm$ 11 <sup>d</sup>	281 $\pm$ 30 <sup>d</sup>
SINETROL	20 mg/ml	213 $\pm$ 13 <sup>c</sup>	592 $\pm$ 36 <sup>c</sup>

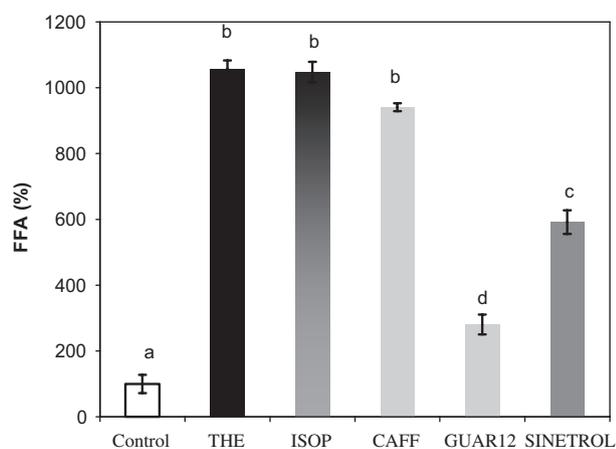
Values are mean  $\pm$  SE,  $n = 3$ , for each tested product.

Means within rows followed by the same superscript are not significantly different ( $p < 0.05$ ).

affinity as PDE inhibitor and can also stimulate lipolysis in this way.

The analysis of the results (Fig. 2) confirms that theophyllin and isoproterenol stimulate significantly ( $p < 0.01$ ) the lipolysis in a range of 10 fold greater than control, which represent a liberation of FFA of 36  $\mu\text{M}$  in 2 h. Purified caffeine at 0.5 mM stimulates also the liberation of FFA in a range of 9.5 fold greater than control ( $p < 0.05$ ).

In the same order of magnitude, the SINETROL stimulates significantly ( $p < 0.05$ ) the lipolytic activity in a range of 6 fold greater than the control (Table 1, Fig. 2). For guarana 12% (standardised naturally in caffeine) the measurement of its lipolytic activity (FFA



**Fig. 2.** FFA release after treatment of human adipocytes by various lipolytic products. Fat human adipocytes solution (540  $\mu\text{l}$ ) was added to 60  $\mu\text{l}$  solution of tested compounds and each reaction mixture was incubated for 2 h at 37 °C. FFA released from adipocytes were measured as combined FFA-BSA in 30  $\mu\text{l}$  assay medium as described in Materials and methods (method 1). Values are expressed as mean  $\pm$  SE. Bars with different index letters are significantly different ( $p < 0.05$ ). *Tested products:* *THE*: theophylline at 1 mM final concentration; *ISOP*: isoproterenol at 1  $\mu\text{M}$ ; *CAFF*: caffeine at 0.5  $\mu\text{M}$ ; *GUAR12*: guarana dry extract standardised at 12% of caffeine at final concentration 20 mg/ml; *SINETROL*: citrus-based dry extract standardised at 70% polyphenols at final concentration 20 mg/ml.

release) showed an increase in a range of 2.8 fold greater compared to control ( $p < 0.05$ ). Moreover, guarana 12% and SINETROL have been tested at the same assay concentration (0.2%) and the results showed that SINETROL has 2.1 greater activity than guarana 12% ( $p < 0.05$ ), while SINETROL content in caffeine (3.6%) is 3 times lower.

These results suggest that SINETROL showed potent lipolytic activity via PDE inhibition. Some dietary supplements (rich in flavonoids) has been related for their lipolytic effect. Pycnogenol, a pin bark extract that contains a mixture of proanthocyanidins, has strong lipolytic activity and effects via stimulation of beta receptor-mediated activity (Mochizuki and Hasegawa, 2004a). Green tea extract, which contains (+)-catechin and (–)- epigallocatechin-3-gallate (EGCG), has strong lipolytic activity related to EGCG, while catechin did not produce a significant increase ((Mochizuki and Hasegawa, 2004b).

