TECHNISCHE UNIVERSITÄT MÜNCHEN

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Anti-obesity effects of *Iwong* (*Ipomoea alba* L., Convolvulaceae)

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DEDICATIONS

To my deceased father,

To my mother.

To my wife, Josiane and my children, Lovely, Kiara and Emma-Marie.

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µg: microgram

µl: microliter

4-AAP: 4-aminoantipyrine

5'-AMP: 5'-adenosine monophosphate ;

AAF-Glo: alanyl-alanyl-Phenylalanyl-aminoluciferin

AC: adenylate cyclase;

ACC: acetyl-CoA carboxylase;

ACLY: ATP citrate lyase;

ACO: Acetyl co-enzyme A

ACRP30: Adipocyte Complement-Related Protein of 30 kDa

ACS: acyl-CoA synthetase

AGPAT: 1-acylglycerol-3-phosphate O-acyltransferase;

AgRP: agouti-related protein

ALT: alanine transaminase

AMP1: most abundant gene transcript 1

AMPK: adenosine monophosphate-activated protein kinase

ANOVA: one way analysis of variance

ANP: atrial natriuretic peptide;

Apo: apoliporotein

ARC: hypothalamic arcuate nucleus

AST: aspartate transaminase

ATGL: adipose triglyceride lipase;

ATP: adenosine triphosphate

BAT: brown adipose tissue

BCA: bicinchoninic acid

BMD: bone mineral density

BMI: body mass index

BNP: brain natriuretic peptide;

BPD: biliopancreatic diversion

BSA: Bovine serum albumin

C/EBP: CCAAT-enhancer-binding protein

cAMP: cyclic adenosine monophosphate;

CART: cocaine amphetamine regulated transcript

CB-1: endocannabinoid receptor 1

CCK: cholecystokinin

CD36: cluster differentiation 36

cDNA: complementary Deoxyribonucleic acid

CETP: cholesteryl esters transfer protein

cGMP: cyclic guanosine monophosphate;

CHYL: chylomicron

CM: culture medium

CSF: cerebrospinal fluid

CVD: cardiovascular disease

DAG: diacylglycerol;

DAP: dihydroxyacetone phosphate

DEX: Dexamethasone

DGAT: diacylglycerol acyltransferase

DHAP: dihydroxyacetone-3-phosphate

DIO: diet-induced obese mice

DM: Differentiation medium

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DSA: DNase Stop Solution

EDTA: ethylendiamintetaacetat

EGCG: epigallocatechin gallate

ELISA: Enzyme-linked Immunosorbent assay

ER: endoplasmic reticulum

ESPA: sodium N-ethyl-N-(3-sulfopropyl)

eWAT: epididymal white adipose tissue

F1, 6BP: fructose 1, 6 bisphosphate;

FAS: fatty acid synthase;

FBS: Fetal Buffer Serum

FFA: free fatty acid;

G-1-P: glycerol-1-phosphate

G3P: glycerol-3-phosphate;

G6P: glucose 6 phosphate;

GADH: glyceraldehyde 3-phosphate;

GBP28: gelatin-binding protein of 28 kDa

GC: guanylate cyclase;

GH: growth hormone

Gi: Gαi protein

GIP: insulinotropic polypeptide

GK: glycerol kinase

GLP-I: glucagon-like peptide 1

Glu: Glycemia

GLUT4: glucose transporter 4;

GPAT: glycerol 3-phosphate acyltransferase;

GPDH: glycerol-3-phosphate dehydrogenase;

GPO: glycerol phosphate oxidase

Gs: Gαi protein

GTC: Guanidine thiocyanate

HCl: hydrochloric acid or hydrogen chloride

HDL High density lipoproteins

HFD: High Fat Diet

HSD: High Sucrose Diet

HSL: hormone sensitive lipase;

IDL: intermediate lipoproteins

IGF-1: insulin-like growth factor-1

IL-6: interleukine-6

IR: insulin receptor;

IRS: insulin receptor substrate;

Iwong4 and Iwong8: Iwong 4and 8%

Iwong5 and Iwong10: Iwong 5 and 10%

JAK/STAT: janus-activated kinases/signal transducers and activators of transcription

Kb: kilo base

KJ: Kilojoules

LCAT: cholesterol acyltransferase

LDL: Low density lipoprotein

LPA: lysophosphatidic acid;

LPL: lipoprotein lipase;

M: molar

MAG: monoacylglycerol;

MC3R and MC4R: melanocortin receptors 3 and 4

mg: milligram

MGL: monoacylglycerol lipase;

MIX: Methylisobutylxanthine

ml: milliliter

Mm: millimolar

$MnCl_2$: manganese chloride

MOPS: 3-(N-morpholino) propanesulfonic acid

mRNA: Messenger ribonucleic acid

mU: milli unit

MW: Molecular weight

NaCl: Sodium chloride

NAD: nicotinamide adenine dinucleotide

NADH: nicotinamide adenine dinucleotide

NaOH: Sodium hydroxide

NEFAs: Non esterified fatty acids

nM: nanometer

NPY: orexigenic neuropeptide Y

NTS: Nucleus tractus solitarii

OA: oxaloacetate;

Ob-R or Lepr-B: leptin receptor

ORO: Oil red O

PA: phosphatidic acid;

PAP: phosphatidic acid phosphatase;

PBS: Phosphate buffer saline

PD: pyruvate dehydrogenase;

PDE: phosphodiesterase

PDE3B: phosphodiesterase 3B;

pH: potential of hydrogen

PI3K: phosphatidylinositol 3-kinase;

PKA: cAMP-dependent protein kinase;

PKG: cGMP-dependent protein kinase;

PLIN: perilipin;

POD: peroxidase

POMC: pro-opiomelanocortin

PPAR: peroxisome proliferator-acitived receptor

Pyr: pyruvate;

RLA: RNA lysis buffer

RNA: Ribonucleic acid

RT-PCR: Real time-polymerase chain reaction

RWS: RNA Wash Solution

RYGB: Roux-en-Y Gastric Bypass

SDS: Sodium Dodecyl Sulfate

SR-BI: scavenger receptor class BI

T2DM: type 2 diabetes mellitus

TAE: Tris-acetate/EDTA

TAG: triacylglycerol;

T-C: total cholesterol

TG(s): Triglycerides

TNF-α: tumor necrosis factor-α

TOOS: N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, dehydrate

Tris-HCl: Tris(hydroxymethyl)aminomethane hydrochloride

TTT: tripartite tricarboxylate transporter

UCP-1: uncoupling protein-1

UCP2: Uncoupling Protein 2

VBG: Vertical Banded Gastroplasty

VLDL: very low density lipoprotein

WAT: white adipose tissue

WHO: World Health Organization

WHR: waist-hip ratio

WR: working reagent

α**2-AR**: α2-adrenergic receptor;

β**-AR**: β-adrenergic receptor;

α **-MSH**: α-melanocyte stimulating hormone

ABSTRACT:

The prevalence of obesity is increasing worldwide, but pharmacologic treatment has poor long-term success and severe side effects. Medicinal plants are considered as an important source of drug discovery and could therefore be an interesting alternative approach to develop new obesity treatments. *Ipomoea alba* (*Iwong*) is a plant used in Cameroonian traditional medicine for its ability to control glucose homeostasis and body weight. It is also applied as a laxative and to improve the quality of breast milk.

The aim of this study was to evaluate the anti-obesity effect of *Iwong* feeding in lean rodents and on the prevention and the regression of obesity in diet induced obese (DIO) mice; as well as to investigate the effect of the ethanolic extract of *Iwong* on lipid accumulation in murine 3T3L1 adipocytes.

Lean *Wistar* rats and NMRI mice were fed a standard chow diet or the same diet supplemented with *Iwong* powder (5 or 10 % w/w) for 4 weeks, whereas for the prevention and the regression of obesity, C57BL/6J mice were fed either a HFD (High Fat Diet) or HFD supplemented with 4 and 8 weight % (*Iwong4* and *Iwong8,* respectively) of *Iwong* powder during 4 or 6 weeks. Body weight and food intake were recorded weekly. At the end of *Iwong* feeding several tissues and fat pads were weighed and blood samples were collected for clinical chemistry and hormone assays.

For the *in vitro* study, 3T3L1 cells were treated with the ethanolic extract of *Iwong* for 9 days. Lipid accumulation, GPDH activity, as well as PPARγ and C/EBPα mRNA levels were assessed.

Iwong feeding led to a dose dependent reduction in body weight gain and fat mass despite increased food intake in both rats and mice. Plasma concentrations of HDL-C and LDL-C were slightly changed in both rodents, whereas other biochemical parameters remained unchanged. In the obesity prevention study, *Iwong* supplementation also increased food intake. In spite of this effect on food intake, a dose dependent reduction in body weight gain associated with a fat mass reduction was observed in both *Iwong* groups. The plasma LDL-cholesterol level in *Iwong* groups was decreased. For the obesity regression study, DIO mice fed *Iwong* also showed increased food intake, associated with a dose dependent decrease in body weight gain and fat mass. Triglyceride content in the liver, total-cholesterol, LDL-cholesterol, glucose and leptin plasma levels were reduced in both *Iwong* groups. Treatment of 3T3-L1 cells with the ethanolic extract of *Iwong* reduced both lipid accumulation and GPDH activity and down regulated PPARγ and C/EBPα gene expression.

In conclusion, our results indicate that *Iwong* is a candidate natural substance for the treatment of obesity via inhibition of lipid accumulation in adipose tissue.

ZUSAMMENFASSUNG:

Weltweit stellt die Prävalenz von Adipositas ein gravierendes Problem dar. Bislang war es nicht möglich eine zuverlässige medikamentöse Behandlung zu entwickeln. Pflanzen aus der traditionellen Naturmedizin stellen eine wichtige Quelle für neue, erfolgsversprechende therapeutische Ansätze einer Adipositas-Behandlung dar. *Ipomoea alba (Iwong)* ist eine traditionelle Medizinpflanze in Kamerun, welche seit langem wegen ihrer abführenden Wirkung Anwendung findet. Auch stillenden Frauen wird diese Heilpflanze verabreicht, um die Qualität der Muttermilch zu verbessern. Nicht zuletzt wird *Iwong* als Antidiabetikum eingesetzt, da ihr ein positiver Effekt auf die Reduktion des Körpergewichts zugeschrieben wird.

Das Ziel dieser Arbeit war es, diesen Effekt von *Iwong* auf die Entstehung, Prävention und Regression von Adipositas zu charakterisieren. Dazu wurden Fütterungsstudien in Ratten und Mäusen durchgeführt, um die Wirkung einer *Iwong*-haltigen Diät zum einen in normalgewichtigen *Wistar* Ratten und NMRI Mäusen und zum anderen in einem ernährungsbedingt fettleibigen Mausmodell zu untersuchen.

Außerdem wurden wirksame Bestandteile von *Iwong* in einer ethanolischen Phase extrahiert. *In vitro* wurde der Effekt dieses ethanolischen Extrakts auf die Akkumulierung von Triglyzeriden während der Differenzierung von 3T3-L1 Adipozyten erforscht.

Für die Fütterungsexperimente wurde *Iwong* trocknen Pulver hergestellt und der jeweiligen Experimentaldiät zu dem angegebenen Prozentsatz zugemischt. *Wistar* Ratten und NMRI Mäuse wurden mit Standard-Diät oder mit einer Standard-Diät, der 5% bzw. 10% *Iwong* beigemischt war, über 4 Wochen gefüttert. C57BL/6J Mäuse wurden mit einer Hochfett-Diät (Kontrollgruppe) bzw. einer Hochfett-Diät, der 4% oder 8% *Iwong* zugemischt war gefüttert. Zur Prävention einer Gewichtszunahme wurden Mäuse mit *Iwong*-haltiger Hochfett-Diät über 4 Wochen gefüttert. Die Wirkung von *Iwong* auf eine Gewichtsregression wurde in Mäusen gemessen, die zuerst 9 Wochen eine Hochfett-Diät und danach 6 Wochen eine *Iwong*haltige Hochfett-Diät erhielten. Körpergewicht und Futteraufnahme wurden einmal pro Woche aufgezeichnet. Am Ende der Experimente wurden verschiedene Fettdepots und Organe entnommen und gewogen. Zur Bestimmung von klinischer Chemie, Leptin- und Insulinspiegel wurden Blutproben entnommen.

In vitro wurden 3T3-L1 Zellen mit dem ethanolischen Extrakt, der aus *Iwong* gewonnen wurde, behandelt. Lipid Akkumulation, GPDH Aktivität und die Genexpression von PPARγ und C/EBPα wurden bestimmt.

Bei Fütterung einer *Iwong*-haltigen Standard Diät war das Körpergewicht und die Fettmenge in diesen Tieren reduziert, obwohl die Futteraufnahme erhöht war. Durch die Fütterung einer *Iwong*-haltigen Diät war die Plasma-Konzentration von HDL-C erhöht, während die PlasmaKonzentration von LDL-C reduziert war. Alle übrigen biochemischen Blutparameter waren gleich.

Im Fütterungsexperiment, welches die präventive Wirkung von *Iwong* auf die Gewichtszunahme untersuchte, zeigte sich, dass in Mäusen, die eine *Iwong*-haltige Hochfett Diät erhielten, die Futteraufnahme erhöht war. Die Körpergewichtszunahme und das Gewicht der Fettgewebe waren in dieser Gruppe im Vergleich zur Kontrollgruppe verringert. Mäusen, die eine Diät-induzierte Adipositas zeigten, wurden auf eine fetthaltige Diät mit *Iwong* umgestellt um den Effekt von *Iwong* auf eine Gewichtsregression zu untersuchen. Diese Mäuse zeigen ebenfalls eine Steigerung der Futteraufnahme aber eine Reduzierung des Körpergewichts und des Gewicht der Fettdepots. Nach einer 6 wöchigen Fütterung war der Triglyzeridgehalt in der Leber gesunken ebenso wie das Gesamtcholesterin, LDL-Cholesterin und die Blutglukose. Außerdem waren Leptin- und Insulinspiegel in der *Iwong*-gefütterten Gruppe im Vergleich zu den Kontrollen reduziert.

In vitro konnte gezeigt werden, dass der ethanolische Extrakt aus *Iwong* die Akkumulierung von Lipiden in 3T3L1-Adipozyten sowie die GPDH Aktivität verringert und zwar sowohl nach Kurz- als auch nach Langzeitbehandlung. Nach 9-tägiger Behandlung war die Genesxpression der Transkriptionsfaktoren PPARγ und C/EPBα in 3T3L1 Adipozyten herunterreguliert.

Unsere Ergebnisse zeigen, dass *Iwong* als traditionelle Heilpflanze ein vielversprechender Kandidat zur Behandlung von Adipositas durch Hemmung der Lipidakkumulation im Fettgewerbe ist.

RÉSUMÉ:

Malgré l'augmentation exponentielle de la prévalence de l'obésité de par le monde, les traitements pharmacologiques actuellement disponibles demeurent toutefois inefficaces à cause notamment de multiples effets secondaires observés, rendant ainsi tout traitement à long terme quasi impossible. Les plantes médicinales étant considérées comme une importante source de nouvelles molécules pouvant être exploitées par l'industrie pharmaceutique pour la synthèse et l'élaboration de nouveaux médicaments. Elles constituent pour ainsi dire une excellente alternative pour l'élaboration de nouveaux médicaments contre l'obésité. *Iwong* est une plante utilisée en médecine traditionnelle au Cameroun pour ses effets laxatifs, son activité antidiabétique, ses propriétés amincissantes et sa capacité à améliorer la qualité du lait maternel.

L'objectif de ce travail était d'évaluer les effets anti-obésité d'*Iwong* d'abord sur des rongeurs non obèses et par la suite sur la prévention et le traitement de l'obésité chez la souris obèse. L'étude *In vitro* quant-à-elle consistait à tester l'effet de l'extrait à l'éthanol d'*Iwong* sur la lipogenèse dans les adipocytes 3T3-L1.

Les rats *Wistar* et les souris NMRI ont été nourries à une alimentation standard ou à cette alimentation enrichie à la poudre d'*Iwong* (5 ou 10 % w/w) pendant 4 semaines. Dans la seconde partie du travail, l'effet préventif d'*Iwong* sur l'apparition de l'obésité a été testé sur des souris C57BL/6J nourries pendant 5 semaines ; à une alimentation standard et à une alimentation grasse enrichie ou non de 4 ou 8 % w/w (*Iwong4* et *Iwong8,* respectivement) de poudre d'*Iwong*. D'autre part afin d'évaluer la capacité d'*Iwong* à traiter l'obésité, nous avons soumis des souris C57BL/6J à une alimentation hypercalorique riche en graisse pendant 9 semaines ou à une alimentation standard. Par la suite, les animaux obèses ont été subdivisés en 3 groupes nourris à une alimentation hypercalorique grasse enrichie ou non de 4 ou 8 % w/w de poudre d'*Iwong* (*Iwong4* et *Iwong8,* respectivement), pendant que le groupe contrôle a été continuellement nourri à une alimentation standard. Le poids corporel et la prise alimentaire ont été mesurés une fois par semaine. Á la fin de la période expérimentale, différents dépôts de graisse ont été prélevés puis pesés, le sang a parallèlement été recueilli et utilisé pour la quantification des paramètres biochimiques et du taux d'hormones plasmatiques. Pour l'étude *in vitro*, les adipocytes 3T3-L1 ont été traités avec l'extrait à l'éthanol d'*Iwong*, la quantité de lipides accumulés, l'activité de l'enzyme GPDH aussi bien que les niveaux d'expression des ARNm des facteurs de transcription PPARγ et C/EBPα ont été mesurés.

Les résultats obtenus ont montrés une réduction dose dépendante du gain de poids corporel et de la masse de graisse, malgré l'augmentation de la consommation alimentaire aussi bien chez la souris que chez le rat. Une légère diminution de la concentration plasmatique de HDL-C et de LDL-C a été observée dans les groupes de rongeurs ayant reçus une alimentation enrichie à la poudre d*'Iwong.* Les autres paramètres biochimiques (TG, T-C et glucose) demeurant quant-à-eux inchangés. Pour l'évaluation de l'effet d'*Iwong* sur la prévention de l'obésité, une hyperphagie paradoxalement associée à une réduction du gain de poids corporel et de la masse de graisse a été observée dans les groupes *Iwong4 et Iwong8*. Nous avons au niveau du plasma noté une réduction du taux de LDL-C et de la concentration de leptine particulièrement dans le groupe *Iwong8*. Chez les souris obèses traitées avec *Iwong*, accessoirement à la tendance à une hyperphagie nous avons remarqué une réduction dose dépendante du poids corporel et de la masse de graisse. La concentration hépatique en triglycérides, les taux de cholestérol total, LDL-C et de glucose ont été réduits de manière significative dans les groupes *Iwong4* et *Iwong8*.

L'étude *in vitro* conduite sur les adipocytes 3T3-L1 a démontré que l'extrait à l'éthanol pouvait inhiber de manière dose dépendante l'accumulation des lipides ainsi que l'activité de l'enzyme GPDH. Cet extrait a agi au niveau moléculaire en réduisant de manière significative l'expression des gènes PPARγ et C/EBPα

Nous pouvons dès lors conclure qu'*Iwong* serait un excellent candidat dans la recherche d'une substance naturelle pouvant traiter et/ou prévenir l'obésité via l'inhibition de l'accumulation des lipides dans le tissu adipeux.

I-)

INTRODUCTION

I-1) OBESITY AND OVERWEIGHT

I-1-1) Definition of obesity

Obesity is the result of a prolonged imbalance between energy intake and energy expenditure[1]. Excessive energy is stored as triacylglycerol in adipocytes. The consequence of lipid accumulation is an increase in adipose tissue mass, which generally involves two processes: increased fat cell size (hypertrophy) and increased fat cell number (hyperplasia) [2].

Body size and the prevalence of obesity within a population are commonly determined using the Body Mass Index (BMI). This index is obtained by dividing body mass (kg) by height (m^2). Subjects can therefore be classified from underweight with a BMI < 18.5 kg/m^2 to morbid obese with BMI ≥ 40kg/m^2, as shown in table1 [3]. Although BMI has been found to be strongly associated with an increase in cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM), it does not take into account the percentage of total fat mass in the body [4]. Therefore, the use of waist-hip ratio (WHR) has been suggested as an additional index for the measurement of obesity and its risk factors. The WHR, obtained by dividing the waist circumference by the hip circumference, is an excellent indicator to identify increased risk of obesity related morbidities due to accumulation of regional fat depots [5, 6]. According to the World Health Organization (WHO), subjects with a WHR > 0.95 cm for men and 0.85 cm for women are considered to develop abdominal obesity [7].

Obesity is associated with an increased risk of T2DM, hypertension, dyslipidaemia, CVD and cancer. In addition, obesity is often associated with a number of negative mental, physical and social consequences [8].

I-1-2) Causes of obesity

Obesity is considered to be a multifactorial disease caused by genetic, environmental and behavioral factors.

A critical environmental factor is the lack of adequate physical activity. A sedentary lifestyle decreases energy expenditure and promotes weight gain in both animals and human subjects [9, 10]. In addition, dietary habits play a critical role in the onset of obesity. Animals fed high calorie diets such as High Fat Diet (HFD) or High Sucrose Diet (HSD) develop obesity [11, 12]. In human

beings, voluntary overeating leads to increased body weight in normal overweight men and women (see review [13]). Furthermore night eating at a frequency of three meals per week is also known to promote body weight [14]. Additionally, numerous studies in animal models as well as in humans have shown association between obesity and variations on gut microbiota; therefore microbiota seems to be a non-negligible factor for the pathogenesis of obesity [15].

Table 1: WHO classification of obesity [3]

Classification	BMI (Kg/m2)	Risk of comorbidities
Underweight	< 18.5	Low (but risk of other clinical problems increased)
Normal range	18.5 to 24.9	Average
Pre-obese	25.0 to 29.9	Increased
Obese class 1	30.0 to 34.9	Moderate
Obese class 2	35.0 to 39.9	Severe
Obese class 3	≥40.0	Very severe

Comorbidity risk	Waist circumference (cm)	
	Women	Men
Above action level 1	≥80	≥94
Above action level 2	≥88	≥102

The link between obesity and genomic variation is well established. Common obesity is caused by an interaction of multiple genetic and behavioral factors. A meta-study on monozygotic and dizygotic twins in 1986 has shown that human obesity is under genetic control with an heritable contribution of 40-70% [16, 17].

I-1-3) Prevalence of obesity

In 2008, another report from the WHO estimated that approximately 1.6 billion of >15-year-old subjects are overweight and 500 million adults are obese [18].

Originally considered to be a disease restricted to developed countries, the prevalence of obesity has been increasing worldwide. This pandemic is now appearing in the poorest developing countries, where urban middle-aged adults are most affected, compared to populations in semi-urban and rural areas [19].

I-2) TREATMENT OF OBESITY

The current recommendations for treating overweight and obese subjects rely on changes in lifestyle, including balanced diets low in calories and increased physical activity. Very often, long-term maintenance of body weight loss is a challenge. Despite some commercial programs, such as the Weightwatchers program, have proven to be useful to help people staying on track [20], behavioral changes to lower body weight and reduce long-term risks associated with obesity remain insufficient and pharmacological approaches are needed [21]. In addition surgical approaches can be also included in the management of morbid obese subjects. Bariatric surgeries are essentially based on two main modes of action, the restrictive and the malabsorptive procedures, or a combination of both. All these procedures result in a restriction of food and nutrient intake, and thus promote body weight loss [22].

I-2-1) Pharmacological treatment

Actual pharmaco-therapeutical approaches are based on three different mechanisms of action: 1) reduction of food intake; 2) reduction of intestinal nutrients absorption; and 3) regulation of metabolism and increase of energy expenditure [23].

I-2-2) Anti-obesity medication withdrawn from the international market

Numerous anti-obesity drugs have been elaborated although most of them have been withdrawn from the international market because of the poor safety profile. One of the first anti-obesity drugs was Dinitrophenol which was used as a thermogenic agent and was banned from the market in 1933, alike Aminorex in 1968 due to its life-threatening complications [24]. Several other remedies such as Amphetamines, Phenylpropanol-amine, and Fenfluramine have been also removed from the international market.

Recently Rimonabant™, which is a selective endocannabinoid receptor 1 (CB-1) antagonist inducing central and peripheral effect on fat metabolism. The central action of Rimonabant™ consists on inhibition of food intake or increased satiety. Its peripheral action is mediated via the regulation of metabolic functions in numerous peripheral organs such as liver, adipose tissue, skeletal muscle and gut [25]. This drug which was one of the most promising anti-obesity drugs has been taken out of the market in 2009 due to its potential serious psychiatric side effects [26].

I-2-3) Anti-obesity medications commonly used

Phentermine and Diethylpropion are the only two appetite-suppressant substances of the phenethylamine family, approved in the USA for short term treatment (≤12 weeks) of obesity [27]. Since June 2013 lorcaserin, a novel appetite-suppressant drug acting as a serotoninergic agonist has been approved by the FDA [28]. Actually phentermine, a dopaminergic agonist is combined with a GABAergic agonist, topiramate, with the aim to reduce the side effect of both, and therefore increase their hypophagic effect by using their synergic action at low dose [29]. Another combination of bupropion and naltrexone, acting on pro-opiomelanocortin (POMC) and opioid pathways respectively is used to promote satiety [30]. Liraglutide, a glucagon-like-peptide 1(GLP-1) analog is also used to stimulate satiety [31]. Orlistat also known as tetrahydrolipstatin, is a derivative of lipstatin isolated from the bacterium *Streptomyces toxytricini.* It is currently the only available anti-obesity drug globally approved for a long-term treatment of obesity [32]. The drug acts as a gastric and pancreatic lipase inhibitor in the gut lumen by forming covalent bonds with these enzymes. Orlistat can therefore prevent the breakdown of ingested fat into free fatty acid and monoglycerides (Fig1) [21]. It reduces the ingested of amount fat up to 30%. This reduction of the intestinal lipid absorption leads to a decrease in caloric absorption and thereby promotes weight loss [33]. The use of Orlistat is usually associated with a high rate of gastrointestinal side effects including steatorrhea, flatulence, fecal incontinence, oily spotting and impaired absorption of lipophylic vitamins such as vitamins: A, K, and D [32].

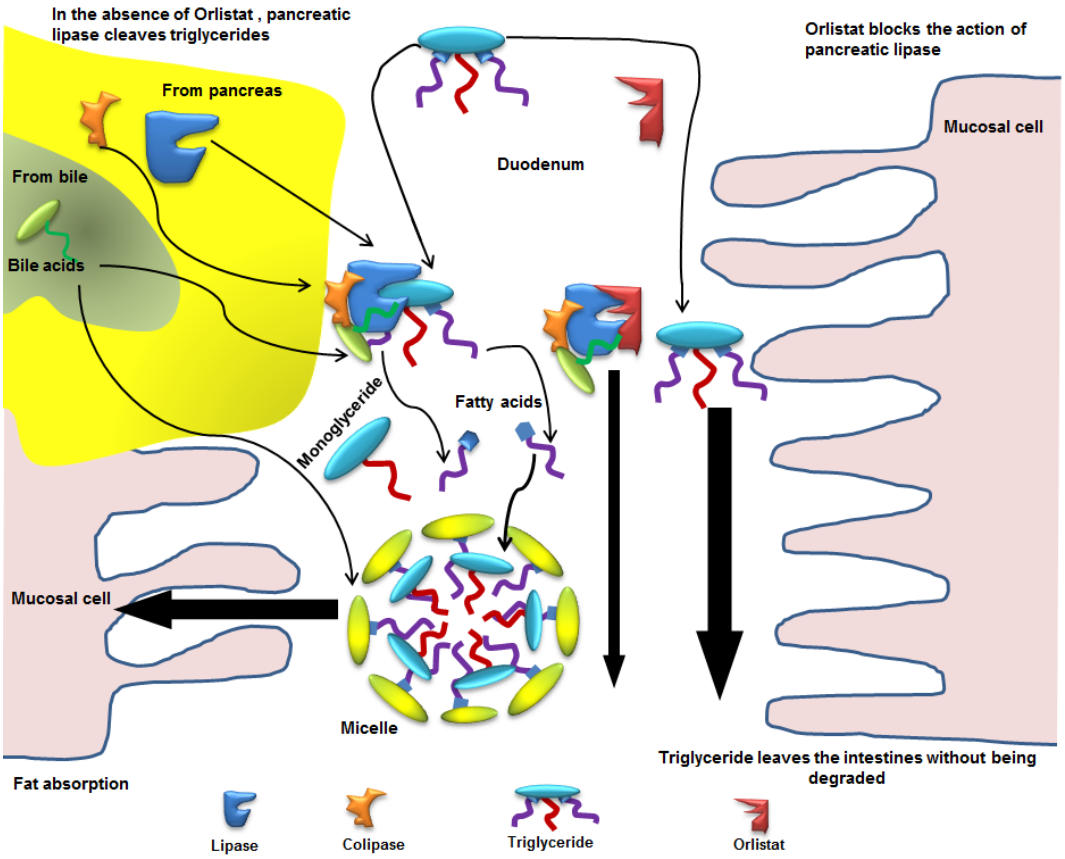


Fig. 1: Mode of action of Orlistat in gut lumen. Dietary triglycerides are hydrolyzed in the intestinal lumen by pancreatic lipase in fatty acids and monoglycerides. In the presence of bile acids from bile monoglycerides form micelle. Fatty acids and micelle are absorbed by enterocytes. Due to its structure xenical (Orlistat) binds to pancreatic lipase and thus inhibits its activity. This inhibition is lead to a decrease of a breakdown of dietary triglycerides and consequently a reduction of intestinal fat absorption. Around 30% of dietary which cannot be degraded leaves the intestine. Adapted from Faure, S [34]

I-2-4) Natural compounds used for the treatment of obesity

A large body of literature indicates that substantial progress has been made concerning the knowledge on bioactive components in plant foods and their link to obesity [35]. Depending on their mechanism of action, natural compounds with anti-obesity effect can be categorized in five groups: appetite suppressants, stimulators of energy expenditure, lipid absorption inhibitors, preadipocyte proliferation inhibitors or anti-lipogenic and lipolytic agents.

Table2: Anti-obesity drugs withdrawn to the market

Drug	Introduced	Mechanism of action	Status
Dinitrophenol	1930	Increases metabolic rate(uncouples oxidative phosphorylation)	Withdrawn 1933-risk of neuropathy and cataracts
Amphetamines Dexamphetamine methamphetamine	1936	Appetite suppression (NE, DA and 5HT releaser)	Banned, restricted or discourageddependency and abuse potential, cardiovascular adverse effects
Amphetamine-like Analogues: Phentermine, diethylpropion, phenylpropanolamine	1959	Appetite suppression (NE, DA and 5HT releaser)	Diethylpropion-available for short-term use (≤ 12 weeks) Phentermine-available for short-term use (≤ 12 weeks) in some countries withdrawn 2000 (UK) Phenylpropanolamine-withdrawn 2000-increased risk haemorrhagic stroke
Aminorex	1965	Appetite suppression (5HT releaser and reuptake inhibitor)	Withdrawn 1968 -pulmonary hypertension
Mazindol	1970	Appetite suppression (NE reuptake inhibitor)	Discontinued 1993 - Australia
Fenfluramine	1963-Europe 1973-USA	Appetite suppression	Withdrawn 1997 - valvular heart disease, pulmonary hypertension
Dexfenfluramine	1985-Europe 1996-USA	Appetite suppression (5HT releaser and re-uptake inhibitor)	Withdrawn 1997 - valvular heart disease, pulmonary hypertension
Sibutramine	1997-USA 2001-Europe	Appetite suppression (NE and 5HT reuptake inhibitor)	Temporarily withdrawn 2002 Italyconcerns of raised risk of heart attacks and strokes Increase in contraindications 2010 - US,Australia Suspension of market authorization 2010
Rimonabant	2006-Europe	Appetite suppression (cannabinoid Receptor CB1 antagonist)	Withdrawn 2009 -potential of serious psychiatric disorders

DA 5 dopamine; NE 5 norepinephrine

Adapted from Elangbam 2009 and Vetter et al. 2010 [21, 36]

I-2-4-1) Appetite suppressants

Plant extracts or herbal supplements such as Ephedra, *Caralluma fimbriata* and *Citrus aurantium* possess appetite-suppressing properties [37, 38]. The most important in this category is a plant from South Africa: *Hoodia gordonii*. The bioactive constituent of this plant known as P57 reduces food intake [39].

I-2-4-2) Metabolic stimulants

For a long time caffeine and ephedrine (Ephedra) were used to increase the energy expenditure. Recently, epigallocatechin gallate (EGCG) a bioactive compound isolated from green tea and capsaicin an alkaloid derived from Chili peppers have also been shown to possess the ability to increase energy expenditure and thermogenesis in humans [40, 41].

I-2-4-3) Intestinal lipase and gastric inhibitors

Numerous medicinal plants possess pancreatic lipase inhibitory activity. Most of them act similar to Orlistat with irreversible inhibitory action on this enzyme and can inactivate it by forming a stable covalent intermediate [42], [43]. Other natural compounds are reversible inhibitors of pancreatic lipase and therefore differ from Orlistat [44].

I-2-4-5) Lipid and glucose metabolism

It is well established that human obesity is accompanied by abnormalities in both glucose and lipid metabolism. Several plant extracts have been successfully investigated either for their effect on cholesterol and triglyceride levels or for their ability to lower serum glucose level.

Phytosterols for instance are commonly used for their cholesterol-lowering effects. In addition guggulsterone, one of the most important sterol isolated from *Commiphora mukul*, is used to treat obesity, arthritis and lipid disorders [45]. Likewise numerous other plant sterols such as diosgenin, campesterol and stigmasterol are known for their cholesterol lowering properties [46]. The effects of several plant extracts on the lipid metabolism have also been evaluated in rodents and in human as well as in cultured adipocytes, e.g. *Salix matsudana* and Korean fermented red pepper paste [47, 48].

Furthermore plants have been widely investigated for their capacity to lower serum glucose level. Several plants including *Gynostemma pentaphyllum*, *Allium sativum*, and *Platycodi radix* have shown hypoglycemic or antihyperglycemic effects [49-51]. Plants are also a source of antidiabetic drugs, the best known in this category is Biguanide, an antihyperglycemic molecule isolated from *Galega officinalis* extract [52].

I-3) ADIPOSE TISSUE

Adipose tissue is one of the most prevalent tissues in the human body, it is widely found in the subcutaneous area and around several organs. Two subtypes of adipose tissue are known: brown and white adipose tissue (BAT and WAT).

The BAT represents around 0.05 to 1% of the total body mass in adult subjects and 5% in newborns [53, 54]. It is anatomically located in several areas such as subcutaneous (interscapular and subscapular), dorsal-cervical and suprasternal. Together these depots form a “heating jacket” around the upper part of the body [55]. Some depots are found around the kidneys (perirenal) and adrenals (suprarenal) as well as around the mediastinal blood vessels, heart and esophagus. Brown adipocytes are ellipsoidal and polygonal with a diameter of 15 to 20 µm for the minimum and 30 to 40µm for the maximum [56]. These cells are multilocular and characterized by the presence of abundant small lipids droplets; and a large amount of mitochondria responsible for the brown color of the BAT. In addition, brown adipocytes exclusively express uncoupling protein-1 (UCP-1), a mitochondrial proton transporter that uncouples oxygen consumption from ATP production, dissipating energy as heat [57]. Therefore the main function of the brown adipocyte is heat production.

The WAT is a widely distributed type of adipose tissue; its distribution varies greatly between species as well as between individuals of the same species. In general WAT can be found in to anatomical compartments: the subcutaneous adipose tissue found beneath the skin and the intra-abdominal adipose tissue which surround the intestine, kidneys and gonads (in rodents) forming visceral, perirenal or epididymal fat. The shape of white adipocytes is spherical, their diameter depends on the region of the body; for subcutaneous fat it ranges from 30 to 160µm, and from 20 to 100µm for abdominal fat [58]. The main function of

WAT is to store excess energy in the form of triglycerides; it is also an active endocrine tissue sensing metabolic signals and secreting factors and hormones called adipokines that contribute to the regulation of energy homeostasis. Among these adipokines are hormones like leptin, adiponectin, resistin and the cytokines TNF-α (tumor necrosis factor-α), and interleukine-6 (IL-6) [57].

I-3-1) Metabolic functions of white adipose tissue

The white adipose tissue has key functions in triglycerides synthesis and lipolysis; it therefore plays a critical role for energy balance in the body.

I-3-1-1) Lipogenesis

Lipogenesis is a process ensuring the novo fatty acid synthesis and consequently the synthesis of triglycerides from glucose in adipose tissue and liver [59]. The synthesis of triglycerides from glucose is a complex cascade of molecular events. Glucose from bloodstream enters the adipocyte through glucose transporters (GLUTs) and is converted into pyruvate by the process of glycolysis. In mitochondria, pyruvate is decarboxylated to form Acetyl-CoA which enters the Krebs cycle so synthesize citrate obtained by condensation of Acetate-CoA with oxaloacetate. Fatty acids are synthesized from Acetyl CoA under the action of two enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) as shown in detail in Fig 2.

