Université de Montréal

Étude des propriétés antidiabétiques de *Nigella sativa* : sites d'action cellulaires et moléculaires

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Résumé

Nigella sativa ou cumin noir est une plante et un condiment populaires. Les graines de *N. sativa* sont très utilisées en médecine traditionnelle des pays nord africains pour le traitement du diabète. Cependant, les mécanismes d'actions cellulaires et moléculaires via lesquels cette plante exerce son effet euglycémiant restent encore mal compris. Le but de notre étude est d'examiner l'effet de *N. sativa* sur la sécrétion d'insuline, le transport de glucose et sur les voies de signalisation impliquées dans l'homéostasie et le métabolisme de glucose, en utilisant des essais biologiques sur des cultures cellulaires murines (cellules β pancréatiques βTC, myoblastes C2C12, hépatocytes H4IIE et adipocytes 3T3-L1) et des études in vivo chez le rat normoglycémique et le *Meriones shawi* (rongeur) diabétique.

Chez les cellules β pancréatiques, *N. sativa* a augmenté leur prolifération ainsi que la sécrétion basale et gluco-stimulée de l'insuline. *N. sativa* a augmenté aussi la prise de glucose de 50% chez les cellules musculaires alors que chez les cellules graisseuses, la prise de glucose est augmentée jusqu'au 400%. Les expériences d'immunobuvardage de type western ont montré que *N. sativa* stimule les voies de signalisation de l'insuline (Akt et ERKs) et aussi celle insulino-indépendante (AMPK) chez les cellules C2C12. Par contre, chez les 3T3-L1, l'augmentation de transport de glucose est plutôt reliée à une activation de la voie de *peroxisome proliferator activated receptor* γ (PPARγ). Chez les hépatocytes, *N. sativa* augmente la stimulation des protéines intracellulaires Akt et *5′ adenosine monophosphate-activated protein kinase* (AMPK). Cette activation de l'AMPK est associée à un effet découpleur de la plante au niveau de la phosphorylation oxydative mitochondriale.

Par ailleurs, chez les *Meriones shawi* diabétiques, *N. sativa* diminue graduellement la glycémie à jeun ainsi que la réponse glycémique (AUC) à une charge orale en glucose (OGTT) pour atteindre des valeurs semblables aux animaux témoins après quatre semaines de traitement. Une amélioration du profile lipidique est observée autant chez les *Meriones shawi* diabétiques que chez les rats normaux. Au niveau moléculaire, *N. sativa* augmente le contenu musculaire en *glucose transporter 4* Glut4 et la phosphorylation de l'acetyl-coenzyme A carboxylase ACC dans le muscle soléaire et le foie chez les *Mériones shawi* diabétiques. Par contre, chez le rat normal, on assiste à une stimulation des voies de signalisation de l'insuline (Akt et ERK) au niveau hépatique.

En conclusion, nous avons confirmé l'action insulinotropique de *N. sativa* au niveau des cellules β pancréatiques et mis en évidence un effet proliférateur pouvant potentiellement s'avérer utile pour contrecarrer la perte de masse cellulaire observée chez les diabétiques. Notre étude a également mis en évidence pour la première fois que *N. sativa* exerce son activité antidiabétique par une combinaison d'effets insulino-mimétiques et insulino-sensibilisateurs directs permettant ainsi d'augmenter le transport de glucose des tissus périphériques. Cette action de *N. sativa* est liée à une stimulation des voies de signalisation intracellulaires insulinodépendantes et -indépendantes (AMPK) chez le muscle squelettique et le foie alors qu'elle passe par la voie des PPARγ au niveau du tissu adipeux. Finalement, l'étude *in vivo* vient confirmer l'effet antidiabétique de *N. sativa*. Notre apport novateur se situe au niveau de la démonstration que l'activité antidiabétique de *N. sativa* chez le *Meriones shawi* diabétique est la résultante des mêmes activités que celles déterminées au niveau de

l'étude *in vitro*. En effet, *N. sativa* active la voie de l'AMPK, améliore la sensibilité à l'insuline et augmente l'insulinémie. Notre étude montre aussi que *N. sativa* possède une activité antilipidémiante. Ces résultats confirment le bien-fondé de l'utilisation ethnopharmacologique de *N. sativa* comme traitement du diabète et des perturbations du métabolisme lipidique qui y sont associées. De plus, les actions pléiotropiques de *N. sativa* en font un traitement alternatif ou complémentaire du diabète très prometteur qui encouragent à présent la tenue d'études cliniques de bonne qualité.

Mots clés: *Nigella sativa*, diabète, résistance à l'insuline, *Meriones shawi*, AMPK, Glut4, HDL, foie, graisse, muscle squelettique, sécrétion de l'insuline, respiration mitochondriale.

Abstract

Nigella sativa or black cumin is a medicinal plant and a popular condiment. The seeds of *N. sativa* are widely used in the traditional medicine of North African countries for the treatment of diabetes. However, the cellular and molecular mechanisms of action through which the plant exerts its hypoglycemic effect remain unclear. The aim of our study is to determine the effect of *N. sativa* on insulin secretion, glucose transport and signaling pathways involved in the regulation of glucose homeostasis and metabolism. We carried out *in vitro* murine cell-based bioassays (βTC pancreatic β cells, C2C12 myoblasts, H4IIE hepatocytes and 3T3-L1 adipocytes) and *in vivo* studies in normoglycemic rats and diabetic *Meriones shawi* (rodent).

In pancreatic β cells, *N. sativa* increased cell proliferation as well as basal and glucose stimulated insulin secretion. It also enhanced glucose uptake in muscle cells by 50%. Moreover, the increase of glucose uptake in fat cells reached levels up to 400%. The experiments using Western immunoblot analysis showed that *N. sativa* stimulated insulin-dependent (Akt and ERK) as well as -independent (AMPK) pathways in C2C12 cells. In 3T3-L1 cells, the increase of glucose uptake was attributed to the activation of the peroxisome proliferator activated receptor γ (PPARγ) pathway. Similarly to C2C12 cells, N. sativa activated Akt and 5' adenosine monophosphate-activated protein kinase (AMPK) in hepatocytes. This activation of AMPK was associated with an uncoupling effect on mitochondrial oxidative phosphorylation.

In diabetic Meriones, *N. sativa* gradually decreased fasting blood glucose and the glycemic response to an oral glucose load (OGTT) to values similar to normal animals at the end of treatment. Improved lipid profile is observed in both animal models. At the molecular level, N. *sativa* increased muscle glucose transporter 4 (Glut4) content and acetyl-coenzyme A carboxylase (ACC) phosphorylation in soleus muscle and liver in diabetic *Meriones shawi*. In normal rats, the plant extract induced a stimulation of insulin signaling pathways (Akt and ERK) in the liver.

In conclusion, *N. sativa* has an insulinotropic effect on pancreatic β cells. Our study has revealed for the first time that *N. sativa* exerts its antidiabetic activity by a combination of insulino-mimetic and insulin-sensitizing effects, thereby increasing glucose uptake in peripheral tissues. This effect of *N. sativa* is linked to the stimulation of insulin-dependent and -independent (AMPK) pathway in skeletal muscle and liver, while in adipose tissue, the effect was attributed to the activation of PPARγ. Finally, the *in vivo* study confirms the antidiabetic and antihyperlipidemic effects of *N. sativa*. Our original contribution lies in the demonstration that the *in vivo* antidiabetic action of *N. sativa* is exerted though the same mechanisms identified by our *in vitro* studies. These data support the soundness of the ethnobotanical use of this plant for the treatment of diabetes and its associated dyslipidemia. Moreover, the pleiotropic actions of *N. sativa* make it a very promising alternative or complementary treatment for diabetes, which calls for immediate high quality clinical trials.

Key words: diabetes, insulin resistance, *Nigella sativa*, *Meriones shawi*, AMPK, Glut4, HDL, liver, fat tissue, skeletal muscle, insulin secretion, mitochondrial respiratory.

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Liste des abréviations

aa	:	Acide aminé
ACC	:	Acetyl-coenzyme A carboxylase
ADN	:	Acide désoxyribonucléique
AGL	:	Acide gras libre
ALAT	:	Alanine amino transférase
AMP	:	Adenosine-5'-monophosphate
AMPK	:	5' adenosine monophosphate-activated protein kinase
ARNm	:	Acide ribonucléique messager
ASAT	:	Aspartate amino transférase
ATP	:	Adenosine-5'-triphosphate
AUC	:	Areas under the curve
CAP	:	c-Cbl associating protein
CDA	:	Canadian diabetes association
cm	:	Centimètre
DL50	:	Dose létale 50
DMSO	:	Dimethyl sulfoxide
EMC	:	Encephalomyocarditis
eq	:	Équivalant
ERK	:	Extracellular signal-regulated kinase
g	:	Gramme
GDP	:	Guanosine-5'-diphosphate

GGT	:	Gamma glutamyl transférase
GLP-1	:	Glucagon-like peptide 1
Glut	:	Glucose transporter
Grb2	:	Growth receptor binding protein-2
GSH	:	Glutathion
GTP	:	Guanosine-5'-triphosphate
h	:	Heure
HDL	:	High density lipoprotein
IDF	:	International Diabetes Federation
ip	:	Intra-péritonéal
IRS	:	Insulin substrate receptor
JC	:	Jésus Christ
kg	:	Kilogramme
MAPK	:	Mitogen-activated protein kinases
MCV	:	Maladies cardio-vasculaires
mg	:	Milligramme
min	:	Minute
mL	:	Millilitre
mmol	:	Millimole
ng	:	Nanogramme
NK	:	Natural Killer
NO	:	Monoxyde d'azote
N. sativa	:	*Nigella sativa*

NZO	:	New Zealand Obese
LDH	:	Lactate déshydrogénase
LDL	:	Low density lipoprotein
LHS	:	Lipase hormono-sensible
LPL	:	Lipoprotéine lipase
OGTT	:	Oral glucose tolerance test
OMS	:	Organisation mondiale de la santé
pg	:	Picogramme
PI-3 Kinase	:	Phosphatidyl-inositol-3 phosphate kinase
PKB	:	Protein kinase B
PKC	:	Protein kinase C
po	:	Per os
PPAR	:	Peroxisome proliferator activated receptor
PPAR-RE	:	Peroxisome proliferator activated receptor response element
RER	:	Réticulum endoplasmique rigueur
Shc	:	Src homology 2 domain and collagen protein
SREBP-1C	:	Sterol regulatory element binding protein- 1C
TBHP	:	Tert-Butylhydroperoxide
WHO	:	World health organization
µg	:	Microgramme
µL	:	Microlitre

À la mémoire de ma mère

À mon père

À ma femme

À mon fils

À mes frères

À tous mes amis

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INTRODUCTION

1. Introduction

Parmi les plantes médicinales les plus utilisées et qui ont suscité un grand intérêt pour les pays méditerranéens et asiatiques, on trouve *Nigella sativa Linn.* Cette plante appartenant à la famille des renonculacées, est communément connue sous le nom de cumin noir ou nigelle. À travers le monde, on ne dénombre pas moins d'une soixantaine de noms communs et trois noms latins. Les graines de *N. sativa* sont cultivées dans les pays de l'Afrique du nord et en Inde. C'est surtout en Turquie que *N. sativa* est largement cultivé et commercialisée en direction des pays du Moyen-Orient où elle est utilisée comme condiment (entrant dans la confection du pain et de la pâtisserie) et comme épice dans la préparation des sauces. Par ailleurs, *N. sativa* est surtout employé comme remède naturel, et ceci depuis plus de 2000 ans. En effet, *N. sativa* est recommandé pour le traitement de plusieurs maladies. Ces actions thérapeutiques ont été plus ou moins vérifiées récemment. Parmi les plus importantes, on retrouve les activités antimicrobienne, antifongique, antitumorale, analgésique, anti-inflammatoire, hypotensive et hypoglycémiante. Celles-ci seront discutées plus en détail à la section 1.3.

Le diabète est une maladie caractérisée par une défaillance de l'organisme dans la régulation de la glycémie. Cette maladie est l'une des premières causes de mortalité et de morbidité dans la plupart des pays occidentaux et son incidence devient aussi importante dans les pays en voie de développement. Le diabète, en particulier le diabète de type II, s'associe fréquemment à d'autres anomalies métaboliques et hémodynamiques. Plusieurs études expérimentales et cliniques

montrent que l'obésité semble être la cause principale de la résistance à l'insuline qui à son tour joue un rôle pathogénique majeur dans le développement du diabète de type II. Ceci est discuté en détail à la section 1.1.

Le traitement moderne du diabète fait appel à plusieurs familles d'hypoglycémiants oraux comme les biguanides et les thiazolidinediones qui agissent sur l'insulino-résistance et les sulfamides et les glitinides qui agissent en stimulant l'insulino-sécrétion. Cependant aucun des médicaments utilisé pour le traitement du diabète n'agit simultanément sur les deux facteurs pathogéniques du diabète ou améliore aussi les anomalies du métabolisme lipidique associées le plus souvent au diabète de type II. La section 1.1.8 discute plus en détail ces approches thérapeutiques.

Le but de notre étude débutera par la détermination des mécanismes cellulaires qu'emprunte *N. sativa* afin de régulariser la glycémie. Plus précisément, nous nous sommes penchés sur l'évaluation de l'action de *N. sativa* au niveau de la sécrétion insulinémique et de la prolifération chez des cellules β pancréatiques ainsi que sur le transport de glucose chez les adipocytes et cellules musculaires squelettiques (Chapitre 2). Une fois cette étape terminée, nous avons déterminé les voies de signalisation intracellulaire impliquées dans la régulation de l'homéostasie et du métabolisme glucidique (voies de PI-3 Kinase, ERKs, AMPK) induit par *N. sativa* chez les cellules périphériques insulino-sensibles (hépatocytes, myotubes, adipocytes - Chapitre 3). Nous avons également vérifié l'effet de l'extrait total de *N. sativa* sur

le fonctionnement mitochondrial et plus précisément sur la chaîne de respiration chez des mitochondries isolées. Les deux étapes suivantes ont consisté à évaluer l'effet antidiabétique de *N. sativa* et à déterminer les mécanismes moléculaires mis en jeux chez les rats normaux (Chapitre 4) puis chez un modèle de diabète de type II (*Meriones shawi* - Chapitre 5), tout en tenant compte des effets de cette plante médicinale sur les autres anomalies métaboliques associées au diabète de type II.

1.1. Diabète

Le diabète est un désordre métabolique caractérisé par la présence d'une hyperglycémie chronique. En 2007, le diabète affecte 246 millions de personnes dans le monde entier et affectera 333 millions de personnes en 2025 (IDF, 2007).

1.1.1. Historique

Le diabète est une des maladies les plus anciennement connues. Des documents faisant mention de prescriptions médicales pour corriger la polyurie ont été retrouvés dans les tombeaux de Thèbes en Égypte. Plus tard, plusieurs médecins dont l'hindou Siscruta ($5^{ème}$ siècle avant JC), le grec Hippocrate (377 ans avant JC) et le chinois Tchang-Tchoug-King (200 ans avant JC) avaient décrit les symptômes de la maladie de la soif. De trente à 90 ans avant JC, Aretee fût le premier à utiliser le mot "*diabainein*" qui en grec signifie " couler à travers".

Avicenne (960-1037 après JC) donna une excellente description du diabète. Il fît mention de l'appétit exagéré, de l'impuissance sexuelle et du goût sucré des

urines. Ce qui suit présente les principales découvertes qui ont permis la compréhension de la physiopathologie du diabète au cours du 19ème et 20ème siècle :

- Au 17ème siècle, William Cullen différencia le diabète sucré du diabète insipide (affection rénale où l'hyperglycémie résulte de la concentration sanguine causée par la perte de fluides induite par la polyurie).
- En 1797, John Rollo signala l'hyperglycémie du diabétique.
- En 1815, Chevreul a montré que le sucre contenu dans les urines était du glucose.
- En 1848, Claude Bernard a découvert la fonction glycogénique du foie.
- En 1874, Minkovski et Vonmering ont confirmé le rôle du pancréas dans la pathogénèse du diabète.
- En 1921, Best et Banting ont isolé l'insuline.
- En 1955, grâce aux travaux de Loubatiers les premiers sulfamides ont vu le jour.
- 1970-1975 : période au cours de laquelle l'éducation du diabétique prend la première place dans le traitement du diabète (Peumery, 1990).

1.1.2. Régulation physiologique de la glycémie

Le glucose est le substrat énergétique des cellules, il est métabolisé en CO_2 et H_2O et permet la formation des molécules d'ATP. Le glucose sanguin a deux origines : une origine exogène représentée par l'apport alimentaire et une origine endogène constituée essentiellement par le glycogène hépatique et musculaire qui est dégradé par la voie de la glycogénolyse. Au niveau hépatique, le glucose peut se

former également à partir des molécules telles que le glycérol, le lactate et les acides aminés glucoformateurs. On parle alors de la néoglucogenèse.

En général, la glycémie est régulée par l'insuline, hormone hypoglycémiante sécrétée par les cellules β des îlots de Langerhans pancréatiques. Le niveau de la glycémie constitue le facteur essentiel de la régulation de la sécrétion de l'insuline. L'insuline assure son action hypoglycémiante par l'activation de la glycolyse et de la glycogénogenèse et par l'inhibition de la glyconéogenèse. La résultante de ces effets est une stimulation de l'emmagasinage du glucose sanguin par les cellules insulino-sensibles (cellules du muscle squelettique, du foie et du tissu adipeux). Cette augmentation de la mobilisation du glucose sanguin vers les tissus périphériques permet d'atteindre l'euglycémie qui, à son tour, freine la sécrétion de l'insuline déclenchée par l'hyperglycémie initiale.

La glycémie est également régulée par d'autres hormones antagonistes de l'insuline telles que le glucagon, les catécholamines, les glucocorticoïdes, les minéralocorticoïdes et les hormones thyroïdiennes. Le glucagon, sécrété par les cellules α pancréatiques, est la plus importante de ces hormones hyperglycémiantes. Il exerce son action en stimulant la gluconéogenèse hépatique, en augmentant la glycogénolyse musculaire et hépatique, et en inhibant la glycolyse au niveau de plusieurs tissus.

Le foie joue un rôle principal dans la régulation de la glycémie; il est très sensible aux variations de la glycémie. Quand celle-ci est élevée, il capte et stocke le

glucose sous forme de glycogène, et quand elle est baisse, il libère le glucose dans le courant sanguin (Pieri et kirkiacharin, 1992; Meyer, 1977; Williams, 1980).

1.1.3. Régulation moléculaire du transport de glucose

Dans les tissus, la captation de glucose est assurée par divers transporteurs de glucose (voir tableau I). Le transport de glucose indépendant de l'insuline (captation basale) augmente de façon linéaire avec l'augmentation de la glycémie par un effet d'action de masse. Cependant, si le glucose-6-phosphate s'accumule, à cause d'une entrée du glucose dans la cellule supérieure à celle requise pour le métabolisme, l'augmentation du glucose-6-phosphate qui résulte va inhiber l'hexokinase et donc la phosphorylation du glucose (rétrocontrôle). Le glucose libre dans la cellule augmente ensuite et réduit le gradient de concentration, ce qui freine le transport de glucose (Andreelli et Girard, 2005).