Recently, it has been demonstrated (Tsuda et al., 2005) that anthocyanins have the potency of anti-obesity in mice by the enhancement adipocytokine secretion and adipocyte gene expression in adipocytes. Based on the gene expression profile, up-regulation of hormone-sensitive lipase and enhancement of the lipolytic activity by the treatment of adipocytes with cyanidin 3-glucoside, have been demonstrated.

## Human clinical study

The results of supplementation with placebo and SINETROL on body mass index (BMI), body weight and body fat evolution in 20 healthy volunteers during 4 and 12 weeks is presented in Table 2 and Figs. 3 and 4.

At the clinical level, intake of SINETROL as compared to placebo revealed a rapid, starting at 4 weeks, and pronounced body weight and fat loss at 12 weeks.

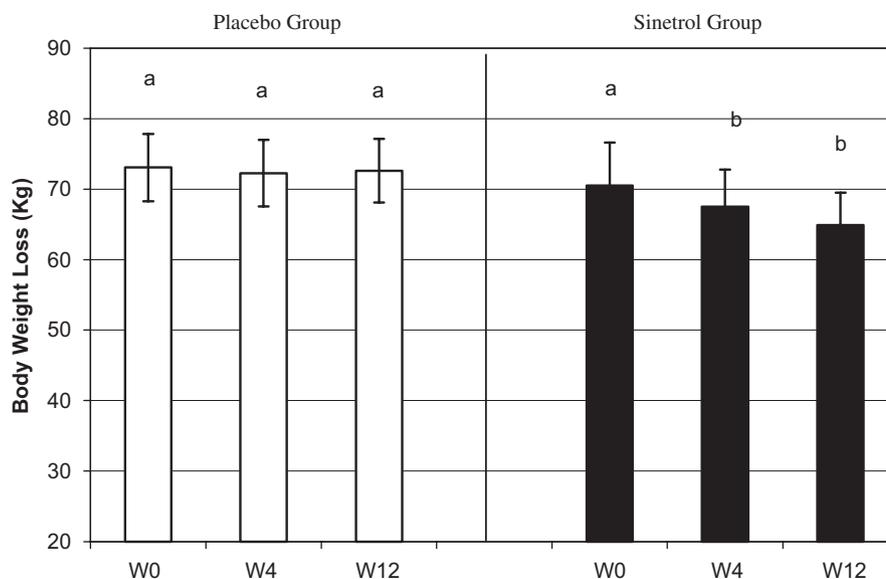
The analysis of the results of the *body weight loss* (Fig. 3) showed that the placebo group have reached a stable level of weight at W0, W4 and W12 (73, 72.2 and 72.6 kg, respectively) and the score stopped to decrease significantly (Table 2).

**Table 2.** Effect of supplementation with placebo and SINETROL on BMI, body weight and body fat evolution in 20 volunteers after 4 and 12 weeks of treatment