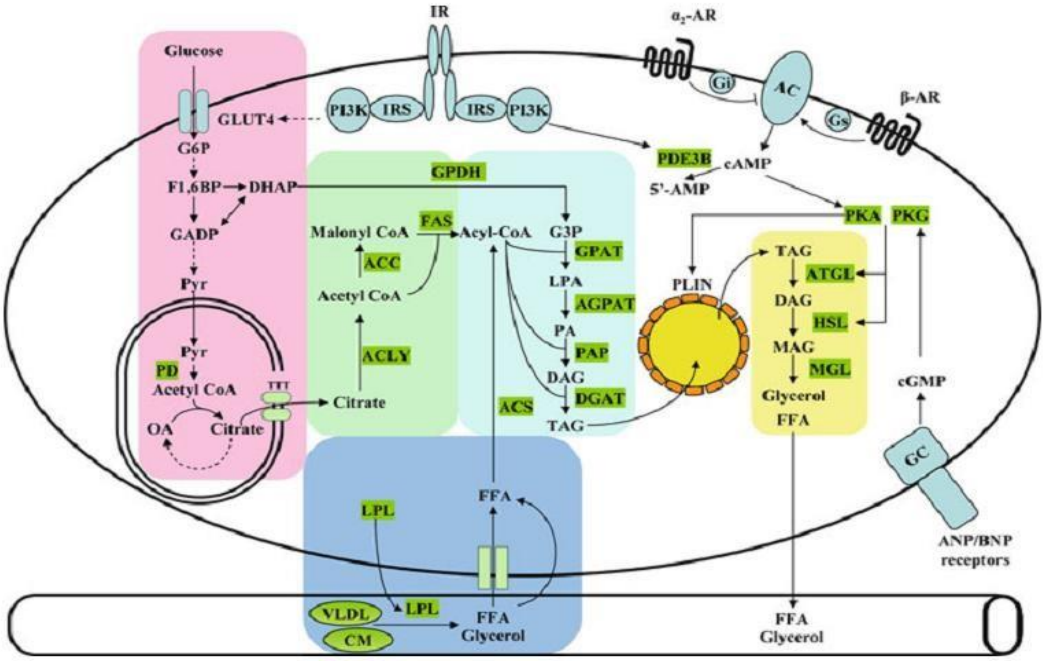


Fig 2: **Principal metabolic functions of WAT**. WAT ensures the function of storage of energy in the form of triglycerides and their hydrolysis to generate energy when it is required by the body. The anabolism of triglycerides (turquoise box) is depends on the uptake and the metabolism of glucose (pink box) followed by the process of de novo lipogenesis (green box) and the uptake of free fatty acid from the bloodstream (blue box). By the process of lipolysis (yellow box) free fatty acids are generated and released to the circulation after the hydrolysis of the triglycerides previously stored. All these processes are regulated by insulin, adrenergic hormones and atrial natriuetic peptide (ANP). α2-AR: α2-adrenergic receptor; α-AR α-adrenergic receptor; 5'-AMP: 5'-adenosine monophosphate ; AC: adenylate cyclase; ACC: acetyl-CoA carboxylase; ACLY: ATP citrate lyase; ACS: acyl-CoA synthetase; AGPAT: 1acylglycerol-3-phosphate O-acyltransferase; ANP: atrial natriuretic peptide; ATGL: adipose triglyceride lipase; BNP: brain natriuretic peptide; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; CM: chylomicron; DAG: diacylglycerol; DGAT: diacylglycerol acyltransferase; DHAP: dihydroxyacetone phosphate; F1,6BP: fructose 1,6 bisphosphate; FAS: fatty acid synthase; FFA: free fatty acid; G3P: glycerol-3-phosphate; G6P: glucose 6 phosphate; GADH: glyceraldehyde 3-phosphate; GC: guanylate cyclase; Gi: Gαi protein; GLUT4: glucose transporter 4; GPAT: glycerol 3-phosphate acyltransferase; GPDH: glycerol-3-phosphate dehydrogenase; Gs: Gαs protein; HSL: hormone sensitive lipase; IR: insulin receptor; IRS: insulin receptor substrate; LPA: lysophosphatidic acid; LPL: lipoprotein lipase; MAG: monoacylglycerol; MGL: monoacylglycerol lipase; OA: oxaloacetate; PA: phosphatidic acid; PAP: phosphatidic acid phosphatase; PDE3B: phosphodiesterase 3B; PI3K: phosphatidylinositol 3-kinase; PKA: cAMP-dependent protein kinase; PKG: cGMP-dependent protein kinase; PLIN: perilipin; Pyr pyruvate; PD: pyruvate dehydrogenase; TAG: triacylglycerol; TTT: tripartite tricarboxylate transporter; VLDL: very low density lipoprotein[59].

Regulation of lipogenesis is performed by several factors including hormones and nutrients. Dietary polyunsaturated fatty acids decrease expression of lipogenic genes [60]. Likewise, carbohydrates also mediate lipogenesis by increasing postprandial plasma glucose and triglycerides levels. In addition to diets, hormones are also regulating lipogenesis. The most important hormonal regulator is insulin, which increases glucose uptake by stimulating the translocation of glucose transporters to the plasma membrane. A further

hormone influencing triglyceride synthesis is growth hormone (GH). Its action is mediated principally by the down regulation of FAS expression in adipose tissue (see review [61]). In addition leptin is a critical hormone which inhibits lipogenesis by stimulating both glycerol release and fatty acid oxidation [62].

I-3-1-2) Lipolysis

Lipolysis is the breakdown of triacylglycerol (TAG) into glycerol and non-esterified fatty acids (NEFA), which are released into circulation for further usage by other organs as a source of energy. TAG are progressively hydrolyzed by specific enzymes. In adipocyte initiation of hydrolysis of TAG is controlled by ATGL which produces diacylglycerol (DAG). This step is followed by further hydrolysis of DAG resulting in the production of monoacylglycerol (MAG), which produces glycerol after the third hydrolysis step catalyzed by monoacylglycerol lipase (MGL). Each hydrolysis is accompanied by the liberation of one molecule of free fatty acid (Fig2).

- **Regulation of lipolysis**

Several factors including, nutrients, hormones and biochemical signals regulate the complex process of hydrolysis of TAG to glycerol and NEFAs.

Hormones play a critical role in the regulation of lipolysis. Catecholamines such as adrenalin and noradrenalin can stimulate lipolysis via β-adrenergic receptors. Binding to β-adrenergic receptors leads to an increase of cyclic adenosine monophosphate (cAMP) followed by activation of protein kinase A (PKA) and finally activation of HSL. An opposite effect is observed when the α2 adrenoceptors are activated. Adrenalin and noradrenalin lead to a decrease of intracellular cAMP level and consequently exert an antilipolytic effect (see review [56]).

Other powerful lipolytic agents are natriuretic peptide and GH. The first one activates guanylyl cyclase by increasing cGMP level, followed by a signal cascade resulting in the activation of HSL. The activity of GH is mediated via β1 and β3 adrenergic receptors and leads to increase of adenylyl cyclase and HSL expression [63].

The antilipolytic effect of insulin via the insulin receptor is well established. Binding of insulin on its specific receptor is followed by the phosphorylation

and activation of phosphodiesterase (PDE), which decreases cAMP levels and thereby inhibits PKA activation. Consequently, a diminution of perilipin and HSL phosphorylation provoks a decrease in lipolysis (see review [64][61]). Nutrients such as calcium, caffeine and ethanol are also known to regulate lipolysis. Increase of intracellular calcium level has an inhibitory effect on lipolysis by downregulating cAMP which leads to a decrease of HSL phosphorylation and subsequently, a reduction of lipolysis [61]. The same is true with acute consumption of ethanol which has an antilipolytic effect, while a chronic intake of ethanol has been reported to decrease lipolysis [65]. Finally, caffeine and other compounds derived from tea and coffee are also known to stimulate lipolysis by increasing the intracellular level of cAMP in mature adipocytes [56].

- **Lipolysis and obesity**

The basal lipolysis rate is higher in obese subjects compared to non-obese both in humans and in animal models. This may be explained by an increased secretion of various hormones and factors associated with downregulation or desensitization of specific receptors involved in lipolysis in obese subjects. Insulin and its receptors are playing an important role in this context. Impaired sensitivity of mature adipocytes to insulin signaling as observed in obese subject favors elevation of basal lipolysis [64].

The concentration of circulating leptin level is known to be higher in obese subjects leading to overexpression of leptin genes in adipocytes that enhance basal lipolysis in obesity [66].

Adipocytes from obese subjects secrete more cytokines as compared to adipocytes from lean subjects. An increased level of TNFα has been observed. This upregulation could consequently increase basal lipolysis in obese subjects by downregulating perilipin levels.

Obesity is also associated with a decrease of catecholamine activity on adipocytes, which is mainly explained by an impaired β-adrenergic-receptor stimulating lipolysis of adipocytes from obese subjects and lower level of cAMP activity compared to lean subjects [67].

I-4) ADIPOGENESIS

I-4-1) Preadipocyte cell models

The process of adipocyte differentiation can be mimicked in vitro and numerous cell lines have been developed. Cells line can be grouped in two classes: pluripotent fibroblasts and unipotent preadipocytes [68].

a) Pluripotent fibroblasts such as 10T1/2, Balb/c 3T3, RCJ3.1 and CHEF/18; can undergo determination into adipocytes, myocytes or chondrocytes. They are consequently good models to study and understand events responsible for cellular determination of the separate cells fates [68, 69].

b) Unipotent preadipocytes include 3T3-L1, 3T3-F422A, Ob1771, TA1 and 30A5. These cells have already undergone determination and can either remain as preadipocytes or differentiated to mature adipocyte in specific conditions. They offer the advantage to help to understand the molecular mechanism of the conversion of preadipocytes into mature adipocytes.

The most widely used unipotent cells are 3T3L1 and 3T3-F442A; they were derived from disaggregated Swiss 3T3 mouse embryos [70].

I-4-2) The 3T3L1 cell line

To study the conversion of preadipocytes into mature adipocytes and understand the molecular events responsible for this process, several unipotent cells have been used. One of the best characterized models is the 3T3L1 cell line [68, 69]. During the process of adipogenesis the appearance and development of lipid droplets mimic live of adipose tissue [70]. In addition, the electron microscopic study of this cell line reveals morphological, ultrastructural and biochemical similarities with mature white adipocytes [71]. Injection of these cells into mice is associated with the formation of fat pads similar to preexisting normal adipose tissue [70]. Confluent 3T3L1 preadipocytes can be differentiated to mature adipocytes in the presence of a specific cocktail. This cocktail is composed by insulin, which acts through the insulin-like growth factor1 (IGF-1) receptor as well as Dexamethasone (DEX) a synthetic glucocorticoide agonist which stimulates the glucocorticoid pathway and Methylisobutylxanthine (MIX), a cAMP-phosphodiesterase inhibitor.

Therefore those cells are frequently used to *in vitro* assess the effect of various agents on the process of lipid accumulation [72].

I-4-3) Description of the adipocyte life cycle in 3T3L1 unipotent cells

Obesity is characterized by an increase in adipose tissue mass as a consequence of two main phenomena: hypertrophy, which is considered as an increase in adipocyte size and hyperplasia, which is due to an increased number of adipocytes [2]. The number and the size of adipocytes in the body largely depend on the process of adipocyte differentiation, also known as adipogenesis.

Adipogenesis is the development of mature fat cells from preadipocytes. It is a complex process which includes alteration of cell shape, growth arrest, clonal expansion, and a complex sequence of changes in gene expression resulting in increased of lipid storage [73].

Initially, 3T3L1 preadipocytes morphologically resemble fibroblasts and are characterized by the expression of a specific marker known as preadipocyte factor-1 (Pref-1). The level of this protein is higher in preadipocytes and is strongly decreased during the process of adipogenesis until it reaches an undetectable level in mature adipocytes. During their conversion to mature adipocytes, preadipocytes undergo morphological and molecular events that can be grouped in three phases: growth arrest, clonal expansion and transcriptional regulation.

I-4-3-1) Growth arrest

Stem cells respond to specific signals to undergo determination to the adipocyte lineage; this process is known as commitment. In culture, committed preadipocytes have to withdraw at G_0/G_1 from the cell cycle boundary [2]. This process of growth arrest is crucial for preadipocyte differentiation, because even in the absence of cell-cell contact cells can differentiate into mature adipocytes [74]. Several changes at the molecular level take place during this first stage of conversion of preadipocytes to mature adipocytes. Two key transcription factors, peroxisome proliferator-acitived receptor-γ (PPARγ) and

CCAAT enhancer-binding proteinα (C/EBPα), are implicated in the growth arrest that is required for adipocyte differentiation[73].

I-4-3-2) Clonal expansion

After growth arrest, cells receiving the appropriate combination of mitogenic and adipogenic signals will re-enter the cell cycle and undergo one or two rounds of cell division before starting terminal differentiation. During the mitotic clonal expansion phase there is an increase of DNA replication and changes in chromatin structure that increase the accessibility of cis-elements to transacting factors, which activate or repress transcription of genes that give rise to adipocyte phenotype [75]. The importance of mitotic clonal expansion as a prerequisite step for the maturation of adipocytes has been well demonstrated. The inhibition of the mitotic clonal expansion with rapamycin, a potent immunosuppressant causing G_1 arrest was able to prevent adipogenesis [76]. This step is not required in vitro by primary preadipocytes derived from human adipose tissue, because these cells undergo critical cell division *in vivo* [77].

I-4-3-3) Transcriptional regulation of adipogenesis

Many classes of transcription factors have been reported to influence adipogenesis, but two of them, PPARs and C/EBPs, are known to play a central role during this process [78]. As depicted in Fig. 3, the initiation event appearing around one hour after induction is the early expression of C/EBPβ and δ. Overexpression of these two transcription factors enhances adipogenesis, whereas knock out of C/EBPβ and δ in mice is associated with a reduction in fat mass demonstrating a synergistic role of these transcription factors during adipocyte differentiation (see review [79]). During the process of adipogenesis in 3T3L1, the expression of C/EBP δ is rapidly decreased to undetectable levels after 48, whereas the decrease of C/EBPβ is gradual and reaches undetectable levels after 8 days [77]. The activity of C/EBPβ and δ also mediated the expression of C/EBPα and PPARγ, which both are critical for the maintained differentiation until the late stage.

PPARγ is a member of the steroid/thyroid receptor superfamily and one subtype of PPARs. It is widely expressed in adipose tissue compared to the other two

subtypes PPARα and PPARδ which are ubiquitously expressed in others tissue. Two isoforms of PPARγ have been identified PPARγ1 and PPARγ2, but expression of the latter isoform is more specific to adipose tissue [80]. C/EBPα and PPARγ cross-regulate each other to maintain their expression and thus promote adipocyte differentiation. In addition, both can bind and transactivate the expression of various genes encoding for example aP2, leptin, insulin receptor and lipogenic enzymes, which are necessary for the differentiation of adipocytes [74].

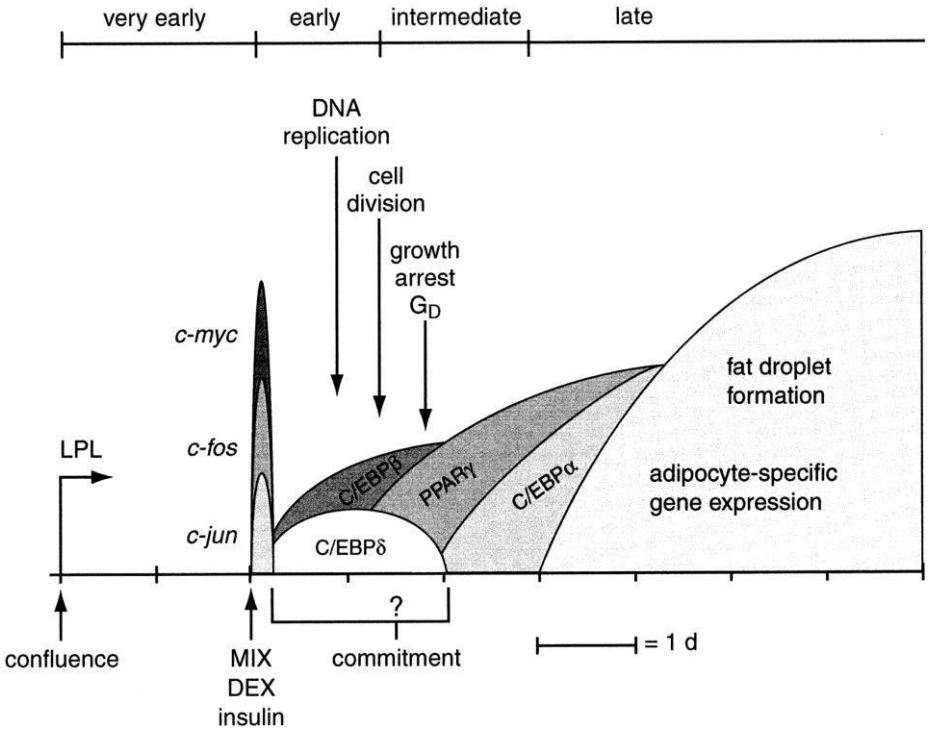


Fig.3: Molecular events during 3T3-L1 differentiation. The major identified events of preadipocyte differentiation are presented chronologically. Areas labeled by gene names represent periods of gene expression during the differentiation program. The distinct stages of differentiation (very early, early, intermediate and late) are also provided. LPL, lipoprotein lipase; C/EBP, CCAAT/enhancer binding protein, PPAR, peroxisome proliferator activated receptor; MIX, methylisobutylxanthine; DEX, dexamethasone. This figure was adapted from Ntambi et al [69].

I-5) METABOLISM OF LIPOPROTEINS

Lipoproteins are water-soluble macromolecules, composed by assembling lipids (triglycerides, cholesterols and phospholipids) and apolipoproteins, containing in their center the hydrophobic lipids.

The role of lipoproteins is essential for dietary fat absorption of cholesterol, fatty acids and lipophilic vitamins for their transport from liver to peripheral tissues and vice versa.

I-5-1) Classification of lipoproteins

Lipoproteins are divided in five classes, depending by their density and size (Table 3)

Table3: Major lipoprotein classes [81]

Lipoproteins	Density (G/ML)	Size NM	Apolipoproteins
Chylomicrons	0.930	75-1200	ApoB-48
Chylomicron remnants	0.930 -1.006	30-80	ApoB-48
VLDL	0.930 -1.006	30-80	ApoB-100
IDL	1.006 -1.019	25-35	ApoB-100
LDL	1.019 -1.063	18-25	ApoB-100
HDL	1.063 -1.210	5-12	ApoA-I

- Chylomicrons are synthesized in enterocytes and ensure the transport of dietary lipids from the lumen intestinal to bloodstream. The maturation of chylomicrons takes place in plasma with the incorporation of apoliporotein C and E (Apo C and Apo E). Two apolipoproteins including apoB-48 and apo E are necessary for the binding of chylomicrons with hepatic receptors, whereas Apo C acts as activator of the lipoprotein lipase. This enzyme hydrolyzes triacylglycerol into fatty acids and glycerol in peripheral tissues. Chylomicrons remnants are smaller, have a higher density and are degraded in the liver.

-Very low density lipoproteins (VLDL) are composed of triacyglycerol, cholesterol and apolipoproteins (apoB-100 and apoA-I) which ensures the transport and redistribution of triglycerides and cholesterol from the liver to other tissues. In plasma apoE and apoC-II from HDL essential for the binding in peripheral tissues receptors and activation of the lipoprotein lipase, respectively, are incorporated into VLDL. In the plasma under the action of lipoprotein lipase triglycerides of VLDL are hydrolyzed into free fatty acids and intermediate lipoproteins (IDL) and later to low density lipoproteins (LDL) [82].

-Low density lipoproteins (LDL). The main function of this lipoprotein is the transport of cholesterol from the liver to peripheral organs, where it is recognized due to the presence of ApoB-100 on its surface and will be internalized by the process of endocytosis [83]. Protein receptors can be recycled or hydrolyzed into amino acids, whereas triacylglycerol and phospholipids will be completely degraded and form fatty acids, or other fatty acids derived metabolites. Additionally the hydrolysis of cholesterol esters will generate free cholesterol. All these products can be later utilized by cells [84].

-High density lipoproteins (HDL). Its biosynthesis is complex, nascent HDL particles are synthesized by the intestine and the liver contains apoA-I and phospholipids but will rapidly be enriched in unesterified cholesterol and more phospholipids from peripheral tissue. The maturation of HDL which became spherical requires esterification of cholesterol to cholesteryl esters by cholesterol acyltransferase (LCAT) and hydrophobic lipid core [81]. The transport of HDL to the liver cholesterol is ensured by both indirect and direct pathways. For the indirect transport HDL cholesteryl esters to apoB containing lipoproteins in exchange to triglycerides by the cholesteryl esters transfer protein (CETP), cholesteryls esters are then removed from the circulation by LDL receptors mediated endocytosis. The second pathway is mediated via scavenger receptor class BI (SR-BI) which allows the direct up take of cholesterol by hepatocytes. After hydrolysis cholesteryl esters can be recycled and formed other lipoproteins or for the synthesis of hormones and finally can be excreted via bile [85].

I-5-2) Lipoproteins and obesity

In non-obese subjects, dietary triacylglycerols are first digested by gastric pancreatic and lipase and then absorbed and transported into the bloodstream as chylomicrons. These are distributed to peripheral tissues, where they are hydrolyzed by LPL to nonesterified fatty acids (NEFA) and stored as triglycerides (TGs) or serve as sources of energy for muscles. In addition fatty acids can be taken up by the liver and used to synthesize TGs, which can be released into blood circulation as VLDL. When more fatty acids are uptake than required, the surplus is stored in the form of fat droplets.

Obesity is commonly associated with dyslipidemia which is characterized by increased TG levels, decreased HDL levels and changes in LDL to a prominent pro-atherogenic composition (small dense LDL) [86].

I-6) HORMONES AND OBESITY

I-6-1) Leptin

Leptin is a peptide hormone of 16 kDa and one of the most central adipocyte derived hormones [87]. The leptin receptor (Ob-R or Lepr-B); is mostly expressed in the arcuate nucleus (ARC), the dorsomedial nucleus of the hypothalamus, the lateral hypothalamic area and the ventromedial hypothalamic nucleus. The Ob-R was also detected in numerous tissues including liver, kidney, heart, small intestine, pancreas and adipose tissue. Six isoforms of leptin receptor have been reported in human (ObRa to ObRf), they can be divided in three classes: long, short and secreted [88, 89]. The longest form Ob-Rb which is widely distributed in brain and involved in the hypothalamic regulatory weight action of leptin, whereas the four short isoforms found in brain microvessels played a role in blood-brain barrier [90].

I-6-1-1) Regulation of leptin expression

The plasma leptin level is positively correlated with total fat mass and the nutritional state. Thus obesity is associated with an increase in the plasma leptin levels, while leptin is undetectable in lipodystrophic patients. The postprandial state and the high fat meal increase leptin level, whereas it is strongly decreased in the fasting state [90].The expression and secretion of leptin also depends on the white adipose tissue depot; subcutaneous fat for instance is more active compared to the visceral fat. This regional difference may be explained by the higher ß- adrenergic sensitivity of visceral WAT [91].

I-6-1-2) Physiological function of leptin

Leptin exerts its physiological function on both central and peripheral actions. In the hypothalamus the central action of leptin is mediated via anorexigenic and orexigenic populations of neurons. Leptin inhibits orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP) expressing neurons and in parallel activates anorexigenic pro-opiomelanocortin (POMC) and cocaine amphetamine regulated transcript (CART) neurons [92].

The peripheral action of leptin is well established since the evidence of its secretion from the gastric mucosa is now recognized [93]. In addition, the leptin receptor Ob-Rb is expressed in gastric epithelial cells and jejunal epithelium of the rat, suggesting that leptin regulates body weight and adiposity by acting directly in the gut [94]. Several studies have confirmed this hypothesis, Barrachina et al. for instance have demonstrated that leptin acts synergically with cholecystokinin (CCK) to reduce food intake and subsequently body weight in mice[95]. Alternatively leptin increases the activity of NTS neurons responsive to gastric vagal stimulation. Taken together these observations confirm the peripheral effect of leptin in the gut, but the complete mode of action remains unclear.

Further peripheral targets of leptin include the immune system, inflammation processes, and hematopoiesis. For instance an increased leptin production is observed during infection and inflammation. Leptin plays an inhibitory role on monocyte/macrophages – mediated responses and a permissive role on lymphocyte mediated inflammation. Leptin also stimulates the proliferation of different cells types of immune system, including T lymphocytes, $CD34^{+}$, leukemia cells and endothelial cells [96].

The bone mineral density (BMD) seems to be influenced by the plasma leptin level, but results remain contradictory and the mode of action is still unclear. A positive correlation between both parameters has been found in women, whereas other studies showed a negative correlation or did not find any relationship [97-99].

I-6-2) Adiponectin

Adiponectin (ACRP30 AMP1 AdipoQ GBP28) is an adipokine secreted by white adipose tissue, with a molecular weight of 28 kDa was identified by four independent groups using different technical approaches [100-103]. The effect of adiponectin is mediated via two receptors AdipoR1 and AdipoR2, which are ubiquitously expressed and have opposite functions in the control of homeostasis. The first one AdipoR1 is mostly found in skeletal muscle and participates in the decrease of the energy expenditure, while AdipoR2 is highly expressed in the liver and contributes to increase energy expenditure [59]. These two receptors have been also recently found to be abundantly expressed in the hypothalamus suggesting a potential role of adiponectin in the central regulation of energy intake and expenditure [104]. Others studies have shown that AdipoR1 and AdipoR2 are both expressed in endothelial cells, whereas only the first one is known to be expressed in cardiomyocytes and pancreatic ß cells [105].

I-6-2-1) Regulation of adiponectin expression

Initially known to be synthesized only by adipocytes, cardiomyocytes are nowadays also suspected to be a site of adiponectin synthesis and secretion [93]. The expression and secretion of adiponectin depend on the fat depot. It is generally accepted that there is a negative correlation between visceral WAT and the plasma level of this hormone. This observation remains conflicting, as some others studies have reported a high expression and secretion in subcutaneous WAT compared to visceral WAT [106]. Adiponectin blood circulating level strongly depends on the sex in human, with a higher plasma adiponectin levels observed in women. This sexual dimorphism could be a consequence of adiponectin regulation by androgens. Nishizawa et al have shown that ovariectomy does not affect adiponectin plasma level, while castrated mice showed a high level of circulating blood adiponectin; this elevation can be abolished by testosterone treatment [107]. The synthesis and secretion of adiponectin also depends on various factors. β-adrenergic activity, TNFα and glucocorticoids decreases expression, whereas thiazolidinediones stimulate the expression of this hormone [108-111].

I-6-2-2) Physiological function of adiponectin

Adiponectin plays a critical role in the control of energy homeostasis by regulating glucose and fatty acid metabolism in peripheral tissues such as liver and skeletal muscle. Although the action of adiponectin is confined in the peripheral tissues, several studies also evoked potential central effects on the hypothalamus [104, 112].

In the liver, adiponectin decreased gluconeogenesis, by repressing the activity of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase, resulting in a decrease of hepatic glucose production. This hormone also increases fatty acid oxidation and therefore prevents triacyglycerol accumulation as well as lipotoxicity in hepatocytes [113].

In skeletal muscle, adiponectin enhanced lipid catabolism and reduced TG content in skeletal muscle; as consequence there is an improvement of insulin sensitivity [114]. Energy metabolism by adiponectin is also mediated by the activation of PPARγ which is associated with a decrease of TG content in myocytes and therefore contributes to decreased insulin resistance [113]. It has also been shown that adiponectin stimulates glucose uptake and β-oxidation through phosphorylation and activation of AMPK [115].

Adiponectin protects against atherosclerosis mainly by suppressing two processes. It can suppress the formation of neo-intimal by inhibiting the expression of inflammatory cytokines in endothelial cells including leukocytic colony formation and phagocytic activity. The second antiartherosclerosis pathway of adiponectin is mediated via the suppression of uptake of cholesterol by inhibiting scavenger receptors and the production of reactive oxygen species [112].

Although adiponectin essential acts in the peripheral level; AdipoR1 and AdipoR2 are located in the central nervous system in the hypothalamus and brain stem suggesting a possible effect of adiponectin on energy intake [116]. Adiponectin also increases ARC activity and thereby stimulates food intake and decreases energy expenditure without affecting the expression of both orexigenic (NPY/AgRP) and anorexigenic (POMC/CART) hypothalamic neuropeptides [117].

I-6-3) Insulin

Insulin is a hormone playing a key role in the regulation of glucose metabolism. It is secreted from β cells and is formed by two peptides: A (21 amino acids) and B (30 amino acids) which are linked with two disulfides bonds. The insulin receptor is a member of the tyrosine kinase class of membrane receptors. Two isoforms of the insulin receptor have been identified, the isoform "A" exclusively found in tumor cells and the isoform "B" expressed in normal cells. The insulin receptor is formed by two extracellular α-subunits containing the insulin binding sites and two membrane-spanning β-subunits with intrinsic tyrosine protein kinase activity [118]. Insulin receptors are ubiquitously expressed in vertebrate cells, but the number of receptors varies from cell to cell. Thus only around 40 receptors are found in red blood cells and this amount can increase up to 200,000 receptors on adipocytes and liver cells [119].

I-6-3-1) Regulation of insulin expression

The synthesis of insulin takes place in pancreas, principally in β cells found in the islets of Langerhans. The preproinsulin, a precursor of insulin, is released into the cytoplasm and transferred to the endoplasmic reticulum (ER). It is cleaved at the ER membrane to form proinsulin, which is translocated to the Golgi apparatus, enclosed into secretory granules. After the action of enzymes the proinsulin is cleaved in peptide C and the mature insulin. The mature insulin which is the active form of this hormone is secreted in the bloodstream when glucose levels increase [120].

Insulin secretion is regulated by nutrients status, other hormones, growth factors and pharmacological agents.

The insulin concentration in the blood is elevated during postprandial period. This fluctuation is commonly observed after a meal rich in simple or complex sugars. In fact glucose is the key regulator of insulin secretion; it is known to increase the ATP/ADP ratio followed by closure of ATP-sensitive K^+-channels and membrane depolarization. Consequently to this depolarization, the voltage-dependent Ca^{2+} channel opens immediately followed by an influx Ca^{2+} resulting in a secretion of mature insulin. In parallel to this glucose stimulatory action other small molecules can promote insulin secretion from β-cells,

including FFA, amino acids and keto-acids. As mentioned above hormones also play an important role in the regulation of insulin secretion. Incretins like glucagon-like peptide 1(GLP-I), glucose dependent insulinotropic polypeptide (GIP) and CCK are known to enhance insulin secretion. Prolactin, leptin and growth hormone (GH) are able to modulate insulin gene expression and therefore stimulate insulin secretion. Other hormones such as noradrenalin and somatostatin decrease insulin secretion [121].

Pharmacological agents are also recognized to regulate insulin secretion. Metformin for instance is a glucose lowering drug of the biguanide class which enhances insulin sensitivity mostly in the muscle and the liver [122].

I-6-3-2) Insulin and obesity

The relationship between body mass index (BMI) and the development of insulin resistance is well established, demonstrating the negative impact of total body fat for insulin sensitivity. In addition hyperinsulinemia as a consequence of insulin resistance can contribute to the development of obesity [123]. In fact insulin is one of the most important regulators of adipocyte development, as it promotes adipocyte triglyceride storage by stimulating glucose transport and TG synthesis. The antilipolytic role of insulin also contributes to development of obesity.

I-7) INTRODUCTION TO THE IMPORTANCE AND SCOPE OF MEDICINAL PLANTS

A medicinal plant could be defined as: “any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which is a precursor for synthesis of useful drugs” [124]. Historically, natural products have provided an endless source of medicine. Plant-derived products have dominated the human pharmacopoeia for thousands of years almost unchallenged [24]. China, India, ancient Egypt and Assyria appear to have been the place which cradles the use of herbs, but herbalism was common in Europe by medieval times. This is still true today. In terms of world health, herbal medicines continue to play a central role in the healthcare systems of large portions of the world’s population, particularly in developing countries, where 80% of the population depends on traditional medicine for primary

health care [125]. Towards the end of the 20th century, there is an enormous resurgence of interest in all herbal products and in traditional medicine in many industrialized countries. In the United States it is estimated that herbal usage increased by 380% between 1990 and 1997 [126]. In the European Union about 1400 herbal preparations are widely used, mostly in Belgium, France, Germany and the Netherlands. Germany for instance is recognized as one of the major exporters of plant-based medicinal products with over 1500 plant species encountered in some 200 families and 800 genera processed into medicinal products [127].

In developing countries, the use of medicinal plants is based primarily on empirical knowledge [128]. They are commonly used as crude herbs, powders and dried decoctions. Thus many plants have not been scientifically evaluated for their safety and efficacy. In developed countries, the industrial uses of medicinal plants are many. These range from traditional medicines, herbal teas, and health foods such as nutriceuticals to galenicals [128].

In 1897, Aspirin (acetylsalicylic acid) was synthesized from salicylic acid, an active ingredient of an herbal preparation; therefore the importance of plants as source of medicine was established. Drugs such as quinine (*Cinchona sp*), pilocarpine (*Pilocarpus jaborandi*), forskolin (*coleus forskohlii*) and vinblantine (*Catharanthus roseus*) were later formulated from medicinal plants. Recently it has been demonstrated by Newman et al that from 1981 to 2002 about 49% of 877 novel medicines developed are based on natural products [129]. Thus, despite drug discovery technology diversification it is clear that natural products from plants and other biological sources remain an undiminished source of new drugs. According to Balandrin et al, at least 25 % of the active compounds in currently prescribed synthetic drugs were first identified in plant sources [130].

I-8-) *Iwong*: *Ipomoea alba*

I-8-1) Description

The genus Ipomoea L represents one of the largest in terms of the number within species of the Convolvulaceae family [131]. Ipomoea sp are widely distributed throughout the South and Central America, and the subtropical parts of the world, including several African countries, among them Cameroon. To this day at least 600 species of *Ipomoea* have been itemized [132-134].

Ipomoea alba, also known as white morning glory or moon vine is a native of tropical and subtropical regions; it is nowadays widely distributed around the world.

Fig. 4:*Ipomoea alba* (https://www.edenbrothers.com/store/moonflower_seeds.html)

I-8-2) Taxonomy [135]

- **Kingdom**: Plantae
- **Subkingdom**: Viridaeplantae
- **Infrakingdom**: Streptophyta
- **Division**: Tracheophyta
- **Subdivision**: Spermatophytina

- **Infradivision**: Angiospermae
- **Class**: Magnoliopsida
- **Superorder**: Asteridae
- **Order**: Solanales
- **Family**: Convolvulaceae
- **Genus**: Ipomoea, Linn
- **Species**: *Ipomoea alba*, Linn
- **Common name** : Morning glory flower or moon vine
- **Vernacular name**: *Iwong* (Southern region of Cameroon)

I-8-3) Ethnopharmacological knowledges

The plants of the genus *Ipomoea* have been used since immemorial times for nutritional, medicinal, ritual and agricultural purposes [136]. *Ipomoea batatas* for instance is used around the world as food; the same is true for *Ipomoea aquatica* which is consumed as a vegetable in some Asian countries [137]. Others are used to treat various pathologies such as inflammations, kidney aliments, constipation, colic and digestive disorders. *Ipomoea batatas*, *Ipomoea pes-caprae* and *Ipomoea cairica* are recognized for their antinociceptive and anti-inflammatory values, whereas species like *Ipomoea digitata*, *Ipomoea indica* and *Ipomoea purge* have shown purgative properties [138, 139]. Since few years the use of Ipomoea genus to treat metabolic diseases and to slim body weight has been also proved [140]. The hypoglycemic or anti-hyperglycemic effects as well as antioxidant properties of *I*. *batatas* and *I. aquatica* have been verified [140]. Although a large body of scientific literature is available on plants of the *Ipomoea* genus, several promising species are still partially or not studied. Therefore the scientific interest in this genus has recently increased.

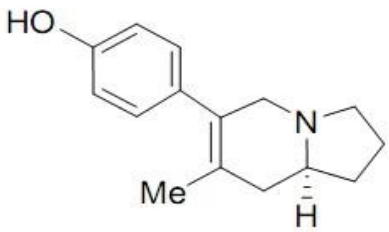
Ipomoea alba is commonly known as *Iwong* in vernacular language of the center and the southern regions of Cameroon. It is a plant used in Cameroonian traditional medicine for its ability to control glucose homeostasis and body weight. It is also applied as a laxative and to improve the quality of breast milk. In India the whole plant is used a as snake-bite remedy, whereas in Nigeria it is used against headaches. It has been demonstrated that *Ipomoea alba* has non-

addictive analgesic properties as well as inhibitory effects on respiratory burst of leukocytes and scavengs oxygen-free radicals [136, 141]. Calystegines B1 and B2, two compounds isolated from this plant, are known for their potential to inhibit the lysosomal ß-glucosidase activity in rat [142].