Par contre, la régulation de l'hyperglycémie fait appel au transport de glucose contrôlé par l'insuline qui dépend des transporteurs de glucose Glut4 exprimés au niveau du muscle squelettique, des adipocytes et du muscle cardiaque. À l'équilibre, ces transporteurs sont localisés dans des vésicules intracellulaires, alors qu'en présence d'insuline, les Glut4 se retrouvent dans la membrane plasmique. Ce processus est appelé translocation (Suzuki et Kono, 1980). L'insuline augmente aussi l'activité des Glut4. L'insuline exerce ses actions cellulaires, incluant la translocation et l'activation des Glut4, par le biais de différentes voies de signalisation qui sont détaillées dans les sections suivantes.

Tableau I : Transport facilité de glucose chez les mammifères (Mueckler, 1993; Ducluzeau et coll., 2002).

Nom	Distribution tissulaire	Fonction
Glut1	Large distribution, abondant dans les érythrocytes, cellules endothéliales et les lignées cellulaires immortalisées	Transport basal de glucose, augmente le transport de glucose au cours de la croissance et la division cellulaire et assure le transport de glucose à travers la barrière hémato-encéphalique
Glut2	Hépatocytes, cellules β pancréatiques, intestin, rein	Assure la fonction gluco-sensitive et le transport transépithélial
Glut3	Distribution très large chez l'humain; présent dans le cerveau chez d'autres espèces	Transport basal chez plusieurs cellules humaines, transport du liquide encéphalique vers les cellules cérébrales
Glut4	Muscle squelettique, cœur, adipocytes	Assure l'augmentation rapide de transport en réponse à l'insuline
Glut5	Intestin (jéjunum), présence faible dans les adipocytes, Muscle, cerveau et rein	Transport intestinal de fructose et autre hexoses
Glut7	Tissu néoglucogénique : foie	Libération de glucose néoformé au niveau de la lumière du RE
Glut8	Blastocytes, possiblement dans d'autres tissus	Transport de glucose insulinodépendant chez les blastocytes
GlutX1	très abondant dans les testicules, moyennement dans le SNC et faiblement dans les tissus insulino-sensibles	?

1.1.3.1. Insuline

L'insuline joue un rôle fondamental dans la physiopathologie de l'obésité, de la résistance à l'insuline et du diabète. L'insuline a été initialement connue comme une hormone hypoglycémiante dont la déficience provoque une augmentation de la concentration plasmatique de glucose et sa diminution au niveau cellulaire. Cette hormone est un peptide de 51 acides aminés (AA). Elle est constituée de 2 chaînes: une chaîne A (21 AA) et une chaîne B (30 AA) liées par des ponts disulfures, indispensables à son activité biologique (Kahn, 1994).

1.1.3.1.1. Biosynthèse de l’insuline

Cette biosynthèse s’effectue uniquement au niveau des cellules β des îlots de Langerhans. En fait, le produit initial de la traduction des ARNm de l'insuline est la pré-pro-insuline; les 25 premiers AA de la molécule représentent une séquence riche en résidus hydrosolubles qui permettent la pénétration rapide du peptide en cours de synthèse dans le réticulum endoplasmique rugueux (RER). Dès que cette séquence est passée dans le RER, elle est éliminée par des peptidases spécifiques. La pro-insuline quitte ensuite le RER, stockée dans des microvésicules; elle rejoint l'appareil de Golgi. Elle est alors spécifiquement reconnue par un récepteur Golgien et est dirigée vers des vésicules de type dit "contrôlé", c'est à dire libérées seulement en réponse à un signal. Elle quitte donc l'appareil de Golgi dans des vésicules, revêtues de clathrine. Ces vésicules subissent une acidification de leur contenu et la pro-insuline est clivée par deux enzymes pour produire l’insuline et le peptide C. Au même moment, la granule perd son revêtement de clathrine et devient un granule mature lisse. Les granules matures sont ensuite soit dégradées par les lysosomes, soit stockés et sécrétés par exocytose. Dans ce dernier cas, il y a alors libération molaire de peptide C et d'insuline. Le peptide C ne possède aucune fonction physiologique; la mesure de sa concentration plasmatique sert à évaluer le potentiel sécréteur de la cellule β (Porte et Sherwin, 1997).

1.1.3.1.2. Sécrétion de l'insuline

La sécrétion d'insuline est étroitement contrôlée par la cellule β qui intègre divers informations métaboliques et physiologiques dans le but d'établir le taux de la sécrétion nécessaire à la régulation du métabolisme systémique. L'insuline est secrétée en réponse à différents stimuli, l'augmentation de la concentration plasmatique de glucose étant le facteur régulateur le plus important (Hutton, 1994). En outre, la réponse insulinémique est contrôlée par une variété d'autres facteurs, de moindre intensité que le glucose, incluant les acides aminés, les acides gras, des hormones gastro-intestinales et de stress, et certains neurotransmetteurs (Porte et Sherwin, 1997). Bien que les connaissances des mécanismes précis déclenchant la sécrétion d'insuline soient encore fragmentaires, certains éléments de ce processus ont été clairement identifiés. Le modèle suivant fait présentement consensus; Le transfert du glucose aux cellules β par diffusion passive faisant intervenir le transporteur Glut2 est suivi d'une étape de phosphorylation du glucose en glucose-6-phosphate par la glucokinase. Cette entrée de glucose dans les cellules β est proportionnelle au niveau du glucose sanguin (Matschinsky, 1990; Takeda et coll., 1993; Newgard et McGarry, 1995). Une augmentation du glucose intracellulaire favorise la production d'ATP intracellulaire par la phosphorylation oxydative faisant intervenir les mitochondries et le cycle de Krebs. L'ATP bloque à son tour les canaux potassiques ATP dépendant, ce qui conduit ultimement à une dépolarisation de la membrane et résulte en un influx du Ca^{2+} via les canaux calciques voltage dépendant. L'augmentation du Ca^{2+} intracellulaire semble représenter le signal déclencheur de l'exocytose des granules sécrétoires contenant l'insuline (Lang, 1999;

Newgard et McGarry, 1995). L'amplitude de la réponse insulinémique au glucose n'est pas médiée uniquement par la concentration plasmatique de glucose, mais aussi par le taux du changement du niveau de glucose, constituant ainsi deux phases dans la sécrétion d'insuline (Porte et Sherwin, 1997). La première phase de la sécrétion d'insuline, appelée aussi phase aiguë ou phase précoce, représente la réponse insulinémique immédiate à l'augmentation abrupte de glucose sanguin. Cette phase dure de 5 à 10 min. Par contre, la deuxième phase de la sécrétion insulinémique est caractérisée par une augmentation lente et progressive du glucose résultant en une sécrétion graduelle d'insuline. La sécrétion basale d'insuline, quant à elle, a lieu en absence d'une stimulation exogène. En outre, la sécrétion d'insuline ne se fait pas de façon continue, mais elle est de nature pulsatile (Porte et Sherwin, 1994). En général, la réponse à un signal approprié est bi-phasique: une phase aiguë (sécrétion d'un pool présent rapidement) suivie d'un deuxième pic plus prolongé si la stimulation persiste (synthèse de novo).

1.1.3.1.3. Récepteur de l'insuline

L'action d'insuline est médiée par un récepteur, décrit pour la première fois par Freychet et isolé par Cuatrecasa (Cuatrecasa, 1971; Freychet et Roth, 1971). Ce récepteur est une glycoprotéine transmembranaire et il est exprimé sur presque tous les types cellulaires des vertébrés. Son nombre varie d'environ 40 récepteurs par cellules dans les érythrocytes circulants à plus que 200 000 récepteurs par cellules dans les adipocytes (Kahn et Folli, 1993; White et Kahn, 1994). Le récepteur à l'insuline est un récepteur à activité tyrosine Kinase. (Dorrestijn et coll., 1998). Il est

composé de 2 sous-unités α et de 2 sous-unités β, unis ensemble par 3 liens disulfures. Les sous-unités α sont entièrement extracellulaires et contiennent les domaines de liaison de l'insuline. Les sous-unités β sont constituées par un domaine extracellulaire, une région transmembranaire et une partie intracellulaire. Cette dernière possède les domaines de liaison de l'ATP et de la tyrosine kinase (Dorrestijn et coll., 1998; Hunter et Garvey, 1998; Perz et Torlinska, 2001).

1.1.3.1.4. Action de l'insuline

L'action de l'insuline sur les tissus cibles résulte d'une cascade d'agent de signalisation qui s'initie par la liaison de l'insuline à son récepteur et qui prend fin par un certain nombre de réponses biologiques. Tous les processus impliqués ne sont pas encore très bien identifiés. Parmi les principaux événements biochimiques établis s'inscrit la liaison de l'insuline avec les sous-unités α, ce qui induit leur changement conformationnel. Une autophosphorylation des sous-unités β s'en suit, stimulant ainsi l'activité catalytique du récepteur, représentée par l'activité tyrosine kinase intrinsèque.

Le récepteur activé catalyse à son tour la phosphorylation sur des résidus tyrosine d'un certain nombre de protéines cytosoliques cibles surtout celles de la famille des IRS. Deux voies majeures de signalisation existent. La première implique la « *mitogen-activated protein kinase* » (MAPK) qui est activée par la liaison d'IRS ou Shc avec Grb2. Cette voie est principalement impliquée dans la médiation des

effets mitogéniques de l'insuline, quoiqu'un rôle au niveau du contrôle de la synthèse de glycogène hépatique ait été suggéré (Carlsen et coll., 1997).

La deuxième voie majeure est celle de PI-3 Kinase. Cette voie est principalement responsable des actions métaboliques de l'insuline. L'association d'IRS avec PI-3 kinase semble jouer un rôle primordial dans la stimulation des effets biologiques de l'insuline. Par ailleurs, les molécules stimulant la phosphorylation sur les résidus sérine/thréonine de l'IRS ou autres protéines effectrices en aval des voies de signalisation, inhibent l'action de l'insuline (Le Roith et Zick, 2001; Litherland et coll., 2001). Par exemple, l'activation de la protéine kinase C (PKC) se traduit par la phosphorylation des résidus sérine/thréonine et de l'inhibition de l'activité tyrosine kinase. D'autre part, plusieurs études ont constaté que l'hyperglycémie active la PKC (Roith et Zick, 2001; Williams et Schrier, 1992). Ces observations sont très importantes, car une réduction de l'activité tyrosine kinase est observée chez les sujets diabétiques et semble être à l'origine de la résistance à l'insuline (Arner et coll., 1987). En général l'activation de la voie de PI-3kinase ainsi que celle de TC10 sont responsables de la plupart des effets biologiques de l'insuline

1.1.3.1.4.1 Voie de signalisation de PI-3 kinase de l'insuline

Après la liaison de l'insuline à la sous-unité α du récepteur, la sous-unité β s'auto-phosphoryle et acquiert une activité tyrosine kinase accrue envers les substrats exogènes lui permettant de phosphoryler les protéines IRS-1 et 2 (*insulin receptor substrate*). Les IRS phosphorylés sur leurs résidus tyrosines interagissent avec la

sous-unité régulatrice p85 de la phosphatidyl-inositol 3 kinase (PI-3 kinase) ce qui conduit à l'activation de la sous-unité catalytique p110 de l'enzyme. L'activation des protéines kinases situées en aval de la PI-3 kinase, notamment les formes atypiques de la protéine kinase C (PKCζ et PKCλ) et la protéine kinase B (PKB ou Akt), permet le déclenchement du processus de la translocation des Glut4 (Tirosh et coll., 2000).

1.1.3.1.4.2 Voie de signalisation de TC10 de l'insuline

c-Cbl est une protéine de 120 kDa qui est phosphorylée par le récepteur à l'insuline. Cette phosphorylation permet à la protéine de se fixer à la protéine CAP (*c-Cbl associated protein*). CAP n'est exprimé que chez les adipocytes, renforçant le rôle spécifique du complexe CAP-c-Cbl dans la signalisation insulinique dans ces cellules. Une fois le complexe CAP-c-Cbl formé, celui-ci se dissocie du récepteur à l'insuline et migre vers les cavéoles qui sont des invaginations de la membrane plasmique hébergeant de nombreux récepteurs membranaires, des protéines G et des lipides membranaires spécialisés dans la signalisation cellulaire. Au niveau du cavéole, le complexe recrute la protéine Crk II qui est lié constitutivement à un facteur d'échange de GTP, C3G. Celui-ci catalyse l'échange du GDP pour le GTP sur la protéine TC10. Lors de son activation, la TC10 interagit avec plusieurs molécules effectrices en provoquant ainsi le déclenchement de la translocation de Glut4 (Baumann et coll., 2000; Chang et coll., 2004).

1.1.3.1.5. Effets biologiques de l'insuline

Le maintien de l'homéostasie du métabolisme du glucose implique une interaction complexe entre les niveaux de glucose circulant, les hormones pancréatiques, les nutriments ainsi que la distribution et l'échange des divers substrats entre les différents tissus. L'insuline est l'hormone clé de la régulation du métabolisme du glucose à la fois dans les conditions de jeûne et postprandiale. Par ailleurs, elle s'inscrit comme modulateur de la régulation du métabolisme intermédiaire. Toutefois, les effets sur le métabolisme des lipides et des protéines sont associés ou résultent de la régulation du glucose. Les tissus musculaire, hépatique et adipeux représentent les trois cibles principales des effets métaboliques de l'insuline (Le Roith et Zick, 2001; Saltiel et Kahn, 2001).

Dans les conditions postprandiales, la sécrétion de l'insuline diminue le taux du glucose sanguin en agissant en premier lieu sur son transport membranaire. En effet, le glucose est transporté dans les cellules cardiaques, adipeuses, et musculaires lisses par diffusion facilitée par le transporteur Glut4. C'est à ce niveau que l'insuline agit en stimulant la translocation du Glut4, localisé dans les nombreux pools intracellulaires, à la membrane plasmique (Hunter et Garvey, 1998). Environ 70 à 80% de ce glucose est pris par le muscle squelettique (Klip et Paquet, 1990). Nous pouvons facilement comprendre qu'une perturbation de l'effet de l'insuline à ce niveau, comme c'est le cas dans la résistance à l'insuline, aura des portées graves sur la glycémie plasmatique.

Quand au métabolisme cellulaire, l'insuline stimule l'oxydation du glucose et son stockage sous forme de glycogène. L'implication directe de l'insuline dans le contrôle de la glycogénogénèse a été démontrée par divers investigateurs, et ce dans les cellules musculaires et hépatiques. D'après ces travaux, l'insuline augmente l'activité de la glycogène synthase, une enzyme clé dans la synthèse du glycogène (Halse et coll., 1999; Saltiel et Kahn, 2001; Schudt, 1980). En parallèle, l'insuline inhibe la production et la sécrétion hépatique du glucose en bloquant le processus de gluconéogenèse et de glycogénolyse. Cette action est exercée par un effet direct sur le foie ou indirectement en jouant sur la disponibilité des substrats gluconéogéniques. L'effet direct est vraisemblablement via la modulation de l'activité de diverses enzymes et de l'expression des gènes codant pour les enzymes hépatiques de la gluconéogenèse et de la glycolyse (Saltiel et Kahn, 2001). Des études élégantes ont en effet confirmé l'inhibition par l'insuline de la transcription des gènes codant pour la phosphoenolpyruvate kinase (enzyme limitante de la gluconéogenèse), le fructose-1,6-bisphosphatase, et le glucose-6-phosphatase ainsi que l'augmentation de la transcription des enzymes glycolytiques (la glucokinase et la pyruvate kinase) et des enzymes lipogéniques (*fatty acid synthase* et acetyl-CoA carboxylase) (Claycombe et coll., 1998; El-Maghrabi et coll., 1991, 1988; Halse et coll., 1999; Hunter et Garvey, 1998; Katsurada et coll., 1990a b; Noupikel et Iynedjian, 1992; O'Brien et coll., 1990; O'Callaghan et coll., 2001).

Le métabolisme lipidique est, lui aussi, fortement influencé par l'insuline. Cet aspect reste malheureusement encore peu connu. Pourtant, connaissant l'association

entre la résistance à l'insuline et les dyslipidémies, l'insuline est à la fois un puissant activateur de la lipogenèse et un puissant inhibiteur de la lipolyse. En effet, l'insuline augmente l'activité de la lipoprotéine lipase (LPL) ainsi que sa sécrétion par le foie (Ashby et Robinson, 1980; Chiappe de Cingalani et coll., 1996; Picard et coll., 1999; Pradines-Figueres et coll., 1988). Simultanément, au niveau des adipocytes, l'insuline inhibe l'activité de la lipase hormono-sensible (LHS) l'enzyme clé de la lipolyse, limitant ainsi la libération des acides gras et du glycérol (Kraemer et Shen, 2002). Par ce double mécanisme, l'insuline diminue la concentration des acides gras libres et permet l'entreposage des triglycérides. De plus, l'insuline peut rehausser l'activité des enzymes impliquées dans la synthèse des lipides. Le traitement des adipocytes humains en culture avec l'insuline, a démontré clairement une augmentation de l'activité de la *fatty acid synthase* et de la glycérol-3-phosphate déshydrogénase (Moustaid et coll., 1996).

En ce qui concerne les protéines, nous pouvons dire que l'insuline avantage leur anabolisme. Tout d'abord, l'insuline stimule le taux de synthèse des protéines (Kimball et coll, 1994). Elle agit probablement à l'étape de l'initiation de la synthèse, en augmentant le nombre initial des ribosomes (Lyons et coll, 1980). Simultanément, elle bloque la protéolyse (Saltiel et Kahn, 2001).

1.1.3.2. Voie de signalisation impliquant l'AMPK

L'*AMP-activated protein kinase* (AMPK) est une enzyme ubiquitaire décrite comme le senseur métabolique de la cellule lui permettant de s'adapter aux modifications de son environnement (Hardie et coll., 1998). L'AMPK est un complexe trimérique constitué d'une sous-unité catalytique α et de deux sous-unités régulatrices β et γ. Cette protéine est activée physiologiquement par l'augmentation de la concentration intracellulaire en AMP lors des situations de carence énergétique cellulaire surtout lors d'une hypoxie, d'une hypoglycémie, d'un exercice physique et lors de l'inhibition ou le découplage de la chaîne de respiration mitochondriale (Winder et Hardie, 1999). Cette activation réduit les voies métaboliques consommatrices d'énergie (comme lipogenèse ou la synthèse stéroïdienne) et augmente les voies productrices d'ATP (comme l'oxydation des acides gras). L'AMPK est en effet activée dans le muscle en réponse à la contraction musculaire, à d'autres stress cellulaires ou à la stimulation par la leptine et l'adiponectine, deux hormones secrétées par l'adipocyte. Cette activation de l'AMPK entraîne une stimulation du transport insulino-indépendant de glucose suite à une activation de la translocation des Glut4 ainsi que l'oxydation des acides gras (Andreelli et Girard, 2005; Winder et Hardie, 1999). Dans le foie, l'activation de l'AMPK diminue la néoglucogenèse et la production hépatique de glucose (Bergeron et coll., 2001; Zhou et coll., 2001). Les effets métaboliques de l'AMPK déjà cités dépendent surtout de l'activation de l'isoforme catalytique α2 de l'AMPK. Ainsi, en stimulant le captage de glucose insulino-indépendant et l'oxydation lipidique des acides gras dans le muscle, et en diminuant la production hépatique de glucose, l'activation de l'AMPK

pourrait avoir des effets bénéfiques sur les anomalies métaboliques observées au cours du diabète de type II et de l'obésité (Winder et Hardie, 1999). En effet, des études récentes montrent que les deux principales classes des médicaments antidiabétiques à savoir les biguanides (la metformine et la phenformine) et les thiazolidinediones (la rosiglitazone, la troglitazone, la pioglitazone) exercent leurs effet antidiabétiques en bonne partie par l'activation de l'AMPK (Viollet et coll., 2007).