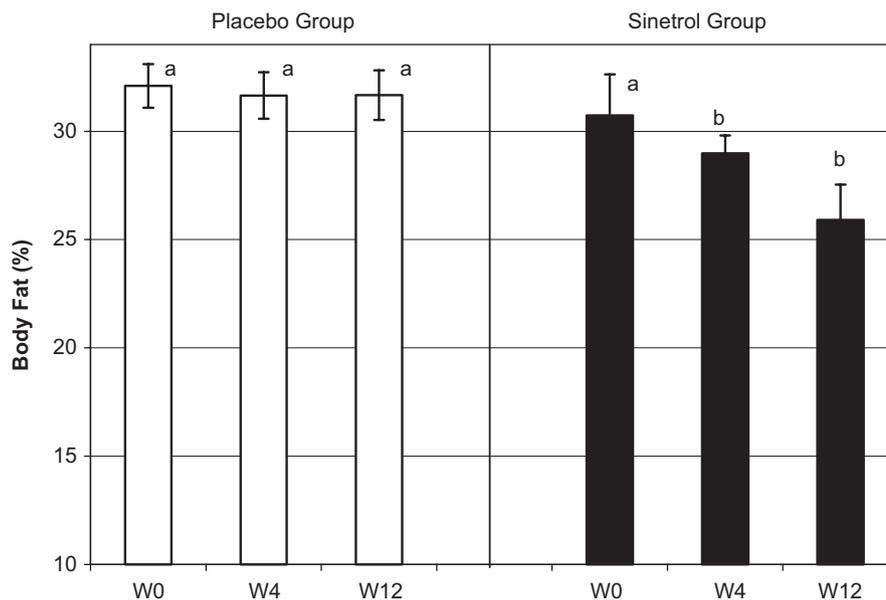
Groups	BMI		Body weight evolution (kg)			Body fat evolution (%)		
	Initial	Variation (%) after 12 weeks	Initial 0 weeks (W0)	After 4 weeks (W4)	After 12 weeks (W12)	Initial 0 weeks (W0)	After 4 weeks (W4)	After 12 weeks (W12)
Placebo	28.5 $\pm$ 0.7 <sup>a</sup>	–0.2 $\pm$ 0.5 <sup>a</sup>	73.0 $\pm$ 4.8 <sup>a</sup>	72.2 $\pm$ 4.7 <sup>a</sup>	72.6 $\pm$ 4.5 <sup>a</sup>	32.0 $\pm$ 1.0 <sup>a</sup>	31.6 $\pm$ 1.0 <sup>a</sup>	31.6 $\pm$ 1.0 <sup>a</sup>
SINETROL <sup>®</sup>	28.1 $\pm$ 2.45 <sup>a</sup>	–2.2 $\pm$ 0.9 <sup>b</sup>	70.5 $\pm$ 6.0 <sup>a</sup>	67.5 $\pm$ 5.2 <sup>b</sup>	64.9 $\pm$ 4.5 <sup>b</sup>	30.7 $\pm$ 1.9 <sup>a</sup>	29.0 $\pm$ 0.8 <sup>b</sup>	25.9 $\pm$ 1.0 <sup>b</sup>

Values are mean  $\pm$  SE,  $n = 10$ , for each placebo and SINETROL<sup>®</sup> tested group.

Means within rows followed by the same superscript are not significantly different ( $p < 0.05$ ).



**Fig. 3.** Effects of supplementation with *placebo* and *SINETROL* on *body weight loss* (kg) in 20 healthy volunteers before (0 weeks), during (4 weeks) and after 12 weeks of treatment. The placebo and *SINETROL* products were administrated as 4 pieces hard capsules containing 350 mg each (1.4 g/day). Two capsules were administrated in the morning and 2 during the main meal. Measurements and treatment protocol were realised as described in Materials and methods (part III). Placebo: maltodextrin; *SINETROL*: citrus extract standardised at 60% of polyphenols and 15% maltodextrin. Values are expressed as mean  $\pm$  SE. Bars with different index letters are significantly different ( $p < 0.05$ ).



**Fig. 4.** Effects of supplementation with *placebo* and *SINETROL* on *body fat loss* (%) in 20 healthy volunteers before (0 weeks), during (4 weeks) and after 12 weeks of treatment. The tested products were administrated as 4 pieces hard capsules containing 350 mg each (1.4 g/day). Two capsules were administrated in the morning and 2 during the main meal. Measurements and treatment protocol were realised as described in materials and Methods (part III). Placebo: maltodextrin; *SINETROL*: citrus extract standardised at 60% of polyphenols and 15% maltodextrin. Values are expressed as mean  $\pm$  SE. Bars with different index letters are significantly different ( $p < 0.05$ ).

However, in the *SINETROL* Group the body weight (kg) decreased with a significant difference ( $p < 0.05$ ) of 3 kg after W4 and 5.6 kg after W12 weeks compared to W0 *SINETROL* Group.

The analysis of the results of the *body fat evolution* (Fig. 4) showed that the Placebo group leads to a similar reaction as on the body weight with a stable, non-significant difference ( $p < 0.05$ ) in body fat (%) at W0,

W4 and W12 (32%, 31.6% and 31.6%, respectively) (Table 2).