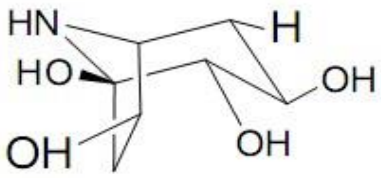
Ipomoea alba is also eaten as a vegetable in Sierra-Leone and in China where young leaves and fleshy calyces are cooked in soups and stews or used as curries[143].

I-8-4) Phytochemistry

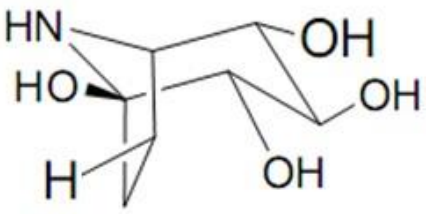
The phytochemical analysis of *I. alba* has shown that the seed of this plant is rich in alkaloids and at least 9 compounds have been isolated among them we can cite: Ipalbine Ipalbidine, Isoipomine, Ipalbidinium, E-ipomine, Z-ipomine, Methoxyipomine, Dimethoxyipomine and Ipohardine. In addition further compounds such as Calystegines A5, B1 and B2 were isolated from the roots [136]



Ipalbidine



Calystegines B1



Calystegines B2

Fig 5: Chemical structure of selected molecules isolated from *Ipomoea alba*

I-9) RESEARCH HYPOTHESIS AND AIM OF THE DOCTORAL THESIS

According to the WHO the prevalence of obesity has been drastically increased either in developed or developing countries and the situation is likely to get worse [144]. The obese and overweight have a high risk to develop several diseases including type 2 diabetes, hypertension and cardiovascular disease [145]. Despite this alarming situation only few medications against obesity are available, and most of them develop severe side effects or are ineffective for a long term treatment [144]. Therefore, an effective alternative therapeutic approach is urgently required. Several medicinal plants have been successfully used in drug discovery to develop new medications against numerous diseases [129]. The well and recent known case is artemisinin a potent antimalarial molecule isolated from *Artemisia annua* [146]. The same is true in the field of anticancer drugs development, where compounds like vinblastine, vincristine, camptothecin as well as paclitaxel derived from medicinal plants or are semi-synthetic analogues of medicinal plant (topotecan, irinotecan, docetaxel) [147]. Medicinal plants could also be an excellent opportunity to meet the demand of the current obesity epidemic. Some medicinal plants with anti-obesity effect such as *Caralluma fimbriata* and *Hoodia gordonii* have already been studied [35, 37, 39]. Recently, epigallocatechin gallate (EGCG) an active compound from green tea showed promising anti-obesity effects [40, 148].

In view of the above:

- Could we identify a medicinal plant used in Cameroonian traditional medicine to manage body weight reduction?
- Could we scientifically prove this empiric declarations or observations on body weight reduction and related diseases observed in obese subjects?
- Could this medicinal plant also be a source of an efficient drug against obesity?

The aim of this doctoral thesis was therefore to assess the effect of *Iwong* on food consumption, body weight and fat mass in rodents. As obesity is associated with dyslipidemia and changes in hormone profiles, we additionally evaluated the ability of *Iwong* feeding to normalize or prevent all these alterations. In parallel murine preadipocytes were used to evaluate the effect of Iwong's ethanolic extract and several fractions on lipid accumulation as well as on lipolysis.

II-)

MATERIAL AND METHODS

II-1) Plant material and preparation of the ethanolic extract

II-1-1) Plant material

The plant *Ipomoea alba* (convolvulaceae) was harvested twice in the region of Yaoundé–Cameroon from April to June for the first batch (A), then two years later from September to October for the second batch (B) and deposited in the national herbarium of Cameroon for identification (n° 44695). The aerial part of the plant was air dried for three weeks. The dried plant material was crushed, and the powder obtained was kept at room temperature for future usage.

II-1-2) Preparation of the ethanolic extract

One kilogram of the powder of *Iwong* was incubated with 3000 ml of ethanol 70% at room temperature for 24 hours. The liquid extract was filtrated (Wathman paper number 1), and the filtrate was concentrated in a rotary evaporator (R-210, BUCHI Laboratory Equipment), with a final yield of 13.5%. Ethanolic extracts were stored at 4°C. For treatment of 3T3-L1 preadipocytes the extract was diluted in 0.1 % DMSO. During the course of the experiments two batches of *Iwong* powder were used for ethanolic extraction which differed in their biological activity as judged from cell culture experiments (batch A:100-300 µg/mL; batch B: 25-75 µg/mL)

II-2) In vitro assays

II-2-1) Cell culture

- **Material**

- 3T3-L1 mouse embryo fibroblasts obtained from the American Type
- Culture Collection (Manassas, VA)
- Incubator (Hera cell 240, Thermo scientific)
- Fetal Buffer Serum (FBS) Superior (Gibco)

- Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (Sigma)
- penicillin-streptomycin (10,000 kU/L) (Biochrom)
- 75 cm^2 dishes
- 6 and 12 wells plate
- Insulin
- Dexamethasone (Sigma)
- 1-methyl-3-isobutylxanthine (IBMX) (Sigma)
- PBS (Biochrom Ag L182-50)
- Trypsin (Biochrom)

➢ **Medium**

Three different medium were used for the proliferation and differentiation of cells.

- Culture medium (CM) was composed by 89%DMEM, 10 % FBS and 1% penicillin/streptomycin.
- Induction medium (IM) was made by CM supplemented with 5µg/ml insulin, 0.25 µM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine (IBMX)
- Differentiation medium (DM): composed by CM supplemented with 5µg/ml insulin.

➢ **Procedure**

- **Handling**

A volume of 10 ml was added in two dishes and placed into the incubator at 37°C for 15 min. Afterwards the vial was thawed at 37°C in water bath for 2 min. The content (1ml) of the vial was mixed with 9 ml of CM and transferred into a 15ml flacon tube. After 5 min of centrifugation at 130 X g the supernatant was discarded and the pellet was resuspended in 10ml of CM. Dishes were removed from the incubator and 5ml of the cell suspension was dispensed into each dish. Cells were incubated for 2 to 3 days until they reached 70 to 80% confluence.

- **Proliferation**

After 2 or 3 days cells dishes were removed from the incubator, the CM was aspirated and discarded; cells were then rinsed twice with 10 ml of PBS (37°C). 2 or 3 ml of trypsin was added into dishes and placed in the incubator until cell layer is destroyed. 7 or 8 ml were added, the medium was transferred aspirated into a 15 ml flacon tube and centrifuged as described below. Appropriate aliquots of the cells suspension was frozen (2 to 3 $X10^3$ cells/cm^2) or seeded in 6 wells plate (50,000 cells/well) or 12 wells plate (25,000 cells/well).

- **Differentiation**

Post confluent preadipocytes (70 to 80% confluence) were induced to differentiate with the IM for 3 days; afterwards the medium was switched to DM and changed every 2 or 3 days until the end of 9 to 14 days differentiation period.

II-2-2) Cytotoxicity assay

The cytotoxicity of *Iwong* was determined at day 3, 5 and 8 of differentiation using a commercial test system CytoTox-Glo kit, (Promega).

- **Principle**

The principle is based on the reaction between protease from dead cells which has been liberated from cells that loss membrane integrity and a luminogenic substrate. This reaction will cleave the luminogenic substrate and produce a new substrate for luciferase (aminoluciferin), resulting in the luciferase mediated emission of light.

- **Reagents and equipment**

Luminescence reader (Sirius, Berthold Detection Systems GmbH, Germany).

- Orbital plate shaker (Hiedolph instruments Unimax 200, Germany)
- Tube
- An assay Buffer
- A CytoTox-Glo reagent containing the alanyl-alanyl-Phenylalanylaminoluciferin (AAF-Glo) ™ Substrate

To prepare the working solution, the CytoTox-Glo reagent was thawed at 37°C after what it was gently mixed to homogenize the components, and then transferred into the assay buffer.

- **Procedure**

One hundred µl of the medium was pipetted from each well to a flacon tube and mixed with 50µl of the working solution. The mixture was orbitaly briefly shacked and incubated at room temperature for 15 minutes. The luminescence was then recorded with a luminometer (Sirius, Berthold Detection Systems GmbH, Germany).

II-2-3) Effect of *Iwong* on adipogenesis

II-2-3-1) Cells treatment

To evaluate the effect of *Iwong* on adipocyte differentiation, the ethanolic extract of *Iwong* was dissolved in DMSO and filtered with a syringe filter (Ø=0.2µm). Cells were treated either with DMSO 0.1 % or with different concentrations of *Iwong* ethanolic extract dissolved in DMSO 0.1 % during 9 days of adipocyte differentiation.

Moreover to determine during which phase of adipogenesis 3T3-L1 cells are most responsive to *Iwong*, cells were treated during early (day 0-2), intermediate (day 3-5) and late (day 6-8) phases of adipogenesis with *Iwong* ethanolic extract. All cells were exposed to 0.1% DMSO if not treated with *Iwong*. Medium was changed every three days.

II-2-3-2) Oil red O (ORO) staining and lipid quantification

- **Material**

- PBS (Biochrom Ag L182-50)
- Formaldehyde 37% (Roth 4979.1)
- Oil Red O (Sigma O0625)
- Isopropanol 99% (Roth 6752.1)
- Syringe filter (Ø=0.2µm)
- Parafilm (Pechiney plastic packaging, Menasha, USA)
- Spectrophotometer (Tecan infinite M200, Austria)

- **Solutions**

- ORO stock solution: 0.5 g of ORO was dissolved in 100 ml of isopropanol 99%.
- ORO working solution: Two parts of ORO stock solution were dissolved in 3 parts of distilled water and kept at room temperature for 10 min. The suspension was then filtrated with a syringe filter (Ø=0.2mm) and stored at room temperature for further usage.
- Formaldehyde working solution (3.7%). A solution of formaldehyde was prepared by mixing 1 part of formaldehyde 37% with 9 parts of distilled water.

- **Staining procedure**

This procedure was conducted under a fume hood. First of all plates were removed from the incubator, then the CM was aspirated and cells were washed twice with 1 ml PBS. Afterwards 1 ml of formaldehyde working solution was added and plates were incubated for 1 hour at room temperature. After fixation the formaldehyde was discarded into a formaldehyde waste container, and each well was rinsed twice with 1 ml PBS. A volume of 1 ml of the ORO working solution was added into each well and incubated for 1 hour at room temperature. The remaining ORO solution from each well was discarded and rinsed once with 1 ml PBS. Finally to keep cells humid 1ml of PBS was added into each well, plates can be immediately viewed on a phase contrast microscope or closed with parafilm and stored at 4°C.

After staining lipids droplet appeared red and the degree of differentiation can be appreciated.

- **Quantification procedure**

For lipid quantification, PBS was removed and 1 ml isopropanol was added and incubated at room temperature for 15 min. After the extraction of the dye the absorbance was read with a spectrophotometer (Tecan infinite M200) at 492 nm.

II-2-3-3) Glycerol-3-Phosphate Dehydrogenase (GPDH) activity

- **Measurement of the activity**

3T3-L1 adipocytes were differentiated in 6 wells plate and treated either with *Iwong* ethanolic extract or DMSO 0.1 %. After 8 days of differentiation cells were washed twice with ice-cold PBS and lysed with a buffer.

- **Principle:**

The principle was based on the measure of the depletion of the nicotinamide adenine dinucleotide (NADH) in the presence of dihydroxyacetone-3-phosphate (DHAP) using GPDH as enzyme with a spectrophotometric method at 340 nm [143]

$$Dihydroxyacetone\ phosphate\ +\ NADH\ +\ H \xrightarrow{GPDH} Glycerol-3-phosphate\ +\ NAD$$

The GPDH activity in each sample was calculated according this formula:

$$GPDH\ activity\ (unit/ml) = \frac{\Delta OD340\ X\ A(ml)\ X\ dilution\ ratio\ of\ the\ test\ sample}{6.22\ X\ C(cm)}$$

Δ OD340: Decrease in the absorbance at 340 nm per minute

A (ml): Total reaction volume

B (ml): The volume of enzyme solution (diluted sample) added

C (cm): Optical path length of the cell used*

6.22: Millimolar absorption coefficient of NADH molecules

- **materiel and solutions**

- Ultrasonic device
- Beckman Photometer
- NADH
- NaOH
- β- Mercaptoethanol
- **Lysis buffer composition**:

0.05 M Tris/HCl (MW157, 60)	788 mg
1mM EDTA (without Na, MW292, 24)	29, 2 mg
1mM 2-Mercaptoethanol (MW78, 13 98% stock)……	7, 8 µl
Adjusted pH	7.4

- **Tram-Buffer composition**:

1M Triethanolamin-HCl (MW185.65)	18, 57 g
25 mM EDTA (without Na MW292, 24)	0,731 g
Adjusted pH	7.5

- **NADH-solution**:

12mM NADH (8, 51 mg/ml) dissolved in Tram-Buffer always prepare in situ and kept on ice.

- **β-Mercaptoethanol-stock solution (BM1)**:

1M of 2-Mercaptoethanol (stock MW 78, 13 98%) e.i a volume of 7,102 ml was dissolved in 100ml of distilled water and cooled store.

- **β-Mercaptoethanol-working solution (BM2)**

5mM Mercaptoethanol (from -stock 1 M)

25 µl stock solution / 5ml of distilled water. Always prepare this solution in situ and keep on ice.

- **DHAP-solution:**

20mM Dihydroxyacetonphosphat (5mg in 1440µl of distilled water). Always prepare this solution fresh and keep on ice.

➢ **Procedure**

To harvest cells, the plate was placed on ice therefore 1 ml of the lysis buffer was added. The cell layer of each well was destroyed with a scraper and the lysate was transferred in a 1.5 ml tube a kept at -20°C.

The extraction of cells was made by an ultrasonic devise for 7 sec with 29% of capacity. The extract was then centrifuged at 10,000 for 10 min at 4°C. The enzyme sample was located in the aqueous fraction.

For the measurement of the GPDH activity, a blank was prepared by 430µl of Tram-Buffer. Whereas samples were composed by a mixture of 40µl of cell suspension with 390µl of distilled water for a final volume of 430µl. Blank and each sample were transferred in a cuvet and mixed with a master mix composed for each sample as follows:

50µl Tram-Buffer

10 µl BM2

5µl NaOH (12 mM)

Later one each cuvet was placed in a photometer and 5µl of DHAP solution were added, measurements were then performed during 5 min results obtained was expressed as mU/ml.

The results were then normalized with the proteins level.

II-2-3-4) Protein quantification

For the protein quantification we used a bicinchoninic acid (BCA) protein kit assay (Pierce Biotechnology, USA) based on the buiret reaction.

- **Principle**

The principle is based on two reactions:

- The reduction of cupric ions (Cu^{2+}) to cuprous ions (Cu^{+}) by peptide bounds.
- The second one is the chelation of one cuprous ion (Cu^{+}) by two BCA molecules. This complex will develop a purple coloration detectable at 562nm.

- **Reagents**

BCA Reagent A containing:

- Sodium carbonate,
- Sodium bicarbonate,
- Bicinchoninic acid
- Sodium tartrate

BCA Reagent B composed by cupric sulfate at 4%

Bovine serum albumin (BSA) at the concentration 2mg/ml

The working reagent prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B

- **Procedure**

A volume of 25µl each standard or unknown sample was pipetted into a microplate well, and then 200µl of working reagent (WR) were added into each of them and mixed on a plate shaker for 30 seconds. The plate was cover and

incubated at 37°C for 30 minutes, afterwards 75µl from each well were transferred into a 96 wells plate and the absorbance was measured at 562 nm on a plate reader.

II-2-3-5) Genes expression analysis

- **Material and solutions:**
  - Centrifuge (5417 R, eppendorf, Hamburg)
  - Scarper (Saerstedt, Newton, USA)
  - Cooled PBS
  - SV total RNA isolation system (Promega)
  - Heater (Thermomixer compact, Eppendorf AG,Hamburg)
  - QuantiTect reverse transcription kit (Quiagen)
  - Syringe (Becton Dickinson SA, Fraga, Spain)

  - **RNA lysis buffer (RLA) composition:**

  4M GTC
  0.01 M Tris (pH 7.5)
  0.97% β-Mercaptoethanol (added)

  - **DNase Stop Solution (DSA) composition:**

  2M GTC
  4mM Tris-HCl (pH 7.5) 57%
  ethanol.

  - **RNA Wash Solution (RWS) composition:**

  60 mM potassium acetate
  10 mM Tris-HCl (pH 7.5 at 25°C)
  60% ethanol

  - **Yellow Core Buffer composition:**

  0.0225M Tris (pH 7.5)
  1.125M NaCl
  0.0025% yellow dye (w/v)

  - **Master mix DNA (concentration for one sample X n samples)**

  Yellow core buffer ….40µl
  $MnCl_2$ ……..5µl
  DNase I……...5µl

- **10 x MOPS running buffer:**

400 mM MOPS (pH 7.0)
100 mM sodium acetate
10 mM ethylendiamintetaacetat (EDTA)

- **RNA loading buffer:**

200 mL formamide
64µl formaldehyde
40µl 10xMOPS
8µl distilled water
8µl ethidiumbromide

- **DNA-loading buffer (6x):**

10 mM Tris-HCl (pH 7.6)
0.2% orange G 60%
glycerol
60 mM EDTA

- **Tris-acetate/EDTA (TAE) buffer (x1)**

2ml EDTA(0.5M)/pH=8.0
39.95 mM tris
1.14 ml acetic acid (pure)
Distilled water to adjust the volume to 1 liter (pH 8.3)

➢ **Total cells lysate**

3T3L1 cells were treated either with several concentrations of the ethanolic extract of *Iwong* or with DMSO during the first 3 days of differentiation. Afterwards the medium was changed and and cells for all groups were treated with DMSO alone until the end of the differentiation phase. After 9 days of differentiation plates were placed on ice and each well was washed twice with 1ml of cooled PBS. 200 ml of RNA lysis buffer (in vitrogen) were added into each well and scraped using a cell scraper to disrupt the cell layer. The suspension of cells obtained was homogenized by pipetting and mixing three or four times with a syringe. The homogenate of each well was transferred into a 2ml tube and stored at -80°C.

- **RNA isolation**

Samples were removed from -80°C and thawed on ice. The RNA were isolated using a commercial kit (invitrogen) according the supplier recommendations. Briefly 350µl of RNA dilution buffer were added into each sample and incubated in a heater at 70 °C for 3 min. This step allowed the denaturation of proteins, nucleoprotein complexes and ribonucleases by GTC, Sodium Dodecyl Sulfate (SDS) and β-mercaptoethanol respectively. To stop the reaction samples were removed from the heater and directly placed on ice and bounded RNA were liberated.

Tubes were the centrifuged at 12000 x g for 10 min at 4°C. The cleared lysate obtained was transferred into a spin basket composed by a columm and a collection tube. To precipitate the RNA, 200µl of 96% ethanol were added into each spin basket. The mixture was then centrifuged as described previously for 1 min.

600 ml of the RNA washing buffer were added into each sample and centrifuged as described above for 1 min. The elute was discarded and 50 µl of DNA master mix were added and samples were incubated at room temperature for 15 min. The reaction was stopped by adding 200µl DNase stop solution. The mixture was centrifuged for 1 min. Afterwards two washing steps were performed by adding 600µl and 250µl of RNA washing buffer respectively. Each washing step was followed by a centrifugation at 12000x g for 1 min.

Finally each spin basket containing bounded RNA was placed into a new elution tube and 100 ml of RNase free water were added. The mixture was centrifuged for 1 min and the isolated RNA obtained was stored at -80°C.

- **Quantification and quality control of isolated RNA**

To measure the concentration and checked the quality of isolated RNA, samples were removed from -80°C and thawed on ice; 1.5µl per sample were used and placed into a spectrophotometer (Nanodrop 2000, Thermo scientific). The concentration was directly calculated and assumed acceptable at 50 ng/µl. The purity of the RNA was estimated by the value of the ratios

A_{260}/A_{280} and A_{260}/A_{230}. The first one evaluated the proteins contamination and the second is used to check the guanidine thiocyanate (GTC) contamination. The RNA was considered as pure when the ratio A_{260}/A_{280} situated between 1.7 and 2.0 and the value of A_{260}/A_{230} superior to 1.8. Samples with a good RNA quality were used to run a gel agarose electrophoresis in order to check the integrity of the RNA samples.

The gel was prepared by using 2 mg of agarose displaced with 144 ml of distilled water. The mixture was placed into a microwave oven until completely melted. Then to facilitate the visualization of the RNA after electrophoresis 2 ml of ethidium bromide was added. Afterwards 20ml of 10x MOPS running buffer and 36 ml of formaldehyde were added, the gel was allowed to solidify at room temperature.

In parallel to the solidification of the gel 1.5mg of RNA solution were mixed with 8 µl of the RNA loading buffer and heated at 70°C for 10 min to allow the denaturation of the RNA. After heating, samples were removed and immediately placed on ice for 1 min, then 2µl of DNA loading buffer (6X) were added into each tube.

The solidified gel was placed into the apparatus containing 1XMOPS as running buffer and allowed to run for around 30 min at 100 volts. Afterwards the gel was removed and put into the illuminator and pictures were taken using a digital camera, two bands were observed. The first one represents 28S and the second 18S sections of the ribosomal RNA. The ratio between the two sections should be equal to 2:1 for RNA with a good integrity.

- **cDNA synthesis**

The QuantiTect reverse Transcription-kit (Quiagen) was used to synthesize cDNAs as described by the provider with minor modifications. All reagents were divided by two with a final volume of 10 µl. Reagents were mixed as shown in the following for one sample and multiplied by the number of samples to prepare a master mix solution.

Reverse transcriptase buffer…….. 2µl

Reverse transcriptase…… 0.5µl

Primer mix…… 0.5µl

A volume correspond to 250 ng of RNA was pipeted and blended with RNasefree water for a final volume of 6µl, and then 1µl of genomic DNA Wipeout buffer was added. The mixture was heated for 2 min at 42°C and samples were immediately cooled on ice and 3µl of the master mix was lastly added. Samples were thus twice consecutively heated at 42°C for 15 min at immediately at 95°C for 3 min.

- **Semi quantitative RT-PCR**

To check the expression of genes of interest, the real time polymerase chain reaction was performed with a commercial kit Immomix (Bioline) according the provider's instructions. The expression of PPAR□, C/EBP□ was then assessed and normalized by GAPDH used as house-keeping gene. Briefly a volume of 1µl of each primer was diluted 1:10, a pooled of all cDNA samples was prepared by mixing 1µl of each sample and diluted in series to reach a final dilution of 1/16. Afterwards a master mix was prepared for each sample and multiplied by the number of samples as followed:

Immomix…….....12.5µl

Nuclease-free water…...…….....9.5µl

cDNA…….....1µl

A volume of 23µl of master mix was pipeted into each tube and mixed with 1µl of each forward and reverse primer of gene of interest. Primer sequences used are given in table 4 and the following PCR program was used for the thermal cycling (Flex)

94°C……10 min

94°C……15 sec

53°C……20 sec } X 40 cycles for C/EBPα and GAPDH

72°C……25 sec and X 35 cycles for PPARγ

72°C……3 min

4°C……∞

Table 4: Primer sequences used for gene expression analysis

Gene	**Orientation**	**Primer sequence**
PPARγ	forward	TTTTCAAGGGTGCCAGTTTC
	reverse	AATCCTTGGCCCTCTGAGAT
C/EBPα	forward	TGCTGG AGTTGACCAGTGAC
	reverse	AAACCATCCTCTGGGTCTCC
GAPDH	forward	TGTGTCCGTCGTGGATCTGA
	reverse	CCTGCTTCACCACCTTCTTGAT

The final products of RT-PCR were removed from the thermocycler and used to visualize the expression of genes of interest. For this step another agarose gel was prepared with 0.8g agarose displaced in 80 ml of TEA, the mixture was boiled into the micro wave oven until absolutely melted and incubated at room temperature pending the total solidification. Samples and markers (1kbp-DNA and 100 bp.DNA ladders) were loaded into individual wells of the gel, and ran at 100 volts for 45 min.

II-2-4) Effect of the ethanolic *Iwong* on lipolysis

Preadipocytes were differentiated to mature adipocytes in 12 wells plate for 14 days, the medium was changed every two days during the entire differentiation period. Mature adipocytes were then incubated in the presence of *Iwong* (50 and 75µg/ml), isoproterenol (1µM) or DMSO (0.1%) for 24h.

➢ Principe

The principle was based on the measure of the depletion of the nicotinamide adenine dinucleotide (NADH) in the presence of glycerol. The amount of NADH oxidized is stoichiometric to the amount of glycerol, as described below:

$$\textbf{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{L} - \text{glycerol} - 3 - \text{phosphate} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \textbf{NADH} + \text{H} + \xrightarrow{\text{L-LDH}} \text{L} - \text{lactate} + \text{NAD}^{+}$$

GK: glycerolkinase;

PK: pyruvate kinase;

PEP: Phosphoenolpyruvate

L-LDH: L-lactate dehydrogenase

➢ Materiel and reagents

- Spectrophotometer
- Cuvettes
- A kit for the determination of glycerol (r-biopharm, Darmstadt, Germany) containing 4 bottles

B1: 2 g coenzyme/buffer mixture (glycyglycine buffer, pH ≈ 7.4; NADH ≈ 7 mg; ATP ≈ 22 mg; PEP-CHA ≈ 11 mg and magnesium sulfate)

B2: 0.4 ml of enzyme suspension (pyruvate kinase ≈ 240 U; L-lactate dehydrogenase ≈ 220 U)

B3: 0.4 ml of glycerokinase suspension ≈ 34 U

B4: Glycerol standard.

NAD^{+}

➢ **Preparation of working solutions**

- Solution 1: was prepared by mixing contents of B1 with 11 ml of distilled water and stand for approximately 10 min at 20-25°C.
- Suspension1 was composed by undiluted contents of B2
- Suspension 2 was composed by undiluted contents of

➢ **Procedure**

To evaluate the effect on lipolysis 250 µl of the media from each well were pipetted and transferred into a cuvette, 475µl of distilled water, 250µl of solution 1 and 0.25µl of suspension 2 were added. For the blank 500µl of distilled water, 250µl of solution 1 and 0.25µl of suspension 2 were mixed. After 7 min at room temperature the absorbance (A1) was read. A volume of 0.25µl of the suspension 3 was immediately added and incubated for 10 min and the absorbance (A2) was read at 340 nm.

The absorbance differences (ΔA) were determined as followed:

$\Delta A = (A_1\text{-}A_2)_{sample} - (A_1\text{-}A_2)_{blank}$

The concentration was calculated by this formula:

$$c\ [g/l] = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A$$

V=final volume [ml]

V= sample volume [ml]

MW = molecular weight of the substance to be assayed [g/mol]

d = light path [cm]

ε= extinction coefficient of NADH = 6.3 at 340 nm

II-3) Animal experiment 1

II-3-1) Animals and feeding procedure

Fifteen male *Wistar* rats, 2-3 months of age, with a mean body mass of 260 ± 30 g were obtained from the institutional animal facility of the Laboratory of Toxicology and Pharmacology at the National Institute for Medical Research and Medicinal Plants (IMPM) of Cameroon. Rats were housed 2-3 animals per cage at room temperature (22-25°C) in a light-dark cycle of LD 12:12. The

Wistar rats were randomly assigned to three diet groups. The first group (control) was fed a standard diet containing 50 kJ% carbohydrates, 30 kJ% proteins and 20 kJ% fat. In the two dietary intervention groups this standard diet was supplemented with powdered plant material of *Iwong* with either 5% w/w (Iwong5) or 10% w/w (Iwong10), respectively.

Table 5: Composition of standard diet used in rat study

	Quantity / Kg	Kcal%
Maize	250 g	25.5
Wheat	400g	40.8
Soya bean	150g	15.3
Fish flour	100g	10.2
Cabbage palm cake	80g	8.2
Bones flour	10g	-
Vitamins complex	10g	-
Energy	≈ 3000 Kcal	-

II-3-2) Food intake and body weight

All rats had free access to food and water during the 4 weeks feeding trial. Food intake per cage was monitored three times every week whereas body weight trajectories were recorded once per week.

II-3-3) Sample collection and assays:

Animals were anesthetized with ether and killed by cervical dislocation at the end of the dietary intervention. Trunk blood was collected into heparinized tubes and immediately centrifuge at 3000 g for 10 min at 4°C. Plasma triglycerides, total-cholesterol, HDL-cholesterol and glucose levels were determined using commercial kit systems (Sigma Diagnostics,). The level of LDL-cholesterol was determined by the formula of Friedewald [149].

Following blood sampling, mesenteric, epididymal and perirenal fat depots were excised and weighed the sum of these several fat pads was assumed as total fat mass.

II-3-3-1) Glucose assay

- **Principle**

The principle is based on the first on the phosphorylation of glucose by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase. This reaction is followed by the oxidation of Glucose 6 phosphate (G6P) in the presence of nicotinamide adenine dinucleotide (NAD) using Glucose 6 phosphate dehydrogenase (G6P) as enzyme.

$$Glucose + ATP \xrightarrow{Hexokinase} Glucose - 6 - phosphate + ADP$$

$$G6P + NAD \xrightarrow{G6PDH} 6 - Phosphogluconate + NADH$$

- **Equipment and reagent**

- Glucose (HK) reagents containing each of them when reconstituted in 20 ml distilled water: 1.5 mM NAD, 1.0 mM ATP, 1.0 unit/ml of hexokinase, and 1.0 unit/ml of glucose-6-phosphate dehydrogenase with sodium benzoate and potassium sorbate.
- A glucose standard solution (1mg/ml in 0.1% benzoic acid)
- Spectrophotometer
- Cuvets

- **Procedure**

Samples and blank prepared as described by the supplier were read at 340 nm.

The concentration of glucose was calculated by the formula:

$$mg\ glucose/ml = \frac{(\Delta A)(TV)(F)(0.029)}{SV}$$

∆A = ATest - ATotal Blank
TV = Total Assay Volume (ml)
SV = Sample Volume (ml)
Glucose MW = 180.2 g/mole or equivalently 180.2 µg/µmoles
F = Dilution Factor from Sample Preparation

II-3-3-2) Triglycerides assay

- **Principle**

The procedure is based on enzymatic hydrolysis of triglyceride to glycerol by lipase. Afterwards the glycerol will enter a cascade of reaction in the presence of several enzymes and the quinoneimine dye produced at the last state shows an absorbance maximum at 540 nm. The amount of triglycerides is positively correlated with the absorbance at 540 nm.

$$Triglycerides \xrightarrow{Lipoprotein\ lipase} Glycerol + fatty\ acids$$

$$Glycerol + ATP \xrightarrow{GK} G-1-P + ADP$$

$$G-1-P + O2 \xrightarrow{GPO} +DAP + H2O2$$

$$H2O2 + 4-AAP + ESPA \xrightarrow{POD} \text{Quinoneimine dye} + H2O$$

G-1-P = glycerol-1-phosphate
GK = glycerol kinase

GPO = glycerol phosphate oxidase
DAP = dihydroxyacetone phosphate
POD = Peroxidase
4-AAP = 4-aminoantipyrine
ESPA = sodium N-ethyl-N-(3-sulfopropyl)

- **Reagents and equipment**

- Spectrophotometer at 540 nm
- Cuvets
- A serum triglyceride determination kit (Sigma) containing triglycerides reagent composed by lipase (250,000 units/L and 0.05% sodium azide)

- **Procedure**

Free glycerol and triglycerides reagent were reconstituted by adding 10 ml of distilled water into each of them.

To prepare the triglycerides working solution 4 ml of glycerol reconstituted reagent were mixed with 1 ml of triglyceride reconstituted reagent, whereas

the blank was prepared by mixing 4 ml of glycerol with 1 ml of distilled water. The triglyceride working reagent was warmed at room temperature. 10 µl of the sample or blank and 1 ml triglyceride working solution was pipette into a cuvet, mixed gently and incubated for 5 min at 37°C. The absorbance was then read at 540 nm versus water as reference.

After the subtraction of the blank from the absorbance of the sample and the standard, the total triglyceride concentration (mg/ml) of samples was calculated by the formula:

$$Concentration = \frac{(\mathrm{A_{sample}}) - (\mathrm{A_{Blank}})}{(\mathrm{A_{standard}} - (\mathrm{A_{Blank}})} \mathrm{x\ concentration\ of\ standard}$$

II-3-3-3) HDL-C concentration

HDL-C molecules were separated to other lipoproteins after a precipitation in the presence of magnesium sulfate (MgSO4). The solubilized HDL-C entered a cascade of reactions in the presence of specific enzymes as described below. The reaction ended with the development of a color depending of the amount of HDL-C presents in the medium. The absorbance was read at 540 nm.

$$HDL - cholesterol\ esters + H_2O \xrightarrow{PGE-CE} Cholesterol + fatty\ acids$$

$$cholesterol + O_2 \xrightarrow{PGE-CO} Cholest - 4 - en - 3 - one + H_2O_2$$

$$H_2O_2 + TOOS + 4 - AAP \xrightarrow{Peroxidase} Color\ development$$

II-3-3-4) LDL-C concentration

The LDL-C concentration was calculated by the formula of [149]

$$LDL - C(g/L) = \frac{(\mathrm{TC}) - (\mathrm{HDL - C}) - (\mathrm{TG})}{5}$$

TC = total cholesterol
HDL-C = HDL cholesterol
LDL-L = LDL cholesterol
TG = triglycerides

II-4) Animal experiment 2

II-4-1) Animals and dietary intervention groups

Male mice (n=18) at the age of 6 weeks weighing 35 ± 1.4 g were obtained from a commercial breeder (outbred strain NMRI, Charles River Laboratories, Germany). Mice were housed in the Small Animal Research Center at the Technische Universität München for one week prior to the start of the feeding trial. They were single-caged at room temperature (22°C) in a light-dark cycle of LD 12:12.

Mice were weighed before the start of the feeding trial and assigned to three diet groups matched for body mass. The control group was fed a standard diet (table 6), composed of 53 kJ% carbohydrates, 36 kJ% protein and 11 kJ% fat (ssniff M-Z, Spezialdiäten GmbH, Soest, Germany), whereas the two dietary intervention groups received the same diet supplemented with 5% (Iwong5) and 10% (Iwong10) *Iwong* powder for four weeks.

II-4-2) Body weight and food intake

During the feeding trial all animals had free access to food and water. Food intake and the body mass were regularly recorded at the frequency of three times per week.

Table 6: composition of chow diet used for MNRI mice

Crude Nutrients	**[%]**
Dry matter	88.3
Crude protein (N x 6.25)	23
Crude fat	6
Crude fibre	3.3
Crude ash	6.8
N free extracts	49.2
Starch	34.4
Sugar	5.2

II-4-3) Sample collections

II-4-3-1) Feces

In the mouse feeding trial feces were collected weekly, dried for 24 hours at 55°C and crushed in a steel ball mill (Tissue lyser, Retsch, Germany). Energy density in food and feces samples was determined by adiabatic bomb calorimetry (6300 calorimeter, Parr Instruments, USA). The assimilated energy (E_{ass}) was calculated by subtracting the total energy intake (E_{in}) by the total amount of energy excreted in feces (E_{out}), the assimilation efficiency (E_{ass}) was calculated as followed:

$$\text{Eass}(\%) = \left[\frac{\text{Ein(kJ/day)} - \text{Eout (kJ/day)}}{\text{Ein (kJ/day)}}\right] \times 100$$

II-4-3-2) Blood

At the end of the feeding trial mice were killed by decapitation under CO_2 anesthesia. Blood was collected in lithium heparin tubes (Sarstedt, Germany), centrifuged at 2000 g for 10 min (4°C) and plasma cooled on ice at 4°C. Blood metabolites were measured immediately using an automated blood analyzer (Abaxis, Union City, CA, USA).

II-4-3-3) Fat pads

Following blood sampling, animals were dissected; mesenteric, epididymal and perirenal fat depots were excised and weighed.

II-4-4) Plasma blood analysis

At the end of the feeding trial mice were killed by decapitation under CO_2 anesthesia. Blood was collected in lithium heparin tubes (Sarstedt, Germany), centrifuged at 2000 g for 10 min (4°C) and plasma cooled on ice at 4°C. Blood metabolites were measured immediately using an automated blood analyzer (Abaxis, Union City, CA, USA).