1.1.4. Définition du diabète

Selon la définition de l'OMS, le diabète est une affection métabolique caractérisée par la présence d'une hyperglycémie chronique résultant d'une déficience de sécrétion d'insuline, d'anomalies de l'action de l'insuline sur les tissus cibles, ou de l'association des deux (WHO, 1999).

1.1.5. Classification

La classification du diabète a évolué au cours des 50 dernières années. En effet, en 1980, l'OMS considérait, comme la plupart des cliniciens, qu'il y avait deux classes principales de diabète, le diabète insulinodépendant ou diabète de type I et le diabète non insulinodépendant ou de type II; des « diabètes d'autres types » et le diabète gestationnel étant aussi reconnus (WHO, comité d'expert, 1980). Les recommandations de l'OMS de 1999 proposent de supprimer les dénominations « insulinodépendant » et « non insulinodépendant », pour garder uniquement les termes « type I » et « type II » et détailler les différentes formes de « diabète d'autre type », tout en continuant d'individualiser le diabète gestationnel (WHO, 1999).

1.1.5.1. Diabète de type I

Le diabète de type I est un terme qui englobe le diabète secondaire à une destruction des cellules β pancréatiques et qui a tendance à provoquer l'acidocétose. Ce diabète représente 5 à 10% de tous les diabètes diagnostiqués. Le diabète de type I est subdivisé en deux sous-types; d'une part, le diabète auto-immun et d'autre part, le diabète idiopathique. Ainsi le diabète de type I est le plus souvent causé par une destruction auto-immune des cellules β. L'interaction entre les facteurs génétiques et environnementaux est à l'origine de ce processus (WHO, 1985; WHO, 1999). Une des caractéristiques majeures de diabète de type I est l'évolution très fréquente vers la dépendance envers l'administration exogène de l'insuline due à une déficience absolue de l'insuline. Certaines formes de diabète de type I n'ont pas d'étiologie connue; elles sont classées sous l'appellation de diabète idiopathique (WHO, 1999).

1.1.5.2. Diabète de type II

Le diabète de type II englobe les diabétiques présentant une insulino-déficience accompagnée par une résistance à l'insuline. En général, les individus atteints de ce type de diabète ne sont pas dépendants de l'insuline exogène. Toutefois, ils peuvent en avoir besoin pour le contrôle de la glycémie plasmatique, si les autres ressources telles que la diète, l'activité physique ou les agents oraux hypoglycémiants ne donnent pas les résultats escomptés (WHO, 2003). Le diabète de type II représente environ 90 à 95% de tous les cas diabétiques (WHO, 2003). Le diabète de type II représente le sujet plus particulier de cette thèse et est discuté en détail à la section 1.1.6

1.1.5.3. Diabète gestationnel

Le diabète gestationnel se caractérise par l'apparition ou la reconnaissance de l'intolérance au glucose observée au cours de la grossesse. Ce diabète, présent dans 2 à 4% des grossesses, peut parfois avoir des conséquences néfastes aussi bien sur le bébé que sur la mère (WHO, comité d'expert, 1980; WHO, 1999).

1.1.5.4. Autres types de diabète

La classe des autres types particuliers de diabètes secondaires est associée à une cause bien définie. Il s'agit des diabètes pancréatiques, endocriniens, des formes monogéniques de diabète ou des diabètes associés à un syndrome génétique ou provoqués par des agents chimiques. Toutefois, ces affections sont relativement peu connues (WHO, 1999).

1.1.6. Diabète de type II

Le diabète de type II n'est pas une entité distincte, mais plutôt un regroupement des divers désordres chroniques et progressifs menant au développement d'une hyperglycémie (WHO, 1999).

1.1.6.1. Physiopathologie du diabète de type II

Pendant de nombreuses années, le diabète de type II a été considéré comme une maladie lié à l'insulino-résistance, alors que la fonction pancréatique ne devenait anormale qu'à partir du moment où la glycémie à jeun commençait à s'élever (De Fronzo et coll., 1979). Aujourd'hui, il semble que les deux processus de perte de

fonction pancréatique et d'aggravation de l'insulino-résistance évoluent de façon parallèle, plus au moins indépendamment l'un de l'autre. En effet, la perte précoce de la fonction pancréatique devient un acteur à part entière apparaissant très tôt dans la maladie et joue un rôle majeur dans la physiopathologie du diabète de type II (Kahn, 1999; Pratley et Weyer, 2001). De même, les connaissances sur l'insulino-résistance ont été approfondies. Ainsi, d'une notion générale, on est passé à une analyse plus fine du processus dissociant les sites hépatique, musculaire, et adipocytaire de l'entrave à l'action de l'insuline.

Si l'insulino-déficience est l'élément majeur responsable de l'apparition de l'hyperglycémie, il faut cependant admettre que l'épidémie annoncée et reconnue de diabète de type II n'est certainement pas reliée à une détérioration soudaine de l'insulino-sécrétion chez les individus, mais bien une conséquence des changements de société auxquels nous assistons : sédentarité, modification des comportements alimentaires, urbanisation.

Les principales anomalies impliquées dans l'étiologie du diabète de type II sont : 1) une résistance aux diverses actions de l'insuline; 2) une perturbation de la sécrétion d'hormones pancréatiques; 3) une augmentation de la production hépatique de glucose. Il est à noter que toutes ces perturbations peuvent varier en fonction de la sévérité et la durée de l'obésité, de la résistance à l'insuline et du diabète (Girard, 1999; Porte et Sherwin, 1997).

1.1.6.2. Résistance à l'insuline

L'insuline produit tout un éventail d'effets biologiques sur les processus métaboliques et mitogéniques. Cependant, le terme de résistance à l'insuline s'applique plus spécifiquement à l'effet amoindri de l'insuline sur l'entrée de glucose dans les tissus insulino-sensibles, tels que le muscle et le tissu adipeux, et sur l'utilisation subséquente de ce glucose. La résistance à l'insuline est donc un état physiopathologique dans lequel les niveaux normaux ou élevés d'insuline produisent des effets biologiques atténués. Les principales manifestations cliniques de la résistance à l'insuline sont l'hyperinsulinémie et l'intolérance au glucose (Cefalu, 2001).

Au niveau musculaire, la résistance à l'insuline est attribuée principalement à une diminution de la synthèse du glycogène (Damsbo et coll., 1991; Shulman et coll., 1990). Un autre défaut métabolique est l'oxydation réduite des glucides (Kelly et coll., 1992).

Chez les adipocytes, l'action inhibitrice de l'insuline sur la lipolyse est diminue. Ceci résulte en une augmentation des acides gras libres (AGL) circulants qui, une fois captés par les autres tissus, contribuent au développement de l'hyperglycémie. Ainsi, au niveau du foie, les AGL stimulent la gluconéogenèse. Dans le muscle squelettique, ils augmentent l'oxydation lipidique et diminuent l'utilisation du glucose (Matschinsky, 1990). Enfin, dans le pancréas, l'accumulation des triglycérides peut induire l'apoptose des cellules β et affecter la sécrétion de

l'insuline (Girard, 2000). Il est donc clair que la résistance à l'action anti-lipolytique de l'insuline dans le tissu adipeux a d'importantes répercussions sur d'autres organes.

1.1.6.3. Dysfonctionnement des cellules β

Le dysfonctionnement des cellules β se traduit par des anomalies qualitatives et quantitatives de la sécrétion de l'insuline. L'un des premiers défauts dans la fonction des cellules, détecté déjà chez les sujets intolérants au glucose, se traduit par la diminution de la phase précoce de la sécrétion insulinique en réponse au glucose. L'absence complète de cette phase caractérise, par ailleurs, l'état de diabète. Un amoindrissement de la deuxième phase de la sécrétion d'insuline est, elle aussi, un des traits typiques de l'intolérance au glucose et du diabète. Les causes exactes de ces anomalies ne sont pas encore connues. Nous pouvons envisager l'hypothèse du défaut inné, mais aussi l'impact négatif de certains facteurs, tels que la glucotoxicité et la lipotoxicité (Girard, 1999; Boden, 2001).

De plus, il a été souvent rapporté que le pourcentage de pro-insuline plasmatique est plus élevé chez les sujets diabétiques de type II (Saad et coll., 1992). Cette anomalie reflète un défaut de clivage de la pro-insuline en insuline (Girard, 1999). Une constatation très intéressante est le fait que ce défaut semble précéder le développement du diabète. D'ailleurs, certains investigateurs ont rapporté qu'une augmentation de pro-insuline, absolue ou relative, est étroitement associée au risque de développer le diabète (Wareham et coll., 1999; Yoshioka et coll., 1988).

1.1.6.4. Production hépatique du glucose

La production basale du glucose hépatique est augmentée chez les diabétiques de type II et de nombreuses études ont observé une corrélation positive entre la production hépatique du glucose et le degré de l'hyperglycémie à jeun. L'augmentation de la production hépatique de glucose serait principalement due à une stimulation de la gluconéogenèse (Defronzo et coll., 1992). Chez les diabétiques, parmi les facteurs principaux responsables de la sur-activation de cette voie métabolique, nous pouvons mentionner la présence de l'hyperglucagonémie et les taux élevés des acides gras libres circulants (Defronzo et coll., 1992; Girard, 1999).

1.1.7. Complications

Le but principal du praticien face à un patient diabétique sera d'éviter l'apparition des complications inhérentes à la maladie. Ces complications sont de deux types : dégénératives et métaboliques.

Les complications dégénératives sont le résultat d'une évolution chronique de la maladie. Ces complications se divisent en fonction du calibre des vaisseaux sanguins en macro-angiopathies et en micro-angiopathies.

1.1.7.1. Micro-angiopathies

Le diabète de type II évolue généralement 7 à 8 ans avant d'être dépisté. Il en découle, qu'au moment du diagnostique, près de 30% des diabétiques de type II présentent une ou plusieurs des complications micro-vasculaires (Long, 2001). Les

mécanismes précis sous-jacents aux dommages induits par l'hyperglycémie ne sont pas très bien compris. Les hypothèses proposées aujourd'hui suggèrent qu'une accumulation de glucose dans les tissus librement perméables au glucose, tels que la rétine, le rein et les nerfs, induit quatre voies métaboliques qui sont à l'origine des dommages observés (Brownlee, 2001; Clark et Lee, 1995). Plus précisément, une élévation de la glycémie plasmatique entraîne 1) une augmentation intracellulaire de sorbitol par la voie d'aldose réductase; 2) la formation des produits terminaux de glucosylation avancé (*advanced glycation endproducts*); 3) l'activation de certaines isoformes de la protéine kinase C et 4) une stimulation de la voie d'hexosamine. Par divers mécanismes, ces changements induisent l'œdème, l'ischémie, la néo-vascularisation de la rétine provoquée par l'hypoxie, la protéinurie, l'expansion de la matrice mésangiale, la sclérose glomérulaire du rein et la dégénération multifocale des axones des nerfs périphériques (Brownlee, 2001). Ces mécanismes se traduisent par l'apparition des pathologies suivantes : la rétinopathie, la néphropathie et la neuropathie.

1.1.7.2. Macro-angiopathies

Le diabète de type II est un facteur de risque majeur et indépendant de diverses formes de maladies cardiovasculaires (MCV). Ces dernières représentent, d'ailleurs, la cause majeure de décès dans la population des diabétiques, étant responsable de 65% de la mortalité totale (Isomaa et coll., 2001; Koskinen et coll., 1992). De même, la coronopathie se développe à un âge plus jeune dans la population diabétique (Nathan, 1993). Le diabète est aussi associé à des dyslipidémies athérogènes et à une

dysfonction endothéliale. Toutefois, il faut mentionner le fait que les facteurs de risque de diabète, soit l'obésité et la résistance à l'insuline, sont eux mêmes impliqués dans l'étiologie des MCV (Assmann et coll., 1999; Ginsberg, 1991). Il se peut donc que le diabète n'apporte qu'une nouvelle composante, l'hyperglycémie, à des perturbations déclenchées bien avant. En effet, le diabète et les anomalies métaboliques impliquées dans le développement des MCV sont très liées, ce qui a engendré la reconnaissance d'un nouvel état pathologique, appelé le syndrome métabolique. En général, la définition du syndrome métabolique comporte autre que la présence de diabète et/ou la résistance à l'insuline, la présence d'au moins deux aux facteurs, tels que l'hypertension, la dyslipidémie, l'obésité et la microalbuminurie. Selon l'OMS, le syndrome métabolique est caractérisé par une glycémie à jeun supérieure à 6,1 mmoL/L, une triglycéridémie supérieure à 1,7 mmoL/L, un HDL-cholestérol plasmatique inférieur à 0,9 mmoL/L, un tour de taille supérieur à 94 cm et une microalbuminurie supérieure à 20 mg/min (WHO, 1998; 1999).

1.1.7.3. Autres complications

Les complications métaboliques comprennent l'acidocétose, le coma hyperosmolaire et l'acidose lactique (Aubert et Guittard, 1990; Fattorusso et Ritter, 1990). Le diabète est un facteur de risque d'infection (Fari, 1990) ainsi que d'anomalies articulaires et dermatologiques (Aubert et Guittard, 1990; Fattorusso et Ritter, 2006).

1.1.8. Traitement

Le traitement du diabète de type II repose sur un ensemble de mesures diététiques et d'hygiène de vie mais aussi sur des hypoglycémiants oraux. Le traitement de cette maladie est donc devisé en deux parties, couvertes dans les sections suivantes.

1.1.8.1. Traitement non médicamenteux

Le traitement du diabète de type 2 repose d'abord sur des modifications du style de vie (régime, perte de poids et exercice physique) et sur la prise en charge des facteurs de risque cardiovasculaire associés.

1.1.8.1.1. Alimentation

Des modifications nutritionnelles peuvent améliorer de nombreux aspects du diabète de type II. Le conseil principal est de réduire les calories afin d'amener à une diminution du poids. L'amélioration du contrôle glycémique induite par la perte de poids est associée à une réduction de l'insulino-résistance et à une augmentation de la sensibilité de la cellule β à répondre par une sécrétion d'insuline adaptée à la glycémie.

1.1.8.1.2. Exercice

L'exercice régulier est bénéfique dans le diabète de type II; il améliore le contrôle glycémique grâce à une augmentation de la sensibilité à l'insuline. De plus, la pratique de l'exercice physique associé à des conseils diététiques retarde la

progression de l'état pré-diabétique vers le diabète. Lorsque les mesures précédentes n'apportent pas un contrôle suffisant de la glycémie, l'introduction d'un hypoglycémiant oral s'avère nécessaire (Ducobu, 2003).

1.1.8.2. Traitement médicamenteux

Il y a actuellement 5 options thérapeutiques principales dans le diabète de type II : 1) augmenter la sécrétion d'insuline avec les sulfonylurées ou les glitinides; 2) augmenter la sensibilité à l'insuline avec un biguanide ou une thiazolidinedione (glitazone); 3) modifier l'absorption intestinale d'hydrates de carbone par un inhibiteur de l'alpha-glucosidase; 4) associer ces médicaments ou utiliser de nouveaux agents thérapeutiques tels que les analogues de la *Glucagon-like peptide-1* (GLP-1) ou les inhibiteurs de la dipeptidase de classe 4 qui dégrade normalement le GLP-1; 5) Administrer de l'insuline exogène.

1.1.8.2.1. Augmenter la sécrétion d'insuline

1.1.8.2.1.1. Les sulfonylurées

Les sulfonylurées sont les médicaments les plus utilisés pour le traitement du diabète de type II. Le récepteur aux sulfonylurées est une composante du canal ATP dépendant du potassium dans les cellules β du pancréas. La liaison des sulfonylurées conduit à l'inhibition de ces canaux qui modifient le potentiel de repos de la cellule, induisant un influx de calcium et une stimulation de la sécrétion d'insuline. Les sulfonylurées sont donc uniquement utiles chez les patients qui ont encore une fonction résiduelle des cellules β (Ducobu, 2003).

1.1.8.2.1.2. Les glitinides (ou glinides)

Ce sont des médicaments hypoglycémiants, de très courte durée d'action, utilisés soit seuls, soit en combinaison. Ils sont différents structurellement des sulfonylurées, mais agissent sur le même récepteur au niveau d'un site différent. Ils se fixent avec une très grande affinité et se détachent très rapidement de leur liaison. Ceci explique qu'ils stimulent plus vite la sécrétion d'insuline pendant un temps plus court (Ducobu, 2003).

1.1.8.2.2. Augmenter la sensibilité à l'insuline

1.1.8.2.2.1. Les biguanides

La metformine (Glucophage®) est le seul biguanide qui est actuellement disponible. Elle agit principalement en augmentant l'action de l'insuline au niveau du foie et en diminuant ainsi la production hépatique de glucose. Elle exerce aussi un effet anti-lipolytique qui diminue la concentration d'acides gras libres et réduit ainsi la disponibilité de substrat pour la néoglucogenèse. La metformine accroît aussi la captation et l'utilisation périphérique de glucose en augmentant, d'une part, la translocation ou la synthèse des transporteurs du glucose (Glut1 et Glut4) et, d'autre part, en favorisant la synthèse de glycogène. L'activation de la protéine kinase AMPK ainsi que la réduction de l'activité du récepteur nucléaire SREBP1C semblent être les mécanismes moléculaires par lesquels la metformine abaisse le glucose et les concentrations lipidiques (Ducobu, 2003).

1.1.8.2.2.2. Les thiazolidinediones ou glitazones

Les glitazones constituent une nouvelle classe d'hypoglycémiants oraux actuellement représentés par la rosiglitazone (Avandia®) et la pioglitazone (Actos®). Ces médicaments agissent en stimulant les récepteurs nucléaires PPARγ. Ils induisent un stockage plus efficace des acides gras libres dans le tissu adipeux. Dès lors, il y a moins d'acides gras libres libérés dans le sang. Cette diminution des acides gras libres entraîne trois conséquences : une diminution de la néoglucogénèse hépatique, une augmentation de l'action de l'insuline au niveau des muscles, du foie et du tissu adipeux (avec un accroissement conséquent de la captation périphérique de glucose) et une amélioration de la fonction des cellules β pancréatiques (par réduction de la lipotoxicité) (Yang et coll, 2002). Les thiazolidinediones accroissent aussi la sensibilité d'insuline dans le muscle squelettique des patients diabétiques de type 2, en facilitant l'activité du transporteur de glucose (Glut4) et en accroissant la synthèse de glycogène musculaire et l'oxydation de glucose. Tous ces effets métaboliques prennent 3 à 4 semaines pour se développer (Ducobu, 2003).