In the SINETROL Group the body fat (%) decreased with a significant difference ( $p < 0.05$ ) of 5.53% after W4 and 15.6% after W12 compared to W0 SINETROL Group.

Finally, the medium value of BMI after 12 weeks of treatment with SINETROL (Table 2) decreased significantly by 2.2% compared to the medium BMI (Placebo Group).

Some natural products have been described to have such physiological effect in the literature. Grapefruit capsules or fresh grapefruit groups (obese patients randomized to placebo) lost significantly more weight (Fujioka et al., 2006). A study (Ballard et al., 2006) indicates that consumption of caffeine with naringin in acute dosage does not affect respiratory exchange ratio, oxygen consumption and prevents the increase of resting energy expenditure in adult humans.

Green tea extract relevant of catechin intake is associated with increased weight loss due to diet-induced thermogenesis. This effect is generally attributed to the catechin epigallocatechin gallate to augment and prolong sympathetic stimulation of thermogenesis (Shixian et al., 2006). In a Japanese study (Yoshikawa et al., 2002), a supplementary food consisting of Salacia Reticula has shown a significant lipolytic effect. These antiobesity effects were exerted by mangiferin, (-)-4'-*O*-methylepigallocatechin and maytenfolic through inhibition of fat-metabolizing enzymes and enhanced lipolysis.

### Mechanism of action by inhibition of PDE activity

The results of inhibition of cAMP-phosphodiesterase (PDE) activity measured by a scintillation assay (SPA) in the presence of different lipolytic products are presented in Table 3 and Fig. 5.

The tested products guarana 12% caffeine, cyanidin-3 glucoside and naringin were selected because of their presence (in smallest concentration) on the polyphenolic composition of SINETROL. Guarana 12% is a natural fruit extract while cyanidin-3 glycoside and naringin are purified polyphenolic pharmaceutical-grade products. Purified caffeine was used as a positive control and DMSO as negative control. All tested substances were diluted to 0.01% in DMSO. With some test products (cyanidin and naringin) quenching could be observed. This reduced the amplitude of scintillation but did not affect the inhibition measured.

The analysis of the results presented in Table 3 showed a decreased efficiency regarding PDE inhibition for the following products: cyanidin = SINETROL > naringin > caffeine > guarana.

SINETROL is a potent inhibitor of PDE product (97% of inhibition;  $p < 0.001$ ). The other two purified polyphenolic compounds naringin (flavanones family)

**Table 3.** Effect of various lipolytic products in vitro PDE inhibition model

Tested products	Concentration (%)	PDE assay		
		PDE assay Cpm	PDE inhibition (%)	Mean (%)
Control	–	2153	–6	0 ± 5a
		2017	3	
		2016	3	
DMSO control	1	2178	–7	–5 ± 9a
		2282	–14	
		1986	5	
Caffeine	0.01	1176	56	56 ± 5b
		1242	51	
		1093	61	
Cyanidin-3 glucoside	0.01	388	105	99 ± 8c
		619	91	
		449	101	
Naringin	0.01	606	91	87 ± 6c
		782	80	
		637	89	
Guarana 12% caffeine	0.01	1989	5	7 ± 3a
		1905	10	
		1943	7	
SINETROL	0.01	625	90	97 ± 1c
		344	108	
		595	92	

Values are mean ± SE,  $n = 3$ , for each tested product.