➢ **Reagents, principles and procedures**

The Piccolo lipid panel disc used with a Piccolo blood analyzer system is an automated system intended for *in vitro* blood determination of totalcholesterol (TC), High density lipoprotein cholesterol (HDL-C), triglycerides
(TG). The (LDL-C) and the very low density lipoprotein cholesterol (VLDLC) are calculated directly by the analyzer.
The list of reagent contained in the panel disc are depicted in the table below (table 7)

Table 7: Detailed list of reagents in one Piccolo disc panel

Components	Quantity/disc
4-Aminoantipyrine	6.7 µg
Adenosine 5'-triphosphate, disodium salt	9.2 µg
Ascorbate oxidase	0.042 U
Cholesterol dehydrogenase	0.080 U
Cholesterol esterase (Genzyme-N)	0.27 U
Cholesterol esterase (Genzyme-P)	0.0080 U
Dextran sulfate	8.4 µg
Diaphorase	0.25 U
N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, dihydrate (TOOS)	79 µg
Glycerol kinase	0.084 U
Glycerol-3-phosphate dehydrogenase	0.21 U
Iodonitrotetrazolium chloride (INT)	8.4 µg
Lipase	16.8 U
Magnesium chloride, hexahydrate	8.6 µg
Magnesium sulfate, heptahydrate	197 µg
Nicotinamide adenine dinucleotide, monosodium salt (NAD)	455 µg
PEG-cholesterol esterase	0.013 U
PEG-cholesterol oxidase	0.089 U
Peroxidase	0.27 U
Buffers, surfactants, excipients, and preservatives	

- **Total cholesterol**

The quantification of total cholesterol is based on an enzymatic procedure which converts cholesterol to NADH in two successive reactions using cholesterol esterase and cholesterol dehydrogenase as enzymes. The NADH produced is measured at 340 and 405 nm; the amount of NADH is directly correlated with the amount of total cholesterol.

$$Cholesterol\ esters + H_2O \xrightarrow{Cholesterol\ esterase} Cholesterol + fatty\ acid$$

$$Cholesterol\ + NAD^+ \xrightarrow{Cholesterol\ \ dehydrogenase} Choles - 4 - ene - 3 - ol\ + NADH + H^+$$

- **HDL-cholesterol**

A precipitation method was used for HDL assay using dextran sulfate and magnesium sulfate (MgSO4) as precipitating agents which form insoluble complexes with chylomicrons (CM) LDL and VLDL. HDL which remains in soluble form will then enter a cascade of reactions with a peroxidation at the final step followed by a development of purple color. HDL concentration is directly proportional to the absorbance read at 540nm with 630nm as reference wavelength.

$$HDL - cholesterol\ esters +\ H_2O \xrightarrow{PGE-CE} Cholesterol + fatty\ acids$$

$$cholesterol +\ O_2 \xrightarrow{PGE-CO} Cholest - 4 - en - 3 - one + H_2O_2$$

$$H_2O_2 + TOOS + 4 - AAP \xrightarrow{Peroxidase} Color\ development$$

TOOS = N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, dihydrate
4-AAP = 4-Aminoantipyrine
Polyethylene glycol-modified cholesterol esterase (PEG-CE)
Cholesterol oxidase (PEG-CO)

- **Triglycerides (TG)**

In the presence of a lipase triglycerides are hydrolyzed into glycerol and fatty acids. The glycerol will enter a chain of reaction which will be end by the production of a formazan. The intensity of colored formazan measured bichromatically at 500nm and 800nm is directly proportional to the concentration of triglycerides in the sample.

$$Triglycerides + 3H_2O \xrightarrow{Lipase} Glycerol + 3fatty\ acids$$

$$Glycerol + ATP \xrightarrow{Glycerol\ kinase Mg^{++}} G-3-P + ADP$$

$$G-3-P + NAD^+ \xrightarrow{G-3-PDH} DAP + NADH + H^+$$

$$NADH + H^+ + INT \xrightarrow{Diaphorase} NAD^+ + Formazan$$

G-3-P = Glycerol-3-Phosphate
G-3-PDH = Glycerol-3-Phosphate Dehydrogenase
DAP = Dihydroxyacetone Phosphate
INT = p-Iodonitrotetrazolium Violet

- **LDL and VLDL cholesterols**

The concentration of LDL was calculated automatically using the equation of Friedewald gave above (see experiment I)

The VLDL concentration was also estimated automatically using by the formula described by Friedewald [149] as followed:

$$VLDL(mg/dL) = \frac{Triglyceride(mg/dL)}{5}$$

II-5) Animal experiment 3: Effect of *Iwong* feeding on prevention and treatment of obesity

Sixty C56BL/6J male mice of 3 weeks aged weighing 15 ± 0.9 g were purchased from a commercial breeder (Charles River Laboratories, Germany). After weighing, animals were single housed and fed a chow diet for 3 weeks. At age of 6 weeks (20 ± 1.07 g) mice were divided in two cohorts of 28 and 32 mice for the prevention and the treatment of obesity respectively.

II-5-1) Obesity prevention

Twenty eight age mice of 6 weeks age were randomized in 4 groups of 7 mice and fed a standard diet, HFD and HFD supplemented with Iwong 4 and 8 % (Iwong4 and Iwong8 respectively). The detail of the composition of diets used is depicted on (table 8).

Table 8: composition of purified standard and high fat diets used for C57BL/6J mice

	S5745-E712	**S5745-E702**
	High Fat Diet (HFD) 20% Palm oil, 5% Soybean oil	Standard Diet (SD) 5% Soybean oil
Gross Energy (GE) [MJ/kg]	19.7	15.5
Dry matter [%]	20.80	20.80
Crude protein (Nx6, 25) [%]	25.10	5.10
Casein	5.00	5.00
Crude fat [%]	5.60	5.60
Soybean oil	40.60	60.10
Palm oil	28.30	47.90
Crude fiber [%]	5.00	5.00
Crude ash [%]	24.00	24.00
Fatty acids [%]		
C14:0	0.22	0.03
C16:0	8.87	0.55
C16:1	0.13	0.03
C17:0	-	-
C18:0	1.19	0.24
C18:1	8.75	1.34
C18:2	4.67	2.65
C18:3	0.43	0.33
C20:0	0.13	0.03
Lysin	0.13	0.03
Threonin	0.92	0.92
Tyrosin	0.63	0.63
Valin	0.19	0.19
Met+Cys	0.21	0.21
Trace elements		
Calcium	0.92	0.92
Phosphor	0.63	0.63
Natrium	0.19	0.19
Magnesium	0.21	0.21
Choline-chloride	0.2%	0.2%

II-5-1-1) Food intake and body weight

During the 5 weeks experimental period, mice were given free access to water and food. Energy and food intake were recorded three times per week.

To assess the preventive effect of *Iwong*, body weight was recorded every week during the entire dietary intervention period.

II-5-1-2) Feces collection

Feces of each individual cage were collected during the last three weeks and treated as described above (section I).

II-5-1-3) Blood collection

After 6 weeks of dietary intervention period, mice were killed by decapitation under CO_2 anesthesia, blood samples were collected in lithium heparin tubes (Sarstedt, Germany), centrifuged at 2000 g for 10 min (4°C) and plasma cooled on ice at 4°C. Blood metabolites were measured immediately using an automated blood analyzer (Abaxis, Union City, CA, USA).

II-5-1-4) Rectal body temperature

Body temperature was recorded from 2 to 3 AM once per week during the first three weeks of dietary intervention feeding period. The mouse was restrained by the neck with a nondominant hand and returned vertically. Afterwards a thermocouple probe first lubricated with Vaseline was inserted to a depth of 2 cm into the rectum and maintained for 5 to 10 seconds until the thermometer beeped. The rectal body temperature was then directly recorded with an electronic thermometer (Almeno 2490, Ahlborn Mess GmbH, Germany) with a measuring range temperature from 0 to 75°C.

II-5-1-5) Fat pads, liver and kidney

At the end of 6 weeks feeding period, mice were killed as described above and dissected; mesenteric, epididymal, perirenal, inguinal, liver and kidney were excised and weighed. For the liver and fat pads two fragments of each were kept either in formaldehyde 4% or immediately transferred into liquid nitrogen and finally stored at – 80°C.

II-5-1-6) Biochemical parameters analysis

Plasma blood samples collected were used for TG, total cholesterol, HDLcholesterol LDL-cholesterol, aspartate, glucose aminotransferase (AST) and alanine aminotransferase (ALT) analyses by an automated analyzer (Abaxis, Union City, CA, USA). The protocol, procedure and reagents used are already been described and given in detail in material and methods section of experiment 1.

II-5-1-7) Plasma leptin level

At the end of feeding experimental period mice were killed as described above, but additionally an aliquot of plasma was stored at – 20°C for hormone concentrations quantification. Plasma hormone levels were quantified using commercial kits

Leptin concentration was quantified using a commercial kit (BioVendor Research and Diagnostics Products)

- **Principle**

The method used was the Enzyme linked ImmunoSorbent Assay (ELISA) based on a sandwich enzyme immunoassay. This method used two different antibodies that can bind to different epitopes of the same specific antigen. The first one, a mouse antibody leptin referred as a captured antibody was directly added into each well by the provider and the second one, a biotin labeled polyclonal anti-mouse leptin antibody which was added later. Samples or standard containing leptin antigen reacted with the first antibody, after several washing the second antibody was added and reacted with the bound antigen. Wells were washed to remove unreacted labeled antibody, afterwards a streptavidin-HRP conjugate was added and washed after an incubation period. To estimate the enzyme bound to the solid support a chromogenic substrate (TMB) and the reaction was stopped of an acidic solution. The yellow product developed was measured spectrophotometrically at 450 nm with the reference length set to 630 nm.

➢ Reagents, equipment and procedure

❖ Equipment required

- Precision pipettes with disposal tips (eppendorf)
- Multichannel pipette wit disposal tips (eppendorf)
- Absorbent material or paper towels (Neolab D 6012)
- Vortex mixer (Zefa laborservice)
- Orbital microplate shaker capable of approximately 300 rpm (Heildolph instruments, Unimax 2010, Germany)
- Microplate reader (Nano quant plate, Tecan, Salzburg, Austria)

❖ Reagents

Kit components	Quantity
Antibody coated Microtiter strips	96 wells
Biotin labeled antibody concentrated (X10)	1.3 ml
Streptavidin-HRP conjugate	1.3 ml
Master Standard Mouse	1 vial
Master Standard rat	1 vial
Quality Control Mouse	2 vials
Quality Control Rat	2 vials
Dilution Buffer	2 X 13 ml
Biotin-Ab Diluent	13 ml
Wash Solution concentrated (X10)	100 ml
Substrate Solution	13 ml
Stop solution	13 ml

❖ Preparation of reagents

Standard: to reconstitute the mouse standard 1 ml of the dilution buffer was mixed with the vial and the final concentration was 4000 pg/ml. the reconstituted standard was then diluted as described below:

Volume of standard	Dilution buffer	Concentration
Mouse Stock solution (SS) 4000 pg/ml	-	4000 pg/ml
250µl M-SS	250µl	2000 pg/ml
250µl of solution 2000 pg/ml	250µl	1000 pg/ml
200µl of solution 1000 pg/ml	300µl	400 pg/ml
250µl of solution 400 pg/ml	250µl	200 pg/ml
250µl of solution 200 pg/ml	250µl	100 pg/ml

Quality control: The mouse quality control was reconstituted by adding 350µl of the dilution buffer into the vial.

Biotin Labeled Antibody: The working labeled antibody solution was obtained by mixing 1 part of Biotin labeled antibody Concentrate with 9 parts of Biotin-Ab Diluent.

Working Wash Solution: the working wash solution was prepared by mixing one part of the concentrated wash solution with 9 part of distilled water.

Samples dilution: 14µl of each sample were diluted in 266µl of dilution buffer.

❖ **Procedure**

A volume of 100 µl of standards, quality control, Dilution Buffer and diluted samples was pipeted in duplicate into each well. The plate was covered and placed on an orbital microplate shaker (300rpm) for 1 hour at 25°C. Furthermore, 350 µl were added to wash each well, the plate was inverted and taped strongly against paper towel. Afterwards 100µl of streptavidin-HRP Conjugate were added into each well and placed on an orbital microplate shaker at 300rpm for 30 min. The washing procedure was the same as described before and was immediately followed by an addition of a volume of 100µl of substrate solution into each well, the plate was covered and incubated for 10 min at room temperature. The color development was stopped by adding 100µl of stop solution, and the absorbance was read at 450 nm with+ the reference wavelength at 630 nm.

II-5-1-8) Triglycerides content in the liver

A piece of liver was excised and directly frozen in liquid nitrogen. It was then stored at – 80°C.

Tissue extraction

- **Equipment and reagents**

❖ **HB-Buffer composition**

10 mM Natriumdihydrogenphosphat

1 mM EDTA

1% v/v Polyoxyetylen 10 tridecyether (Sigma)

❖ **Equipment**

Liquid nitrogen;

Stainless steel spoon, holder and knife;

Balance (Denver instrument);

Thermomixer (eppendorf);

Cooled centrifuge (eppendorf centrifuge 5417 C);

A steel ball mill (Tissue lyser, Retsch, Germany);

2 ml cryotubes (Sarstedt, Germany) and 2 ml microtubes (Sarstedt, Germany)

❖ **Procedure**

40 mg of liver were mixed with 1 ml of cold HB-buffer. After a speed homogenization in a steel ball mill, the homogenate was transferred in a precooled 2 ml microtube and centrifuged at 23000 g (4°C) for 15 min. The supernatant was transferred in a new precooled 2 ml microtube and placed into a thermomixer shaker (600 upm) for 5 min at 70°C. Microbutes were then immediately placed on ice for 5 min, afterwards they were cooled centrifuged (4°C) at 23100g for 15 min. The supernatant obtained was transferred into precooled 1.5 ml microtube, 3 aliquots for each sample were prepared and stored at -20°C.

Triglycerides quantification

The total amount of triglycerides contained in the liver was determined using a commercial kit according information provided by the supplier (Sigma, TR0100).

❖ **Principle**

The principle is based on enzymatic hydrolysis of triglycerides to glycerol and fatty acid by lipase. The glycerol produced was then entered a chain of reaction under several enzymes with a Quinoneimine dye as final product. The absorbance was read at 540nm and was proportional to the concentration of triglycerides.

$$Triglycerides \xrightarrow{Lipoprotein\ lipase} Glycerol + fatty\ acids$$

$$Glycerol + ATP \xrightarrow{GK} G-1-P + ADP$$

$$G-1-P + O2 \xrightarrow{GPO} +DAP + H2O2$$

$$H2O2 + 4-AAP + ESPA \xrightarrow{POD} \text{Quinoneimine dye} + H2O$$

More details concerning reagents and the assay procedure are already given in rats experiment (see triglyceride assay)

II-5-1-9) Histology

➢ **Equipment and chemicals**

- Incubator (Thermo electron corporation, Heraeus)
- Semi-enclosed Benchtop Tissue processor (Leica TP 1020)
- Modulation tissue embedding Center (Leica 1150 C)
- Multi-stainer (Leica ST 5020),
- Fully Automated Rotary Microtome (Leica RM2255)
- Embedding cassettes (Roth)
- Water bath (Memmert GmbH, Schwabach, Germany)
- Oven (Thermo scientific)

➢ **samples collection and fixation**

A portion of epididymal adipose tissue (eWAT) for each animal was collected and immediately kept into a tube containing 4% formaldehyde solution. The fixation process was conducted for at least 48 hours depending on the size of the tissue used.

- **Dehydratation and clearing**

Since paraffin and water are not miscible tissue were first dehydrated and cleared before paraffin inclusion. Thus following fixation each portion of tissue was transferred into individual embedding cassette and incubated in a semienclosed Benchtop tissue processor (Leica TP 1020) for 12 hours. The program used for tissue processing was a conventional one. Briefly to completely removed water, tissues were putted in gradual concentrations of ethanol 70%, 96% and 100%. Afterwards to remove excess alcohol samples were automatically cleared into xylene. This step was followed by paraffin infiltration.

- **Paraffin embedding**

Later than tissue processing, each portion of eWAT was removed from the cassette and placed into a precooled stainless steel base Mold. Each mold was then filled with a sufficient volume of paraffin and directly positioned on a cold surface until a solid block of paraffin was formed. All this embedding process was made using a modulation tissue embedding Center (Leica 1150 C).

- **Sectioning and Mounting**

The sectioning was made using a fully Automated Rotary Microtome (Leica RM2255). The block of paraffin was placed in the block receptacle and slowly moved closer to the blade. Automated trimming was started to reach the appropriate area of the tissue to be cut. A sharp and clean section of the blade was used; the ribbon obtained (5 µm) was picked with a paint brush and transferred directly into a water bath at 40°C. The ribbon was then carefully separated in one or two sections. A glass slide was used to pick one or two sections per slide and directly inclined to remove water. Slides were placed on a paper towel for 15 minutes for drainage and finally putted in an oven for a complete drying.

- **Staining**

After 24 hours of incubation period in the oven, dried slides were transferred into a multi-stainer (Leica ST 5020) and were first automatically rehydrated in

100%, 96% and 70% ethanol successively, the rehydration process was followed by a H/E staining. After staining each slide was covered with a cover glass.

- **Microscopy**

Slides were photographed using a microscope (Zeiss Axiovert 40 CFL) connected with a camera (Zeiss Axiocam), pictures were analyzed with a software (Axiovision) for the macroscopic differences between adipocytes of each group.

II-5-2) Obesity regression

Thirty two mice were fed a standard diet or HFD for 9 weeks to induce obesity. Afterwards 21 diet-induced obese mice were randomized in 3 groups of 7 animals and maintained under a HFD for the HFD group or switched to a HFD supplemented with 4 or 8% of the powder of *Iwong* for the two others (Iwong4 and Iwong8 respectively). The control group was continuously fed a standard diet during the whole experimental period.

II-5-2-1) Food intake and body weight

During the first 9 weeks of the experimental period concerning the induction of obesity phase and 6 weeks of treatment of obesity, the amount of food intake and the body weight of each animal were recorded once per week.

II-5-2-2) Feces collection

Feces were collected each week during the 6 weeks of treatment and treated as described previously in section I.

II-5-2-3) Blood, tissue and organs collection

Blood, liver, kidney and several fat pads were collected and treated as described in obesity prevention section.

II-5-2-4) Biochemical parameters

Plasma blood were used for TG, total cholesterol, HDL-cholesterol LDLcholesterol, aspartate, glucose aminotransferase (AST) and alanine aminotransferase (ALT) analyses by an automated analyzer (Abaxis, Union City, CA, USA). The protocol, procedure and reagents used are already been described and given in detail in material and methods section of experiment 1.

II-5-2-5) Plasma hormone levels

After 15 weeks experimental period mice were killed as described above, but additionally an aliquot of plasma was stored at – 20°C for hormone concentrations quantification. Plasma hormone levels were quantified using commercial kits.

a) Leptin

Blood plasma concentration was determined as described in the obesity prevention section.

b) Adiponectin

Plasma adiponectin concentration was quantified by a commercial kit (BioVendor Research and Diagnostics Products)

- **Principle**

The principle was the same as described for leptin ELISA, with one principal modification. The enzyme used to bind to the second antibody was a horseradish peroxidase-HRP.

- **Reagents, equipment and procedure**

- **Reagents**

Kit components	Quantity
Antibody coated Microtiter strips	96 wells
Conjugate solution	13 ml
Master standard	2 vials
Quality control HIGH	2 vials
Quality control LOW	2 vials

Dilution buffer Concentrated (X10)	22 ml
Wash Buffer Concentrated (X10)	100 ml
Substrate Solution	13 ml
Stop Solution	13 ml

❖ Preparation of reagents

Dilution Buffer working solution: One part of the Dilution Buffer Concentrated was diluted.

Standard: The reconstituted Standard was obtained by adding 1ml of distilled water into the lyophilized Master Standard with a final concentration of 8ng/ml. The set of standards was prepared by using dilution buffer as shown in below.

Volume of standard	Dilution buffer	Concentration
Mouse Stock solution (SS) 8 ng/ml	-	8 ng/ml
500µl M-SS 8 ng/ml	500µl	4 ng/ml
500µl of solution 4 ng/ml	500µl	2 ng/ml
500µl of solution 2 ng/ml	500µl	1 ng/ml
500µl of solution 1 ng/ml	500µl	0.5 ng/ml
500µl of solution 0.5 ng/ml	500µl	0.25 ng/ml

Quality Controls HIGH, LOW solutions: they were reconstituted by adding 1 ml of Dilution Buffer into each lyophilized quality controls followed by at least 15 minutes of incubation period.

Working Wash Solution: this solution was obtained after the dilution of one part of a 10 fold concentrated wash solution with 9 parts of distilled water.

Samples dilution: Two successive dilutions were performed, the first (dilution A) was obtained by mixing 10µl of each sample into 990µl of dilution buffer, followed by a second one (dilution B) made by 10µl of dilution A and 990µl of dilution buffer.

❖ **Procedure**

As in leptin assay 100µl of standards, quality control, dilution buffer and diluted samples all in duplicate were added into predefined wells. The plate was covered and placed on an orbital shaker at 300rpm for 1 hour. After washing three times with 350µl of washing buffer, the plate was inverted and strongly taped against paper towel. Therefore 100µl of conjugate solution were added into each well and incubated, the plate was covered and placed once again on an orbital microplate shaker. Subsequent to one hour of incubation, wells were washed as described previously and 100µl of substrate solution were added into each well, the plate was covered with aluminium foil and incubated for 10 min at room temperature. Furthermore a volume of 100µl of stop solution was added to stop the color development. Afterwards the absorbance was read using a microplate reader set to 450 nm with 630 nm wavelength used as reference.

c) Insulin

The plasmatic insulin concentration was determined using an ELISA commercial kit (Shibayagi Co. Ltd).

➢ **Principle**

Mouse insulin ELISA (S-type) is a solid phase two-site enzyme immunoassay, based on a direct sandwich method. It is using two monoclonal antibodies which are directed against different determinants on the insulin molecule. Wells were coated with a mouse captured insulin antibody by the supplier. The captured insulin molecules reacted with biotin conjugated anti-insulin. Afterwards HRP conjugated streptavidin was added and bound to the second antibody. The bound conjugate was detected by reaction with TMB. The color development was stopped by the addition of acidic solution and the absorbance was read at 450 nm with a reference at 630 nm.

➢ Equipment and reagents

❖ Equipment required

Precision pipettes with disposal tips (eppendorf)

Multichannel pipette with disposal tips (eppendorf)

Absorbent material or paper towels (zifa laborservice)

Vortex mixer (Neolab D6012)

Orbital microplate shaker capable of approximately 300 rpm (Heidolph instruments, unimax 2010)

Microplate reader (Nano quant plate, Tecan, Salzburg, Austria)

❖ Reagents

Components	Amount
Anti-insulin coated plate	1 plate (96 wells)
Standard mouse insulin solution	500 µl/vial
Buffer solution	60 ml /bottle
Biotin conjugated anti-insulin	200 µl/1 vial
HRP conjugated streptavidin	200 µl/1 vial
Substrate chromogen reagent (TMB)	12 ml/1 bottle
Reaction stopper(1M H2SO4)	12 ml/1 bottle
Washing buffer concentrate (10X)	100 ml/1 bottle

❖ Preparation of reagents

Standard solutions: a serial dilution as described below was made from a master standard (5000 pg/ml) solution to obtain each standard solution.

Volume of standard solution	Buffer solution	Concentration
Master standard solution		5000 pg/ml
50 µl of master standard solution	50µl	2500 pg/ml
50 µl of solution 5000 µl	50µl	1250 pg/ml
50 µl of solution 1250 µl	50µl	625 pg/ml
50 µl of solution 625 µl	50µl	313 pg/ml
50 µl of solution 313 µl	50µl	156 pg/ml
50 µl of solution156 µl	50µl	78 pg/ml
Blank	50µl	0

Biotin conjugated anti-insulin working solution was obtained by mixing 9 parts of dilution buffer with 1 part of biotin conjugated anti-insulin.

HRP conjugated streptavidin working solution corresponded to 9 parts of dilution buffer in 1 part of the enzyme.

Washing buffer working solution was prepared by dilution of 1 volume of concentrated washing buffer to 10 volumes with distilled water.

❖ **Assay procedure**

Each well of the anti-insulin coated plate was washed 4 times with washing buffer and the residual buffer was removed by taping the plate on a paper towel. 50 µl of biotin conjugated anti-insulin and 5 µl of every sample or standard solutions were successively putted into individual designated wells.
The plate was covered and placed on a plate shaker for 30 seconds at 600 rpm. After 2 hours of incubation period at room temperature the reaction mixture was discarded and rinsed as described above. Therefore 50µl of HRP conjugated streptavidin were added into all wells and shacked as explained before. This step was followed by an incubation time of 30 min at room temperature, afterwards the reaction mixture was discarded and the plate was washed as previously. A volume of 50µl of substrate chromogen reagent was then added into all wells and shake for 30 seconds at 600 rpm. After 30 min of incubation time at room temperature a volume of 50µl of reaction stopper was incorporated in every well and shacked as formerly. The absorbance was read at 450 nm with a reference wavelength at 620 nm.

II-6) Statistical analysis

Results are expressed like means ± SD and $P<0.05$ was used as level of significance for statistical testing. TIBCO Spotfire S®+8.1 for Windows or SigmaStat 3.0 software were used to perform statistical analysis. The effect of *Iwong* feeding on body weight, body weight gain and cumulative food intake over time was analyzed by Linear Mixed Effects model (LME) using time as repeated measure factor and *Iwong* or control groups as fixed factor, followed

by Bonferroni's post hoc test. One way Analysis of variance (ANOVA) followed by Bonferroni's post hoc test were performed to analyze all others results. All statistics for *in vitro* studies were carried out with Sigmastat 3.0 software by One way Analysis of variance (ANOVA) completed by Bonferroni's post hoc test.

III-)

RESULTS

III-1) Efficacy of *Iwong* feeding in Wistar Rats

III-1-1) Food intake and body weight

A trend of means food intake valuesincreased was noticed in Iwong5 rats during the last two weeks and in Iwong10 rats during entire intervention period, but since food intake data represents the sum of total food consumed of 2 cages per diet group; statistical analysis was not applicable.

Body mass of *Iwong* fed rats was significantly lower as compared to controls (Table 9, $p < 0.05$). Due to an initial difference in body mass between the diet groups, body mass gain was calculated. Both dietary intervention groups, Iwong5 and Iwong10 gained less body mass from week 1 to the end of treatment (Table 9, $p < 0.05$).

Table9: Effect of dietary supplementation of *Iwong* on body mass trajectory, body mass gain and cumulative food intake in male Wistar rats (n=5)

	Diet group	Initial	Week 1	Week 2	Week 3	Week 4
Body mass (g)	Control	291.4 ± 18.5	302.4 ± 18.2	340.0 ± 35.2	360.0 ± 29.4	380.0 ± 28.1
	Iwong5	261.8 ± 41.3	256.2 ± 39.3*	259.8 ± 24.8*	298.6 ± 33.6	295.0 ±36.4**
	Iwong10	237.8 ± 13.0**	233.8 ± 18.4**	238.8± 18.4**	271.3± 14.4**	253.8± 16.0**
Body mass gain (g)	Control	/	11.0 ± 2.2	48.6 ± 22.2	68.6 ± 11.9	88.6 ± 16.3
	Iwong5	/	- 5.6 ±2.9	- 2.0 ± 22.5*	36.8 ± 19.6*	33.2 ± 22.1*
	Iwong10	/	- 4.0 ± 1.7	1.0 ± 7.1*	33.4 ± 7.7*	16.0 ± 12.1*
Cumulative food intake (g)	Control	/	259	477	635	791
	Iwong5	/	217	445	675	831
	Iwong10	/	331	611	874	1062

***P<0.05 and **P<0.01**

III-1-2) Effect of *Iwong* feeding on fat mass and liver weight

Distinct subcutaneous and intraperitoneal adipose tissue depots were dissected and weighed to determine whether body fat content contributes to diminished body weight gain in response to *Iwong*. In rats, a dose-dependent reduction was observed in mesenteric (-43% and -60%), perirenal (-50 % and -80%) and epididymal (-27 % and - 50%) fat mass in Iwong5 and Iwong10 rats, respectively (Fig. 6). The total dissected fat mass was significantly reduced in Iwong10 rats ($p<0.05$, Fig. 6). The effect of *Iwong*

feeding on the liver was also assessed, and a significant reduction on liver weight was observed in Iwong's groups (Fig. 7)

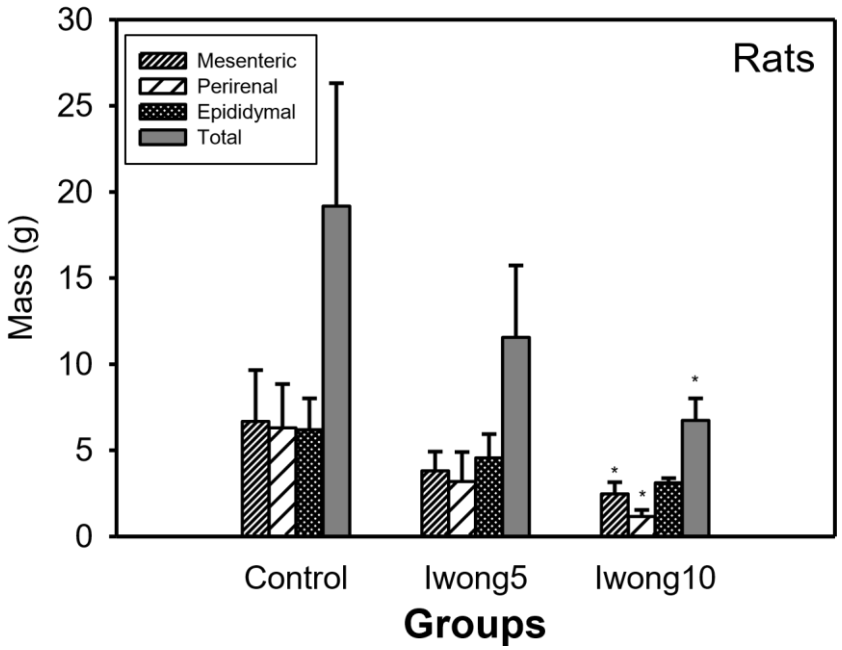


Fig. 6: ***Effect of Iwong on fat mass in rats.*** Animals housed 2 or 3 per groups were fed a standard diet or a standard diet supplemented with Iwong 5 and 10% (Iwong 5% and Iwong 10% respectively) for 28 days. At the last day of feeding experimental period, they were anaesthetized and killed. Several fat pads were excised and weighed, the sum of various fat pads was assumed as total fat mass. Values are means ± SD *significantly different from the control group (n=5).

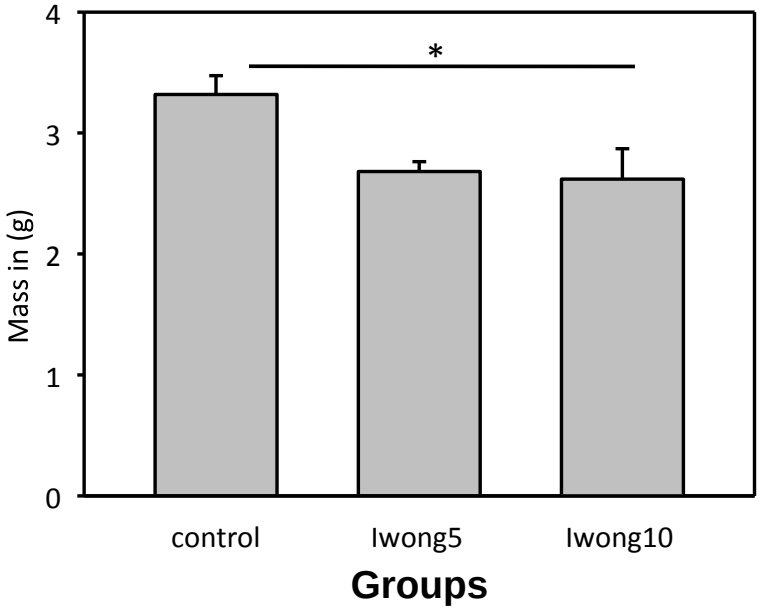


Fig 7: Liver weight in the rat study. After 28 days of *Iwong* feeding, rats were killed and dissected; the liver was excised and weighed. Results shown are mean values of 5 animals per group ± SD. *(P< 0.05) significantly different from the control group.

III-1-3) Blood clinical chemistry parameters

Plasma clinical chemistry revealed a trend towards lower blood glucose, with -21% or -18% for Iwong5 and Iwong10 groups, respectively, compared to the control. No difference was observed in triglyceride (TG) levels; whereas minor alterations were noticed in other lipid parameters, with total cholesterol and HDL-cholesterol significantly elevated in Iwong10 rats but no difference in LDL-cholesterol (Table 10)

Table 10: Plasma glycaemia (Glu), triglyceride (TG), total-cholesterol (T-C), HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C), levels in rat.

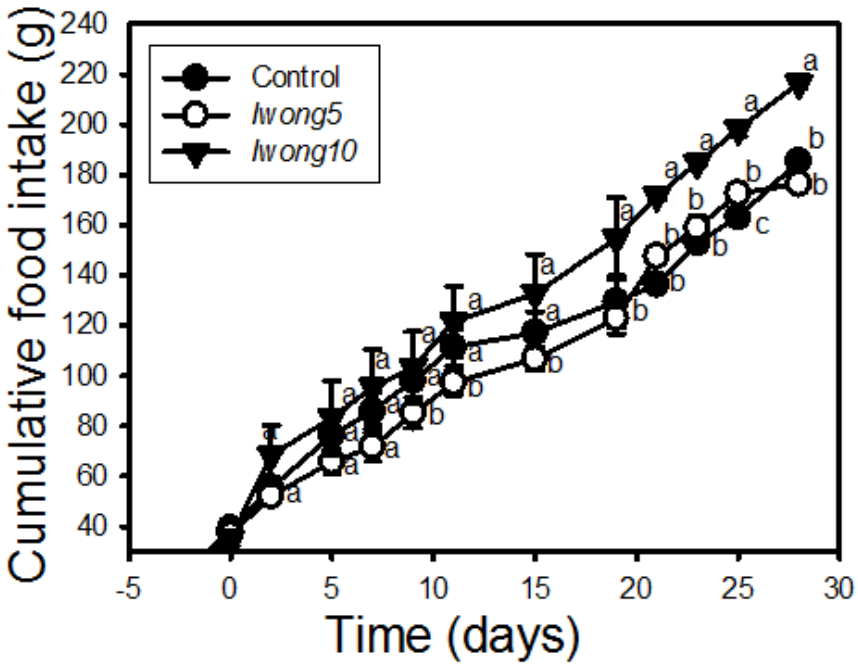
	Control (n=5)	Iwong5 (n=5)	Iwong10 (n=5)
Glu (mg/dL)	76.6 ± 2	60.6 ± 13.0	67.4 ± 3.6
TG (mg/dL)	64.2 ±1 2	67.6 ± 14.0	68.6 ± 4.0
T-C (mg/dL)	109.2 ± 11.0	112.6 ± 9.0	128 ± 4.0*
HDL-C (mg/dL)	46.6 ± 11.0	54.4 ± 10.5	61.6 ± 10.0*
LDL-C (mg/dL)	49.8 ± 11.0	44.7 ± 5.2	52.7 ± 12.4

***P<0.05**

III-2) Efficacy of *Iwong* feeding in mice

III-2-1) Food intake

Based on the effects of *Iwong* on energy balance in rats, we conducted a more detailed analysis in a second feeding trial using an outbred strain of NMRI mice. Similar to the rat feeding trial, *Iwong* feeding increased cumulative food intake in Iwong10 group which gained statistical significance after one week ($p<0.05$, Fig. 8). Food intake of Iwong10 mice remained elevated compared to controls until the end of the feeding trial whereas food intake level of Iwong5 mice was not different to the control group.



Figu 8: ***Effects of Iwong on food intake.*** NMRI mice (n=6) were fed a standard diet (control) or a standard diet supplemented with 5 % (*Iwong5*) or 10% (*Iwong10*) of powdered *Iwong* during 28 days. Food intake was recorded twice per week during the 4 weeks of feeding, data are expressed as mean ± SD. Values with dissimilar letters were significantly different (P<0·05).

III-2-2) Food digestibility

Elevation of food intake could be due to lower energy content or poor digestibility of *Iwong* supplemented diets resulting in increased fecal energy excretion. Therefore we compared the energy content of the diets and the fecal energy content by bomb calorimetry.

Supplementation of the standard diet with *Iwong* did not alter the energy density of the diets. Both Iwong5 and Iwong10 diets contained 17.0 kJ g^{-1} compared 16.9 kJ g^{-1} of the standard diet. Considering these energy densities and the food intake data the daily energy intake was significantly elevated by 17% in Iwong10 mice ($p<0.05$, Table 11) but not affected in Iwong5 mice compared to controls. Taking into account the daily feces production and fecal energy density of individual mice, the fecal energy excretion per day was significantly increased in both *Iwong* groups. The digestibility of food, expressed as the assimilation coefficient, was decreased in Iwong5 and Iwong10 (Table 11). Despite reduced digestibility Iwong10 mice assimilated significantly more energy per day compared to controls (Table 11) due to their higher energy intake. In contrast, daily energy assimilation rather tended to be lower in Iwong5 mice but this trend did not attain statistical significance.