1.1.8.3. Modifier l'absorption intestinale d'hydrates de carbone

Inhibiteur de l'alpha-glucosidase (acarbose) ralentit l'absorption intestinale des hydrates de carbone et diminue aussi l'hyperglycémie postprandiale, mais son effet sur le contrôle glycémique est modéré par rapport aux autres hypoglycémiants oraux. Il peut être éventuellement utilisé en association. Son utilisation est plus répandue pour contrôler les hyperglycémies fonctionnelles postprandiales chez les non diabétiques (Coniff et krol, 1997). Il possède également des effets indésirables sur le

système digestif (flatulence, douleur abdominale et diarrhée) que l'on peut réduire en augmentant la dose très lentement (Ducobu, 2003).

1.1.8.4. Médicaments en investigation

- Des agonistes des PPARγ et α sont en cours d'investigation. Ils pourraient avoir à la fois des effets sur les hydrates de carbone et sur les lipides (Cox, 2006).

- Le GLP-1 est un peptide gastro-intestinal qui stimule la libération d'insuline, inhibe la libération du glucagon et réduit la vidange gastrique. En administration sous-cutanée, il abaisse l'élévation postprandiale du glucose. Comme le GLP-1 possède une très courte demi-vie (moins de 1 minute), son utilité clinique restera limitée. Des analogues du GLP-1 pourraient rendre son usage plus aisé. L'administration orale d'un inhibiteur de la peptidase qui dégrade le GLP-1 est aussi une voie de recherche (Abbatecola et coll., 2008).

1.1.9. Modèles animaux du diabète

Afin d'étudier l'étiologie de diabète et en raison de la gravité de ses nombreuses répercussions métaboliques et dégénératives, l'utilisation de modèles expérimentaux représente autant de voies d'accès dans la compréhension de la genèse et des complications de cette pathologie.

Durant ces dernières années, plusieurs modèles adéquats de diabète animal ont été mis au point, notamment chez le rat et la souris. L'installation du diabète chez les modèles animaux se fait soit spontanément, soit par induction chirurgicale,

endocrine, immunologique, ou encore par sélection ou par génie génétique. Dans les sections suivantes, bon nombre de ces modèles seront brièvement décrits (Cefalu, 2006; Sima et Shafrir, 2001).

1.1.9.1. Modèles animaux de diabète spontané.

La plupart de ces modèles sont étudiés pour leur spontanéité à développer un diabète de type II. Il s'agit de:

- La *souris ob/ob*. La maladie se transmet selon un mode autosomal récessif, l'hyperinsulinémie est provoquée par l'hyperphagie secondaire à une déficience en leptine et l'hyperglycémie apparaît secondairement à l'insulino-résistance.

- La *souris obèse "yellow mice"*. Le mode autosomal de transmission est dominant, l'obésité et l'hyperinsulinémie sont liées à une hypertrophie des adipocytes; une intolérance au glucose induit une hyperglycémie postprandiale.

- La *souris NZO* (*New Zealand Obese*). La maladie est de type polygénique, l'animal est obèse et présente une légère hyperglycémie et une hyperinsulinémie.

- La *souris KK* (japonaise) diabétique. Le diabète est secondaire à l'hyperphagie et l'obésité modérée qui en résulte. Une glycosurie est observée.

1.1.9.2. Modèles animaux de diabète induit par inoculation de virus

L'exemple le plus connu est l'infection de la souris par le virus EMC (Encephalomyocarditis). Ce virus entraîne un diabète en pénétrant dans la cellule β. L'ADN viral s'intègre au génome de la cellule β hôte provoquant ainsi une altération des fonctions de ces cellules et notamment de la synthèse et de la sécrétion d'insuline.

1.1.9.3. Modèles animaux de diabète induit par pancréatectomie

La méthode de pancréatectomie chirurgicale induite chez le rat permet de réaliser une ablation de 90% du pancréas endocrine. La glycémie à jeun reste d'abord normale, mais 6 à 7 semaines après la pancréatectomie, elle s'élève légèrement et il apparaît, chez ces animaux, une intolérance au glucose.

1.1.9.4. Modèles transgéniques

Les techniques de génie génétique ont permis d'obtenir des animaux permettant l'étude du diabète. Le modèle le plus utilisé est le *rat Zucker*. Il présente une obésité, une insulino-résistance, une hyperinsulinémie, une hyperlipidémie mais une glycémie normale. Son pancréas est hypertrophique, hyperplasique et hypersécrétoire.

1.1.9.5. Modèles animaux de diabète induit par le régime alimentaire

Ces modèles ont permis de mettre en évidence le rôle de la consommation hypercalorique et de l'âge associés à un manque d'activité physique.

- *La souris Spiny (Acomys chirinus)*. Cet animal vit dans les régions désertiques et semi-désertiques autour du bassin méditerranéen. Un régime de laboratoire riche en sucrose provoque chez cette souris une réduction des enzymes de la glycolyse, une réduction de la lipogenèse qui entraîne une hyperlipidémie, une intolérance au glucose, et une hyperinsulinémie, mais ne provoque pas d'hyperglycémie ni d'obésité. Un régime riche en lipides induit une obésité, une intolérance au glucose, une

hyperinsulinémie, une augmentation du glucagon plasmatique avec une hyperglycémie mais sans changement dans le contenu pancréatique en insuline.

- *Le rat des sables (Psammomys obesus).* Dans son milieu naturel, cet animal se nourrit de plantes salées pauvres en calories. Lorsque soumis à un régime standard de laboratoire et la sédentarité de la captivité, 40% des animaux deviennent obèses et développent un diabète de type II à partir du 3ème mois.

Tous ces modèles expérimentaux de diabète démontrent bien que cette maladie possède des traits pathologiques complexes et diversifiés. Cependant, chaque modèle permet d'en étudier un aspect particulier, que ce soit au niveau cellulaire, moléculaire, biochimique ou génétique, en référence à ce qui est observé chez l'être humain. La mise au point de ces modèles a permis une meilleure compréhension de la physiopathologie et de l'évolution naturelle du diabète, notamment dans le but de développer des approches thérapeutiques antidiabétiques (Cefalu, 2006; Sima et Shafrir, 2001).

1.1.9.6. *Mériones shawi*

Ce modèle s'apparente à celui du rat des sables et est décrit en détail ici car il a servi plus spécifiquement à certaines études *in vivo* effectuées pour cette thèse. La mérione de shaw est un rongeur de la famille des Gerbillideae. La mérione de shaw est l'espèce la plus grande du genre mériones. La longueur de la queue est inférieure ou égale à celle du corps plus la tête et se termine par un pinceau terminal noir. Le pelage dorsal varie du fauve au gris piqueté de brun. Le pelage ventral est blanc. Les

pattes postérieures et antérieures sont de formes différentes, les pattes postérieures sont allongées et adaptées au saut, les antérieures, sont plus courtes, servent pour la prise d'objets ou de nourriture. Les pattes postérieures ont une sole plantaire (figure 1).

La mérione de shaw est répandue dans la région du nord du Sahara marocain à l'Égypte. Cette espèce habite les régions steppiques arides et des zones pré-désertiques où la période de végétation est brève (2 à 5 mois par ans) sans toutefois s'aventurer dans les régions désertiques. La mérione présente un rythme d'activité crépusculaire et nocturne. Cet animal est à la fois herbivore, granivore et insectivore et peut se passer complètement d'eau libre. La mérione creuse des terriers peu profonds labyrinthiques communiquant entre eux.

Plusieurs méthodes sont utilisées pour la capture de cet animal. Parmi les plus utilisées, on compte la méthode basée sur l'utilisation de pièges appâtés pour la capture de l'animal (l'appât peut être une datte ou un morceau de pain imbibé d'huile d'olive). Non moins fréquente est celle de l'inondation des terriers. Après s'être assuré de l'existence de mérione de shaw dans le terrier par la présence de traces de pattes, de débris de plantes ou d'excréments, on place les cages dans différentes sorties du terrier. Celui-ci est ensuite inondé d'eau afin d'obliger les animaux à sortir pour se trouver piégés dans les cages.

Ces gerbilles développent un diabète de type II lorsqu'ils sont soumis à un régime standard de laboratoire avec une inactivité physique. En effet, 46% des animaux deviennent obèses et développent un diabète de type II à partir du 3ème mois. Ce pourcentage peut atteindre 72% après une durée de 9 mois. Après cette période, ce diabète s'associe à une augmentation des lipides plasmatiques ainsi qu'à une hypertension diastolique (Settaf et coll., 2000).

Figure 1 : Photo de *Mériones shawi*

1.2. Phytothérapie et diabète

1.2.1. Histoire des plantes médicinales

La phytothérapie est une approche thérapeutique ancestrale qui remonte aux fonds des âges les plus reculés de l'histoire de l'humanité. Les plantes médicinales ont toujours été tenues en haute estime pour leurs propriétés actives sur la santé de l'être humain. En effet, pour combattre la maladie et apaiser la souffrance, l'homme devait puiser dans son milieu naturel.

Aux origines des temps, quand l'homme émergeait de la nuit obscure, il devait – pour une question de survie – déjà comprendre la notion de la plante biologiquement active, comprenant aussi bien la plante qui guérit que la plante qui tue. Mais il devait aussi contrôler les quantités et la durée du traitement. Celui qui maîtrisait ce savoir, représentait le sorcier, le mage et le guérisseur. Il détient à la fois la science des poisons et la science des drogues, d'où leur «pouvoir surnaturel».

Le cumul des acquisitions thérapeutiques à travers les âges, continue à se développer et s'enrichir de jour en jour jusqu'à aujourd'hui. De nos jours, la confiance qu'on accordait aux plantes médicinales n'a pas disparue. Au contraire, celles-ci suscitent un vif regain d'intérêt et profitent des progrès technologiques les plus avancés; ceux-là mêmes développés en partie par l'industrie pharmaceutique moderne. Et c'est ainsi que les secrets les plus intimes se dévoilent de manière de plus en plus détaillée.

Les tablettes de signes cunéiformes, datant de l'époque Sumérienne (4000 ans avant JC) et découvertes en 1948, sont considérées par les historiens comme les premiers recueils des formules de "plantes médicinales". On y dénombre 250 plantes utilisées en suspension ou en tisanes. Parmi les civilisations antiques qui ont marqué le plus l'histoire de la phytothérapie, l'Égypte Pharaonique occupe sans doute la première place.

Les égyptiens ont constitué les premiers principes de l'utilisation des plantes médicinales ou "Pharmacopée" dont le nom dérive du mot égyptien "*Farmaké*" (qui guérit). La découverte des dessins de certaines plantes médicinales sur les temples, les sanctuaires et les pyramides des anciens égyptiens prouve tout l'intérêt que ceux-ci accordaient à ces drogues.

En Asie, et plus particulièrement en Chine, l'empereur "Chen-Nong", connu sous le nom de "laboureur" et qui a vécu vers 3000 ou 2500 ans avant JC, accorda beaucoup d'importance à l'agriculture. Il fut le premier à étudier la phytothérapie et à dégager de l'expérimentation certains effets thérapeutiques ou, au contraire nocifs des plantes. La médecine chinoise lui doit la découverte de 365 plantes dont l'opium, le cannabis, la cannelle, la noix de muscade et ainsi de suite, qu'il classa dans un répertoire médical où il fixa les grandes indications de ces plantes.

En Inde, les drogues végétales entraient dans la composition des formules magiques, moins destinées directement à soigner qu'à soumettre les forces occultes de la nature. Ce sont les Sumériens et les Égyptiens qui, forts d'une solide culture pharmacologique, enseignèrent aux Grecs et aux Étrusques la méthode permettant de distinguer les plantes dangereuses des plantes curatives, ainsi que l'art de préparer des infusions et des décoctions médicales.

À l'époque Romaine, du moins dans les premiers siècles, les experts en matière de botanique étaient toujours Grecs et ce n'est que plus tard que les Romains

s'attachèrent à l'étude de la flore, et à l'élaboration des médicaments qu'ils en pouvaient obtenir.

Les arabes furent, eux aussi de grands connaisseurs de plantes. Ils avaient hérité, de la grande culture des perses, de solides préceptes médicaux, tout en se référant à Galien (131-201 après JC) pour l'essentiel de sa doctrine. Les écoles arabes de Bagdad et de Cordoue furent célèbres et c'est encore aux arabes que les Italiens de l'école de Salerne se sont dirigés, au 12ème siècle, pour retrouver des secrets qui avaient été perdus dans les temps barbares.

Depuis cette époque jusqu'à nos jours, les herbes, les baies, les racines et autres produits végétaux n'ont pas cessé d'être l'objet d'étude approfondies, si bien que chaque jour leurs vertus sont reconnues davantage, mieux comprises et plus utilement appliqués. (Confalonière, 1970).

1.2.2. Plantes médicinales antidiabétiques

Outre les approches thérapeutiques décrites plus haut et comprenant la diététique et l'exercice physique, l'insulinothérapie ou les hypoglycémiants oraux, les individus souffrant de diabète de type II ont recours, et ce depuis des temps immémoriaux, à la médecine traditionnelle pour traiter leur maladie, notamment en utilisant une variété de plantes. L'évaluation de l'effet antidiabétique de ces plantes et la recherche de principes actifs est une voie prometteuse de recherche de nouveaux produits antidiabétiques.

À travers le monde, plus de 800 plantes ont été utilisées pour combattre le diabète ou ses principaux symptômes (Alarcon-Aguilara et coll., 1998). Bien que la majorité de ces plantes n'ont pas fait l'objet d'études détaillées, plusieurs de celles qui ont subit une analyse expérimentale ont montré une activité hypoglycémiante. Par ailleurs, certaines autres se sont avérées sans aucune action sur le métabolisme de glucides. Dans certains cas, les études ont même réussi à identifier les principes actifs responsables de l'activité hypoglycémiante. Il s'agit principalement de glycosides isolés des familles des Compositae, Convolvulaceae, Ericaceae, Moraceae, Papaveraceae, Ranunculaceae, et Scrophulaceae. Les glycanes seraient responsables de l'action hypoglycémiante de plantes appartenant aux familles Ranunculaceae et Graminae. D'autre principes actifs auraient une action hypoglycémiante comme certains terpènes, sulfites, polysaccharides, huiles, vitamines, alcaloïdes, saponines, acides aminés, peptides, protéines et autres composés (Atta-ur-Rahman et Zaman, 1989; Ivorra et coll., 1989).

L'action hypoglycémiante des plantes peut s'effectuer selon plusieurs mécanismes. Parmi ceux-ci, les chercheurs ont entre autres identifié la stimulation de l'insulino-sécrétion, l'inhibition du glucagon ou d'une autre hormone hyperglycémiante et l'amplification de l'action de l'insuline au niveau de certaines réactions clés de la glycolyse, de la glycogénogenèse (Eno et coll., 2008; Yin et coll., 2008; Park et coll., 2006).

1.3. *Nigella sativa*

1.3.1. Position systématique (Negre, 1962)

La famille des renonculacées comprend une trentaine de genres et environ 1200 espèces.

Classe : Dicotylédones

Sous classe : Dialypétales

Série : Thalamiflores

Ordre : Ranales

Famille : Renonculacées

Genre : Nigella

Espèce : *N. sativa*

1.3.2. Identification et aspect botanique de *N. sativa*

La nigelle possède trois noms latins *Cuminum nigrum, N. indica et N. sativa*, ce dernier étant le plus employé. Ce sont des noms dérivés du latin "niger" qui signifie noir. *N. sativa* possède aussi une multitude de noms à travers le monde, nous en citons quelques uns dans le tableau II :

Tableau II : Différents nom communs de *N. sativa*

Région	Synonymes de *N. sativa*
Arabique	Sinouj, Sanouz, Shunez, Habbah sauda, Habbet el beraka, Kamun aswad
Arménienne	Shoushma
Allemande	Zwiebelsame,Schwarzkümmel
Anglaise	Devil in the bush, Love in the mist, Fennel flower, Onion seed
Estonienne	Mustköömen
Finlandaise	Neidonkuka
Française	Cheveux de vénus, Nigelle, Poivrette

Hindi	Kalounji, Munga reala
Hongroise	Feketekömény, Parasztbors, Kerti katicavirág, Borzaskata mag
Italienne	Nigella, Melanzis
Norvégienne	Svartkarve
polonaise	Czarnuszkawna
Punjabie	Kalongi
Russe	Charnushka
Singhalaise	Kaluduru
Espagnole	Niguilla, Pasionara
Suédoise	Svartkummin
Tamile	Karun jiragam
Turquie	Çörekottu siyah

Originaire d'Asie occidentale, *N. sativa* est une plante herbacée, annuelle, à tige dressée, côtelée, anguleuse et rameuse d'une soixantaine de centimètres de hauteur, portant des feuilles inférieurs pétiolées et des feuilles supérieurs sessiles. Les pétales des fleurs sont petites, blanches et mêlées de bleu. Les pétales sont au nombre de cinq et présentent une tache verte au sommet (figure 2 panneau A). Le fruit est une capsule largement ovoïde et couverte de tubercules granuleux. Il est formé de 3 à 6 carpelles soudées entre elles et renferment des graines noires triangulaires et ridées transversalement (figure 2 panneau B).

Les espèces voisines de *N. sativa*, sont également utilisées en phytothérapie. Nous en citons trois décrites selon Negre en 1962 (Negre, 1962) : *N. hispanica, N. arvensis, N. damascena.*

Figure 2 : Photos présentant la fleur et les graines de *N. sativa*

Panneau A : fleur de *N. sativa*

Panneau B : graines de *N. sativa*

1.3.3. Origine et implantation

N. sativa est originaire de l'Asie occidentale. Sa culture s'est propagée depuis l'Asie jusqu'en Afrique et en Amérique. Elle se trouve très répondue en Inde, en Iraq et dans certains pays de la méditerranée, notamment la Syrie, la Turquie et les pays d'Afrique du nord. Les principaux pays producteurs de *N. sativa* sont les États-Unis, l'Inde, le Pakistan, l'Iran, l'Iraq, la Syrie et l'Égypte. Dans les pays de l'Afrique du nord, la nigelle est cultivée dans les régions côtières. Elle fleurit au mois de mai et les graines sont récoltées au mois de juillet.