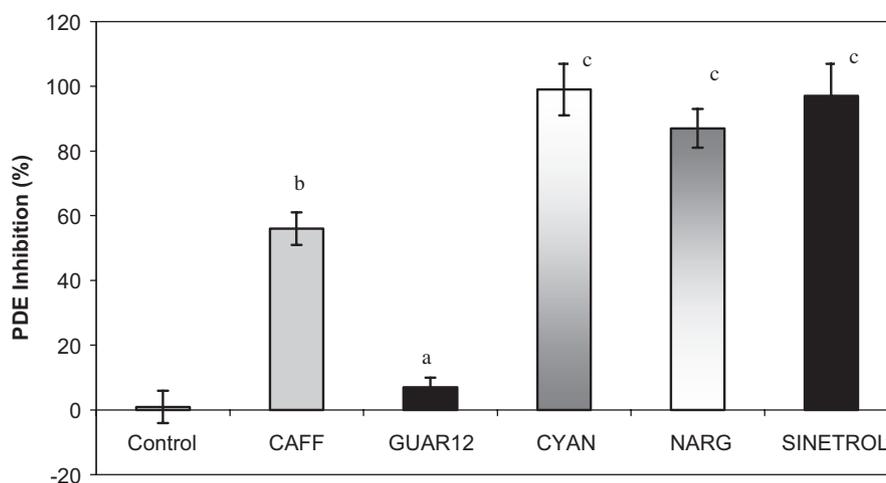
Means within rows followed by the same superscript are not significantly different ( $p < 0.05$ ); Cpm = counts per minute.

and cyanidin-3 glycoside (anthocyanins family) also showed a very strong PDE inhibition (87% and 99%, respectively) ( $p < 0.001$ ).

These data suggested a strong effect of SINETROL on cAMP-PDE inhibition. These results could be attributed to the synergetic polyphenolic complex of SINETROL. In fact, SINETROL contains approximately 10% of naringin and 2% of cyanidin, while the tested purified products contain 96% cyanidine and 70% naringin.

SINETROL synergetic polyphenolic composition is due to cyanidin and naringin but also due to other identified flavanones (such as naringenin, isonaringin, narirutin, hesperidin) present at 5–10% in SINETROL and some yet non-identified polyphenols as well as due to caffeine (present at 3.6% in SINETROL).

It is important to indicate that natural guarana containing 12% caffeine induced almost no PDE inhibition (7%), while purified caffeine inhibits PDE significantly (56%). These results show that caffeine is an inhibitor of PDE activity but only at high concentrations.



**Fig. 5.** Effects of various lipolytic products in *in vitro* PDE inhibition assay. PDE (50  $\mu\text{g/ml}$ ) were incubated during 10 min at 37 °C in the presence or not of tested products and 3',5' cAMP (0.5  $\mu\text{Ci/ml}$ ). The PDE assay was performed using SPA scintillation beads as described in Materials and methods. The scintillation proximity assay (cpm) was determined by liquid scintillation. Values are expressed as mean  $\pm$  SE. Bars with different index letters are significantly different ( $p < 0.05$ ). *Tested products:* *CAFF*: caffeine at 0.01% (0.5 mM); *GUAR12*: guarana dry extract standardised at 12% of caffeine at final concentration 0.01%; *CYAN*: cyanidin-3-*O*-glucoside chloride at 0.01%; *NARG*: naringin at 0.01%, *SINETROL*: citrus dry extract standardised at 60% polyphenols at final concentration 0.01%.

For the first time we described the potent phosphodiesterase inhibition property of these 2 polyphenols (i) cyanidin-3-*O*-glucoside (anthocyanin family) and (ii) naringin glycoside (flavanone family).

Both polyphenols have similar skeletal features: C6-C3-C6 with a C3,4 double bond and hydroxyls groups at C5,7,3',4' for cyanidin and for naringin a keto group at C4 and hydroxyls groups at C5,4'.

## Conclusions

In summary, it has been established that SINETROL has a strong lipolytic activity measured by FFA release. It might be possible that SINETROL lipolytic effect are mediated by cAMP-PDE inhibition and that the subsequent increase in cAMP levels stimulates HSL. Moreover, cyanidin-3 glucoside and naringin (two main flavonoids present in SINETROL composition) showed a strong cAMP-PDE inhibition.

These lipolytic results may be attributed to the synergetic polyphenolic complex of SINETROL (anthocyanins, flavonoids and caffeine).

In addition, the results of the clinical study showed that SINETROL may serve to prevent obesity by decreasing BMI and its synergetic polyphenolic composition may help to decrease body weight and body fat.

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