Table 11: Effect of *Iwong* on dietary digestion after 28 days of feeding per mouse

	Control (n=6)	*Iwong5* (n=6)	*Iwong10* (n=6)
Energy intake (KJ/day)	98.1. ± 8.7	95.1 ± 8.8	114.7 ± 8.2*
Energy excreted (KJ/day)	19.1 ± 2.1	23.9 ± 2.3*	26.4 ± 2.0*
Energy assimilated (KJ/day)	78.6± 8.1	71.1 ± 8.0	89.3 ± 14.4*
Assimilation efficiency (%)	80 ± 2	75 ± 4*	76 ± 2

***P<0.05**

III-2-3) Body weight gain trajectories

To assess whether the effect observed on body weight in rats is a general phenomenon, NMRI mice were fed a standard diet supplemented with Iwong 5 and 10% for 4 weeks.

Body weight gain was significantly lower after one week of feeding Iwong10 group (Fig.9, $p<0.05$). This difference was maintained until the end of the feeding period. The Iwong5 group showed an intermediate trajectory which reached a significant difference from the control group during the second phase of the feeding period (Fig.9, $p<0.05$).

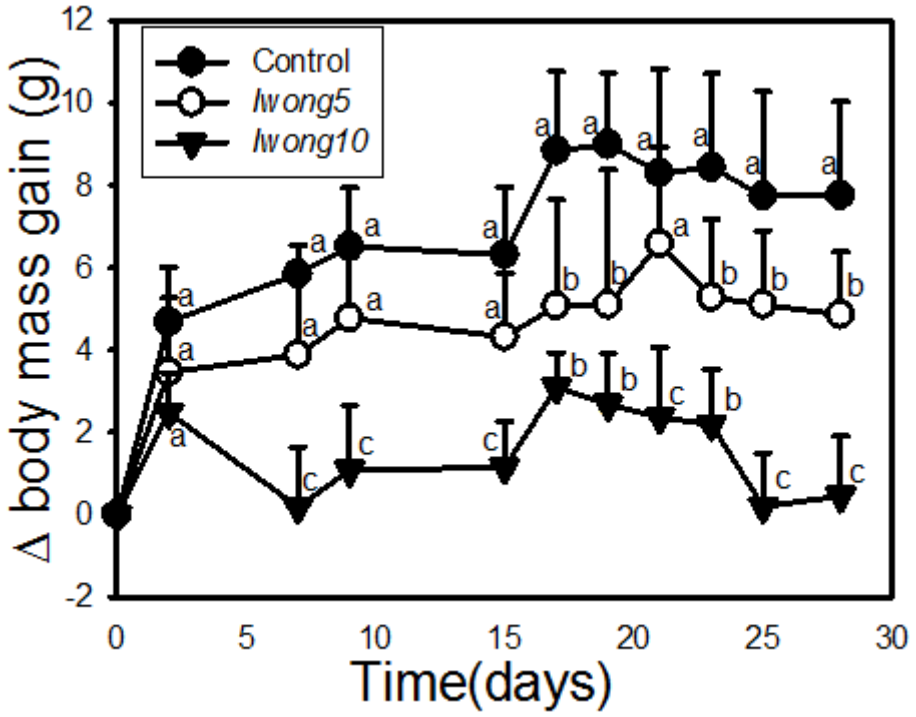


Fig. 9: ***Effects of Iwong on body weight gain in mice*** NMRI mice (n=6) were fed a standard diet (control) or a standard diet supplemented with 5 % (*Iwong5*) or 10% (*Iwong10*) of the powder of *Iwong* during 28 days. Body mass was recorded twice per week during the 4 weeks of feeding. Data are expressed as mean ± SD. values with unlike letters were significantly different (P<0·05).

III-2-4) Effect of *Iwong* feeding on fat mass in mice

After 4 weeks of feeding mice were sacrificed and different fat pads were dissected and weighed. Results as depicted in Fig. 10 showed a dose dependent decrease of mass for different fat pads. In the Iwong5 group, a trend of fat mass reduction was observed in each WAT pad (mesenteric, perirenal, epididymal and inguinal) but not BAT. As a consequence the total fat mass was lower in Iwong5 but did not attain significance. Whereas in Iwong10 group, despite a reduction of total fat mass, only mesenteric, perirenal and BAT were significantly different from the control group. Taken together we observed a significant decrease in total fat mass ($p<0.05$).

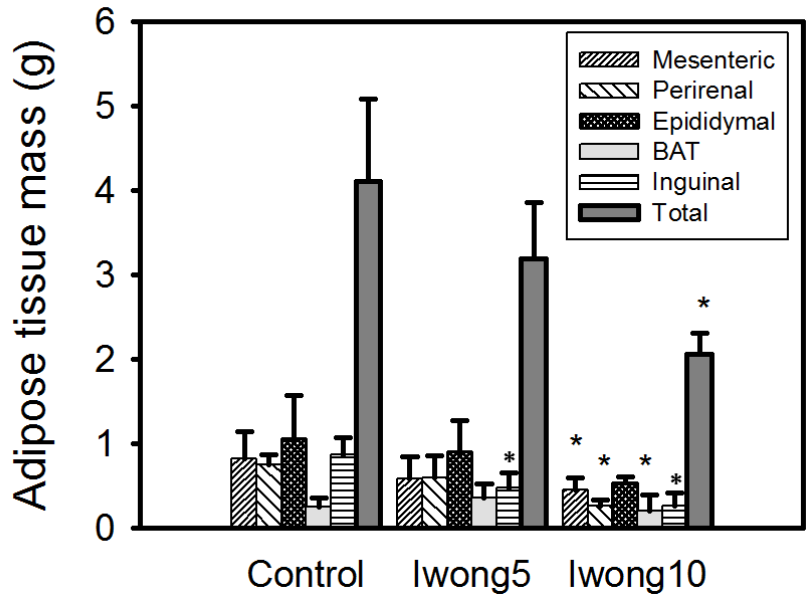


Fig 10: ***Effect of Iwong on fat mass in mice.*** At the end of 28 days experimental period several fat pads were excised and weighed, the sum of various fat pads was assumed as total fat mass. A) represents different fat pad and the sum of fat depot of rats and B) is an illustration of selected fat depot and their total dissected in mice (n=6). Values are means ± SD *significantly different from the control group.

III-2-5) Effect on gastrocnemius muscle, liver and kidney weight

To assess the effect of *Iwong* feeding the gastrocnemius was excised and weighed. As shown in Fig. 11A there was no effect on the gastrocnemius muscle.

In parallel the liver and kidney were also weighed, results obtained showed no difference in both organs (Fig. 11B and Fig. 11C)

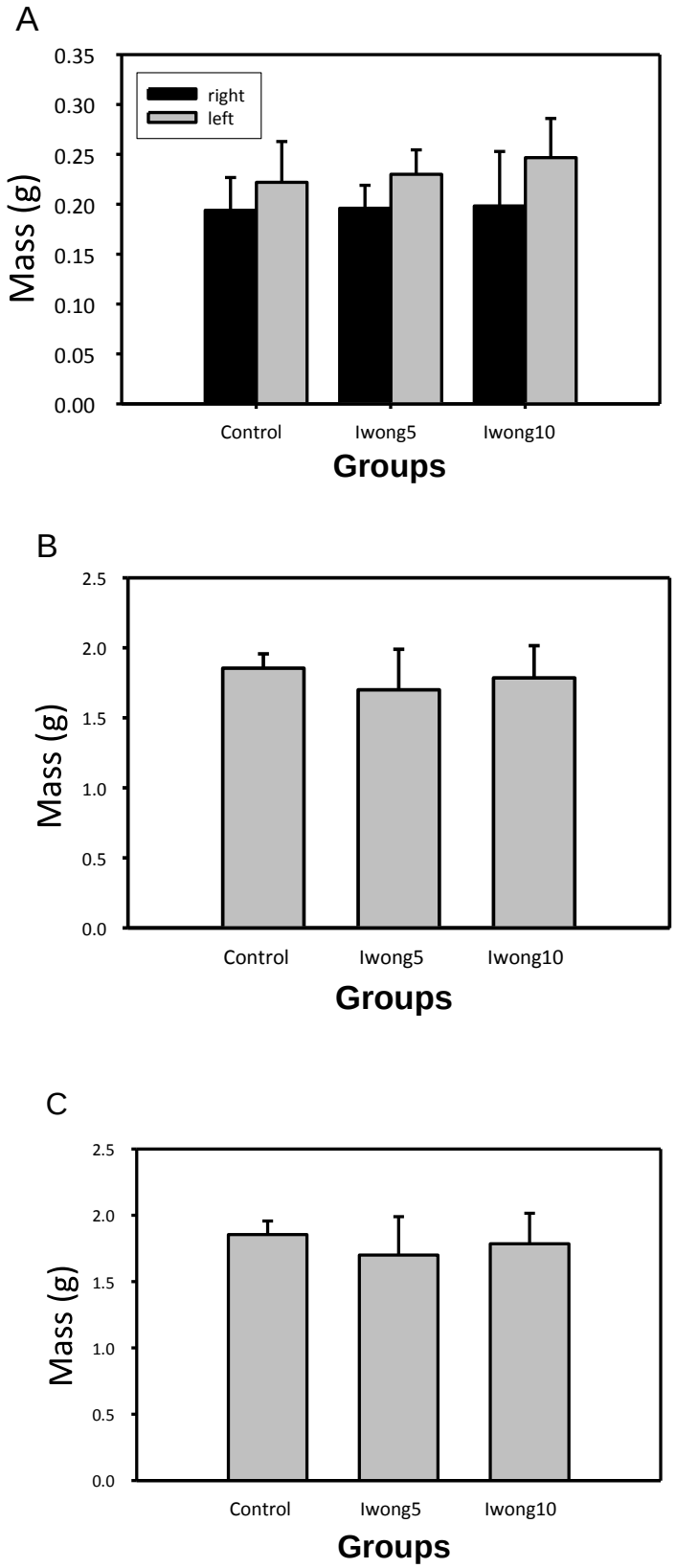


Fig 11: Effect of *Iwong* feeding on the mass of gastrocnemius, liver and kidney. NMRI male mice (n=6) were killed and dissected. Organs and muscles were removed and weighed. (A) represents means values of left and right gastrocnemius , (B) shows the means mass of the liver per groups and (C) illustrates the mean mass of the right and left kidney. All values are means ± SD.

III-2-6) Biochemical parameters

After 4 weeks of feeding, mice were killed and blood samples collected for plasma biochemical analyses. No differences were observed between groups in plasma concentrations of triglycerides (TG), total-cholesterol (T-C) very low density lipoprotein (VLDL). The level of high density lipoprotein-cholesterol (HDL-C) was significantly elevated in Iwong10, but remains at the same level as in control group for Iwong5. In addition a significant decrease of the low lipoprotein density-cholesterol (VLDL-C) was noticed in both *Iwong* groups ($p<0.05$) (Table12).

The plasma concentration of the aminotransferase ALT was not changed, whereas the mean of AST level was increased but did not attain significance (Table 12).

Table 12: Triglyceride (TG), total-cholesterol (T-C), HDL cholesterol (HDL-C) and LDLcholesterol (LDL-C), alanine transaminase (ALT) and aspartate transaminase (AST) levels in mice.

	Control (n=6)	Iwong 5 (n=6)	Iwong 10 (n=6)
TG (mg/dL)	78.2 ± 27.55	86.2 ± 31.98	72.25 ±10.1
T-C (mg/dL)	62.4 ± 5.32	57.4 ± 17.53	61 ± 3.46
HDL-C (mg/dL)	42.8 ± 6.14	42.2 ±13.33	47.75 ± 2.75*
LDL-C (mg/dL)	51.04±5.2	32.44±3.5*	31.5±2.6*
VLDL (mg/dL)	17 ± 3.67	17.4 ± 6.58	14.5 ±1.73
ALT (U/L)	40.2 ± 13.08	42.8±11.81	46.5 ± 18.69
AST (U/L)	102.2 ± 30.26	184.8±.98	233.2 ± 161.80

***P<0.05**

III-3) Effect of *Iwong* supplementation in mice fed a HFD: Obesity prevention

III-3-1) Food intake

C57BL/6J mice were used to assess the ability of *Iwong* to prevent obesity. Mice were fed a standard diet, HFD, or HFD supplemented with powdered *Iwong* for 5 weeks. Cumulative food intake was similar during the first 2 weeks of feeding in all 4 groups (Fig. 12). This trend changed during the second phase of feeding, where food consumption was significantly increased in both *Iwong* groups compared to the control and the HFD group.

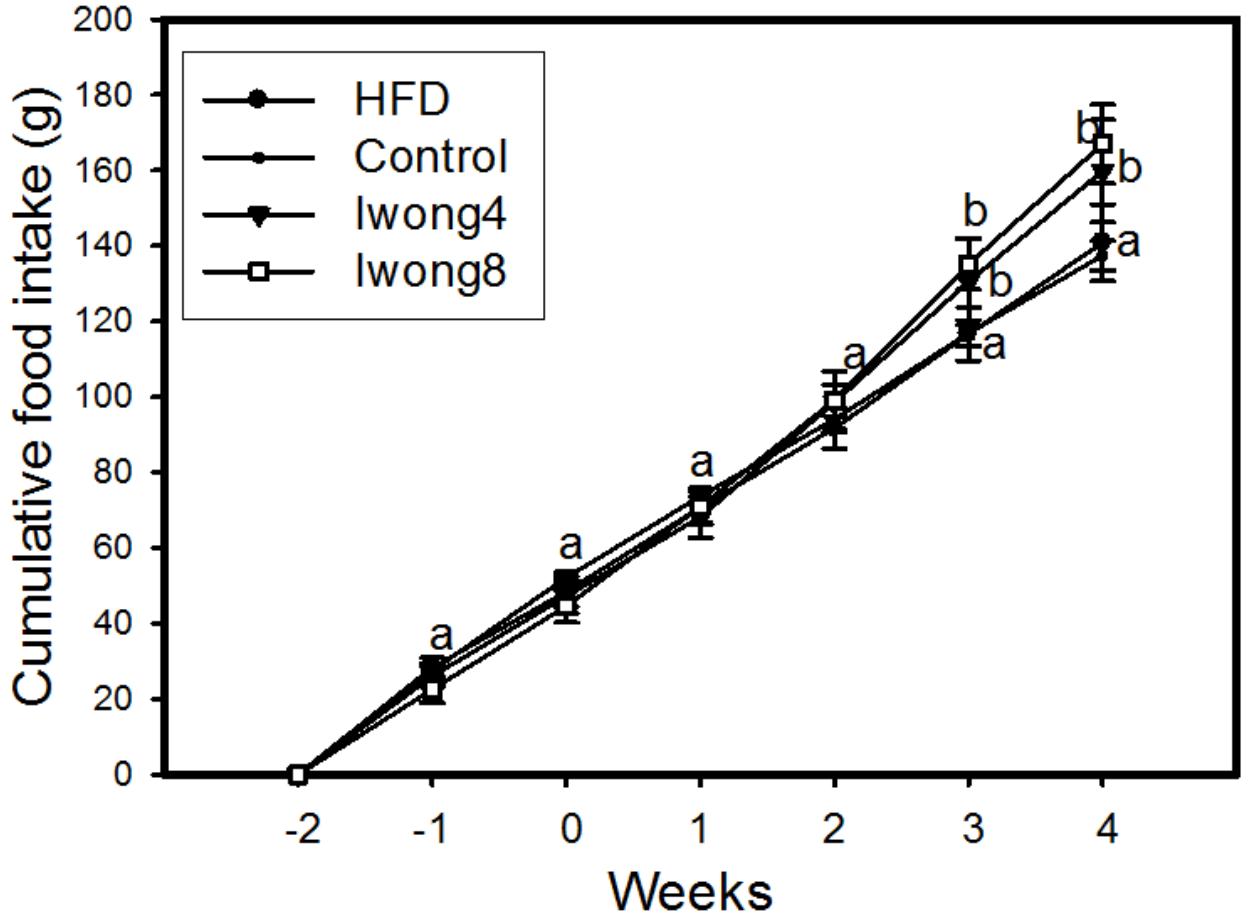


Fig 12: Effect of *Iwong* on food intake in C57Bl/6J mice. Twenty eight mice were divided in 4 groups of 7 and fed a standard diet, a HFD, or a HFD supplemented with Iwong 4 and 8%. Food intake was recorded once per week during the entire feeding process. Results are shown as means ± SD, the asterisk (*) represents statistical difference at $p<0.05$ compared to the HFD.

III-3-2) Body weight

Body weight was recorded each week as depicted in Fig. 13. During the first two weeks of feeding there were no differences between HFD and control groups, but the body weight development changed among both during the second phase of the feeding trial. Therefore a significant ($p<0.05$) variation appeared from week two until the end of the experimental period. This observation suggested that HFD used for this study is able to induce obesity.

For *Iwong* groups despite an increase on food consumption as described below, we noticed a significant variation on body weight compared to the HFD group from week 3 to week 5. Whereas, although the body weight development remained elevated in both Iwong groups compared to the control during the same period, this did not reach

significance. In addition, the drop on body weight observed with Iwong4 after one week of feeding could be considered as an artifact. In fact those mice were first fed a HFD supplemented with 10% (w/w) of *Iwong* powder like in previous experiments, but due to this decrease which could be explain by the fact that the powder of Iwong used here was prepared from a new batch, which seems to be more potent. we switched to HFD enriched with 4% (w/w) of the powder of *Iwong*.

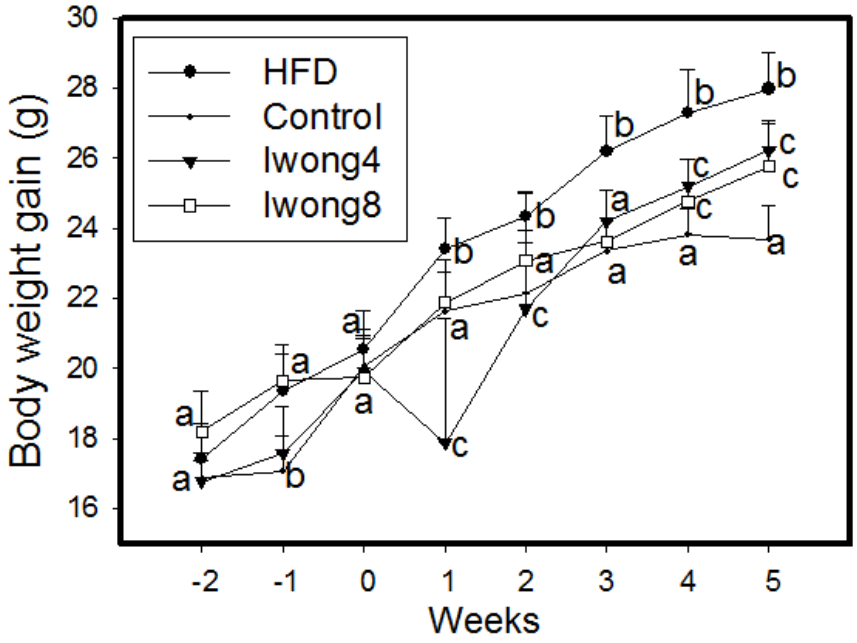


Fig 13: Body weight trajectories of mice in study to assess the prevention of obesity. Young mice aged 6 weeks were randomized in 4 groups of 7 and fed with HFD for 5 weeks, a standard diet or a HFD supplemented with 4 and 8% (w/w) of *Iwong* powder. Body weight was recorded once per week and values are means ± SD. values with unlike letters were significantly different (P<0·05).

III-3-3) Food digestibility

The lower increase in body mass was associated with hyperphagia. In order to assess the food digestibility, feces were collected during the last 3 weeks of feeding and the fecal energy excreted was measured. The total fecal energy excretion in mice fed a HFD compared to the control group was similar (Fig. 14). However, the total fecal energy excretion of both *Iwong* groups was significantly elevated ($p<0.05$) compared to the control group.

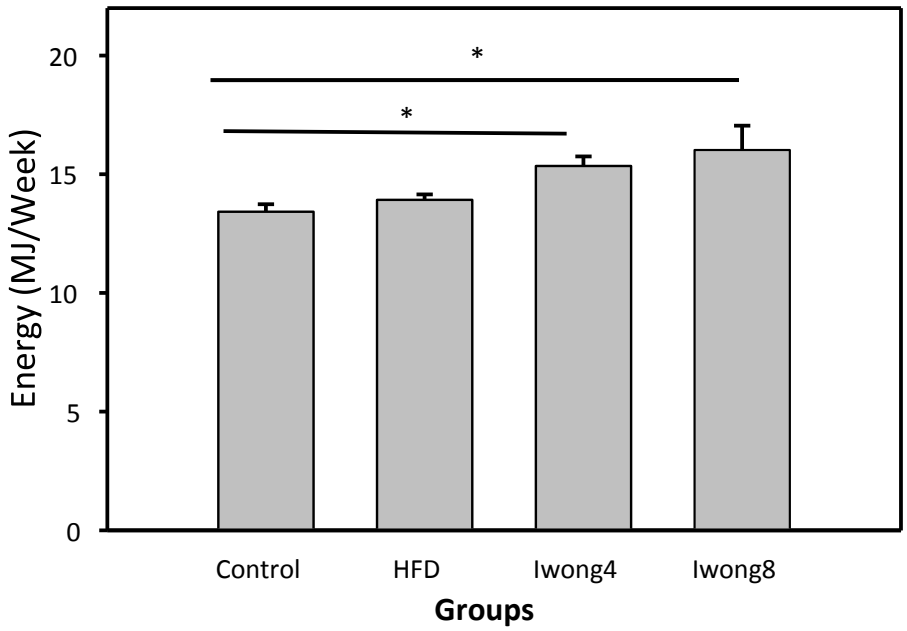


Fig 14: Fecal energy excretion of C57BL/6J male mice. Feces were collected once a week dried and crushed. The powder obtained was used to make pellets of 1 g. The energy content was quantified with an adiabatic bomb calorimeter. Values are expressed as mean feces excreted per week and per group of mice (n=7). *p<0.05

III-3-4) Fat pad mass

Mice fed a HFD had a significantly higher total fat mass (p<0.05) compared to those fed a standard diet, whereas supplementation of HFD with Iwong 4 and 8% (w/w) prevented increased of fat mass. Therefore *Iwong* feeding lead to a significant lowering of total fat mass compared to the HFD group (Fig. 15, p<0.05)

III-3-5) Effect of *Iwong* on liver and blood metabolites after 5 weeks of feeding

After 5 weeks of feeding, mice were killed, blood samples and a fragment of liver tissue were collected for biochemical analysis. In plasma, TG level remained unchanged in all dietary intervention groups (Fig. 16A), as well as in liver triglycerides (Fig.16B). The plasma level of total-cholesterol (T-C) was higher in mice fed a HFD (p<0.05) but not in mice fed *Iwong* supplemented with diet where this level was close to the control group. The same was true for the plasma LDL-C where the concentration in HFD group was significantly elevated compared to the control and Iwong groups (Fig.17A, p<0.05).

A trend of a dose dependent hypoglycemia effect was noticed in Iwong4 (-15%) and in Iwong8 (-20%) groups, but this was not significant compared to the control and HFD groups (Fig. 17B).

A slight but not significant increase was noticed in *Iwong8* compared to other groups (Fig 18).

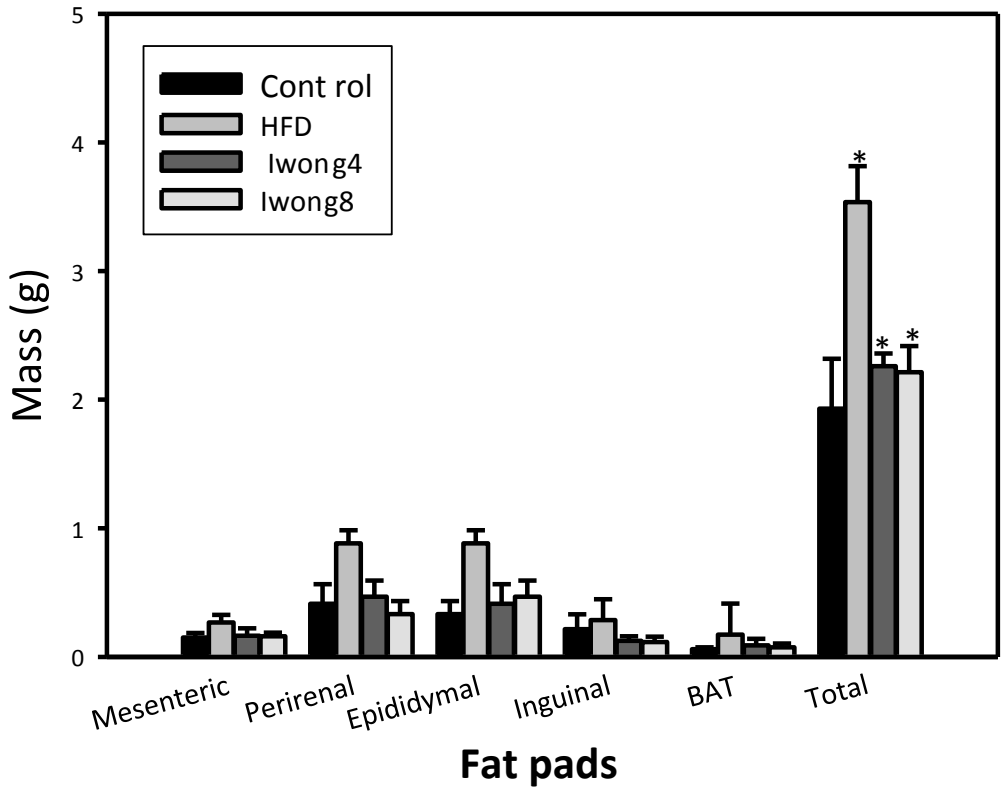


Fig 15: Effect of *Iwong* feeding on fat mass in young male mice fed a HFD. After 5 weeks of dietary intervention experimental period, mice (n=7) were killed and several fats were excised and weighed. The total fat mass was estimated as the sum of all fat pads. *p<0.05, significantly different compared to the HFD group.

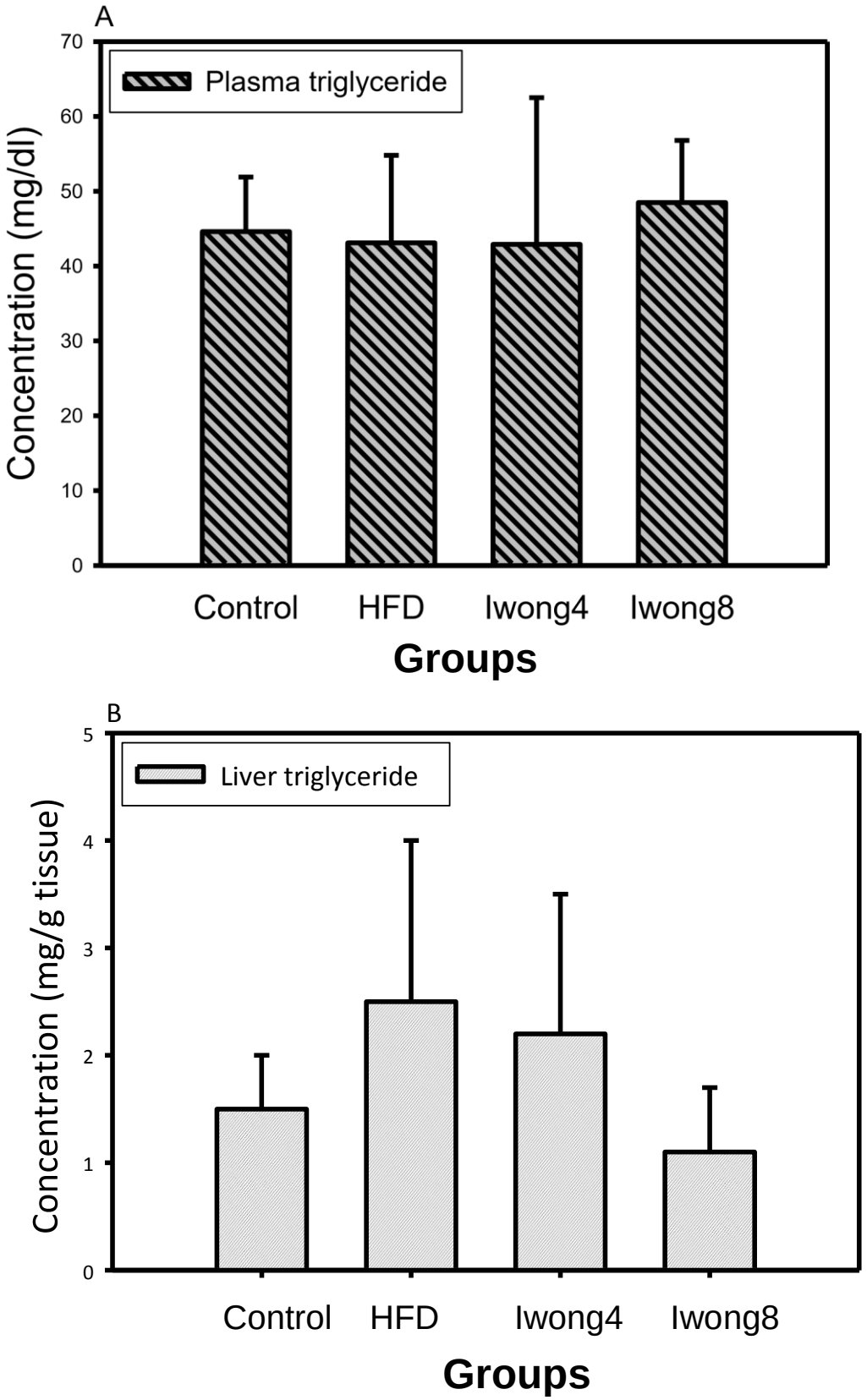


Fig 16: Plasma and liver triglycerides level in young male C57BL6J mice (n=7). Animals were fed a standard diet (control), High fat diet (HFD) and High fat diet supplemented with 4 or 8% of the dried powder of *Iwong* (Iwong4 and Iwong8 respectively). *p<0.05 compared to the HFD.

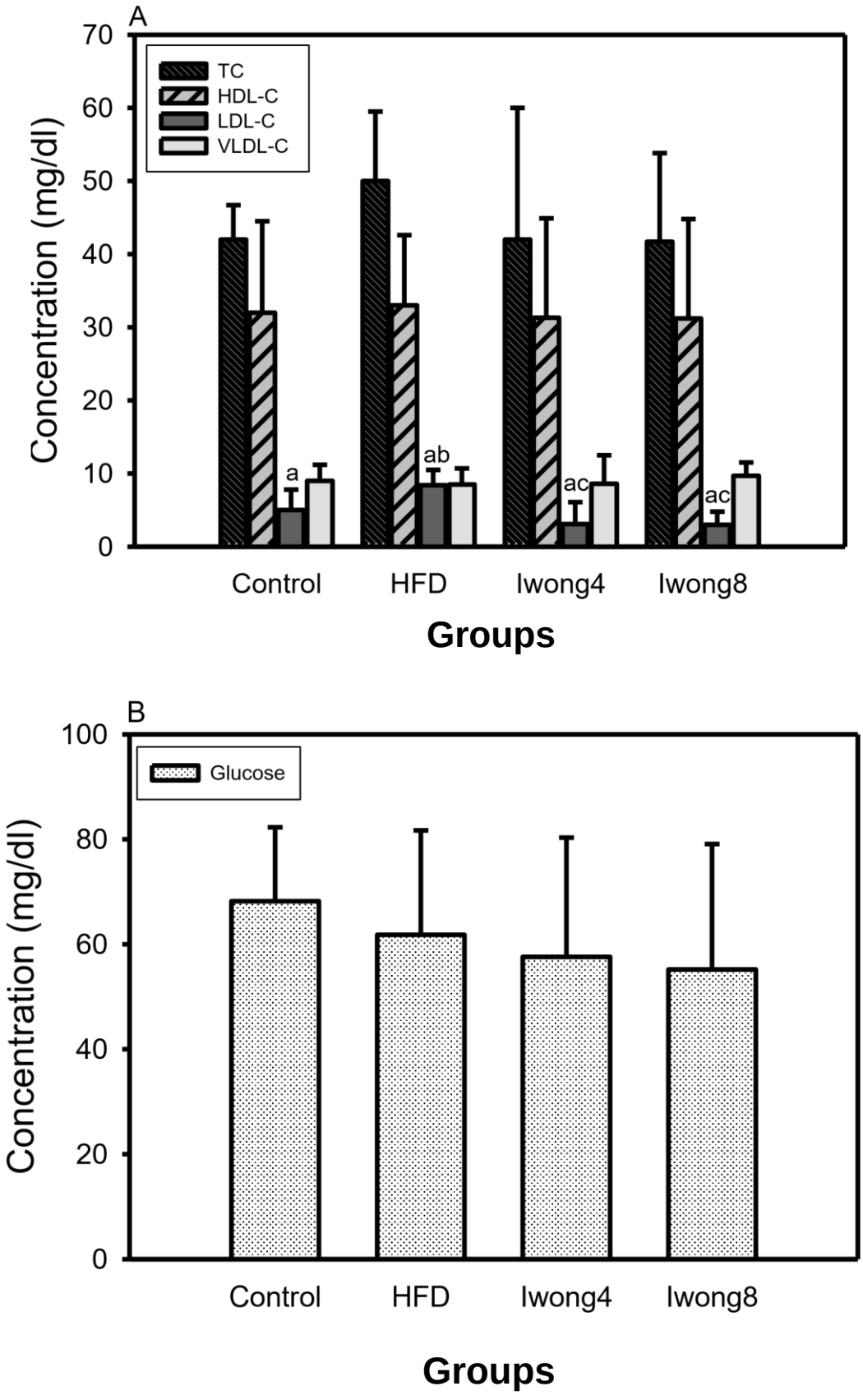


Fig 17: Plasma cholesterol and glucose levels in young C57Bl/6J male mice (n=7)**.** After 5 weeks of feeding intervention period, animals were killed and blood was collected. Plasma obtained after centrifugation was used to quantify the amount of triglyceride (TG), total-cholesterol (TC), high density lipoprotein (HDL-C), low density lipoprotein (LDL-C) and very low density lipoproteins cholesterol (VLDL-C).

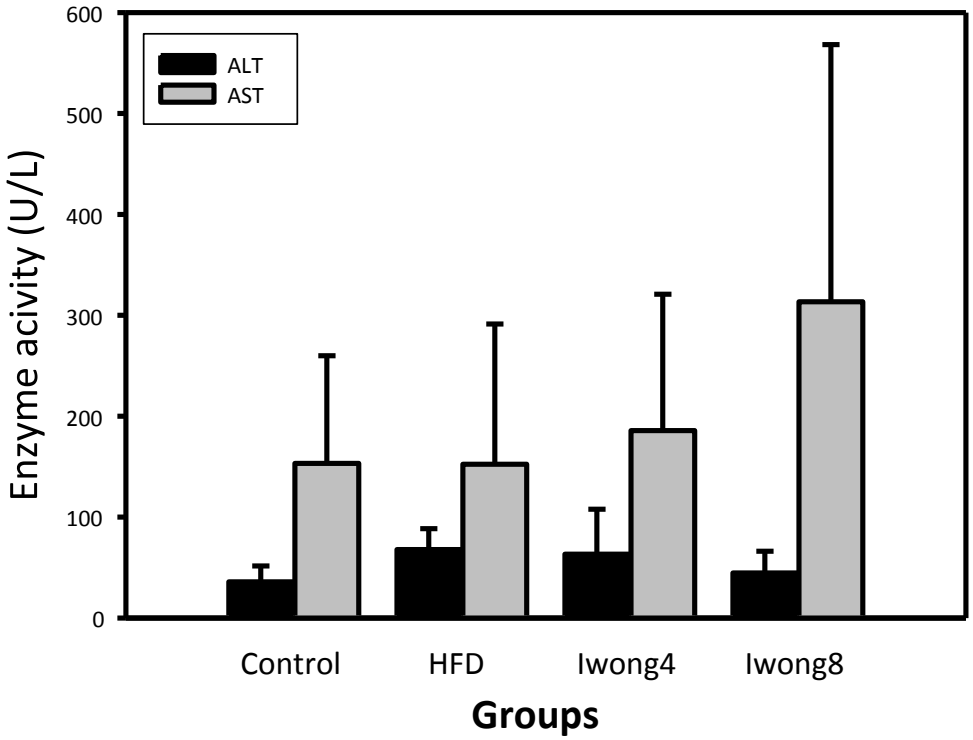


Fig. 18: Plasma aminotransferases level of young male C57Bl/6J mice (n=7). Animals were fed a standard diet (control), high fat diet (HFD) and high fat diet supplemented with 4 or 8% of the powder of *Iwong* (Iwong4 and Iwong8 respectively). After 5 weeks of feeding animals were killed, blood was collected and analyzed for Alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

III-3-6) Effect of *Iwong* feeding on plasma leptin level in younger male mice

Mice were fed a standard diet, HFD, or a HFD supplemented with the powder of *Iwong* for 5 weeks. Afterwards, blood was collected and centrifuged; plasma obtained was used to determine the leptin level. In mice fed a HFD the leptin level was significantly elevated compared to control mice. Leptin level remained elevated in mice fed a HFD supplemented with Iwong 4%, while in the Iwong8 group hyperleptinemia induced by HFD feeding was completely abolished. Leptin levels in Iwong8 mice was significantly reduced compared to the HFD group (Fig. 19).