1.3.4. *N. sativa* et l'histoire de son usage traditionnel

La nigelle tient une place importante parmi les plantes médicinales les plus utilisées et ce, depuis plus que 2000 ans. Elle était le « *Chanquit* » des anciens égyptiens. Elle est citée dans leurs papyrus comme un médicament pour les maladies

pulmonaires et la toux. Elle est aussi citée dans certaines livres sacrés ainsi que dans le traité des simples d'Hippocrate. Discorde, préconisait l'usage des graines de *N. sativa* contre les maux de tête, les affections des yeux, les maux des dents et les morsures d'araignées. Galien conseille de les brûler pour tuer les moucherons et les moustiques et Tragus les employait comme antihelminthiques (Chamss-Edine, 1972).

On crédite à l'excellence des arabes en médecine l'élargissement du champ d'application de cette plante en thérapeutique. Les disciples du prophète Mohamed, tel qu'Ibn Atîq, avait utilisé les graines de *N. sativa* macérées dans l'huile d'olive. On instille trois gouttes de cette huile dans chaque orifice nasal pour traiter la grippe qui s'accompagnait d'éternuements en salves (Alami, 1989).

Avicenne (*Ibn Sina*) conseillait de griller les graines et de les réduire en poudre. Cette préparation est mise dans une bourse en tissu qu'on fait inhaler quotidiennement pour désobstruer les voies nasales, selon le même principe qu'un vaporisateur (Al-Nassimi, 1984). Avicenne préconisait aussi la nigelle dans la dyspnée et dans le traitement de l'asthme et les bronchites. Cette même préparation, prise avec de l'eau bouillie, possède des actions diurétiques et dissolvantes des calculs rénaux (Ibn Sina, 1972). D'autre part, la prise de l'huile de nigelle avec de l'huile de l'olive était très réputée comme aphrodisiaque. À la nigelle on reconnaît aussi des propriétés emménagogues, galactagogues, abortives, vermifuges, et ténicides.

Les graines de *N. sativa* en poudre utilisée en cataplasme avec du vinaigre aurait une action résolutive dans les pustules et la gale surinfectée et aurait une action verrucide, si on lui ajoute de miel (Ibn Sina, 1972; Ibn Al-Qaîm, 1957). Enfin, les médecins arabes connaissaient la toxicité de *N. sativa* à forte dose. De ce fait, ils recommandaient de ne pas dépasser une dose unitaire d'un demi-Dirham (1,62 g) et une dose journalière de deux Dirhams (6,48 g). Le tableau III résume l'ensemble des activités citées dans la médecine populaire de certains pays du pourtour méditerranéen et de l'orient.

Tableau III : Activité des graines de *N. sativa* dans la médecine traditionnelle de certains pays du bassin méditerranéen et de l'orient.

Pays	Activité	Extraction	Voie d'administration	Références
Inde	Abortive	Infusion	Orale	Malhi et Trivedi, 1972
	Diaphorétique	Infusion	Orale	El Zawahry, 1964
	Emménagogue	Infusion	Orale	El Zawahry, 1964
	Galactagogue	Infusion	Orale	Burkil, 1966
	Vermifuge	Infusion	Orale	Burkil, 1966
	Diurétique	Infusion	Orale	Burkil, 1966
	Antimites	Non cité	Externe	Salama, 1973
Jordanie	Vermifuge	Décoction	Orale	Al-Khalil, 1995
	Stomachique	Décoction	Orale	Al-Khalil, 1995
	Carminative	Décoction	Orale	Al-Khalil, 1995
	Dépurative	Décoction	Orale	Al-Khalil, 1995
	Sudorifique	Décoction	Orale	Al-Khalil, 1995
	Emménagogue	Décoction	Orale	Al-Khalil, 1995
	Antiasthmatique	Décoction	Orale	Al-Khalil, 1995
	Antidiabétique	Graines	Orale	AL-Hader et Aqel, 1993
Malaisie	Aménorrhée	Infusion	Orale	Burkil, 1966
Maroc	Antiasthmatique	Graines	Orale	Bellakhdar et coll., 1991
	Antidote de poison	Graines	Orale	Bellakhdar et coll., 1991
	toxique	Graines	Orale	Bellakhdar et coll., 1991
	Abortive	Graines	Orale	Bellakhdar et coll., 1991
	Antigrippe	Graines	Orale	Bellakhdar et coll., 1991
Népal	Emménagogue	Infusion	Orale	Suwal, 1970
	Galactagogue	Infusion	Orale	Bhattaray, 1992

Oman	Anti-paralysie	Graines	Externe	Ghazanfar et Al-Sabahi, 1993
	Anti-congestion	Infusion	Orale	Ghazanfar et Al-Sabahi, 1993
	Antiasthmatique	Infusion	Orale	Ghazanfar et Al-Sabahi, 1993
Turquie	Douleurs	Graines	Orale	Yesilada et coll., 1995
Yémen	Antiémétique	Non cité	Orale	Fleurentin et Pelt, 1982
Ethiopie	Anti-immflamatoire	Non cité	Orale	Deschamps et coll., 1983
Iran	Aménorrhée	Graines	Orale	Zagari, 1993
	Antihelminthique	Graines	Orale	Zagari, 1993
Arabie saoudite	Digestive	Infusion	Orale	Al-Yahya, 1986
	Appétissante	Infusion	Orale	Al-Yahya, 1986
	Antipyrétique	Infusion	Orale	Al-Yahya, 1986
	Anti-diarrhée	Infusion	Orale	Al-Yahya, 1986
	Antihelminthique	Infusion	Orale	Al-Yahya, 1986

1.3.5. Phytochimie de *N. sativa*

La famille des Renonculacées et notamment l'espèce *N. sativa* ont bénéficié de nombreuses études phytochimiques. Des échantillons de graines de *N. sativa* ont été analysés et caractérisés en termes de propriétés physiques, de composés chimiques, de minéraux et de composés lipidiques. Ces études ont montré la présence d'une diversité de substances naturelles regroupant des lipides, des terpènoïdes, des flavonoïdes, des alcaloïdes et des saponines. *N. sativa* constitue une importante source de protéines (21%) et de sels minéraux : phosphore, calcium, potassium, magnésium et sodium (Aboutabl et coll., 1986). Les valeurs et proportions fournies par la littérature diffèrent d'un auteur à l'autre; la variété et l'origine des échantillons peuvent en être partiellement responsables.

1.3.5.1. Lipides et terpènoïdes

Les graines *N. sativa* renferment environ 0,4 – 0,45% d'huile essentielle, plus de 30% d'huiles fixes (Dominiczak et coll., 1991; Hashem et El-Kiey, 1982) et 38%

de lipides totaux (Martin et coll, 2001). Les acides oléique et linoléique sont les deux importants acides gras de l'huile de *N. sativa*, ils constituent 75,2% des acides gras totaux (Abdel-Aal et Attia, 1993). D'autres auteurs fournissent des valeurs différentes : les graines contiendraient 26,6% d'huiles dont 64,6% d'acide linoléique et 20,4% d'acide palmitique (Sener et coll., 1985).

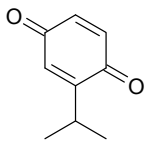
Les huiles de *N. sativa* contiendraient approximativement 46% de monoterpènes et 25% de composés carbonyles dont 24,5% de thymoquinone, 1,7% de phénol, 0,9% d'alcools et 16% d'esters (Aboutabl et coll., 1986). D'autres études montrent que les huiles fixes contiendraient 0,17% de thymoquinone et de nombreux acides gras dont la composition varie peu selon l'origine des échantillons. Le tableau IV présente la composition des échantillons des huiles fixes des graines de *N. sativa* selon l'origine.

Tableau IV : Composition (en pourcentage des huiles fixes) des graines *N. sativa* en acides gras selon l'origine de l'échantillon (Houghton et coll., 1995).

Acide gras	Origine de l'échantillon des graines de *N. sativa*		
	Éthiopie	Inde	Syrie
Mystique	0,21	0,2	0,22
Palmitique	12,07	13,15	14,64
Stéarique	2,7	2,97	2,6
Oléique	23,46	25,67	24,51
Linoléique	58	54,68	54,13
Arachidique	0,2	0,25	0,2
Linolénique	0,47	0,68	0,69
Eicosadienoique	2,87	2,39	3,02
Saturés totaux	15,18	16,57	17,66
Insaturés totaux	84,82	83,43	82,34

La thymoquinone est un monoterpène oxygéné (figure 3). C'est une substance très active, sa polymérisation permet d'obtenir la nigellone. Cette dernière retient la plupart des propriétés pharmacologiques de la thymoquinone et présente l'avantage d'être moins toxique (Mahfouz et coll., 1960).

Figure 3 : Représentation schématique de la structure chimique de la thymoquinone



1.3.5.2. Saponosides

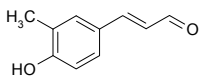
Les saponosides sont des hétérosides de stérols ou de triterpènes. Ce sont des composés très répandus dans le règne végétal. Solubles dans l'eau, il libère par hydrolyse un ou plusieurs oses et une génine (sapogénine). La première saponine isolée par Greenisch en 1882 à partir des graines de *N. sativa* est la mélanthine. Récemment, d'autres saponosides ont pu être isolés à partir d'un extrait éthanolique des graines de *N. sativa* dont le 3-O-[beta-D-xylopyranosyl-(1-3)-alpha-L-rhamnopyranosyl-(1-2)-alpha-L-arabinopyranosyl]-2 identifié par Ansari (Ansari et coll., 1975), alors que de nombreux autres saponosides ont pu être déterminés à partir des huiles de *N. sativa* (Abdel-Aal et Attia, 1993).

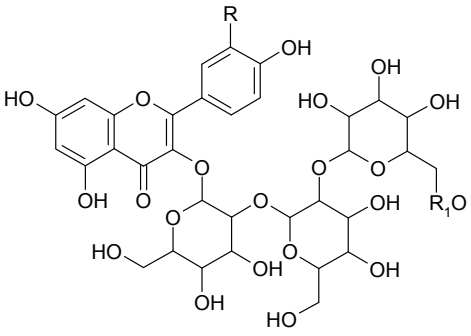
1.3.5.3. Flavonoïdes

Les flavonoïdes sont des composés aromatiques dont la biosynthèse constitue l'un des processus fondamentaux de la phytochimie. Ils font partie de ce que l'on

appelle les composés phénoliques. Les flavonoïdes sont des substances généralement colorés très répandus chez les végétaux. Parmi les flavonoïdes présentant un intérêt, nous citerons les anthocyanes qui présentent des couleurs différentes selon divers pH : du rouge-orange en milieu acide au bleu-mauve en milieu alcalin. Les Renonculacées sont un groupe riche en flavonols et en flavones. En 1997, trois nouveaux flavonoïdes triglycosylées (figure 4) ont été isolés par Merfort à partir des graines de *N. sativa* et leurs structures ont été déterminées (Merfort et coll., 1997).

Figure 4 : Trois flavonoïdes isolés des graines de *N. sativa*

	R	R_1
1	**OH**	**H**
2	**H**	**H**
3	**OH**	

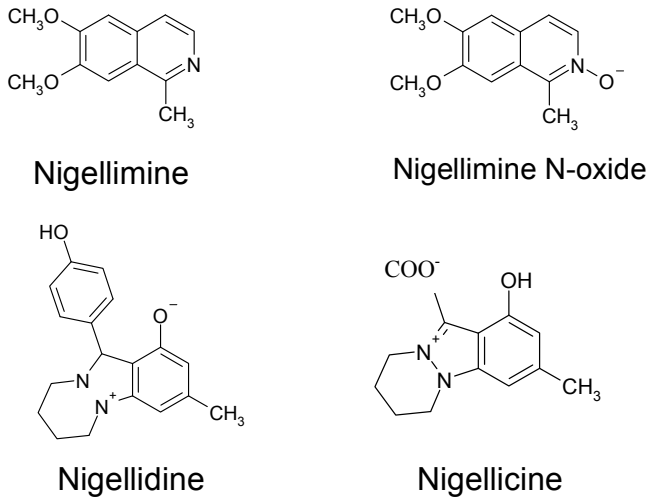


1.3.5.4. Alcaloïdes

Les alcaloïdes ont des substances présentant des caractères alcalins, contenant de l'azote, le plus souvent inclus dans un hétérocycle. Les alcaloïdes ont, pour la plupart, des actions physiologiques et thérapeutiques à faibles doses. Ils deviennent cependant très toxiques à fortes doses; c'est pourquoi nombre d'entre eux figurent sur les Annexes A et B des substances venimeuses de Santé Canada. Les plus importants alcaloïdes de *N. sativa* (figure 5), ont été isolés à partir des graines par

Atta-ur-Rahman entre 1985 et 1995 : Nigellicine (Atta-ur-Rahman et coll., 1985b), Nigellimine (Atta-ur-Rahman et coll., 1992), Nigellimine N-oxyde (Atta-ur-Rahman et coll., 1985a) et Nigellidine (Atta-ur-Rahman et coll., 1995).

Figure 5 : Structure chimiques des plus importants alcaloïdes isolés des graines de *N. sativa*



Pour clore cette section consacrée à l'étude phytochimique de *N. sativa*, nous présenterons les molécules et les composés isolés et identifiés des *N. sativa* ainsi que leurs natures et origines dans le tableau V.

Tableau V : Principaux composés et molécules isolés de *N. sativa*.

Composé	Nature	Partie de la plante	Pays	Auteurs
Alanine	AA	Graine	URSS	Kudryashova et kolobkova, 1953
β-Amyrin	Triterpène	Huile de graine	Grèce	Menounos et coll., 1986
Acide Arachidique	AG	Huile de graine	Inde	Bhakarai et coll., 1992
Acide Arachidonique	AG	Huile de graine	Inde	Bhakarai et coll., 1992
Acide Ascorbique	Vitamine	Feuille	non cité	Scheunert et coll., 1952
Acide Aspartique	AA	Graine	URSS	Kudryashova et kolobkova, 1953
5-dehydro-Avenastérol	Stéroïde	Huile de graine	Grèce	Menounos et coll., 1986
7-dehydro-Avenastérol	Stéroïde	Huile de graine	Grèce	Menounos et coll., 1986
Butyrospermol	Triterpène	Huile de graine	Grèce	Menounos et coll., 1986
Campestanol	Stéroïde	Huile de graine	Grèce	Menounos et coll., 1986
Carvacrol	Stéroïde	Huile de graine		Michelitsch et coll., 2004
Campestérol	Stéroïde	Huile de graine	Grèce	Menounos et coll., 1986
Carvone	Monoterpène	Huile essentielle	Inde	Rathee et coll., 1982
Cholestrol	Stéroïde	Huile de graine	Soudan	Salama, 1973
Citrostadienol	Triterpène	Huile de graine	Grèce	Menounos et coll., 1986
Cycloartanol	Triterpène	Huile de graine	Grèce	Menounos et coll., 1986
Cycloarténol	Triterpène	Huile de graine	Grèce	Menounos et coll., 1986
Cycloeucalénol	Triterpène	Huile de graine	Grèce	Menounos et coll., 1986
Cystine	Alcaloïde	Graine	URSS	Kudryashova et kolobkova, 1953
Damascenine	Alcaloïde	plante entière	URSS	Kudryashova et kolobkova, 1953
Méthyl-Damascenine	Alcaloïde	Graine	Égypte	El Zawahry, 1964
Dithymoquinone	Monoterpène	Huile de graine	Arabie Saoudite	Basha et coll., 1995
Dna	Alcaloïde	Racine	Inde	Banerjee et sharma, 1995
Acide Eicosadienoique	AG	Huile de graine	Égypte	Babayen et coll., 1978
Huile Essentielles	Lipides	Graine	URSS	Jukneviciene et coll., 1977
Huile Fixes	Lipides	Graine	Pakistan	Malik et khan, 1965
Flavonoides		Graine		Merfort et coll., 1997
Glucose	Sucre	Graine	Inde	Tiwari, 1946
Acide Glutamique	AA	Graine	URSS	Kudryashova et kolobkova, 1953
Gramistérol	Triterpène	Huile de graine	Grèce	Menounos et coll., 1986
Hederagénine	Triterpène	Graine	Égypte	Mustafa et Soliman, 1943
Acide Indole-3-acétique	Alcaloïde	Tissu du calice	Inde	Mukherjee et coll., 1981
Leucine	AA	Graine	URSS	Kudryashova et kolobkova, 1953
Acide Linoléique	AG	Huile de graine	Inde	Ansari et coll., 1975
Acide Linolénique	AG	Huile de graine	Égypte	Babayen et coll., 1978
Lophénol	Triterpène	Huile de graine	Grèce	Menounos et coll., 1986
24-Ethyl-Lophénol	Triterpène	Huile de graine	Grèce	Menounos et coll., 1986
24-Méthyl-Lophénol	Triterpène	Huile de graine	Grèce	Menounos et coll., 1986
Lysine	AA	Graine	URSS	Kudryashova et kolobkova, 1953
Melanthine	inconnue	Graine	Égypte	El Zawahry, 1964
Acide Myristique	AG	Huile de graine	Inde	Bhakare et coll., 1992
Nigellicine	Alcaloïde	Graine	Pakistan	Atta-ur-Rahman et coll., 1995
Nigellidine	Alcaloïde	Graine	Pakistan	Atta-ur-Rahman et coll., 1992
Nigellimine	Alcaloïde	Graine	Pakistan	Atta-ur-Rahman et coll., 1985b
Nigellimine-N-Oxide	Alcaloïde	Graine	Pakistan	Atta-ur-Rahman et coll., 1985a
Nigelline	Alcaloïde	Huile essentielle	Égypte	Mahfouz et coll, 1962

Nigellone	Quinoïde	Graine	non cité	Canonica et coll., 1963
Obtusifoliol	Triterpène	Huile de graine	Grèce	Merfort et coll., 1997
Acide Oléique	AG	Huile de graine	Égypte	Babayen et coll., 1978
Acide Palmitique	AG	Huile de graine	Turquie	Singh et coll., 1978
Acide Palmitoléique	AG	Huile de graine	Inde	Bhakare et coll., 1992
Protéines	Protides	Graine	Égypte	Babayen et coll., 1978
Saponine 1	Triterpène	Graine	Pakistan	Ansari et coll., 1988
β Sitostérol	Stéroide	Huile de graine	Turquie	Sener et coll., 1985
α Spinastérol	Stéroïde	Huile de graine	Soudan	Salama, 1973
Acide Stéarique	AG	Huile de graine	Inde	Bhakare et coll., 1992
Stigmastanol	Stéroïde	Huile de graine	Grèce	Menounos et coll., 1986
Stigmastérol	Stéroïde	Huile de graine	Grèce	Menounos et coll., 1986
Taraxérol	Triterpène	Huile de graine	Grèce	Menounos et coll., 1986
Acide Telfarique	AG	Huile de graine	Tchèque	Bures et mladkova, 1930
Thymol	Monoterpène	Huile de graine	Arabie Saoudite	Basha et coll., 1995
Thymoquinone	Monoterpène	Huile de graine	Arabie Saoudite	Basha et coll., 1995
Tirucallol	Triterpène	Huile de graine	Grèce	Menounos et coll., 1986
Tryptophane	AA	Graine	URSS	Kudryashova et kolobkova, 1953
Valine	AA	Graine	URSS	Kudryashova et kolobkova, 1953

1.3.6. Toxicité de *N. sativa*

La toxicité de la nigelle est bien connue par la plupart des herboristes. En effet, elle n'est utilisée qu'à faible dose, que ce soit par la voie interne, externe, en fumigation ou en inhalation. Un surdosage des graines de *N. sativa* peut être mortel. J. Bellakhdar (1978) a rapporté dans son ouvrage que le surdosage thérapeutique peut provoquer des d'avortements. Cette toxicité, comme celles de la plupart des espèces de la famille des renonculacées, est due essentiellement à la présence de forte quantité de saponines et d'alcaloïdes dans les graines de nigelle. Mahfouz et collaborateurs en 1965, et ensuite Tenekoon et collaborateurs en 1991, ont étudié la toxicité des extraits aqueux et alcooliques de *N. sativa*. Les concentrations plasmatiques de γ-glutamyl transférase (GGT) et de l'alanine aminotransférase (ALAT) ont été augmentées chez le rat après un traitement oral durant 14 jours; cependant aucune anomalie histologique n'a été observée chez ces rats (Tenekoon et coll., 1991). Zaoui et collaborateurs ont rapporté que les huiles fixes présentent un

DL50 de 28,8 mL/kg (po) et 2,06 mL/kg (ip). Dans le même sens, la toxicité chronique de 2 mL/kg des huiles durant 12 semaines présente des valeurs normales pour les ALAT, les GGT et l'aspartate aminotransférase (ASAT) (Zaoui et coll., 2002). La plupart de ces études montrent clairement que la nigelle possède un index thérapeutique élevé et une excellente innocuité à des doses inférieures à 4 g/kg/jour de *N. sativa.*

1.3.7. Activité antitoxique de *N. sativa*

Plusieurs études chez les rats et les souris ont montré que les graines de *N. sativa* pourraient avoir un effet protecteur contre certains produits toxiques (Badary et coll., 2000; El Dakhakhny et coll., 2000b; El-Daly, 1998; Nair et coll., 1991). Effectivement, un extrait de *N. sativa* protège contre la chute du niveau d'hémoglobine et du nombre de leucocytes, quand les souris sont intoxiquées par l'administration intra-péritonéale de 2 mg/kg de Cisplatine pendant 5 jours (Nair et coll., 1991). Chez le rat, il a été aussi démontré que la thymoquinone protège les hépatocytes isolés de foie de rat contre la toxicité induite par l'hydroperoxyde du tertabutyl (TBHP), un agent toxique connu pour ses dégâts oxydatifs et la perte progressive du glutathion (GSH) intracellulaire qu'il provoque (Daba et Abdel-Rahman, 1998). Il est aussi rapporté que *N. sativa* protège contre les effets délétères de hyperhomocysteinémie induite par la méthionine chez le rat (El-Saleh et coll., 2004). Un groupe de chercheurs indiens a montré que *N. sativa* diminue les effets secondaires de la chimiothérapie au potassium bromate ($KBrO_3$) (Khan et coll., 2003). L'équipe de Mahmoud a trouvé que *N. sativa* possède des effets

hépatoprotecteurs puisque elle permet de régulariser les taux plasmatiques de tous les enzymes impliqués dans le fonctionnement hépatique chez des souris infectés par *Schistosoma Mansoni* (Mahmoud et coll., 2002).

1.3.8. Activités pharmacologiques de *N. sativa*

1.3.8.1. Activité hypoglycémiante de *N. sativa*

Tel que décrit au Tableau IV, le traitement avec diverses préparations de *N. sativa* engendre systématiquement une diminution de la glycémie chez différents modèles animaux. Plusieurs mécanismes ont été proposés pour expliquer cette activité hypoglycémiante qui sont aussi détaillés au Tableau IV. Certains de ces mécanismes, telle l'implication de l'insuline, demeurent sujets de controverse. Ainsi, chez le lapin rendu diabétique par l'alloxane, *N. sativa* présente une action hypoglycémiante (Al-Hader et coll., 1993). Selon les mêmes auteurs, cet effet serait indépendant des mécanismes insuliniques puisque l'amélioration de la glycémie n'était pas accompagnée par des modifications de l'insulinémie. D'autres études effectuées chez le rat des sables (*Psammomys obesus*) ont montré que le niveau plasmatique de l'insuline pourrait être réduit par le traitement oral par l'extrait aqueux des graines de *N. sativa* (Labhal et coll., 1997). Par opposition, d'autres études ont montré que l'effet hypoglycémiant de *N. sativa* pourrait être lié à une augmentation du niveau d'insuline dans le sang (Eskander et coll., 1995). De même, les études de la sécrétion d'insuline à l'aide de modèles *in vitro* ont aussi donné des résultats contradictoires. Récemment, notre équipe a contribué à la démonstration du fait que *N. sativa* inhibe l'absorption intestinale de glucose chez le rat Sprague

Dawley (Meddah et coll., 2009), ce qui pourrait participer à la réduction de la glycémie, surtout en période postprandiale. Finalement, l'inhibition de la gluconéogenèse hépatique ainsi que la forte activité antioxidante de *N. sativa* ont aussi été mis en cause dans l'activité hypoglycémiante de la plante.

Grâce aux travaux présentés dans la présente thèse, nous avons fourni une contribution significative dans l'élucidation des sites cellulaires et moléculaires d'action de *N. sativa.*

Tableau VI : différentes études montrant l'effet antidiabétique de N. sativa et les différents mécanismes d'action proposés.

Effet	Traitement	Dose	Model biologique	Durée de traitement	Références
↓**glycémie**	Mélange de plantes (po)	1g/kg	Rat streptozotocine	1 semaine	Al Awadi et coll., 1987
	Mélange de plantes (po)	1g/kg	Rat streptozotocine	2 semaines	Al Awadi et coll., 1991
	Huiles volatiles (ip)	50 mg/kg	Lapin de Nouvelle Zélande traité à l'alloxane	4 - 6 h	El-Hader et coll., 1993
	Extrait aqueux (po)	2 g(eq de plante)/kg	*Meriones shawi*	9 mois	Labhal et coll., 1999
	Extrait aqueux (po)	1 g/kg	Lapin de Nouvelle Zélande traité à l'alloxane	2 mois	Meral et coll., 2002
	Huiles fixes (po)	1 mL/kg	Rat normoglycémique	3 mois	Zaoui et coll., 2002
	Huiles volatiles (po)	400 mg/kg	Hamster streptozotocine-nicotinamide	4 semaines	Fararh et coll., 2002
	Huiles fixes (po)	0,4 g/kg	Rat streptozotocine	2 - 6 semaines	El Dakhakhny et coll., 2002
	Huiles volatiles (ip)	200 µL/kg	Rat streptozotocine	30 jours	Kanter et coll., 2003
	Huiles volatiles (ip)	200 µL/kg	Rat streptozotocine	4 semaines	Kanter et coll., 2004
	huiles fixes (po)	400 mg/kg	Hamster streptozotocine	1 à 4 semaines	Fararh et coll., 2004
↑**insulinémie**	Huiles volatiles (po)	400 mg/kg	Hamster streptozotocine-nicotinamide	4 semaines	Fararh et col.l, 2002
	Huiles volatiles (ip)	200 µL/kg	Rat streptozotocine	30 jours	Kanter et coll., 2003
	Huiles volatiles (ip)	200 µL/kg	Rat streptozotocine	4 semaines	Kanter et coll., 2004
↓**insulinémie**	Extrait aqueux (po)	1 g(eq de plante)/kg	*Psammomys obesus*	3 mois	Labhal et coll., 1997
	Extrait aqueux (po)	2 g(eq de plante)/kg	*Meriones shawi*	9 mois	Labhal et coll., 1999
± sécrétion d'insuline	Huiles fixes, Nigellone, thymoquinone	0-100 µg/mL	Îlots de pancréas de rat isolés	1 h	El Dakhakhny et coll., 2002
↑ **sécrétion d'insuline**	Extrait dégraissé, fraction alcaline	0,01 to 5 mg/mL	Îlots de pancréas de rat isolés	30 min	Rchid et coll., 2004
↓**neoglucogenèse**	Mélange de plantes (po)		Hépatocytes de rat isolés	1 semaine	Al Awadi et coll., 1991
	huiles fixes (po)	400 mg/kg	Hépatocytes d'hamster streptozotocine isolés	2 h	Fararh et coll., 2004
↓**absorption intestinal**	Extrait aqueux	0,1 pg to 100 ng/mL	Jéjunum de rat isolé	instantané	Meddah et coll., 2009
antioxydant	Extrait aqueux (po)	1 g/kg	Lapin de Nouvelle Zélande traité à l'alloxane	2 mois	Meral et coll., 2002
	Huiles volatiles (ip)	200 µL/kg	Rat streptozotocine	4 semaines	Kanter et coll., 2004
	Thymoquinone (po)	0,5-1 mg/kg	Rat hypertensive NO déficient	4 semaines	Khattab et Nagi, 2007

1.3.8.2. Activités cardio-vasculaire de *N. sativa*

En 1962 Mahfoud et collaborateurs ont confirmé l'effet antihypertenseur en utilisant des huiles de graines de nigelle. D'autres chercheurs ont montré que les huiles volatiles des graines de *N. sativa*, administrées par voie intraveineuse chez le rat normotendu, pouvaient aussi induire une baisse de la pression artérielle (El-Tahir et coll., 1993). Selon ces chercheurs, l'effet hypotenseur observé serait d'origine cérébrale via des mécanismes autant sérotoninergiques que muscariniques. Un groupe marocain a démontré que les huiles de *N. sativa* réduisent la tension artérielle chez le rat spontanément hypertendu. Cet effet serait partiellement lié à l'activité diurétique des graines de *N. sativa* (Zaoui et coll., 2000). Les mêmes effets ont été observés quand les huiles de *N. sativa* ont été remplacées par la thymoquinone, celle-ci étant un important principe actif des huiles de *N. sativa*. La thymoquinone normalise notamment la pression artérielle chez le rat rendu hypertendu par déficience en NO (Khattab et Nagi, 2007).

Par ailleurs, la nigelle et ses dérivés possèdent aussi des effets antispasmodiques qui se manifestent par une inhibition des contractions spontanées et une baisse du tonus de la musculature lisse *in vitro*, qu'elle soit de nature vasculaire (Aqel, 1992), utérine (Aqel et Shaheen, 1996) ou intestinale (Aqel, 1993). Selon ces auteurs, cet effet antispasmodique serait probablement dû à une activité antagoniste au niveau des canaux calciques membranaires de type L. De même, une étude a montré que les graines de *N. sativa* possèdent un effet broncho-dilatateur engendré par un mécanisme d'antagonisme calcique (Gilani et coll., 2001).

Finalement, une étude japonaise a permis de découvrir que les huiles fixes de *N. Sativa*, ainsi que trois molécules pures isolées de ces huiles, possèdent un effet inhibiteur sur la coagulation sanguine et l'agrégation plaquettaire comparable à celui de l'aspirine (Enomoto et coll., 2001).

1.3.8.3. Activités hypocholestérolémiante et hypolipémiante de *N. sativa*

Le groupe de Labhal ont montré que l'extrait aqueux des graines de *N. sativa* aurait une action hypocholestérolémiante et hypotriglycéridémiante chez le rat des sables et que cette action serait accompagnée d'une baisse de l'insulinémie (Labhel et coll, 1997). Ces effets ont été également soulignés par d'autres auteurs chez le rat diabétique (Al-Awadi et Shoukry, 1988; Eskander et coll., 1995). Chez le rat Wistar, la nigelle diminue significativement les valeurs de cholestérol et des triglycérides au niveau plasmatique (Zaoui et coll., 2002). Une étude biochimique a démontré que les huiles fixes des graines de *N. sativa* pouvaient inhiber la production de leucotriène B-4 et de thromboxane B-2 au niveau des liposomes, une inhibition de la peroxydation des lipides et augmenter la perméabilité membranaire des leucocytes en culture (Houghton et coll., 1994).

1.3.8.4. Action de *N. sativa* sur la masse pondérale

En Afrique de nord, les graines de *N. sativa* sont traditionnellement préconisées dans le traitement de l'obésité et de l'hyperlipidémie qui l'accompagne souvent. Cette action anti-obésité a été confirmée chez le rat des sables traité oralement par un extrait aqueux des graines de *N. sativa* (Labhel et coll., 1997).

Dans le même sens, les huiles fixes de *N. sativa* permettent de diminuer le poids corporel à partir de 6 semaines chez le rat *Wistar* (Zaoui et coll., 2002). Ces résultats sont confirmés par l'étude menée par Meddah et collaborateurs où un traitement de 6 semaines avec l'extrait aqueux des graines de nigelle a réduit la masse pondérale des rats *Wistar* (Meddah et coll., 2009).

1.3.8.5. Activité de *N. sativa* sur la réponse immunitaire

Pour la première fois en 1987, l'effet de *N. sativa* sur la réponse immunitaire a été évalué chez des sujets humains volontaires. L'administration de capsules renfermant la poudre de *N. sativa* a entrainé, après cinq semaines de traitement, une augmentation de la population lymphocytaire *T helper* (CD4) et a contribué à l'amélioration du rapport cellules *T helper*/cellules T suppresseurs (CD4/CD8). Par ailleurs, *N. sativa* augmente de 30% l'activité des cellules tueuses NK (El-Kadi et Kandil, 1987). Une autre étude a montré que l'extrait aqueux de *N. sativa* stimule la réponse lymphocytaire et la production des interleukine-3. *N. sativa* stimule aussi la libération des interleukines-1β. Cela entraîne une stimulation de l'activité phagocytaire des leucocytes polynucléaires et macrophages (Haq et coll., 1995).

1.3.8.6. Activité antitumorale de *N. sativa*

L'extrait de *N. sativa* réduit l'incidence de sarcomes et diminue le diamètre des tumeurs induites par des substances chimiques carcinogènes (Salomi et coll., 1991). Ce même groupe a déterminé que *N. sativa* possède un effet antitumoral envers plusieurs types de cellules malignes. Cette action est due à une inhibition de

l'incorporation de thymidine au niveau de l'ADN (Salomi et coll., 1991). L'extrait d'acétate d'éthyle de *N. sativa* lui aussi agit contre la prolifération chez différentes lignées cellulaires cancéreuses (Swamy et Tam, 2000). Kumara et Huat (2001) ont isolé un principe actif (α-hederine) qui a une forte activité antitumorale chez des souris implantés avec un sarcome de poumon et le lymphome murin P388.

1.3.8.7. Activité antibactérienne et antifongique

De nombreuses études *in vitro* ont montré que l'extrait par le diéthyle éther de graines de *N. sativa* a entraîné, d'une manière dépendante de la concentration, une inhibition de *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* et la levure pathogène *Candida albicans* (Hanafy et Hatem, 1991). L'activité antibactérienne des huiles volatiles de *N. sativa* a été également démontrée sur 37 souches de *Shigella dysenteriae, Shigella flexneri, Shigella sonnei* et *Shigella boydii* ainsi que 10 souches de *Vibrio cholerae* et *Escherichia coli* (Ferdous et coll., 1992).

1.3.8.8. Autres actions biologiques de *N. sativa*

Plusieurs autres activités biologiques de *N. sativa* ont été rapportées dans la littérature. La liste suivante en cite la majeure partie :

Activité chronotrope négative (El-Tahir et coll., 1993b);

Stimulant respiratoire (El-Tahir et coll., 1993a);

Activité anti-nématodes (Abivardi., 1971);

Effet anti-convulsant (Devynck et coll., 1982);

Inhibition de plaque dentaire (Namba et coll., 1985);

Inhibition de la formation de l'aflatoxine (El-Shayeb et Mabrouk, 1984);

Activité analgésique (Khanna et coll., 1993);

Activité hémostatique (Gracza, 1986);

Activité neuroleptique (Devynck et coll., 1982);

Protection contre la fibrose et la cirrhose du foie (Turkdogan et coll., 2001);

Protection contre l'ulcère (El-Dakhakhny et coll., 2000);

Activité anti-oxydante (Burits et Bucar, 2000).

En plus de son action hypoglycémiante, la nigelle possède d'autres propriétés bénéfiques sur la plupart des désordres métaboliques et anomalies associés au diabète de type II, notamment son action antihypertensive, sa capacité à contrôler le poids corporel et son pouvoir de régler l'hypercholestérolémie et hypertriglycéridémie. On peut donc conclure que cette plante possède un potentiel thérapeutique prometteur dans le contexte du syndrome métabolique.

1.4. Prémisses de l'étude

D'après les études effectuées durant les deux dernières décennies sur les effets pharmacologiques de *N. sativa* déterminent avec certitude que cette plante possède un effet anti-hyperglycémique non seulement chez des modèles animaux de diabète de type I mais aussi chez ceux de type II. Cependant, rares sont les études qui ont essayé de déterminer les mécanismes cellulaires et les voies de signalisation sous-jacentes qu'emprunte *N. sativa* pour exercer sont effet antidiabétique soit *in vitro* ou *in vivo*. Afin de répondre combler ce vide, le projet de doctorat a cherché à comprendre l'implication des principaux mécanismes physiologiques impliqués dans

la régulation de la glycémie dans l'action anti-hyperglycémique de la nigelle. En premier lieu, il convenait donc d'examiner l'impact de la nigelle sur la sécrétion de l'insuline chez les cellules β pancréatiques et sur le transport de glucose chez les cellules du muscle squelettique et tissu adipeux. En second lieu, il paraissait important de comprendre les effets de *N. sativa* sur les voies de signalisation impliquées dans le métabolisme du glucose, notamment celle de l'insuline, de l'AMPK et du PPARγ, autant *in vitro* qu'*in vivo.*

2. Article 1

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Anti-diabetic activity of *Nigella sativa* seed extract in cultured pancreatic beta cells, skeletal muscle cells, and adipocytes.

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Short Title : Tissue specific effects of *N. sativa*

Keywords : glucose uptake, insulin secretion, natural health products, traditional medicine, type 2 diabetes mellitus.

ABSTRACT

The seeds of *Nigella sativa* (NS), a plant of the Runanculaceae family, are used in traditional medicine in North Africa and the Middle East for the treatment of diabetes. Despite widespread use and a number of scientific studies, target tissues and cellular mechanisms of action of this plant product are not well understood. This study evaluated the effects of NS seed crude ethanolic extract on insulin secretion in INS832/13 and βTC-tet lines of pancreatic beta cells and on glucose disposal by C2C12 skeletal muscle cells and 3T3-L1 adipocytes. An 18 h treatment with NS amplified glucose-stimulated insulin secretion by more than 35%, without affecting sensitivity to glucose. NS treatment also accelerated beta cell proliferation. An 18 h treatment with NS increased basal glucose uptake by 55% (equivalent to approximately two-fold of the effect of 100 nM insulin) in muscle cells, and approximately by 400% (equal to the effect of 100 nM insulin) in adipocytes; this effect was perfectly additive to that of insulin in adipocytes. Finally, NS treatment of pre-adipocytes undergoing differentiation accelerated triglyceride accumulation comparably to treatment with 10 μM rosiglitazone. It is concluded that the well-documented in-vivo anti-hyperglycaemic effects of NS seed extract are attributable to a combination of therapeutically-relevant insulinotropic and insulinomimetic / insulin-sensitizing properties.