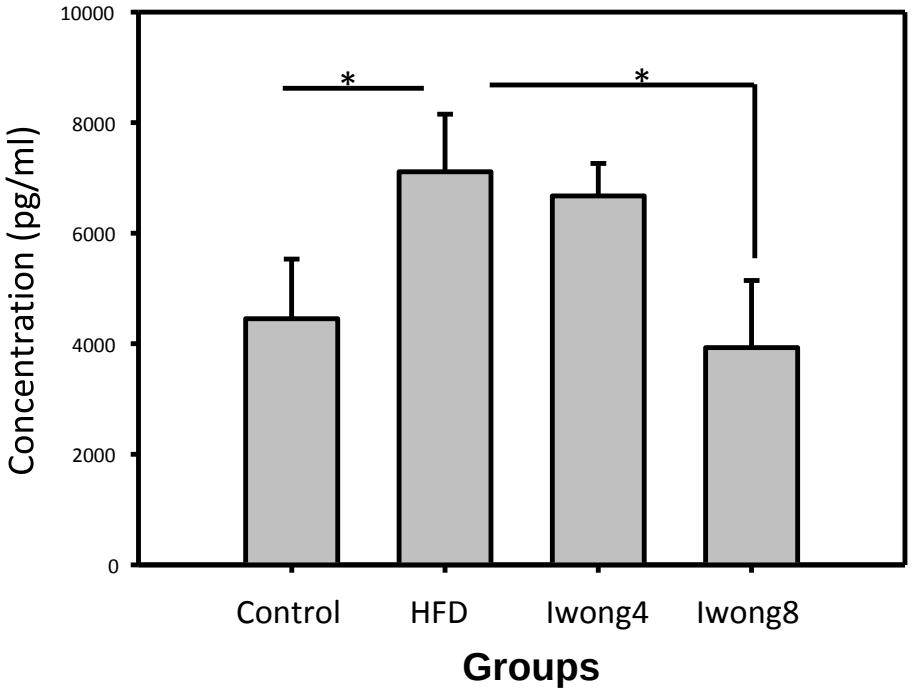


Fig. 19: Plasma leptin level in young mice. Twenty eight young C57BL/6J male mice were randomized in 4 groups of 7, HFD group fed a high fat diet, control group fed a standard diet and *Iwong* groups fed a HFD supplemented with 4 and 8% of the powder of *Iwong* (Iwong4 and Iwong8 respectively). After 5 weeks feeding animals were killed and plasma was used to measure the level of leptin by the ELISA technique. Data are expressed as mean ± SD and (*) represents $p<0.05$.

III-4) Effect of *Iwong* on obese mice: obesity regression

III-4-1) Food intake

Diet induced obese mice (DIO) were used to evaluate the slimming effect of *Iwong*. Food intake recorded once per week was similar in HFD and control groups during the induction of obesity. When switched to *Iwong* feeding the food consumption trajectory remained similar for the first 4 weeks, a slight but not significant increase on food intake was noticed during the last 3 weeks of treatment in Iwong4 and Iwong8 groups compared to the HFD group (Fig. 20).

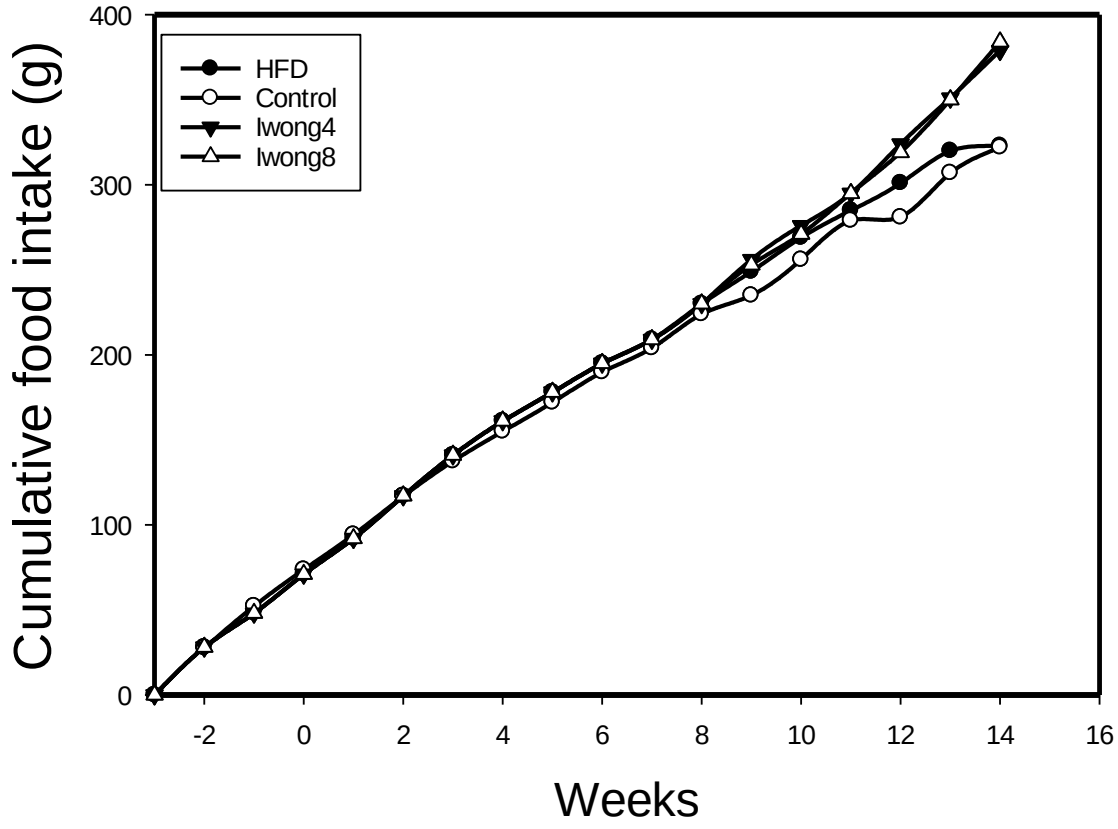


Fig. 20: Cumulative food intake in obesity regression experiment: Male C57Bl/6J mice at the age of 6 weeks were fed a standard diet (control, n=7) or a HFD (n=21) for 9 weeks for the induction of obesity. Afterwards 21 obese mice were selected and randomized in 3 groups of 7 mice. The first group was maintained on HFD, the other two groups were fed a HFD supplemented with 4 %(*Iwong4*) and 8 % (*Iwong8*) of the powder of *Iwong* for 6 weeks. Cumulative food intake recorded from week -3until week 15 are represented as means of 7 mice per group.

III-4-2) Body mass

The HFD used in this experiment successfully induced obesity, as shown in Fig. 21 mice fed a HFD had a significant higher body mass compared to those fed a standard diet (Fig. 21). Supplementation of *Iwong* led to an urge drop in body mass during the first two weeks of dietary intervention in both Iwong4 and Iwong8 compared to the HFD group. In general, the body weight of *Iwong* groups remained lower in mice fed *Iwong* until the end of the treatment.

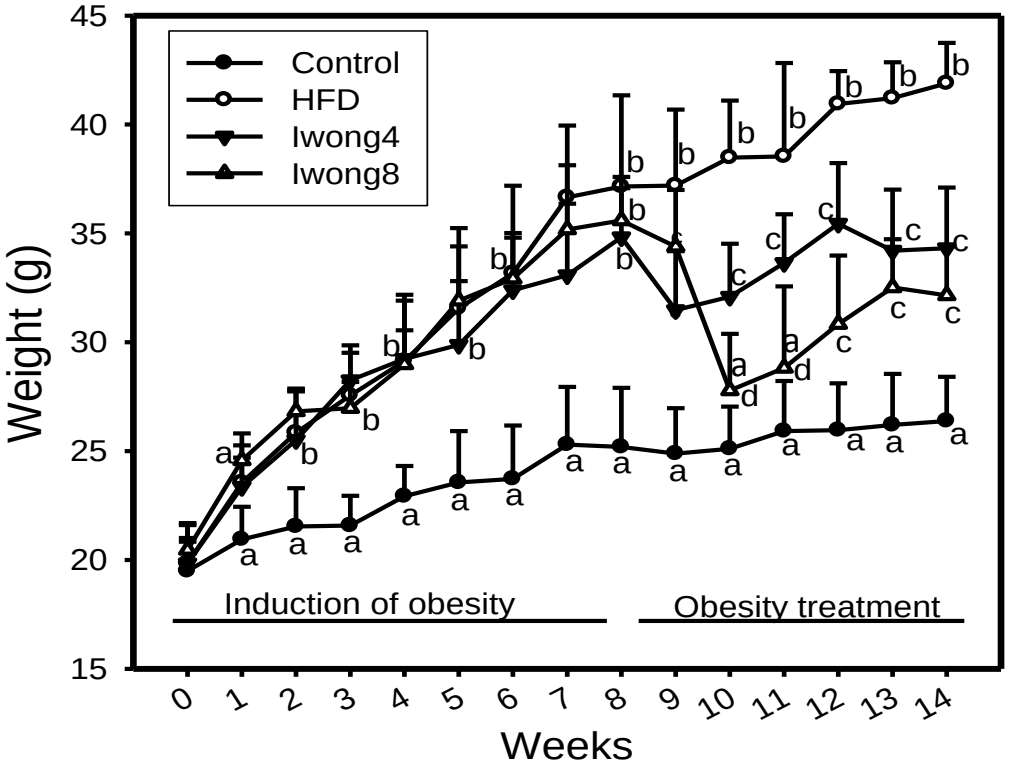


Fig. 21: Body mass trajectories in response to obesity treatment with *Iwong*. C57BL/6J male mice were randomized in two groups, the first group of 7 animals referred as control group was fed a standard diet and the second group of 21 mice was fed a HFD. After 9 weeks of induction of obesity, obese mice were divided in 3 groups of 7 mice; a HFD group maintained under HF feeding, Iwong4 and Iwong8 groups fed a HFD supplemented with 4% and 8% of the powder of *Iwong* respectively. Body mass was recorded once per week during the induction and the treatment phases. Values are represented as means ± SD of 7 mice per group. Values with unlike letters were significantly different (P<0·05).

III-4-3) Fat pads mass

After 9 weeks of feeding mice were killed and several fat pads were weighed. Total fat mass was higher in HFD mice compared to control mice (Fig. 22, p<0.001). In mice fed *Iwong* there was a dose dependent protective effect against the increase in total fat mass when compared to the HFD (Fig. 22, p<0.05).. In addition *Iwong* feeding was associated with a reduction in liver mass compared to the HFD group, whereas no noticeable changes were observed in the kidney mass (Fig. 23)

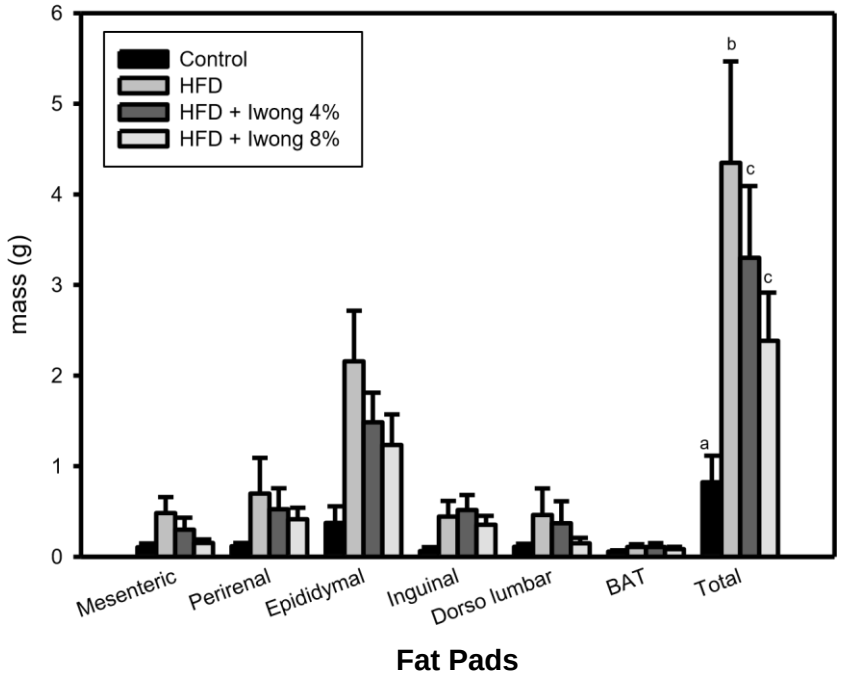


Fig. 22: Effect of *Iwong* feeding on fat pad mass. Seven lean mice fed a standard diet, seven obese mice fed a HFD and HFD supplemented with 4 and 8% of *Iwong* powder (n=7) for 6 weeks and were killed under CO_2 anesthesia. Several fat pads were immediately excised and weighed. Results are depicted as means ± SD.

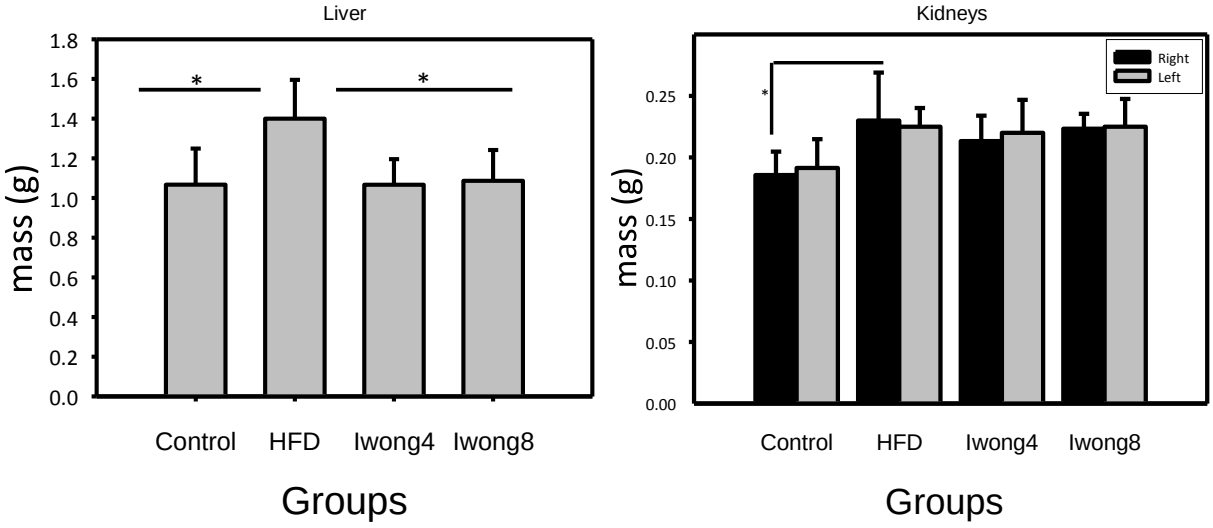


Fig. 23: Absolute weight of liver and kidneys of mice treated or not with *Iwong*. Seven lean mice fed a standard diet, seven obese mice fed a HFD and HFD supplemented with 4 and 8% of *Iwong* powder (n=7) for 6 weeks and were killed under CO_2 anesthesia. Afterwards liver (A) and kidney (B) were excised and weighed. Results are presented as means of 7 mice ± SD. * $p< 0.05$.

III-4-4) Effect of *Iwong* feeding on hepatic and blood metabolites of obese and lean mice

Plasma triglycerides (TG) level were elevated in HFD and Iwong8 and reached significance in the Iwong4 group (Fig. 24A). Liver TG content was significantly elevated in the HFD group compared to the control group, whereas a dose

dependent protective effect against lipid accumulation was found in mice fed a HFD supplemented with *Iwong* (Fig. 24B). High fat feeding was also associated with a significant elevation of total cholesterol (T-C) and LDL-C level compared to low fat (standard diet) feeding. This effect was attenuated in mice fed a HFD supplemented with the powder of *Iwong*. In addition, plasma HDL-C levels were significantly elevated in the HFD group compared to other groups *(*Fig. 25A). Although, there was no significant difference in plasma glucose level; mice of the HFD group showed a high glucose level compared to control and *Iwong's* groups (Fig. 25B).

Measurement of the plasma activities of two key transaminases (ALT and AST) did not show notable difference. The ALT level was nearly similar in all four groups, whereas a notable but not significant increase of approx. 60% of the AST level in HFD and Iwong4 was observed compared to the control and Iwong8 (Fig 26).

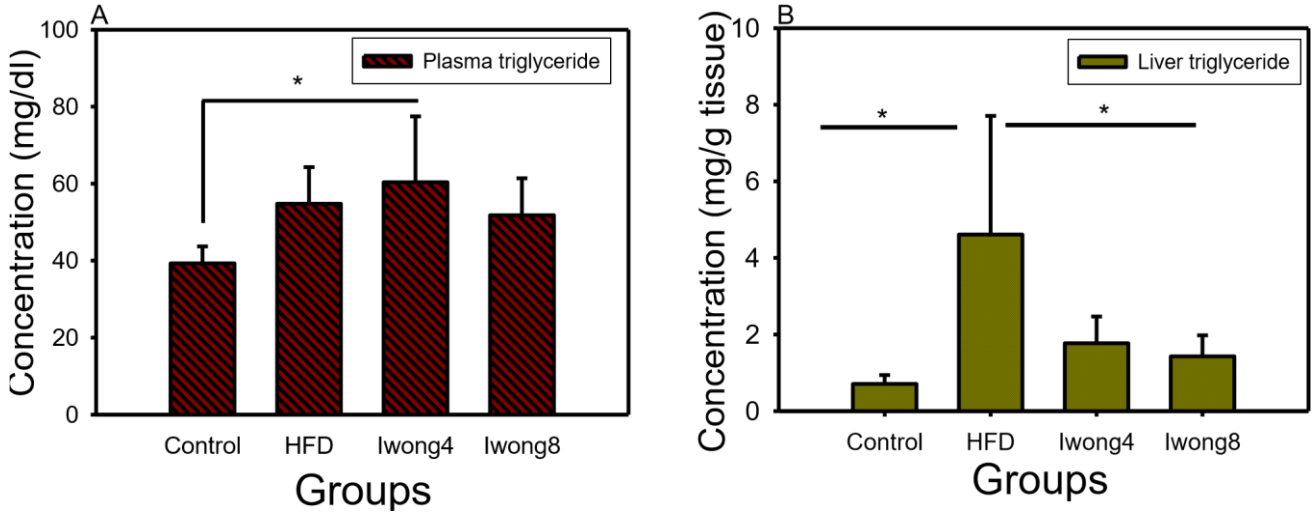


Fig. 24: Plasma and liver triglycerides level in obese and lean male C57BL6J mice treated or not with *Iwong* (n=7). Lean and obese animals were fed a standard diet (control), High fat diet (HFD) and High fat diet supplemented with 4 or 8% of the dried powder of *Iwong* (Iwong4 and Iwong8 respectively) for 6 weeks. Afterwards animals were killed, thus plasma blood and liver were used to quantify circulating TG and total TG content in the liver. *p<0.05 compared to the HFD.

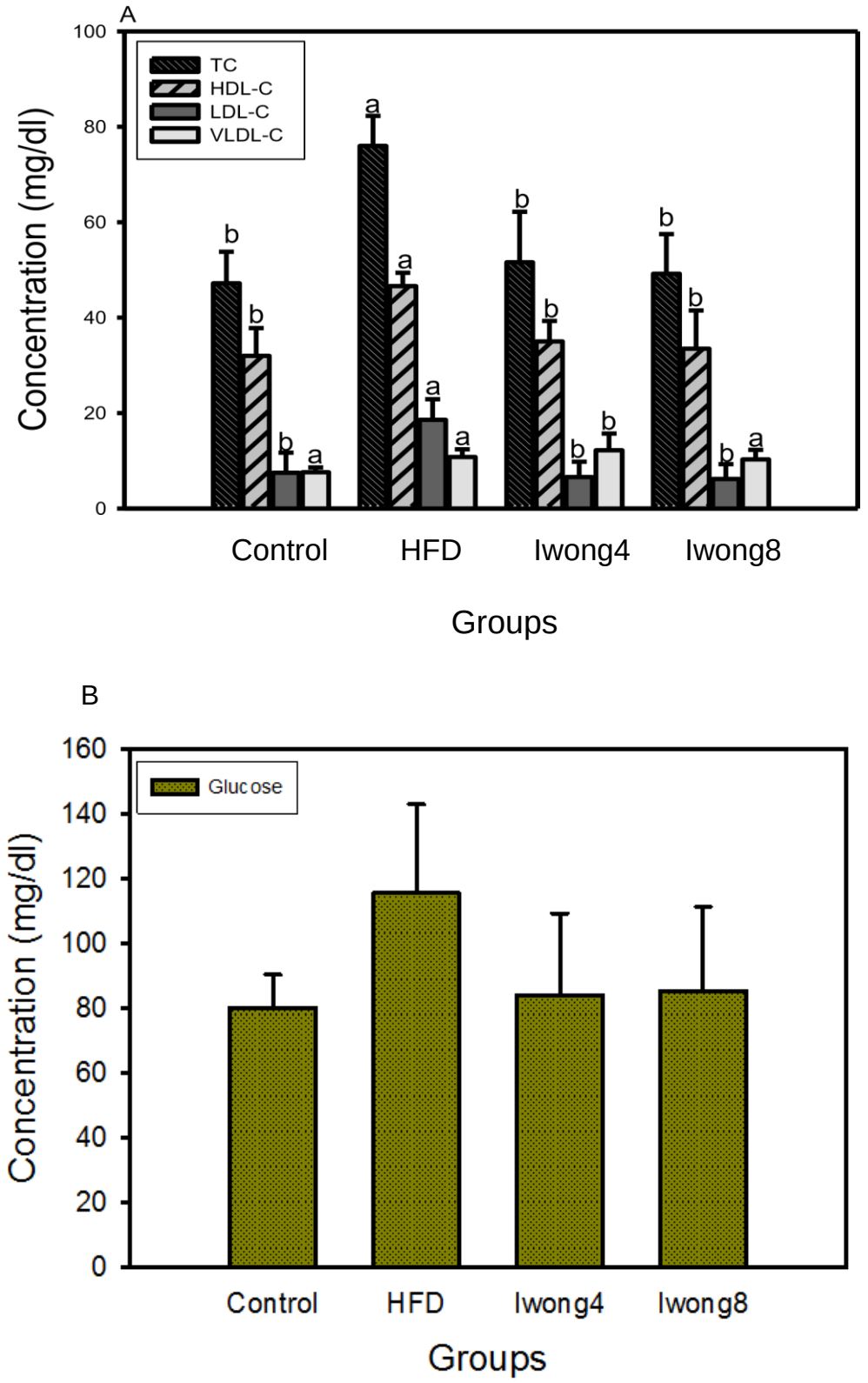


Fig. 25: Plasma cholesterol and glucose levels in obese and lean male C57BL6J mice treated or not with *Iwong* (n=7). After 6 weeks of feeding intervention period animals were killed and blood was collected. Plasma obtained after centrifugation was used to quantify the amount of total-cholesterol (TC), high density lipoprotein (HDL-C), low density lipoprotein cholesterol (LDL-C) and very low density lipoproteins cholesterol (VLDL-C) [**A**] as well as glucose level [**B**]. Date are represented as mean ± SD and (*) means. Values with unlike letters were significantly different (P<0·05).

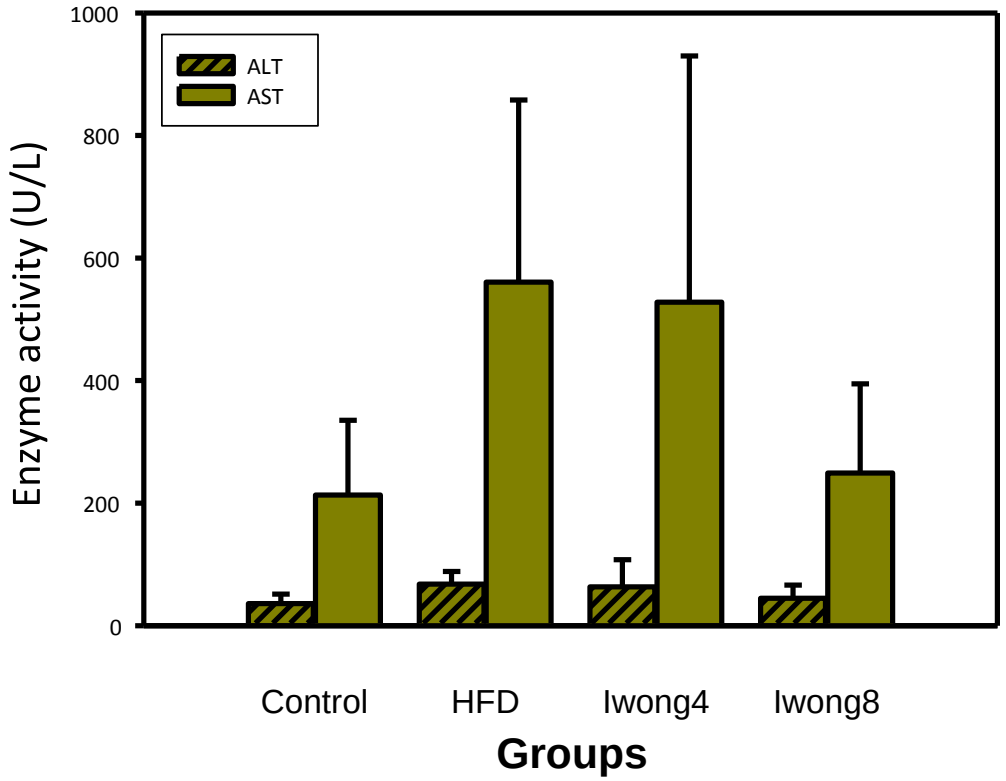


Fig. 26: Plasma aminotransferases activities in obese and lean male C57BL6J mice treated or not with *Iwong* (n=7). Animals were fed a standard diet (control), high fat diet (HFD) and high fat diet supplemented with 4 or 8% of the powder of *Iwong* (Iwong4 and Iwong8 respectively). After 6 weeks of feeding animals were killed, blood was collected and Alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

III-4-5) Histology of eWAT and BAT

Increase of body mass was associated with increased total fat mass in DIO mice compared to those fed a standard diet. We therefore performed histology of selected fat pads including brown adipose tissue (BAT) and white adipose tissue (epididymal) in order to assess lipid infiltration and changes in the size of adipocytes. Mice fed a HFD displayed hypertrophy compared to adipocytes of mice fed a standard diet suggesting that an increase in fat mass could be due to enlargement of adipocytes size and not to an increase in adipocyte number (Fig. 27). This effect on adipocyte size seems to be attenuated in mice treated with *Iwong* for 6 weeks. In the Iwong4 group, adipocytes were smaller compared to adipocytes from HFD group although the size remained larger when compared to those of the control group. Interestingly, the size of adipocytes of mice fed Iwong8 group was similar to those of control. Based on these macroscopic observations, *Iwong* seems to attenuate hypertrophy of adipocytes induced by high fat diet in a dose dependent manner. But these conclusion should be taken with caution, since there is no quantitative data to confirm this statement.

In addition infiltration of white adipocytes was observed in BAT of DIO, whereas this effect was abolished by *Iwong* feeding in both *Iwong* groups.

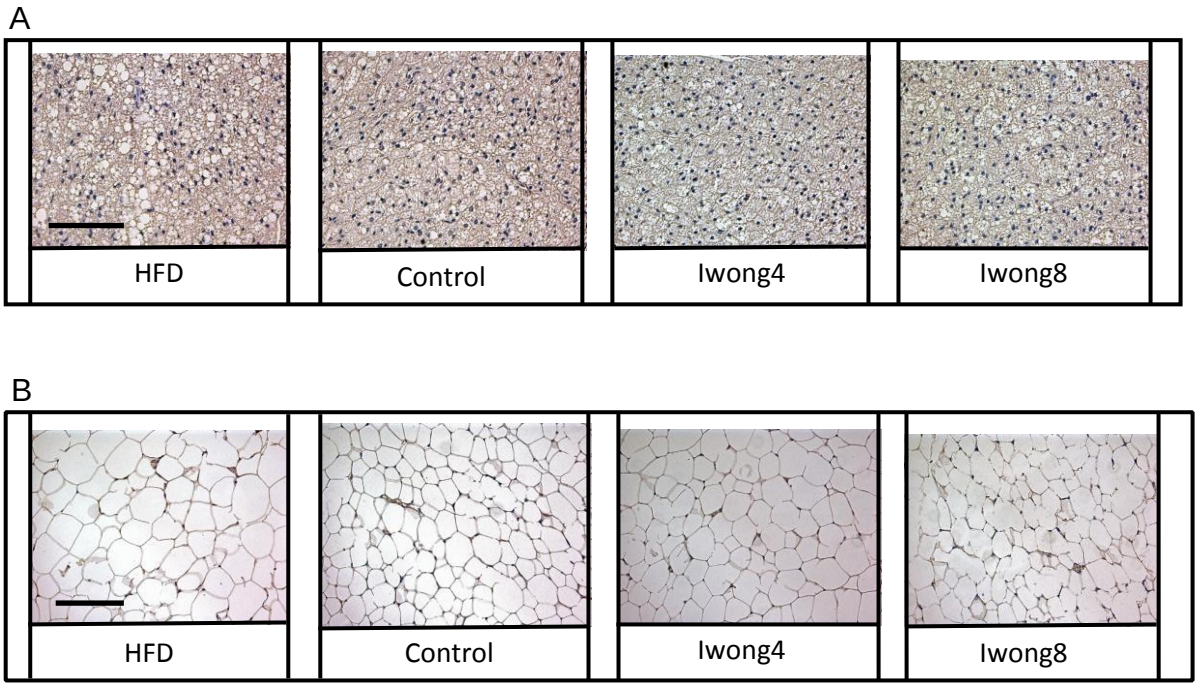


Fig. 27: Histology of BAT (A) and epididymal white adipose tissue (B) of treated and untreated mice (n=7). Animals were fed a High fat diet (HFD), standard diet (control), HFD supplemented with 4% or 8% of the powder of *Iwong* (Iwong4 and Iwong8 respectively). After 6 weeks of feeding mice were killed, epididymal white adipose tissue and BAT were dissected and fixed in 4% neutral-buffer formalin. Tissues were later paraffinized, embedded and stained with Hematoxylin-Eosin (H/E). Images of microscopic sections for each slide were taken and compared macroscopically in order to appreciate the morphological changes of adipocytes.

III-4-6) Rectal body temperature

Rectal body temperatures were recorded once per week during the first three weeks of treatment (Fig 28). During the first week of treatment, we noticed a slight increase in rectal body temperature in the HFD group (37.1°C) compared to the control group (36.5°C), whereas a minor decrease was observed in both Iwong4 and Iwong8 groups compared to the control group. A drop of body temperature was observed in all groups during the second week of treatment. Nevertheless, no significant difference was detectable between groups. The body temperature remained lower in all four groups after 3 weeks of dietary intervention compared to the first week of feeding. Taken together, these results demonstrated that there was no significant effect of diet and *Iwong* on rectal body temperature.

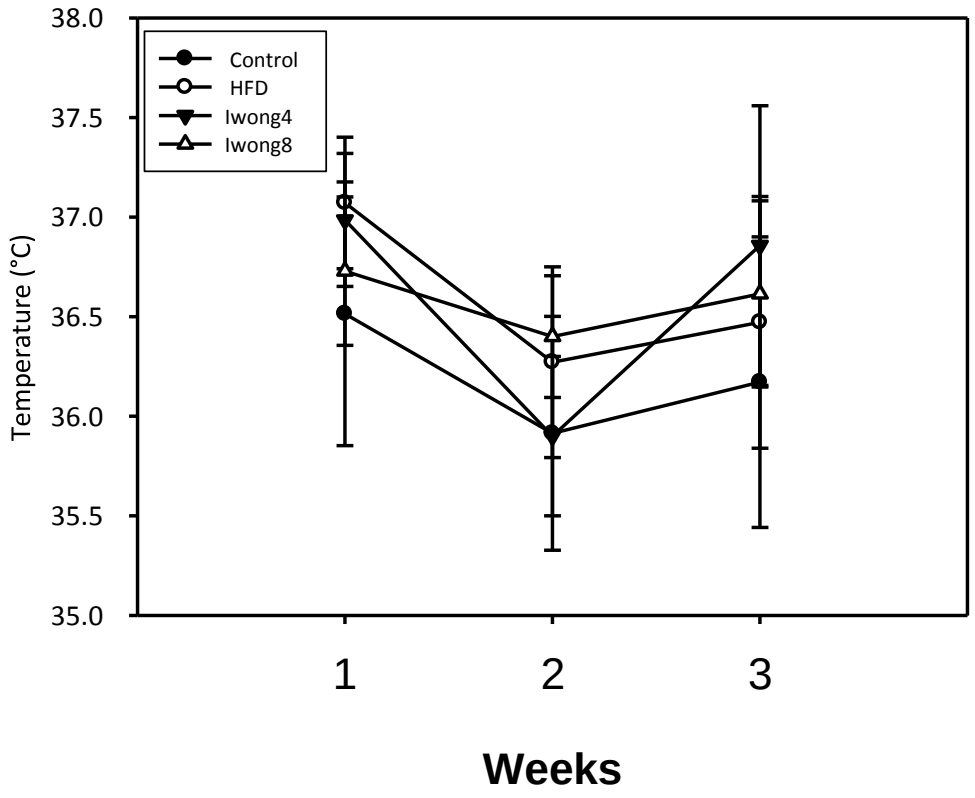


Fig. 28: **Evolution of body temperature during treatment of obesity.** C57BL/6J obese mice were randomized in 3 groups. The HFD group fed a high fat diet and two groups fed a HFD supplemented with 4 and 8% of the powder of *Iwong* (Iwong4 and Iwong8), additionally another group of lean mice was continuously fed a standard diet and was assumed as a control group. The rectal body temperature was recorded each week at the same time (around 2 pm) with an electronic thermometer. Results are shown as mean ± SD of 7 animals per group.

III-4-7) Plasma adiponectin, leptin and insulin

Plasma leptin and adiponectin concentrations from mice killed after 9 weeks of *Iwong* feeding are depicted in Fig. 29A. The plasma leptin level was significantly increased in the HFD; this elevation of leptin was strongly reduced in mice treated with *Iwong*. No differences were found in the adiponectin circulating concentration either in HFD or in *Iwong* groups compared to the control.

We noticed a slight but not significant increase of insulin concentration in mice fed a HFD that was absent in the *Iwong* groups. (Fig. 29B).