INTRODUCTION

Type 2 diabetes mellitus is a disorder characterized by defects in insulin action and insulin secretion resulting in glucose intolerance and chronically elevated glycaemia (Bianchi et al, 2007; Meece, 2007). More than 150 million people worldwide are afflicted by this condition and this figure is expected to double in the next 20 years (Boutayeb et al, 2004). Current medications for the treatment of TIIDM are limited, and no one medication can enhance insulin secretion and sensitivity simultaneously (Cohen et al, 2007); there is a clear need for new and more active therapeutic agents. Products from the plant realm hold tremendous potential for meeting this growing need. Indeed, thousands of plant species are documented to produce anti-hyperglycaemic effects when ingested (Alarcon-Aguilara et al., 1998; Yeh et al, 2003), and metformin, the most widely prescribed insulin-sensitizer, is derived from a metabolite isolated from *Galega officinalis L,* a plant consumed for centuries as a treatment for diabetes (Bailey C.J., 2000)

Nigella sativa (NS*)* is an herbaceous plant of the Ranunculaceae family that grows spontaneously and widely in several southern Mediterranean and Middle Eastern countries (Haddad et al, 2006). The seeds of NS*,* also known as black seed or black cumin, are often used as a spice but are also used extensively in the traditional medicine of many of these countries, including Morocco, to treat diabetes and several other disorders (Haddad et al., 2001; Bellakhdar et al., 1991; Ziyyat et al., 1997). The anti-hyperglycaemic effects of NS have been the object of numerous in-vivo scientific studies using various animal models (Haddad et al, 2006; Le et al.,

2004; Rchid et al., 2004; Kanter et al., 2003; El-Dakhakhny et al., 2002; Farah et al., 2002; Meral et al., 2001; Labhal et al., 1999, 1997; Al Hader et al., 1993; Al-Awadi et al., 1991, 1987, 1985). While in-vivo effects are well-known, few studies have addressed the mechanisms of action of NS extract and there remains controversy regarding its site(s) of action (Haddad et al, 2006). The aim of this study was to elucidate target tissues through which NS extract exerts its anti-hyperglycaemic action by using cell-based assays of anti-diabetic activity. This study demonstrates that NS seed extract possesses the unusual and therapeutically-relevant properties of increasing insulin secretion while simultaneously exerting insulin-like effects in peripheral tissues.

MATERIAL AND METHODS:

Plant material

Seeds of NS were obtained from an herbalist in Rabat, Morocco in August 2003 and were authenticated by an experienced botanist (Prof. Oulyahya, Institut Scientifique, Rabat, Morocco). A voucher specimen has been deposited in the herbarium of the Institut Scientifique of Rabat. Seeds were washed and dried, and then powdered with an electric microniser. Powder was extracted three times with 80% ethanol and the solvent was evaporated at 40 °C under reduced pressure. This procedure resulted in a two-phased extract. The oily and the solid phases were recombined in proportion to their yield (typically 55% and 45%, respectively) and solubilized freshly in DMSO prior to treatment of cells. The seeds of Trigonella foenum-graecum (Fenugreek) were purchased from Lone Wolf Herb (Phippen, SK Sask) for use as a positive control and were extracted similarly to NS. All extracts were conserved at 4 °C and protected from light and humidity.

Reagents and cell-lines

Cell culture reagents were purchased from Invitrogen (Burlington, ON) and Wisent (Saint Bruno, QC). Other reagents were purchased from Sigma-Aldrich (Saint Louis, MO), unless otherwise noted. INS832/13 rat β pancreatic cells and βTC-tet murine β pancreatic cells were generous gifts from B. Newgard (Duke University) and S. Efrat (Tel-Aviv University), respectively. C2C12 murine myoblasts and 3T3-L1 murine pre-adipocytes were purchased from ATCC (Chicago, IL).

Cell culture

C2C12 myoblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, 10% HS and antibiotics (penicillin 100 UI/ml, streptomycin 100 μg/ml) at 37°C and 5% CO_2. Upon 75-80% confluence, serum content was reduced to 2% HS in order to induce differentiation into myotubes. Medium was changed every 48 h. Experiments were performed on well-differentiated multinucleated cells 6 to 7 days later. 3T3-L1 cells were grown in DMEM supplemented with 10% FBS and antibiotics until 75-80% confluent. 3-isobutyl-1-methylxanthine (500 μM), insulin (500 nM) and dexamethasone (10 μM) were then added to the medium for 2 days to induce adipogenesis. Cells were allowed to differentiate for an additional 10 to 14 days in insulin-containing (500 nM) medium until over 90% of cells contained lipid droplets visible by phase-contrast microscopy. INS832/13 cells were grown in RPMI 1640 medium containing 11 mM glucose and supplemented with 10 % FBS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM beta-mercaptoethanol, and antibiotics, until 80% confluent. βTC-tet cells were grown in DMEM supplemented with 15% HS and 2.5% FBS until 80% confluent. All cells were treated with 200 μg/ml of total ethanolic extract in culture medium with a final DMSO concentration of 0.1%, with the exception of INS832/13 treated at a dose of 100 μg/ml. This concentration was based on a maximal solubility of 200 mg/ml in DMSO. The concentrations tested did not induce any morphological changes following 18 h treatment in any of the four cell types employed.

Insulin secretion assay

The INS832/13 and βTC-tet cell lines were used to test for effects of NS on potentiation of glucose-stimulated insulin secretion (GSIS). These pancreatic β cell lines release insulin in response to physiological concentrations of glucose in a dose-dependent manner (Hohmeier et al, 1998; Knaack et al, 1994; Efrat et al, 1995; Fleischer et al, 1998). INS832/13 cells were seeded in 12-well plates at a density of 2-4 x 10^5 cells per well and incubated in growth medium for 24 h in complete RPMI until 80% confluence. Cells were then treated for 18 h with or without NS, and with glucose adjusted to 3 mM to confer glucose sensivity to the cells. βTC-tet cells were seeded in 12 well plates at a density of 2.5 x 10^5. Upon reaching 80% confluence, growth medium was supplemented with 1 μg/ml tetracycline for 48 h to arrest proliferation and to remove any influence of potential proliferative effects on measurement of insulin secretion. The βTC-tet cell line is developed from transgenic mice expressing the SV40 T antigen (Tag) under the control of the insulin II promoter and regulated by the tetracycline conditional gene expression system. This cell line is dependent on Tag expression for its proliferation: in the absence of tetracycline, the Tag transgene is expressed and the cells proliferate normally, while in the presence of tetracycline, Tag expression is shut off and cell replication is arrested. Growth arrested βTC-tet cells were then treated for 18 h with or without NS extract. β pancreatic cell lines were rinsed and pre-incubated with Krebs-Ringer HEPES Buffer (KRHB) (10 mM HEPES, 2 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, pH 7.4, 135 mM NaCl, 3.6 mM KCl, 1.5 mM CaCl, 0.5 mM $MgCl_2$ and 0.07% fatty-acid-free BSA) for 1 h at 37 ºC in presence or absence of the extract. For INS832/13

cells, the medium was then replaced with KRHB containing 2 mM glucose in the presence or absence of the extract (basal insulin secretion) or KRHB containing 4 mM or 11 mM glucose, or 30 mM KCl in the presence or absence of extract for an additional period of 1 h. βTC-tet cells were treated with 2 mM (basal insulin secretion), 6 mM, 8 mM, 10 mM or 16 mM glucose, or 30 mM KCl for 1 h in the presence or absence of extract in buffer supplemented with 0.5% IBMX. Samples of culture medium were taken at the end of this period. Samples were centrifuged 3 min at 4 ºC at 3000 x g, and supernatants were stored at -20 ºC until assayed by radioimmunoassay (RIA). Insulin secretion in INS832/13 was normalized to the total cellular protein content by the bicinchoninic acid method (Pierce, Biolynx, Brockville, ON). Insulin secretion was expressed per well for βTC-tet cells since all wells contained approximately the same number of cells. Total cellular insulin content was also measured in unstimulated (basal state) cells. Insulin was extracted in 1.25% HCl-75% ethanol and samples were kept overnight at 4 ºC. These samples were sonicated and centrifuged at 30 000 g for 5 min before measurement of insulin in the supernatant by RIA. For analysis of basal, stimulated insulin secretion and total cellular insulin content, samples were placed on ice and appropriate dilutions were made with phosphate buffer (50 mM Na_2HPO_4, pH 7.5, 25 mM EDTA, 1% BSA RIA-grade, 0.01% thimerosal). Diluted samples were incubated in 12 x 75 mm polypropylene RIA tubes with rat ^{125}I-insulin (Linco Research, Saint Charles, MO) and a primary antibody against rat insulin (Linco, # 1013) at 4 ºC overnight in the dark. The tubes were then incubated with the precipitating reagent (Linco, # 2020) for 20 min at 4 ºC, and tubes were then centrifuged at 5350 g for 15 min.

Radioactivity in the pellet was measured by gamma counter (Wallac Wizard 1470, Perkin Elmer, Woodbridge, ON). Human insulin was used as a standard. Four to six replicates were performed for each experimental condition.

βTC-tet proliferation assay

To test for a proliferative effect of NS on β cells, extract was applied to replicating (non-growth arrested) βTC-tet cells and incorporation of ^{3}H-thymidine was evaluated. Cells were seeded in 24 well plates at a density of 1.0 x 10^{5} and incubated in growth medium for 24 h. Incubation was continued for another 48 h in growth medium while one group was treated with tetracycline (1 μg/ml) in order to arrest growth, as above. Replicating cells were then incubated 18 h in presence or absence of NS. 1 μCi/ml of methyl ^{3}H-thymidine (4 Ci/mmol; # 2404105, MP Biomedicals, Irvine, CA) was added over the last 6 h of treatment to all cells. Cells were then rinsed 3 times in PBS, lysed with 0.1 M NaOH for 30 min and scraped. The lysates were added to 4 ml of liquid scintillation cocktail (Ready-Gel 586601, Beckman Coulter) and incorporated radioactivity was measured in a scintillation counter (LKB Wallac, model 1219, Perkin Elmer, Woodbridge, ON). Four replicates were performed for each experimental condition.

Glucose transport assay

Differentiated C2C12 and 3T3-L1 cells grown in 12-well culture plates were treated with 200 μg/ml of NS extract for 18 h or 1 h. A vehicle control (DMSO), and positive controls (400 μg/ml metformin or 75 μg/ml fenugreek (Vats et al., 2002; Spoor et al., 2006)) were included in each experiment. Three hours prior to the experiment, the medium was replaced with serum-free DMEM containing extract to establish a baseline insulin-free state. Following the treatment, cells were incubated for 30 min with 0, 1, or 100 nM insulin in Krebs-phosphate buffer (20 mM HEPES, 4.05 mM Na_2HPO_4, 0.95 mM NaH_2PO_4, pH 7.4, 120 mM NaCl, 4.7 mM KCl, 1mM $CaCl_2$ and 1 mM $MgSO_4$) containing 5 mM glucose and NS extract or appropriate positive and vehicle controls at 37 ^oC. Cells were then washed twice in warm glucose-free Krebs-phosphate buffer before incubation for exactly 10 min at 37 ^oC in glucose-free Krebs-phosphate buffer containing 0.5 μCi/ml 2-deoxy-D-[1-^{3}H]glucose (TRK383, Amersham Biosciences, Buckinghamshire, UK). Cells were then placed on ice and immediately washed three times with ice-cold Krebs-phosphate buffer. Cells were lysed and scraped in 0.1 N NaOH. Lysates were transferred to 4 ml of Ready-Gel and radioactivity was counted in a liquid scintillation counter, as above. Five to eight replicates were performed for each experimental condition.

Rate of adipogenesis assay

PPARγ agonists, such as the commonly-prescribed rosiglitazone and other members of thiazolidinedione (glitazone) family are known to increase insulin sensitivity in muscle and adipose tissue (Konrad et al., 2005). One of the hallmarks

of glitazones is their ability to increase rate of adipogenesis. To test NS for glitazone-like activity, we performed a rate of adipogenesis assay, as performed elsewhere (Spoor et al., 2006; Martineau et al., 2005). Briefly, 3T3-L1 pre-adipocytes were seeded in 12-well plates and allowed to proliferate until fully confluent. One day post confluence, proliferation medium was replaced with differentiation medium containing the hormone cocktail, as above, for 2 days and with insulin-supplemented medium, as above, for an addition 4 days, resulting in incomplete differentiation and a submaximal content of intracellular triglycerides at the end of this period. During this entire differentiation period, cells were treated with NS extract or 0.1% DMSO vehicle in differentiation medium. Vehicle in proliferation medium was used as a negative control. Rosiglitazone (Alexis Biochemicals, San Diego, CA) solubilized in DMSO, was used as a positive control at 10 μM. Medium was changed every 24 h during differentiation. At the end of the 6^{th} day, cell morphology was evaluated by phase-contrast microscopy and cellular triglyceride content was determined as a marker of differentiation and used to compare the rate of differentiation between conditions. Triglyceride content was quantified using the AdipoRed dye assay (Cambrex Bioscience Inc, Walkersville, MD). 3T3-L1 adipocytes were washed gently with phosphate-buffered saline before the addition of 60 μl of AdipoRed reagent per well. Fluorescence was measured in a plate reader (Wallac Victor2, Perkin Elmer, Woodbridge, ON) at λ_{ex} = 485 nm, λ_{em} = 572 nm. Four replicates were performed for each experimental condition. The mean value obtained from the negative control condition was considered as background and subtracted from all other readings.

Statistical analysis

All data are reported as the mean ± SEM of the indicated number of experiments. Results were analysed by one-way analysis of variance (ANOVA) and Fisher post-hoc test using StatView software (SAS Institute Inc, Cary, NC). Statistical significance was set at $p \leq 0.05$.

RESULTS

NS treatment potentiates pancreatic insulin secretion.

NS extract was tested for insulinotropic activity in insulin-secreting INS832/13 and βTC-tet cells. Following an 18 h treatment with NS extract, insulin secretion over a period of 1 h was assessed in response to various concentrations of glucose and in response to potassium. In both cell types, insulin secretion was significantly increased under all conditions; in INS832/13 cells, secretion was increased by approximately 400 ng/mg protein/h (Table 1), while in growth-arrested βTC-tet cells, secretion was increased proportionally by 39-49%. The dose-response relationship between glucose and insulin secretion was shifted upwards (Figure 1a), indicating no change in the triggering mechanisms of insulin release, but an increase in the stimulus amplification mechanisms (Henquin, 2000). The insulinotropic effects were not accompanied by a significant change in total cellular insulin content (Table 1 and Figure 1c).

NS treatment accelerates pancreatic beta cell proliferation.

The insulinotropic effect of NS treatment was smaller in growth-arrested βTC-tet cells than in proliferating INS832/13 cells, suggesting that NS may exert a proliferative effect. Indeed, such an effect has been proposed to explain the protective effect of NS treatment against streptozotocin-destruction of beta cells in vivo (Kanter et al, 2003). In order to test whether NS can stimulate β cell proliferation, replicating insulin-secreting βTC-tet cells were incubated 18 h with NS or vehicle and ^{3}H-thymidine incorporation over the last 6 h was assessed. NS

treatment significantly increased DNA synthesis over this period by 13% as compared to the DMSO vehicle control (Figure 2).

NS treatment enhances glucose uptake in muscle cells and adipocytes.

NS extract was tested for insulinomimetic/insulin-sensitizing properties by assessing basal and insulin-stimulated glucose uptake in differentiated C2C12 myotubes and 3T3-L1 adipocytes, two insulin-responsive and Glut-4-containing cell lines (He et al., 2007). Following an 18 h treatment with NS extract, basal glucose uptake was markedly increased in both muscle (Figure 3) and fat (Figure 4) cells. In muscle cells, basal uptake was increased by 55%, corresponding to 2.1 times the effect of 100 nM insulin (a supra-physiological dose). The effect of NS treatment could not be further increased by acute stimulation with insulin (1 or 100 nM); indeed NS treatment combined with 100 nM insulin for 30 min resulted in 2.3 fold more uptake than 100 nM insulin in vehicle-treated cells (Figure 3a). In adipocytes, basal uptake was increased 4-fold, which is equal to the effect of 100 nM insulin. In contrast to muscle cells, the effect of acute insulin stimulation (1 or 100 nM) was perfectly additive to that of NS treatment; consequently, 100 nM insulin in NS-treated cells resulted in an increase of 2.3-fold more than 100 nM insulin in vehicle control cells (Figure 4a). A 1 h treatment with NS extract resulted in smaller increases in basal uptake, corresponding to approximately one half of the effect of 100 nM insulin in either cell type. Furthermore, the effect of acute insulin stimulation (1 or 100 nM) was not additive to that of NS treatment in either cell type (Figure 3b; 4b). Taken together, these data suggest that NS and insulin elicit their acute

responses through a common pathway, and that a longer NS treatment may increase maximal glucose uptake through an upregulation of the content of key proteins involved in uptake.

NS treatment accelerates adipogenesis.

To test for glitazone-like activity, triglyceride accumulation was measured in differentiating 3T3-L1 adipocytes treated with NS extract throughout their differentiation period. At day 6 of differentiation, a time when differentiation is incomplete and lipid droplets are observable in a small percentage of vehicle control cells, chronic NS treatment resulted in 3.5-fold higher triglyceride content than in control cells, indicating a higher rate of adipogenesis. This effect was comparable to the effect of 10 μM rosiglitazone.

DISCUSSION

Nigella sativa seed extract is used in the traditional medicine of numerous North African countries as a treatment for diabetes (Haddad et al., 2006, 2001; Bellakhdar., 1991; Ziyyat et al., 1997). The in-vivo anti-hyperglycaemic activity of this extract is well-known (Kanter et al., 2003; El-Dakhakhny., 2002; Farah et al., 2002; Meral et al., 2001; Labhal et al., 1999; Al Hader et al., 1993; Al-Awadi et al., 1991, 1987, 1985). While this action has been mainly attributed to insulinotropic effects, observed in animal studies as well as in isolated pancreatic islets (Rchid et al., 2004), the contribution of peripheral sites of action has been suggested (Al-Awadi et al., 1991; Farah et al., 2004). The purpose of this study was to use cell-based assays of anti-diabetic activity to elucidate the cellular site(s) of action of NS biological activity. NS was confirmed to exert an important insulinotropic effect on pancreatic β cells and was also found to have significant insulin-like effects in peripheral tissues, namely the stimulation of basal glucose uptake in skeletal muscle cells and adipocytes, in addition to the rosiglitazone-like enhancement of adipogenesis.