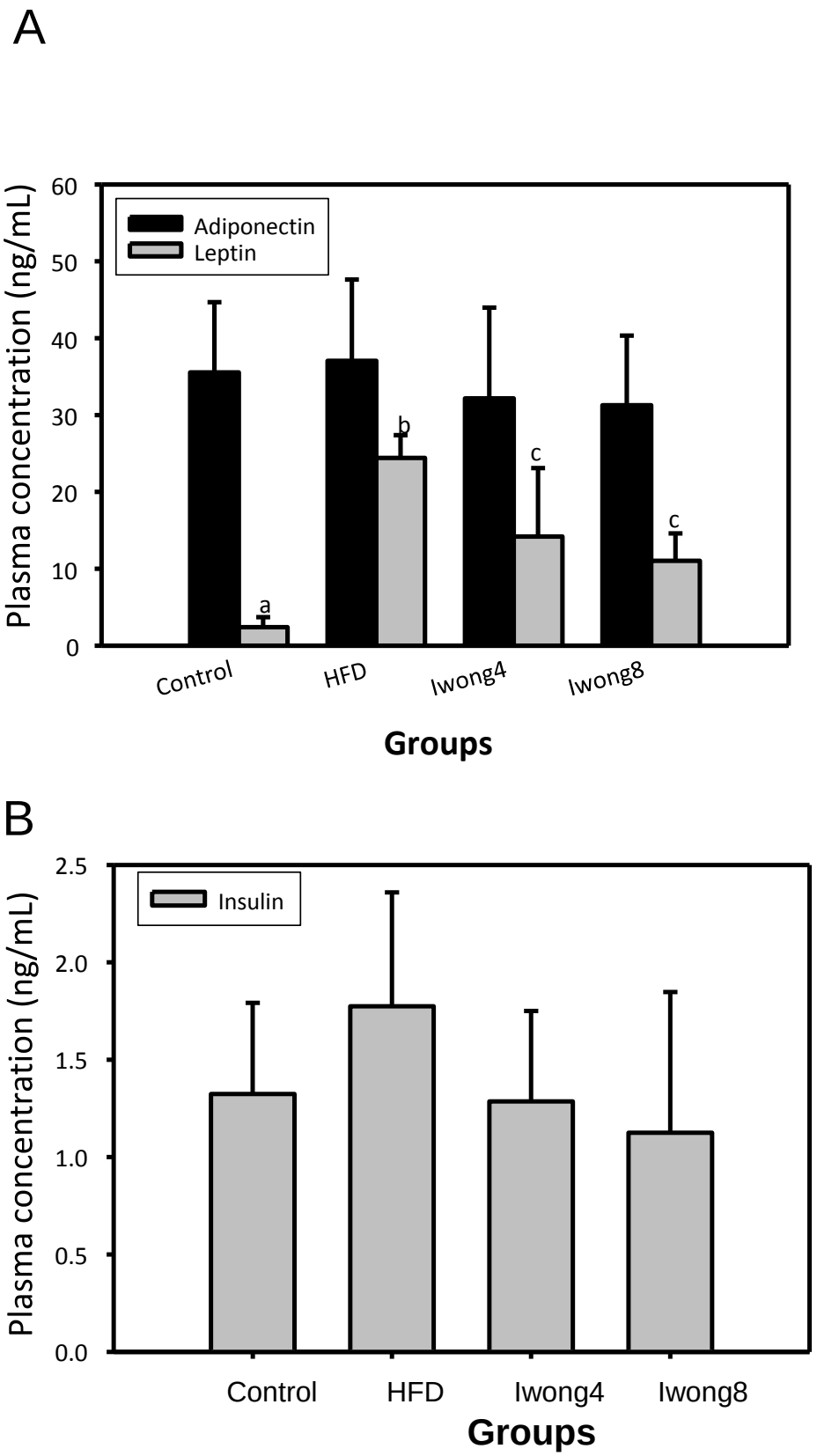


Fig. 29: Plasma hormone levels in obese mice (treated *or not* with *Iwong*) and in lean mice (n=7). Obese mice and lean mice aged 9 weeks were fed a HFD, a HFD supplemented with 4% and 8% of the powder of *Iwong* (Iwong4 and Iwong8 respectively) or standard diet. After 6 weeks of dietary experimental period, mice were killed and blood trunk was collected and centrifuged. (A) Plasma adiponectin and leptin levels, (B) plasma insulin level were measured by ELISA assay. Values with different letters are considered as significantly different with $p<0.05$ compared to the control.

III-5) Evaluation of the cytotoxic effect of the ethanolic extract of *Ipomoea alba* (*Iwong*) and Resveratrol on 3T3L1 cells.

To assess the toxicity of the ethanolic extract of *Iwong*, 3T3L1 preadipocytes were treated either with DMSO 0.1% or with various concentrations of *Iwong* ethanolic extracts. Three different time points were chosen from day 0 to 3 or day 5 to day 8 and from day 0 to day 8 of the differentiation process.

Results of the cytoxicity effect of *Iwong* are confined in fig. 30, *Iwong* had no cytotoxic effect at the concentration tested after 3 days of treatment, despite a trend increase of cytotoxity observed for cells treated either with DMSO or with various concentrations of the ethanolic extract of *Iwong* during this period.

During the intermediate phase of differentiation (day 5) a dose dependent increase of the total number of dead cells was noticed and reached significance at a concentration of 300µg/mL. This was confirmed in cells treated from day 0 to 9 with the ethanolic extract of *Iwong,* the tendency observed for the intermediate phase of the treatment was confirmed and tend to be more pronounced with a significant difference reached at the concentrations 200 and 300µg/mL.

Opposite results were obtained with Resveratrol (Fig 31) A significant increase on cytotoxicity was noticed after 3 days of the treatment ($p<0.05$), whereas at the intermediate (day 5) and late (day 8) phase of treatment a significant decrease of cytotoxicity was observed on all cells treated with Resveratrol ($p<0.05$).

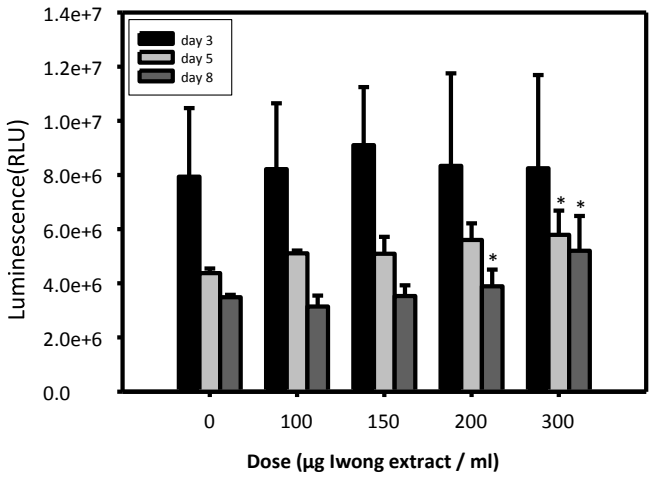


Fig. 30 : ***Cytotoxicity of ethanolic extract of Iwong*** Two-days post confluent 3T3-L1 cells were differentiated according to the MDI protocol in the presence or the absence of 100µg/ml 150µg/ml 200µg/ml and 300µg/ml of *Iwong*, the cytotoxicity of the extract was determined after 3, 5 and 8 days of incubation. Data shown are mean values of 3 wells ± SD of 3 independent experiments. *$p<0.05$ as compared with DMSO 0.1% treated cells. **(**Adapted from[150])

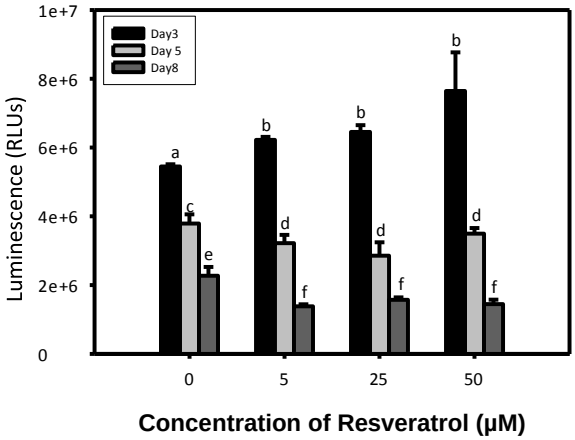


Fig. 31: ***Cytotoxicity of Resveratrol.*** Two-days post confluent 3T3-L1 cells were differentiated according to the MDI protocol in the presence or the absence of 5, 25 and 50 µM of reveratrol, the cytotoxicity of reveratrol was determined after 3, 5 and 8 days of incubation. Data shown are mean values of 3 wells ± SD of 3 independent experiments. *p<0.05 as compared with DMSO 0.1% treated cells

III-6) Effect of the ethanolic extract of *Iwong and* Resveratrol on adipogenesis

II-6-1) Oil red O staining (ORO)

Pictures obtained from 3T3L1 cells treated for 9 days and stained by ORO dye (which indicates the intracellular lipid accumulation) were used for macroscopic analysis of the degree of differentiation. Cells treated with DMSO showed a higher intensity of staining compared to those treated with 200 and 300µg/mL of *Iwong* (fig 32). Since the intensity of the coloration is positively correlated with the amount of accumulated lipids, we can therefore assume the ethanolic extract of *Iwong* reduced lipid accumulation.

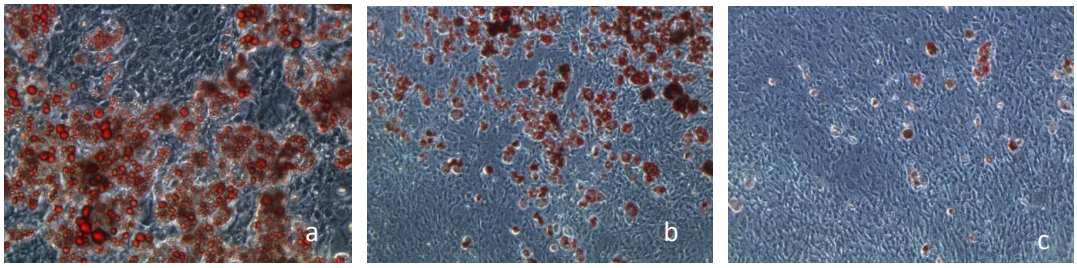


Fig. 32: ***Microscopic images of the effect of ethanolic extract of Iwong on lipid accumulation.*** Two days post confluent 3T3-L1 preadipocytes were differentiated with MDI in the presence either of several concentrations of the ethanolic extracts of *Iwong* or DMSO 0.1%. After 8 days cells were stained with Oil Red O, and photographed at magnitude x100, a) DMSO 0.1%, b) 200µg/ml, c) 300µg/ml. Figure adapted from Mengue et al [150]

II-6-2) Lipid quantification

For the quantitative analysis of neutral lipid content, cells were treated for 9 days either with DMSO or with the ethanolic extract of *Iwong* and stained with ORO, which was removed and read by the absorbance at 492nm. The amount of lipid is related with

absorbance. Cells treated with DMSO and 100µg/mL of *Iwong* showed a higher degree of absorbance, whereas a dose dependent decrease of the absorbance was observed from 150 µg/ml and reached the maximum effect at 300µg/mL (Fig. 33, $p<0.05$).

Resveratrol, a flavonoid known for its antiadipogenic effect was used as positive control. Post confluent 3T3-L1 preadipocytes were differentiated for 9 days in the presence of DMSO 0.2% or various concentrations of Resveratrol (5µM, 25µM and 50µM) and the amount of lipid accumulation was assessed. As depicted in Fig. 34, there was a dose dependent inhibitory effect on lipid accumulation in cells treated with Resveratrol. The optical density was significantly lower at 5µM ($p<0.05$) and more pronounced with 25µM and 50µM ($p<0.001$)

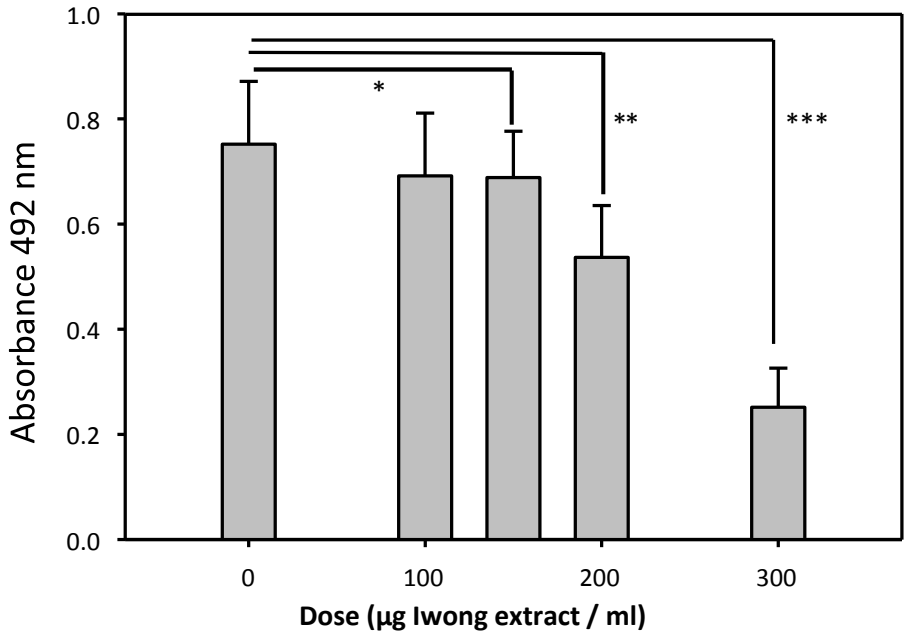


Fig. 33: ***Effect of ethanolic extract of Iwong on lipid accumulation*** Two days post confluent 3T3-L1 preadipocytes were differentiated with MDI in the presence either of several concentrations of the ethanolic extracts of *Iwong* or DMSO 0.1%. At days 9 of differentiation cells were stained by ORO, which was removed by isopropanol for lipid quantification. The amount of lipid was read spectrophotometrically at 492 nm. Each experiment has been done in triplicate and repeated at least 3 times. Data are expressed as means ± SD. *p<0.05, **p<0.01 and ***p<0.001 [150]

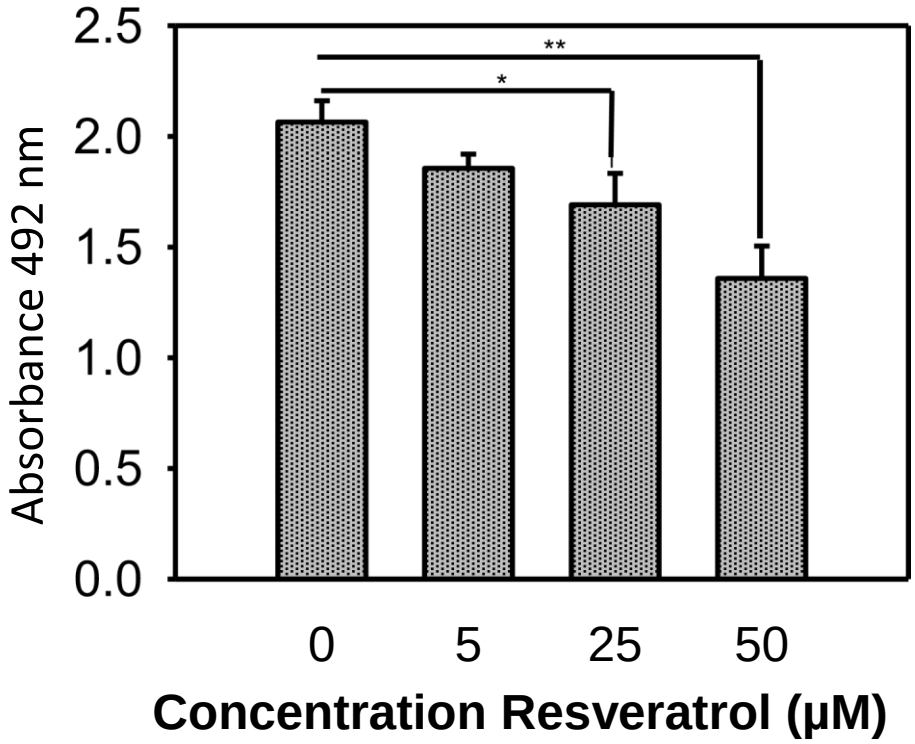


Fig. 34: ***Effect of Resveratrol on lipid accumulation*** Two days postconfluent 3T3-L1 preadipocytes were differentiated with MDI in the presence of various concentrations of Resveratrol 5µM, 25µM and 50µM and with DMSO 0.2%. At day 9 of differentiation cells were stained by ORO, which was removed by isopropanol for lipid quantification. The amount of lipid was read spectrophotometrically at 492 nm. Each experiment has been done in triplicate and repeated at least 3 times. Data are expressed as means ± SD. [150]

II-6-3) Time-dependency effect of *Iwong*

Due to the increased of cytotoxicity with the duration of the treatment, we aimed to determine at which stage of the differentiation process 3T3-L1 cells are most sensitive to the treatment of *Iwong*. We therefore divided the process of differentiation in 3 phases, the early (0-2 days), the intermediate (3-5 days) and the late (6-8 days).

Treatment of 3T3-L1 preadipocytes during different phases of adipogenesis proved that the inhibitory activity of *Iwong* is restricted to the early (day 0-2) phase of differentiation. In fact the presence of *Iwong* during the first three days of differentiation significantly reduced lipid accumulation ($p<0.05$) to the same extent as a chronic treatment (day 0-8). Notably, *Iwong* exposure during intermediate and late phases of adipogenesis had no effect on lipid accumulation (Fig. 35).

II-6-4) GPDH specific activity

GPDH occupies a central position in the pathway of triglyceride synthesis and is commonly used as a marker of adipogenesis. The effect of the ethanolic extract of *Iwong*

on the GPDH activity was thus tested. *Iwong* significantly reduced the GPDH activity in a dose dependent manner, i. e. *Iwong* at the concentration 150 µg/mL ($P<0.05$) this inhibition which was more pronounced with 200 µg/mL ($p<0.005$) reached the maximum effect at 300 µg/mL ($p<0.001$) (Fig 36).

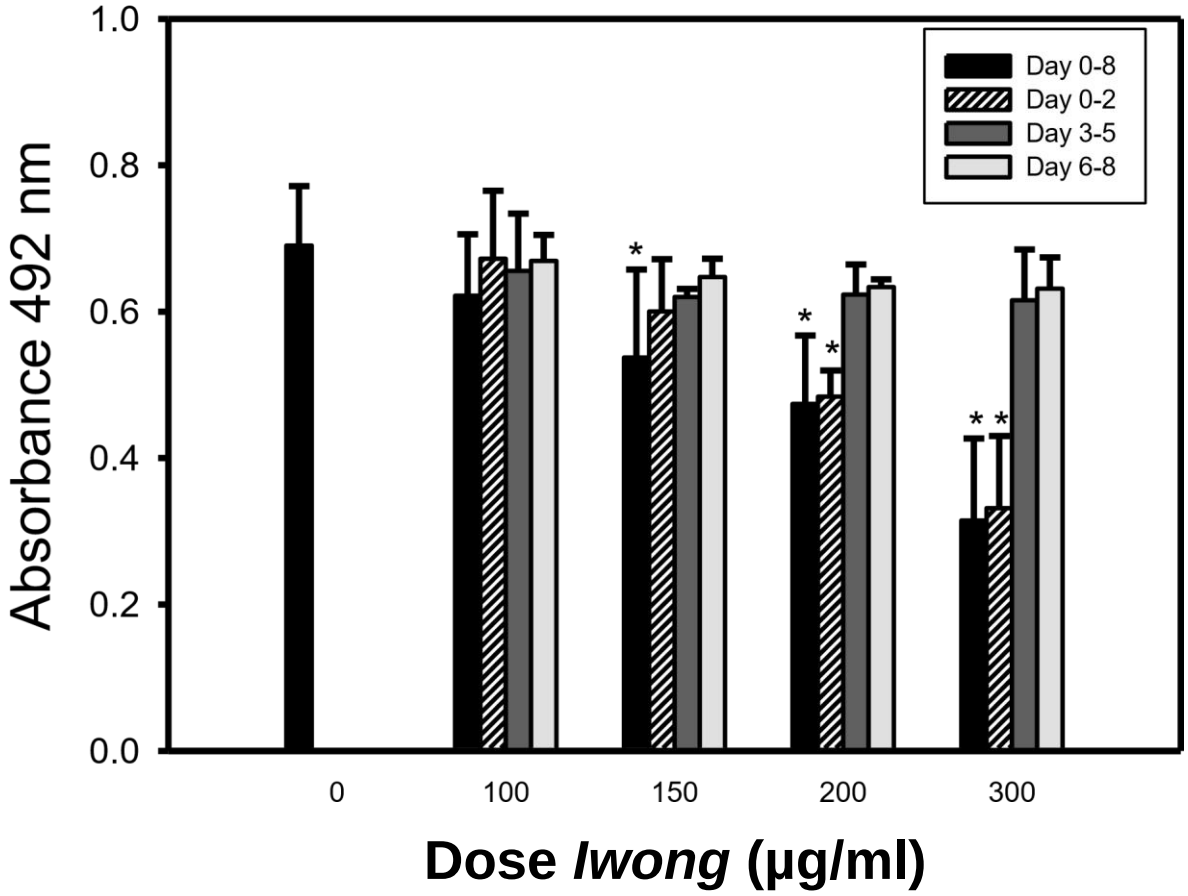


Fig. 35: ***Effect of ethanolic extract of Iwong on lipid accumulation in 3T3-L1 cells in different stages of differentiation*** 3T3-L1 preadipocytes were proliferated for two days, the differentiation of post-confluent preadipocytes was induced by standard adipogenic medium to initiate adipogenesis in the presence of DMSO 0.1 % or 100, 150,200 and 300 µg/ml of *Iwong.* The ethanolic extract of *Iwong* was added at different stages of the differentiation process from day 0 to 2 (early stage), 3 to 5 (intermediate stage), 6 to 8 (late stage) and 0 to 8 (chronic treatment). Data are expressed as means (n =3) ± SD *p <0.05. [150]

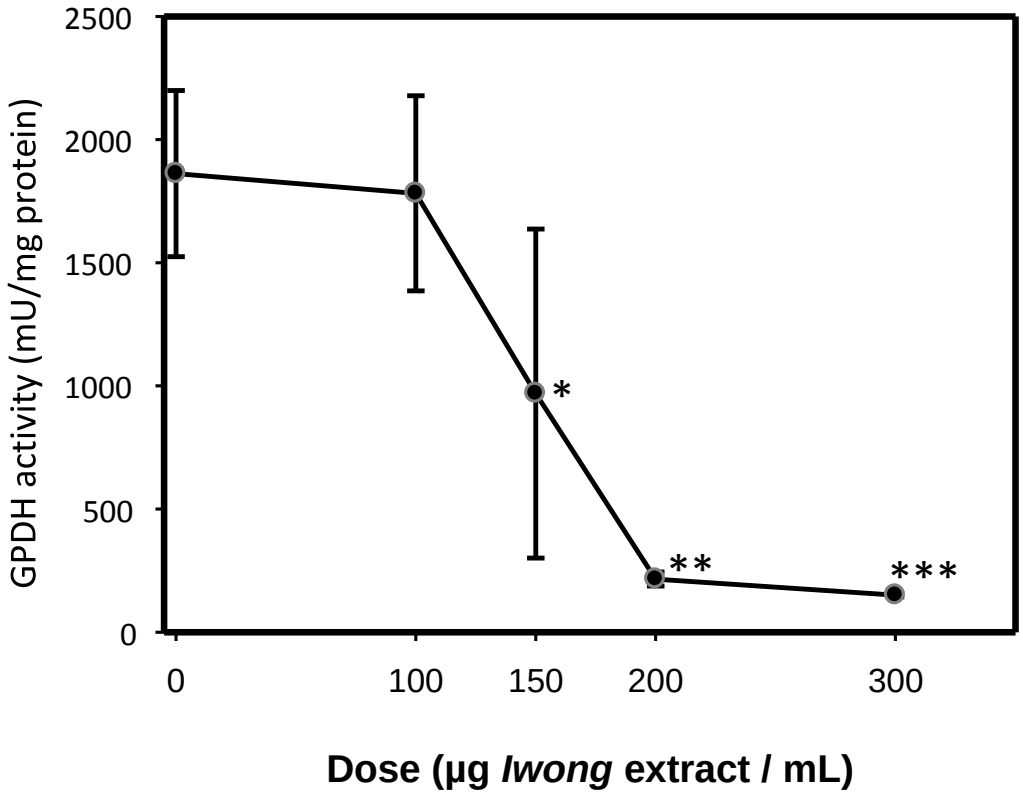


Fig. 36: Effect of *Iwong* on GPDH activity 3T3-L1 preadipocytes were proliferated and reached 80% to 90 % confluence after 2 days. All cells were then treated by a specific adipogenic cocktail for 9 days enriched either with DMSO 0.1% or different concentrations of *Iwong* ethanolic extract (100 to 300µg/ml). At the last day of differentiation cells were harvested and the GDPH activity was measured and normalized by proteins concentration. Results expressed as mU activity/mg proteins are means ± SD of 3 independent experiments did in triplicate. *p<0.05 [150]

III-6-5) Effect of *Iwong* on lipolysis

To assess whether the ethanolic extract of *Iwong* can stimulate lipolysis in differentiated adipocytes, 3T3-L1 preadipocytes were differentiated for 14 days and then treated in the presence of *Iwong* (200 and 300µg/ml), isoproterenol (1µM) or DMSO (0.1%) for 24h. Free glycerol released into the medium was quantified. As summarized in Fig 37, the ethanolic extract of *Iwong* had no effect on glycerol release, whereas isoproterenol (1µM) used as positive control stimulated glycerol release.

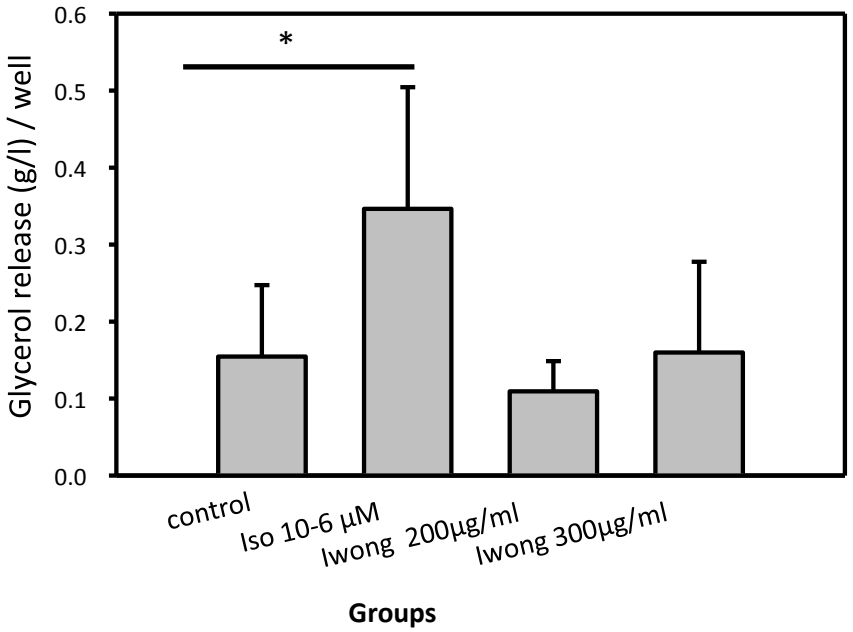


Fig. 37: Effect of ethanolic extract of *Iwong* on glycerol release 3T3-L1 cells were differentiated for 14 days, and then treated for 24h with DMSO 0.1%, isoproterenol (Iso) 10^6µM, 200 and 300µg/ml of *Iwong.* The amount of glycerol release in the medium was determined by the spectrophotometer at 340nm. Each experiment has been done in triplicate and repeated at least 3 times. Data are expressed as means ± SD. *P<0.05 [150]

III-6-6) Effect of the ethanolic extract of Iwong on the expression of key adipogenic transcription factors

To obtain first insight to the molecular mechanism of *Iwong* action, the transcript levels of key adipogenic transcription factors were assessed in 3T3-L1 preadipocytes exposed to *Iwong*. These experiments were conducted with fresh *Iwong* extracts from the second batch of plant material with a high anti-adipogenic activity (See ORO staining procedure in material and methods section), which showed a maximum with the concentration 75 µg/ml. Thus concentrations in the range of 25 –75 µg/ml were chosen.

PPARγ and C/EBPα are two key transcription factors that are expressed and increased during the differentiation process; therefore they are widely used as marker of adipogenesis.

A dose dependent down regulation of the expression of PPARγ and C/EBPα was noticed and reached significance at 50µg/mL and 75µg/mL ($p<0.05$) (Fig. 38).

Taken together *Iwong* inhibited lipid accumulation by down regulating the expression of key transcription factors involved in the process of adipogenesis

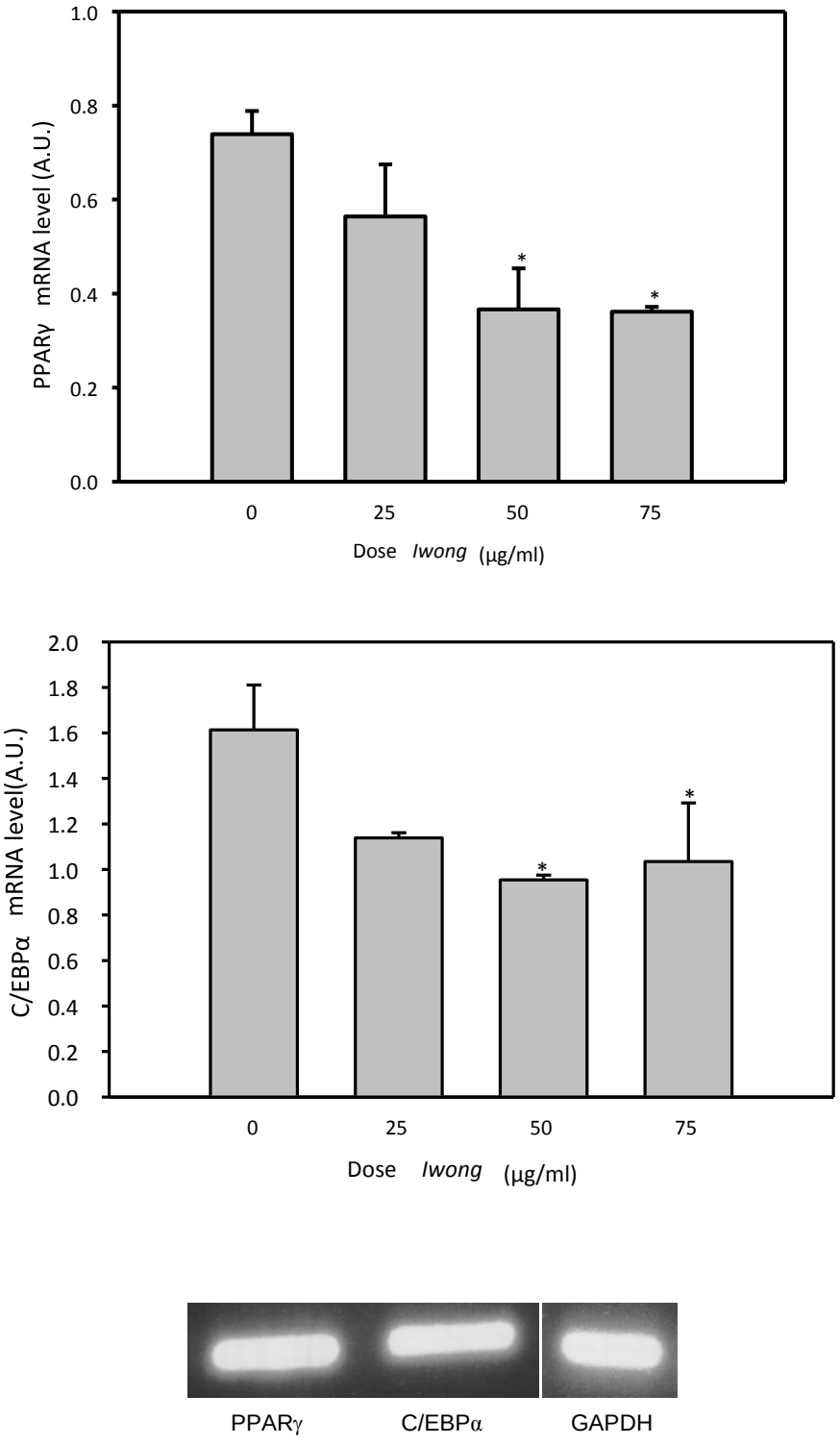


Fig. 38: ***Effect of Ethanolic extract of Iwong on the mRNA level of two key transcription factors during 3T3L1 cells differentiation***. Post confluent 3T3-L1 preadipocytes were treated with DMSO 0.1% or different concentrations of *Iwong* (25-75µg/ml) from day 0 to day 2 and with insulin only from day 3 to day 8. Cells were harvested at day 8. The abundance of mRNA of PPARγ, C/EBPα and GAPDH was measured by quantitative PCR (qPCR). Values are normalized by GAPDH and expressed in arbitrary unit (U.A.) are mean ± S.D. of three different experiments of PPARγ and C/EBPα (*P<0.05). Published by Mengue et al [150]

III-7) Effect of different fractions of *Iwong* on cytotoxicity and lipid accumulation

III-7-1) Cytotoxicity

The cytotoxicity effect of 3 fractions of *Iwong* was assessed at 2 different time-points: day 3 and day 9 of differentiation. Results for cells treated only for the 3 first days of differentiation showed that acetylacetate and water fractions were not harmful at the concentration used for this experiment, i.e. 5 to 15µg/ml for acetylacetate fraction and 10 to 50µg/ml for water fraction. On the contrary to the two others fractions, the diethylether fraction was highly toxic. Therefore cells treated with gradual concentration from 15µg/ml of this fraction killed all cells after 3 days (Fig. 39A).

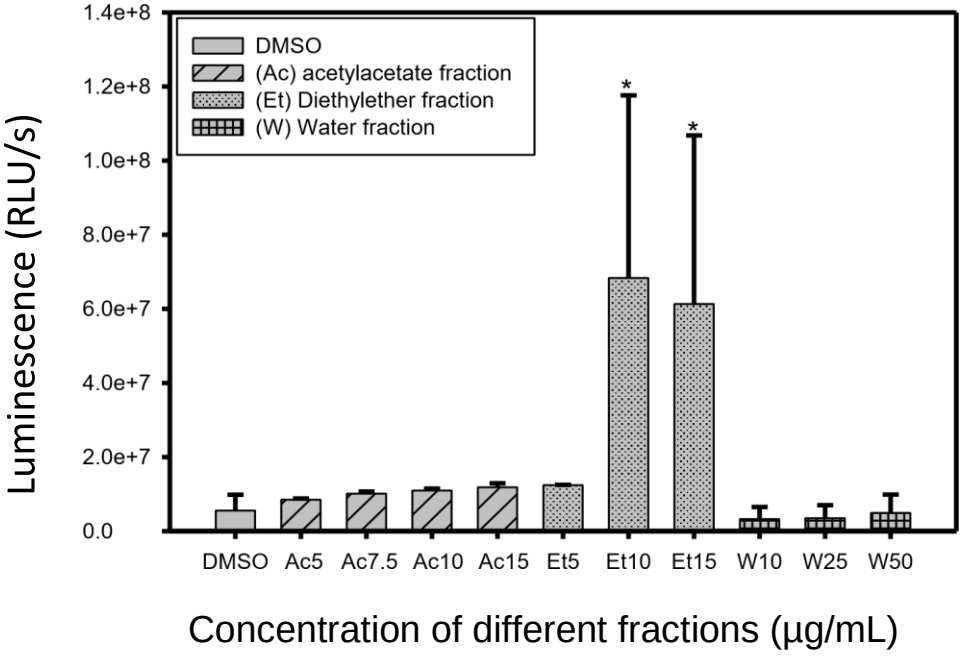
A chronic treatment (9 days) with the same fractions and at identical concentrations as for the short term treatment (3 days) was more harmful. Therefore for all concentrations used with the acetylacetate and diethylether fractions, the cytotoxicity was significantly increased compared to cells treated with DMSO only. In addition despite a slight dose dependent increased of cytotoxicity observed after 9 days of treatment, water fraction was not harmful for cells (Fig. 39B).

III-7-2) Effect of three fractions of *Iwong* ethanolic extract on lipid accumulation

The effect of 3 fractions prepared from the ethanolic extract of *Iwong* on adipogenesis was tested in 3T3L1 adipocytes. Post confluent preadipocytes were induced to differentiate for 9 days, but as demonstrated in cytotoxicity test, cells exposed to various concentrations of each fraction during the early phase (3 first days) of differentiation was not harmful compared to a chronic treatment.

We treated cells during the 3 first days of differentiation. Results of lipid quantification as shown in Fig 40 after ORO staining, demonstrated that at concentrations used, the water and diethylether fractions had no effect on lipid accumulation. A dose dependent decrease on lipid accumulation was observed with the acetylacetate fraction. In fact a slight but not significant inhibition on lipid accumulation was observed with the concentration 5µg/ml and this effect reached significance with at 10 and 15µg/ml ($p<0.05$). Taken together these results suggest that the active fraction of the ethanolic extract of *Iwong* could be the acetylacetate fraction.

A



B

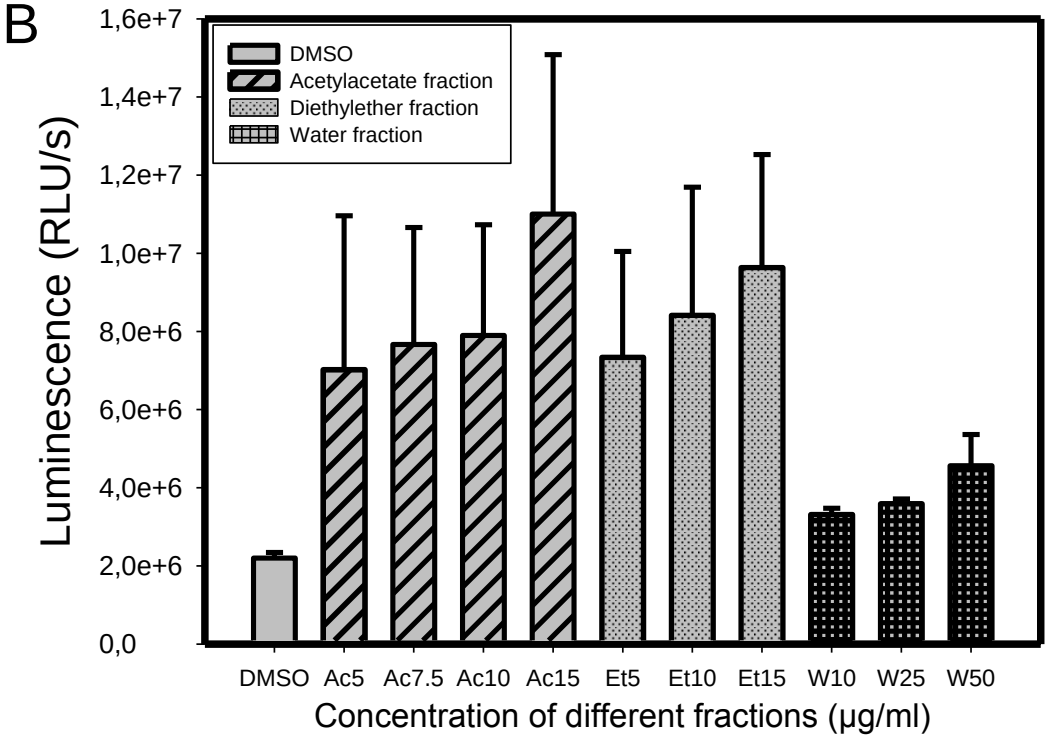


Fig. 39: **Cytotoxicity of different fractions after 3 and 9 days of treatment**. Three different fractions generated from the ethanolic extract of *Iwong* were used and their cytotoxicic effect was evaluated on 3T3-L1. For that purpose preadipocytes were seeded and allowed to proliferate for 2 or 3 days until they reach 90% confluence. Cells were treated during the 3 first days of differentiation with DMSO 0.1% or several concentrations, afterwards the cytotoxicity was tested (Fig 1A). Additionally cells were differentiated for 9 days in the presence of DMSO 0.1% or various concentrations of fractions, at the end of differentiation phase the cytotoxicity was also tested (Fig1B). The concentrations of fractions used were, acetylacetate fraction (5 to15 µg/ml), diethylether fraction (5 to15 µg/ml) and water fraction (10 to 50mg/ml). Results are expressed as means ± SD of 3 independent experiments done in triplicate. *P<0.05

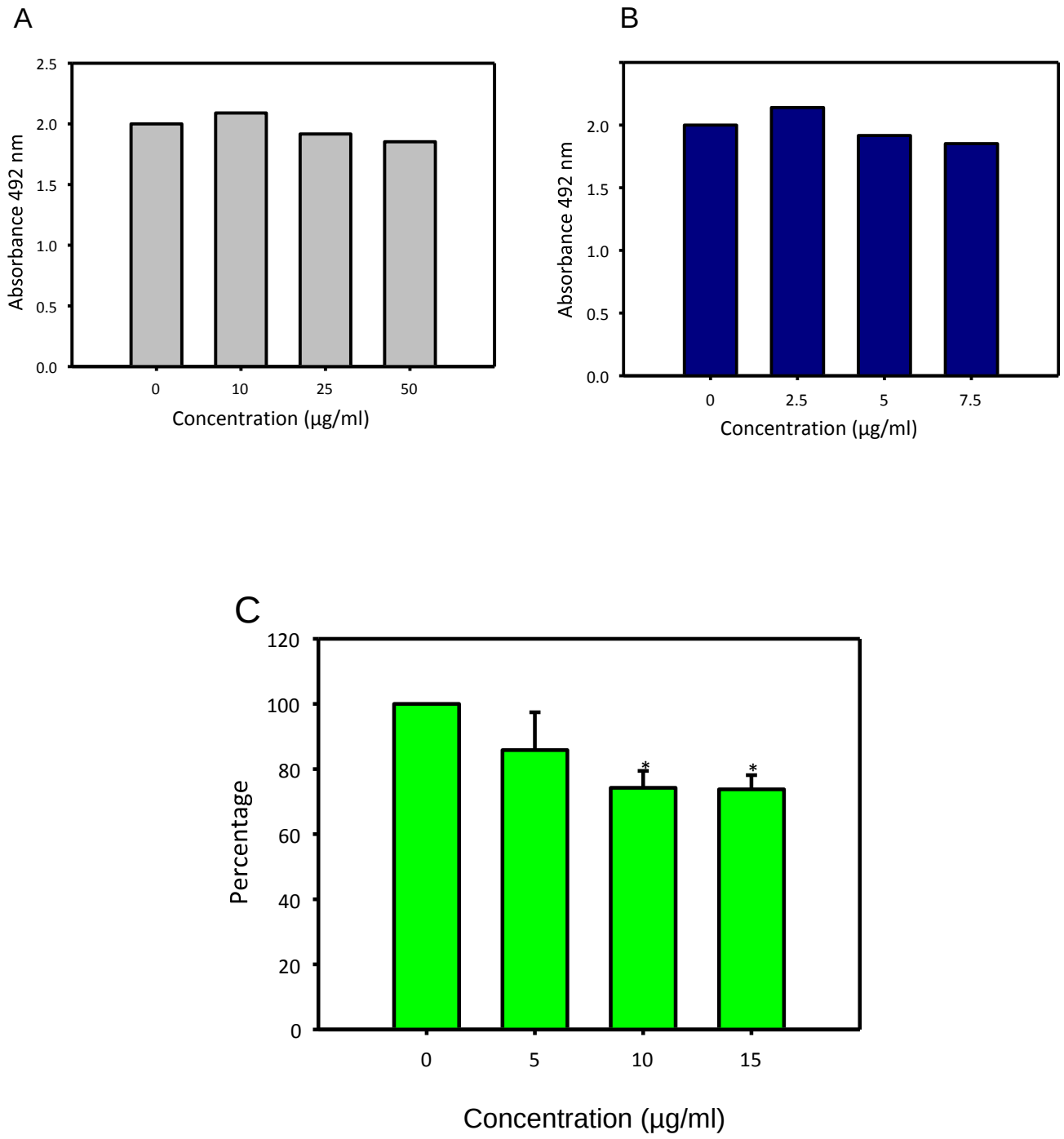


Fig. 40: Effect of three different fractions of *Iwong* on lipid accumulation. 3T3-L1 preadipocytes were induced to differentiate and treated only during the 3 first days of differentiation either with DMSO 0.01% or with several concentrations of fractions, acetylacetate fraction (5 to 15 µg/ml), diethylether fraction (5 to 15 µg/ml) and water fraction (10 to 50mg/ml). Afterwards medium was changed and switched to the standard adipogenic cocktail supplemented with 0.1% DMSO until the end of 9 days differentiation phase. Cells were stained and lipids were quantified as described above by ORO. Results are presented as means ± SD of different fractions, (**A**) water fraction, (**B**) diethylether fraction and (**C**) acetylacetate fraction

IV-)
DISCUSSION
AND
CONCLUSION

IV-1) Discussion

Obesity remains one of the major health problems in the world. A modern strategy to manage overweight and obese patients include lifestyle change, diet and increase of physical activity [151]. For obese subjects with a BMI ≥ 35 kg/m^2, the surgical intervention is an excellent option, but the high cost of surgery, the postoperative complications and anatomic distortion might not make this an universal treatment [152-154]. Pharmacological treatment is then the most important strategy for suitable body weight loss and normalization of metabolic disorders, and appears to be the only available method to address the problem on a huge scale[155]. Despite efforts made to discover an efficient treatment against this pandemic, only few drugs are currently used [156, 157]. Unfortunately, obesity treatment drugs have low rate effect and can show severe side effects [158]. The need of an effective therapeutic approach for the treatment of obesity remains crucial. Currently obesity remedies based on dietary supplements are popular, suggesting that, phytotherapy can be used as strategy to manage obesity [159, 160]. Since scientific studies of medicinal plants are a source of new drug discoveries in obesity treatment and prevention [35, 142, 161], ethnopharmacological approaches have increased over the last decades. Pharmacotherapy is now considered as a an important scientific tool in the selection of new plant candidate for *in vitro* and *in vivo* studies to evaluate the efficacy, safety and quality of anti-obesity pharmacological actions[51, 129, 162-164]. In the present study, the effects of *Iwong* powder prepared from dried plants of *Ipomoea alba* had been therefore assessed, on body weight development and metabolic parameters in conventional mice and rats. Furthermore, the effects of *Iwong* ethanolic extracts on adipogenesis in 3T3L1 cells were also evaluated.

IV-1-1) Effect of *Iwong* feeding on lean rats and mice

The efficacy of a new treatment against obesity can be assessed by the effect on body weight and considered successful if it prevents further weight gain [165]. Obesity is a state of excess adiposity which is the consequence of sustained positive energy balance over time. Several factors interact in a

complex way to influence eating and drinking patterns as well as activity behaviors [166]. The proposed mode of action for the therapeutic treatment of obesity is the control of appetite, satiety, energy homeostasis and body weight evolution [167]. Food intake, body weight, energy intake and energy expenditure were recorded in this first part of the study. Feeding *Iwong* in both mice and rats was associated with a reduction in body weight gain and total fat mass, despite an increase in energy intake. One explanation for this weight loss effect could be a decrease in dietary energy absorption. Hence, the effect of *Iwong* on food digestibility in mice was assessed. Results revealed a higher energy content in feces from mice in both *Iwong* groups, and consequently a significant lowering of the assimilation efficiency in mice fed *Iwong*. Obesity is due to imbalance in energy homeostasis, in which the consequences of excessive food intake are not balanced by increased energy expenditure[1]. The first law of thermodynamics stipulates that body weight cannot change if, over a specified time, energy intake and energy expenditure are equal [168]. Thus results as obtained demonstrate that *Iwong* reduced body weight probably by affecting energy balance.

Although lean animals were used for this first part of the study, the influence of *Iwong* feeding on blood lipid and glucose profiles was after all checked. In fact, additionally to the effect on body weight and fat mass, anti-obesity drugs should also focus on the improvement of dyslipidemia, which often appears in obese subject [169]. In general, obesity accompanying with dyslipidemia consists of increased TGs and FFAs, decreased HDL-C with HDL dysfunction and normal or slightly increased LDL-C with increased small dense LDL [86]. The plasma chemical analysis showed a lower level of LDL-C in mice and no difference in rats fed a standard diet supplemented with *Iwong.* This effect on LDL-C was associated with an increase in HDL-C in both mice and rats that was strongest in the *Iwong* 10% groups. Since it has long been known that the level of HDL-C is negatively correlated with increased cardiovascular risk, whereas this risk is increased with the level of LDL-C; Results as obtained suggest that, *Iwong* might protect against the cardiovascular risk associated with obesity. More studies are then needed in obese model with metabolic disorders to confirm this observation.

The liver is the most important organ for protein production and detoxification, both of which are facilitated by a specific enzyme. The quantification of

enzymes released from hepatocytes and proteins produced by the liver and released into the blood can be used to analyze liver health [170]. Hepatocellular damage results in changed cell membrane permeability and as consequence an excessive outflow of transaminases. Amount of them ALT and AST are considered as good markers of damage of hepatocyte and thus commonly as liver function test [171],[172]. A slight alteration of circulating plasma levels of both ALT and AST as observed in mice fed a standard diet supplemented with *Iwong* powder might thus indicate damage of hepatocytes, although macroscopic structure and weight of the liver remained unchanged.
Taken together all these results demonstrated that *Iwong* feeding reduced body weight gain and fat mass without unwanted side effects on biochemical parameters.

IV-1-2) Prevention of obesity

Observations in lean mice and rats showed a potential benefit effect of *Iwong* feeding on body weight, fat mass and biochemical parameters. These results have to be considered with caution, while animals used in this study were lean mice and rats. Nevertheless, these findings constitute a solid base for more investigations in obese animals. A study in mice fed a HFD in order to evaluate the preventive effect of *Iwong* feeding against obesity was therefore conducted.
Obesity is always associated with an increase in body mass due to accumulation of fat in the body. Therefore, substances with an anti-obesity effect should be able to either prevent fat accumulation or reduce fat mass in obese subjects. The effect of *Iwong* feeding was assessed on both, body weight and total fat mass as well as on food intake. The results showed an increase in food intake and a dose dependent reduction in body weight and total fat mass after 5 weeks of dietary intervention period. Comparing *Iwong* with natural compound or medicinal plants, EGCG supplementation for instance prevents body weight gain and fat mass accumulation in mice without changes on food intake [148]. A study of Han et al. showed similar results on prevention of body weight gain and decreased of adipose tissue depots, but also differs in food consumption compared with *Iwong* supplementation [48]. To our knowledge this finding on food intake has not

been reported for any medicinal plants with anti-obesity activity; *Iwong* seems to be a rare example of a plant which provokes an increase in food intake with a reduction in body weight gain and total fat mass. One possible explanation for this effect on food intake could be compensation of the lower assimilation efficiency as well as of the increase energy amount excreted in feces as found in NMRI mice and confirmed in C57BL/6J mice. More studies are needed in order to elucidate the mode of action by which *Iwong* provokes hyperphagia.

HFD feeding either is known to increase adiposity by hyperplasia (increased total number of adipocytes) or by hypertrophy (increased size of adipocytes). Treatment with numerous anti-obesity medicinal plants such as *Brassica campestris spp* and *Lysimachia foenum-graecum* just to name a few, is recognized to be associated with a decrease of adipocytes size [162, 173]. Similar results were obtained in the epididymal fat depot from mice fed *Iwong*, confirming the protective effect of *Iwong* against lipid accumulation. Adipose tissue is no longer considered as a dormant tissue with a single role to store excess energy in form of triglycerides. In addition, adipose tissue also plays a critical role in the regulation of metabolic homeostasis via the secretion of adipokines. Leptin for instance, is positively correlated with the size and amount of fat mass; therefore its plasma level is drastically elevated in obese subjects. As shown in numerous studies with antiobesity substances, plasma leptin concentration is positively correlated with the reduction of body weight [174]. The same was true with *Iwong*, most clearly in mice fed a diet with 10% of the powder of this plant, demonstrating that *Iwong* downregulates leptin secretion, probably as a second event to fat mass loss. Since leptin is recognized as an anorexigenic hormone, a decline of its secretion might partially explain the hyperphagia as observed in this study.

The development of obesity is constantly associated with progressive changes in the plasma lipid profile including increased TG, total cholesterol which is associated with a rise in pro-atherogenic lipoproteins concentration such as LDL-C and VLDL-C. Additionally a decrease in anti-atherogenic HDL-C is observed. As a whole these changes favor the development of cardiovascular diseases and atherosclerosis usually observed in obese subjects[86]. In lean animals *Iwong* showed a promising benefit effect against dyslipidemia. Therefore, the preventive effect of *Iwong* in mice fed a HFD was

assessed. Results clearly showed that *Iwong* feeding after 5 weeks was led to a decrease in T-C and LDL-C levels and might thus protected against atherosclerosis and risk of CVD.

Abdominal obesity is mostly associated with hepatic steatosis due either to increased delivery of FFA to the liver or increases in hepatic lipogenesis which is sustained by the hyperinsulinemia [175]. Dietary TG hydrolyzed by pancreatic enzymes in the intestine are afterwards transported into the bloodstream in the form of chylomicrons and broken down into FFA, which are transferred to target tissue such as adipose tissue, skeletal muscle and liver [176]. Since mice were fed a diet enriched in lipids, the effects of HFD and *Iwong* feeding on the liver were evaluated. A significant increase in liver weight associated with increase TG content in the liver of the HFD group was found compared to control group. This effect was abolished in *the Iwong10* group suggesting a protective effect of *Iwong* on triglycerides accumulation in the liver and consequently a possible preventive effect against hepatic steatosis in overweight subjects.

The increased risk of type 2 diabetes with obesity is well established; obese patients tend to develop hyperglycemia as well as insulin resistance. This is mainly explained by increased plasma FFAs. The elevation of plasma FFAs concentration impairs the ability of insulin to suppress hepatic glucose production as well as its capability to stimulate glucose uptake into skeletal muscle. In addition higher plasma FFAs concentration is known to decline pancreatic insulin synthesis [123]. One aim of therapeutic weight management and fat mass reduction is accompanied with a normalization of plasma glucose levels. HFD feeding after 5 weeks failed to induce hyperglycemia, although as noticed above body weight and total fat mass were significantly elevated, possibly due to the short dietary intervention period. In fact in a study evaluating the rate of metabolic disorders in sucrose induced obese rats, it has been shown that the development of metabolic disorders is a time and tissue depend process during the development of obesity [177]. Finally, it was not possible to conclude for a beneficial effect or not of *Iwong* on hyperglycemia.

In turn, *Iwong* feeding for 5 weeks reduced body mass gain, fat mass and proatherogenic lipoproteins, demonstrating the ability of this vegetable to

prevent the development of obesity as well as CVD, although that need to be confirmed in animal models of CVD.

IV-1-3) Treatment of obesity

Iwong is able to reduce body mass gain and fat mass in lean rodents as well as to prevent obesity in mice fed a HFD. Experiments in obese mice were performed in order to evaluate the ability of *Iwong* to treat diet-induced obesity. Therefore, obesity was induced in C57BL/6J mice with HFD containing 20% palm oil, an increase in body weight after 9 weeks of HFD feeding was observed. This large rising on body weight was coupled with an enhancement of total fat mass; demonstrating the obesogenic capacity of palm oil.
Iwong supplementation for 6 weeks in these DIO mice was accompanied by suppression in body weight and a significant reduction on total fat mass compared to mice fed a HFD alone. These results were consistent with those observed in lean rodents and therefore confirm the capacity of *Iwong* to reduce body weight.

As noticed by Otto and Lane, increase fat mass in obesity state is due either by hypertrophy or by hyperplasia [2]. The epididymal fat pad is widely used in animal studies to evaluate the growth of fat cells under obesogenic conditions, mainly because of its relative homogeneity, the ease of dissection and its accurately weighed compared to other fat depots [178]. Therefore, histology of the epididymal fat pad was performed to clarify whether increase of fat mass was due to hyperplasia or hypertrophy. The images of microscopic sections showed that adipocytes size was positively correlated with fat mass, whereas macroscopic observations did not show notable changes on adipocytes cell number. Consequently, it can be speculate that increase of fat mass observed was at least due to the enlargement of adipocytes size, but not or slightly to hyperplasia. This observation was not surprising, as it had been reported that during obesity development recruitment of new adipocytes is only needed at the early stage and is not necessary at the late stage even if the animal remains in the obesogenic conditions [179]. Hypertrophy of adipocytes induced by HF feeding was

abolished in adipocytes of both *Iwong* groups mice, which is corroborates by the weight of epididymal fat pads mostly in Iwong8 mice. These findings proved that *Iwong* also has anti-obesity effect in preexisting obesity.

Obesity is associated with metabolic abnormalities, including hypertriglyceridemia, low levels of HDL-C and converts in LDL-C to a more pro atherogenic composition (small dense LDL), The assessment of the effect of HF feeding on the lipid profile revealed a significant increase of the pro-atherogenic parameters (TC, TG and LDLC), suggesting that feeding with high palm oil is able to induce metabolic disorders. This dyslipidemia may be explain by the presence of high amount of palm oil (20%) which has a deleterious effect on the lipid profile in rats [180][156]. In addition the most prominent fatty acid of palm oil is palmitic acid (≈44%), which is recognized for its atherogenic effect. This fatty acid increases risk of cardiovascular disease mostly by increasing the TC and LDL-C levels [181, 182], in part by mediating clearance and down regulating the LDL-receptor activity [183]. LDL-C is present in two circulating types: the large LDL-C and the small LDL-C. The latter form is more artherogenic, due to the lower binding efficiency to the LDL receptors, which consequently increase their circulating level and therefore their infiltration into the arterial wall. Another phenomenon that explains the atherogenecity of small LDL-C is the change in apolipoprotein B on their surface, which facilitates the entry of this LDL-C into the vascular wall [184].

Anti-obesity substances in addition to their lowering effect on body weight and fat mass would thus ideally possess the ability to normalize the blood lipid profile. Orlistat, a well-established pharmaceutical treatment against obesity and numerous medicinal plants such as *Platycodi radix*, *Momordica charantia* and *Nulembo nucifera* for instance are all known for their beneficial effect on lipid profile in obese animals or in humans [185-187]. *Iwong* feeding was associated with a dose dependent decrease of both total cholesterol and LDL-cholesterol compared to HFD groups. This finding suggests that *Iwong* has a hypocholesterolemic effect and could therefore be beneficial to reduce cardiovascular risk associated with obesity.

HFD is also known to increase liver TG content and that can be observed after 10 days of feeding with consequently the development of dyslipidemia, insulin resistance and type II diabetes [188]. In fact, the role of the liver in lipid

metabolism is critical; it ensures fatty acid uptake, the novo fatty acid synthesis, fatty acids and TG uptake as well as fatty acid oxidation [189]. Additionally, according Fabbrini et al increase rate of intrahepatic TGs production compared to the rate of the secreted TGs from the liver is another indicator of hepatic steatosis state [190]. Higher TG content in the liver of mice fed a HFD compared to control animals as observed in this doctoral thesis confirmed the fact that the steady balance of hepatic TGs can be affected by nutritional conditions specially overnutrition and therefore promotes the development of hepatic steatosis. The link between steatosis and basal VLDL has been established in mouse as well as in rat studies [191]. Increase level of VLDL as observed in this study can be explained by different molecular mechanism including the de novo lipogenesis and the alteration of the β-oxidation activity of the liver. Since the same glucose concentration in HFD and standard diet was used in the present study, the hypothesis of potential glucose uptake by the liver should be eliminated. One explanation could thus be the defect on the hepatic β-oxidation, which increases fatty acid availability and in turn hepatic TG accumulation. Although the fatty acid oxidation was not evaluated, it can be nevertheless speculated that elevation of plasma VLDL developed in HFD mice was due to impair hepatic β-oxidation as found in several other studies in humans and rodents [192].

In addition, there was no effect on triglyceridemia in mice fed a HFD supplemented with *Iwong*; plasma TG and VLDL remained significantly elevated and similar to mice fed a HFD alone. This observation is in contradiction with other studies showing that reduction in fat mass is due to hypotriglyceridemia either in animals or in human studies (ref). It is known that TG circulating level is directly linked to the amount of fat mass in the body, thus lowering of body weight with fenofibrate for instance is associated with a decrease in TG level [193].

In mice treated with *Iwong,* the level of hepatic TGs content was VLDL-TG plasmatic levels as observed in mice treated by *Iwong* could be a consequence of a decrease of hepatic uptake or by an overexpression from the liver of this lipoprotein. Such of results have been also observed in human subjects treated by non-statin LDL lowering, but the mechanism of those changes is not yet completely elucidated [194].

Prolonged high fat feeding has been reported to induce moderate hyperglycemia in rats as well as in mice [195, 196]. Fasting plasma glucose level was therefore assessed, and found to be elevated in DIO animals compared to controls on a standard diet. Effect on glycaemia induced by HFD was reduced on a dose depend manner in mice fed *Iwong*, suggesting that this plant protects against hyperglyceamia.

Several hormones including leptin, adiponectin and insulin play a critical role in the maintenance of energy homeostasis; most of them are known to be either up or downregulated in the obese state(see review [197]). Results as observed in this study with high plasma leptin levels in DIO are as expected. Leptin is produced mostly in WAT and its circulating level is positively correlated with the amount of adipose tissue in the body. In addition, plasma insulin level was slightly but not significantly elevated in DIO mice demonstrating a trend to hyperinsulinemia. Insulin plays a key role in the metabolism of glucose and development of adipose tissue and its huge elevation is led with increase insulin resistance and consequently hyperglycemia or later development of type II diabetes. Furthermore the link between BMI and the plasma insulin level and consequently insulin resistance has been well established [198]. The secretion and excretion of this hormone are regulated by several factors such as glucose as well as FFAs (see review [120]). Therefore plasma glucose level, as observed matched with the insulin concentration, but as FFAs level had not be measured, it is not evident to incriminate the increase of blood circulating FFAs as another cause of the trend to insulin resistance although a large body of literatures have reported this fact (see review [120, 199]). A trend of a dose dependent anti-hyperinsulinemia was observed after 6 weeks of *Iwong* feeding, suggesting that *Iwong* tends to improve insulin sensitivity. This finding combined to the tendency to antihyperglycemic effect insinuates that *Iwong* could also be beneficial for diabetic subjects, but more investigation is needed in order to elucidate this assumption.

Leptinemia was significantly elevated in obese mice compared to mice fed a standard diet, and was restored in a dose dependent manner after 6 weeks of *Iwong* feeding. Adiponectin is another critical hormone for the control of energy homeostasis. This hormone is known to decrease in obese subjects as well as in obese animal [200, 201]. The plasma adiponectin level was similar in both obese and lean mice. This observation is in contradiction with expecting results

as this hormone is known to be negatively correlated with the mass of adipose tissue [112][110]. The level of adiponectin in mice fed *Iwong* also remained unchanged during the whole feeding experimental period. Since HFD did not affect adiponectin level, it is difficult to draw an overall conclusion concerning the effect of this vegetable on adiponectinemia. More studies are needed in order to alter adiponectinemia in obese and later evaluate the effect of *Iwong* feeding on these mice.

IV-1-4) Anti-adipogenic effect of ethanolic extracts of *Iwong*

Studies either in lean or in obese rodents showed an anti-obesity effect of *Iwong* including reduction of body weight and fat mass associated with an improvement of biochemical parameters. Natural substances with anti-obesity effect may act by one of the following mechanisms: increasing energy expenditure, suppressing appetite, inhibiting intestinal lipid absorption or affecting directly the metabolism of adipocytes [35], e.g. a decrease in lipogenesis in adipocytes [202]. *In vitro* studies were therefore conducted with the aim to elucidate the direct effect of *Iwong*, on fat cell development and/or metabolism. The assumption that the ethanolic extract of *Iwong* could inhibit triglyceride accumulation in adipocytes, similar to several other extracts of medicinal plants or natural compounds has been made [35]. Preadipocyte cell lines and primary cultures of adipose-derived stromal vascular precursors cells, are several cell culture models used to understand the molecular mechanism of adipogenesis, including proliferation, differentiation, adipokine secretion and gene/protein expression of adipocytes. Moreover, established cell lines are suitable to evaluate the interaction of natural compounds or pharmacological drugs with the process of adipocytes differentiation [158]. Amount of these cell culture models, 3T3L1 cell line established by Green and Kehinde, is widely used to study the adipogenic process and is known to be one of the best characterized models for studying the conversion of preadipocytes into adipocytes [69, 70]. In fact, 3T3L1 cell line compared to other models develop the characteristics close to that observed in animal tissue [72]. The inhibitory effect on lipid accumulation and the down-regulation of genes involved in adipocyte differentiation by Resveratrol and Genistein for instance have been evaluated in the 3T3L1 cell line[203].

Sung et al, using 3T3L1 cells, demonstrated that *Akebia quinata* extract exerted anti-obesity and hypolipidemic effects in mice fed a high-fat diet by regulating adipogenesis and fatty acid oxidation via AMPK activation [163]. The antiobesity effect of an extract of the seeds of *Irvingia gabonesis* observed in rodents was confirmed *in vitro* in 3T3L1 cells. In fact, it has been showed that, this extract reduced lipid accumulation via the down-regulation of PPARγ and leptin genes and up-regulation of adiponectin genes [204]. The ethanolic extract of *Iwong* as observed in this thesis significantly reduced lipid accumulation after 9 days of treatment. Obesity is characterized by increased adipose tissue mass due to both, hypertrophy and hyperplasia. The number and the size of adipocytes in the body largely depend on the process of adipocyte differentiation also known as adipogenesis, thus substances that reduce adipocyte differentiation, may be useful in the development of anti-obesity drugs [35]. Results as observed in the present thesis suggest that *Iwong* reduced fat mass in animal studies by acting directly in adipocytes.

The evaluation of cytotoxicity showed a significant harmful effect of the ethanolic extract of *Iwong* on 3T3L1 cells after 9 days at the high concentration of 300µg/ml. The toxicity of a substance depends on many factors, including dose and duration of the treatment or exposure[205]. The toxic effect on cells viability was evaluated at 3 different time points: day 3, day 5 and day 8 of treatment. Treatment during just the early phase (day 3) of differentiation was not detrimental for cells. Due to this observation the effect on lipid accumulation at the same three time points has been also tested. Interestingly the result demonstrated that the presence of *Iwong* ethanolic extract during the early stage (day 0-2) of differentiation, significantly prevented fat storage in 3T3L1 to the same extent as the chronic treatment (day 0 to 8). This effect was not observed when the plant extract was only added at the intermediate (day 3 to 5) or late (day 6 to 8) stages. Taken together these observations demonstrated that acute administration of *Iwong* could be sufficient to produce a sustained inhibition on lipid accumulation without or with fewer side effects. The first two days of the exposure of 3T3-L1 preadipocytes to differentiation inducers correspond to the period of mitotic clonal expansion [206, 207]. During this stage, early events occur such as expression of C/EBPβ and δ which mediate the expression of PPARγ. Since treatment during the intermediate and the late stages of differentiation failed to inhibit lipid accumulation, it can be hypothesized that

Iwong acts exclusively during the clonal expansion phase. Natural compounds with inhibitory effect on lipid accumulation are also known to decrease the activity of GPDH, which is a key enzyme on adipogenesis necessary for the conversion of dihydroxyacetone phosphate (DAHP) to glycerol-3-phosphate (G3P) [208]. The activity of this enzyme significantly increased during the differentiation process, thus GPDH is widely used as a marker of adipogenesis. The GPDH specific activity was downregulated in a dose dependent manner confirming that *Iwong* affected adipocyte development by inhibiting lipogenesis. This anti-adipogenic effect of *Iwong* corroborates the observation made in adipocytes of obese mice treated with *Iwong* powder and consequently explains the reduction of the size of adipocytes of these mice when compared with non-treated obese mice.

The development of mature fat cells from preadipocytes is a complex process characterized by growth arrest and clonal expansion followed by changes in cell morphology, hormone sensitivity, gene expression and lipid storage [2]. Several transcription factors are expressed during the process of differentiation, so changes in their expression and activity defined the process of differentiation [78]. Consequently the effect of *Iwong* ethanolic extract on the two critical adipogenic transcription factors (PPARγ and C/EBP*α*) involved in the process of adipogenesis was investigated. These transcriptional regulators of adipocyte differentiation act synergistically to generate fully differentiated, insulin responsive adipocytes. The results showed a downregulation of both PPARγ and C/EBPα mRNA expression, suggesting that adipogenesis was repressed by downregulation of the transcription factor cascade upstream of PPARγ and C/EBPα. This effect is in line with several other studies in which it has been demonstrated that natural compounds from plant as EGCG, esculentin, berberine and guggulsterone inhibit lipid accumulation in 3T3L1 cells by down-regulating PPARγ and C/EBPα expression (see review [35]).

IV-1-5) Effect of the ethanolic extract of *Iwong* on lipolysis

Natural compounds such as procyanidin and genistein are known for their lipolytic effect [209, 210]. Since lipolysis is one of the proposed pathways of medicinal plants exert anti-obesity effects [21]. In addition to the antiadipogenic property of *Iwong*, its potential lipolytic effect has been evaluated. Isoproterenol,

a β-adrenergic agonist which is known to stimulate β-adrenergic receptors and in turn activates lipolysis in adipocytes was used as a positive control. The *Iwong* ethanolic extract did not stimulated glycerol release, compared to Isoproterenol which significantly stimulated lipolysis. Thus the ethanolic extract of *Iwong* affected adipogenesis by inhibiting triglyceride accumulation, but not by enhancement of lipolysis in adipocytes.

A summary of the action of *Iwong* in the adipocyte life cycle is described in Fig.41.

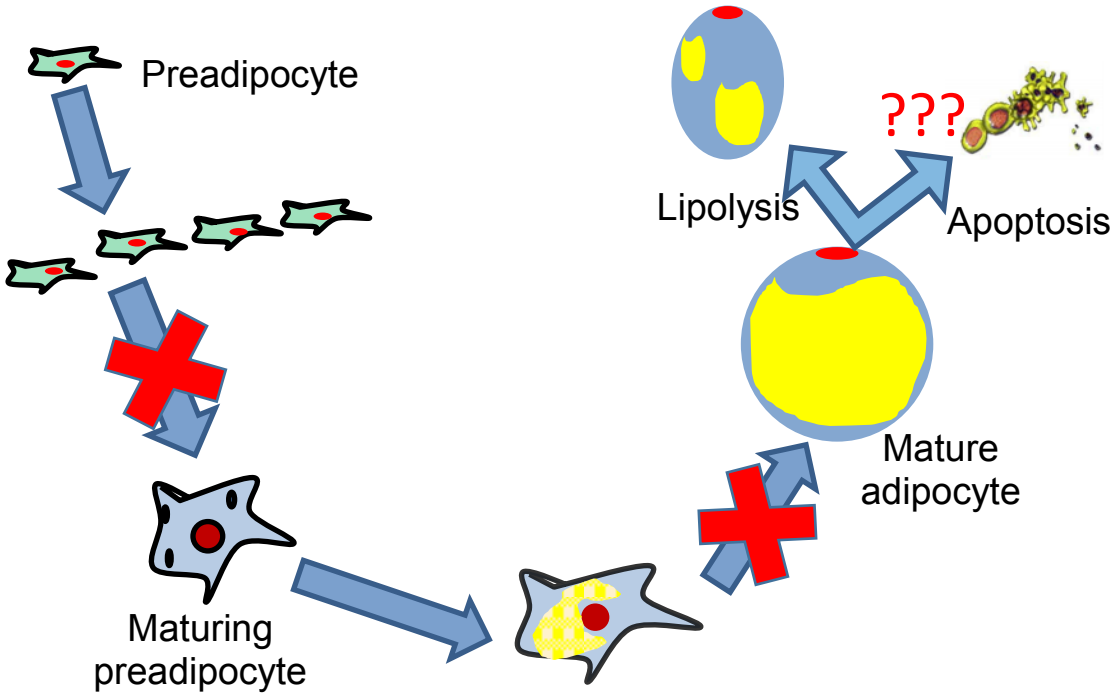


Fig. 41: Potential site of action of *Iwong* in adipocyte life cycle. Preadipocytes can undergo a cascade of events including lipid accumulation and then differentiate in mature adipocytes. Mature adipocytes can finally go through lipolysis or under certain conditions to apoptotic dead cells process. Regarding the results obtained, *Iwong* interacts with adipocyte life cycle by inhibiting conversion of preadipocyte to mature adipocyte mainly at the early stage of adipogenesis, whereas lipolysis was not affected. Additionally the effect of *Iwong* on apoptosis needs to be evaluated in further studies as a slight decrease of total cell number as observed in this study could be due either by the cytotoxic effect or by apoptosis (adapted from Rayalam et al [35]).

IV-2) Conclusion

Obesity is nowadays a public health concern in both developed and developing countries. Numerous efforts were made to develop an efficient treatment against this pandemic, but only few drugs are globally used to manage body loss [156]. For decades there has been an enormous resurgence of interest on medicinal plants with anti-obesity properties. The evaluation of the anti-obesity properties of *Iwong* was conducted both *in vivo* and *in vitro* and clearly demonstrated that, this vegetable can be used to manage body weight. The first part of the *in vivo* studies conducted with lean rodents, showed an increase on food intake, a reduction on body weight gain associated with fat mass decrease in both rats and mice. This finding is corroborated by the study of Kao et al on EGCG, a well-known anti-obesity natural compound isolated from green tea, which was also able to reduce body weight in lean rats [211]. Furthermore in plasma clinical chemistry revealed a decrease on LDL-C and an increase on HDL-C, whereas all others parameters remained unchanged in animals fed *Iwong*. In mice particularly the energy assimilated was reduced in two *Iwong* groups. For the second part of the study, obese mice were used. Results obtained showed that *Iwong* was also able to prevent obesity by reducing both body weight gain and fat mass as shown in young male C57BL/6J mice fed a HFD as well as preventing against dyslipidemia and hyperleptinemia. Moreover Iwong feeding additionally to the benefic effect on body weight and fat mass was associated with an improvement of metabolic disorders including hypocholesterolemia and antihyperglycemia in DIO mice. Interestingly, plasma leptin and insulin level were downregulated in these mice. To get insight into the mode of action, the effect of the ethanolic extract of *Iwong* was tested on the adipocyte life cycle using 3T3L1 preadipocytes. As summarized in fig 41, *Iwong* interacted with the adipocyte life cycle by inhibiting conversion of preadipocytes to mature adipocytes mainly at the early stage of adipogenesis, whereas lipolysis was not affected. This observation was confirmed at the molecular level where the inhibition of lipid accumulation accompanied by a downregulation of PPARγ and C/EBPα expression. Taken together all these results demonstrated that *Iwong* has anti-obesity effects and justified its used in traditional medicine to control glucose homeostasis and body weight.

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