We observed that NS increases insulin secretion by pancreatic β cells while preserving normal glucose sensitivity, thereby resulting in an upshifted relationship between insulin secretion and glucose concentration. Our study is thus in agreement with others that have observed that the basic and nonlipidic sub-fractions of NS seeds increase insulin secretion in isolated islets of Langerhans (Rachid et al., 2004). Others, using the volatile oil fraction, failed to see a pancreatic effect (El-Dakhakhny

et al., 2001). This suggests that the secretaogue activity may be found in the nonlipidic components of our crude ethanolic extract and further studies will be necessary to elucidate this point.

Our study also shows that NS treatment does not affect the intracellular insulin content of the two lines of pancreatic β cells used, but exerts a proliferative effect on βTC-tet cells. A proliferative or regenerative activity has been suggested by others who have observed increased insulinaemia after treatment of rats having undergone partial destruction of their beta cell mass with streptozotocin. (Kanter et al., 2003)

We have also examined the biological activity of NS in extra-pancreatic tissues, notably insulin-responsive skeletal muscle and adipose cells. In both cell types, treatment with NS extract significantly increased basal glucose uptake. In response to a 1 h treatment time, the relative increase in basal uptake corresponded to approximately one half of the effect of 100 nM insulin in either cell type, while in response to an 18 h treatment, the relative increase corresponded to double the effect of 100 nM in muscle cells or to the same effect as 100 nM insulin in adipocytes. The fact that the 18 h treatment resulted in a greater effect, and, that combined with acute insulin stimulation, it increased uptake above that which can be induced by a supra-physiological dose of insulin in untreated cells (2.3 fold the effect of 100 nM insulin in either cell types), strongly suggest that NS affected gene expression of proteins involved in glucose transport. Such an effect would be compatible with stimulation of the AMP-activated protein kinase (AMPK) (Winder., 2000) Interestingly, the

effect of the 1 h treatment was lost or masked by acute insulin stimulation; it is unclear whether a similar phenomenon would have been observed following an 18 h treatment with a lower concentration of NS extract and whether this is indicative of NS and insulin acting through a common signalling pathway. Finally, while the relative magnitude of the effect of NS treatment on basal uptake was more important in muscle cells than in adipocytes, it is unclear whether NS exerts its effects through similar mechanisms in both cell types.

Similar insulinomimetic or insulin-sensitizing actitivites could also extend to hepatocytes, where reduced gluconeogenesis would further contribute to an overall anti-glycaemic effect of NS, as proposed by other investigators (Al-Awadi et al., 1991; Farah et al., 2004). In support of this, our laboratory has previously showed that NS exerts an insulin-sensitizing effect on hepatocytes isolated from normal rats after 4 weeks of *in vivo* treatment (Le et al., 2004).

The effect of NS treatment on rate of adipogenesis was evaluated in order to screen for glitazone-like properties. The glitazones, including the widely-prescribed insulin-sensitizer rosiglitazone, are PPARgamma agonists and a consequence of their action in adipocytes is increased adipogenesis (Lazar., 2005). The anti-diabetic effects of glitazones are attributable to PPARgamma activation in adipocytes as well as to an activation of AMPK in muscle (Lazar., 2005). Chronic treatment with NS extract was found to significantly increase rate of adipogenesis, similarly to rosiglitazone. It remains to be determined whether this is the result of PPARgamma

agonism or of some other mechanism, and whether this activity is related to the observed enhancement of glucose uptake resulting from shorter treatment durations.

In summary, the results of our cell-based functional assays successfully identified sites of action that can explain the documented anti-hyperglycaemic effects of NS seed extract. We have confirmed a proposed insulinotropic action of NS at the level of pancreatic β cells and presented evidence of a proliferative effect of the plant extract on these cells, as well. This holds promise for the maintenance of β cell mass and a slowing of the progression of type II diabetes. Our study further provides novel and direct evidence for an insulin-like or insulin-sensitizing action of NS extract at the level of skeletal muscle and adipose tissue. Part of the action of NS extract may be related to stimulation of insulin-dependent and -independent intracellular signalling pathways, as well as to PPARgamma agonism. In conclusion, NS seed ethanol extract exhibits the remarkable ability to concomittantly increase insulin secretion, induce proliferation of pancreatic β cells, and stimulate glucose uptake in muscle and fat cells. Such beneficial properties warrant further work to elucidate molecular targets of NS seed extract and to isolate and identify the active compound(s).

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TABLES AND FIGURES

Table1: Effect of NS on insulin secretion and insulin content in INS832/13 cells.

	Glucose concentration (mM)				
Treatment	2	4	11	2 + 30 mM KCl	Total insulin content
DMSO 0,1%	221 ± 28	331 ± 13	737 ± 33	411 ± 23	8113 ± 514
Nigella 200 µg/ml	606 ± 40*	638 ± 51*	1213 ± 110*	821 ± 43*	7678 ± 650

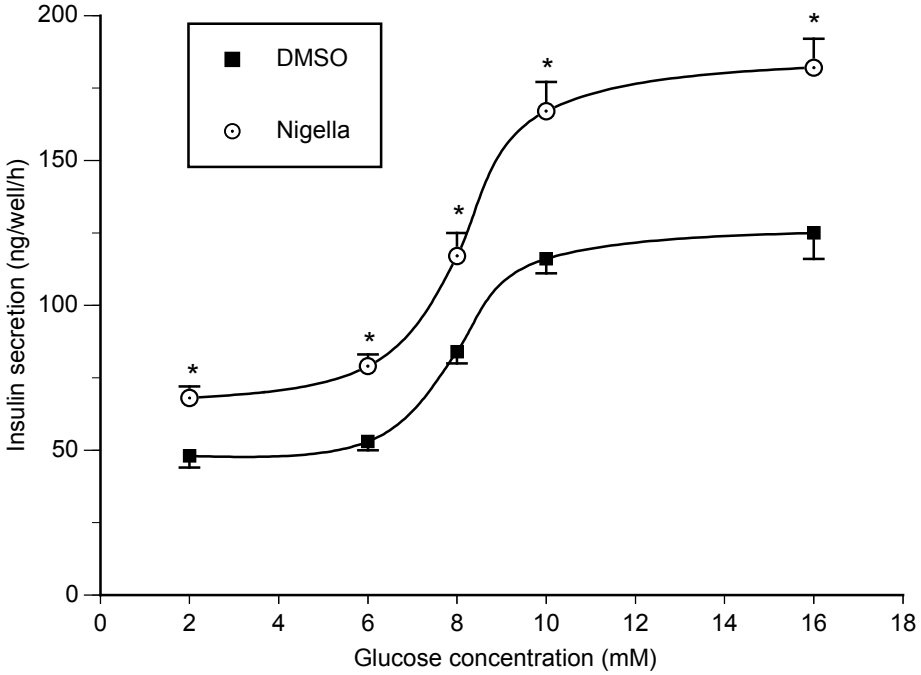
Data expressed as ng of insulin/mg of protein/h.

Result are mean ± SEM for n = 6.

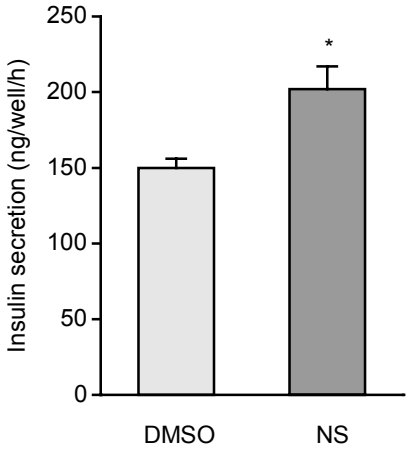
* = significantly different ($p \leq 0.05$) from vehicle control group

Figure 1

a:


DMSO
Nigella
Insulin secretion (ng/well/h)
Glucose concentration (mM)
0
50
100
150
200
0
2
4
6
8
10
12
14
16
18
*

b:


Insulin secretion (ng/well/h)
0
50
100
150
200
250
*
DMSO
NS

c:

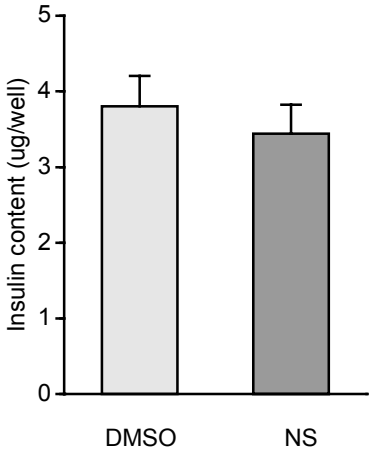


Figure 2:

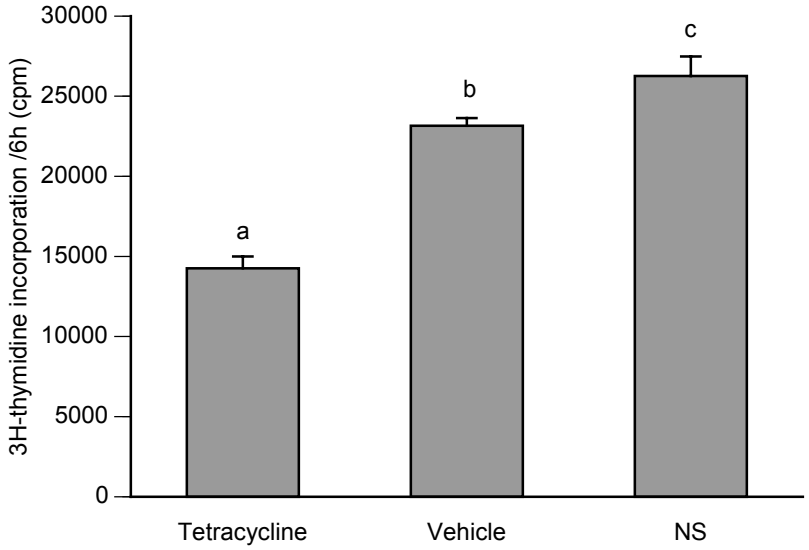
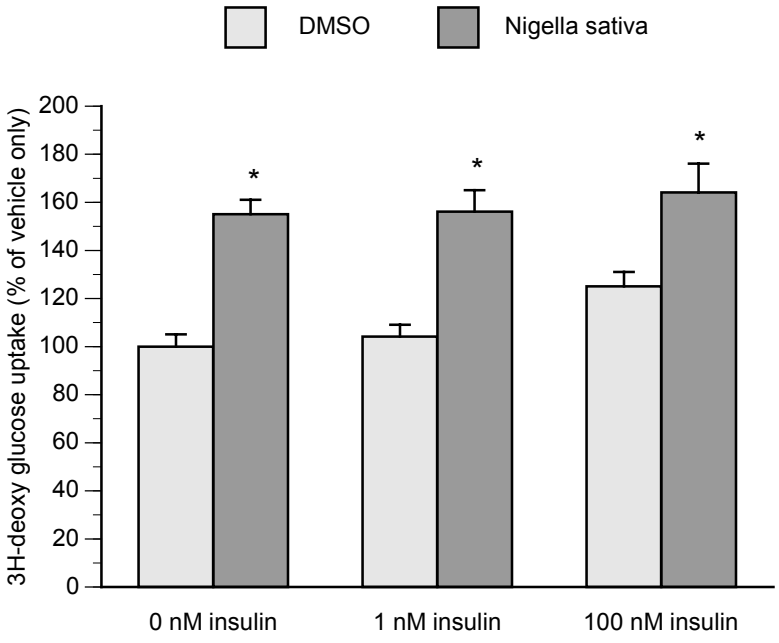

30000
25000
20000
15000
10000
5000
0
3H-thymidine incorporation /6h (cpm)
a
b
c
Tetracycline
Vehicle
NS

Figure 3

a:


DMSO
Nigella sativa
3H-deoxy glucose uptake (% of vehicle only)
200
180
160
140
120
100
80
60
40
20
0
*
*
*
0 nM insulin
1 nM insulin
100 nM insulin

b:

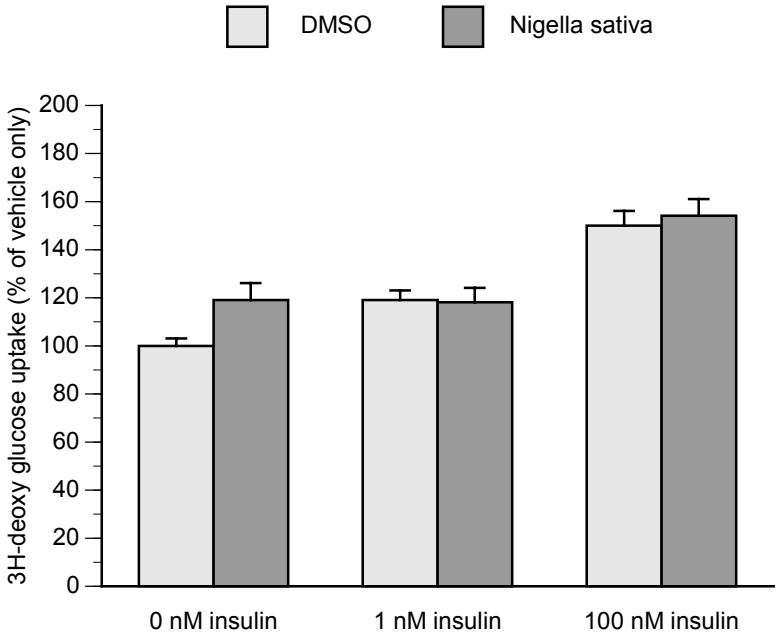
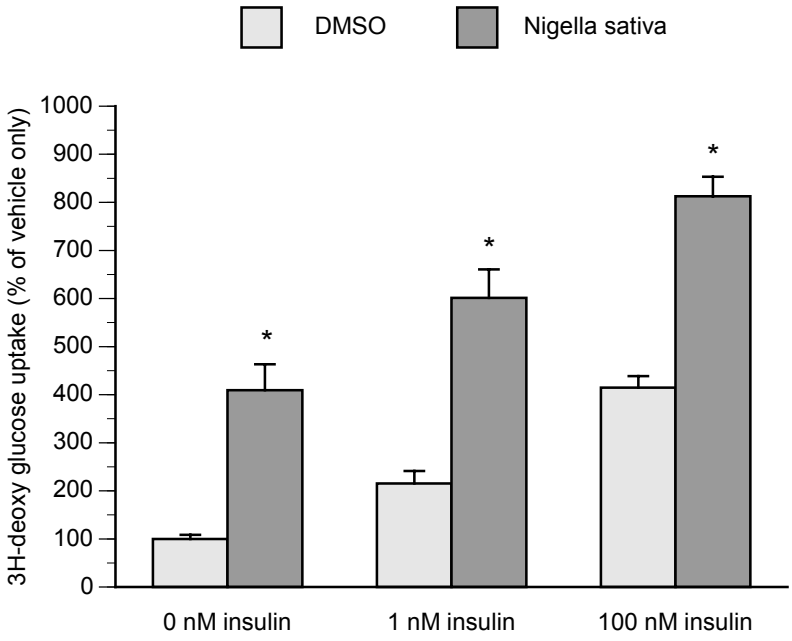

DMSO
Nigella sativa
3H-deoxy glucose uptake (% of vehicle only)
200
180
160
140
120
100
80
60
40
20
0
0 nM insulin
1 nM insulin
100 nM insulin

Figure 4

a:


DMSO
Nigella sativa
3H-deoxy glucose uptake (% of vehicle only)
1000
900
800
700
600
500
400
300
200
100
0
*
*
*
0 nM insulin
1 nM insulin
100 nM insulin

b:

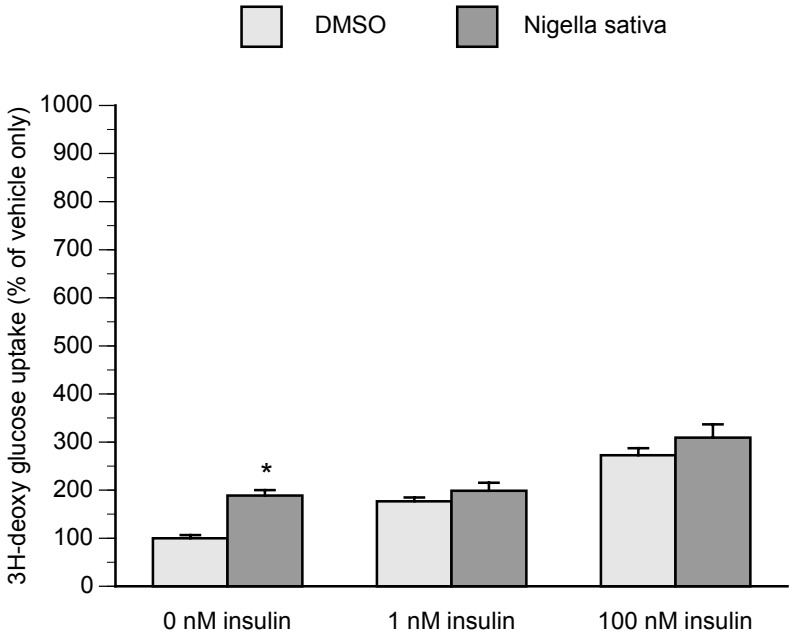

DMSO
Nigella sativa
3H-deoxy glucose uptake (% of vehicle only)
1000
900
800
700
600
500
400
300
200
100
0
*
0 nM insulin
1 nM insulin
100 nM insulin

Figure 5:

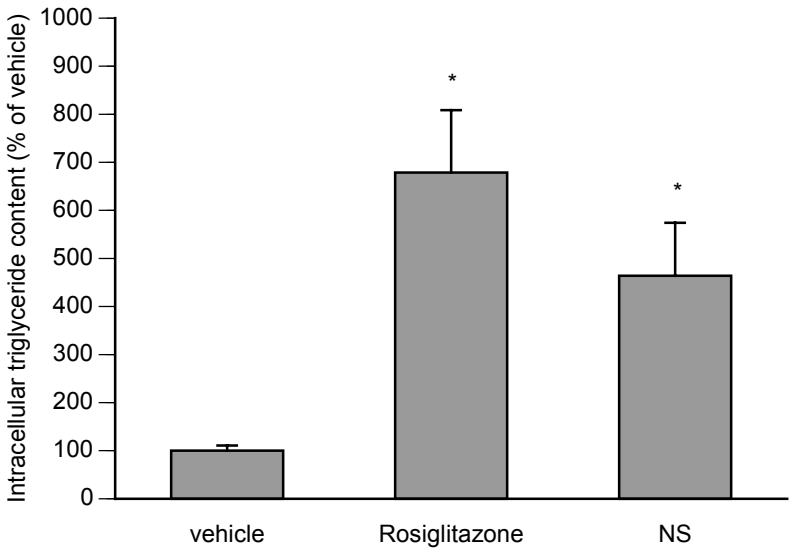

Intracellular triglyceride content (% of vehicle)
1000
900
800
700
600
500
400
300
200
100
0
*
*
vehicle
Rosiglitazone
NS

FIGURE LEGENDS

Figure 1: The Effect of NS seed extract on glucose-stimulated insulin secretion, potassium-stimulated insulin secretion and total insulin content was assessed in growth-arrested βTC-tet pancreatic β cells. Cells were treated with 200 μg/ml of extract or with vehicle only (0.1% DMSO) for 18 h. Insulin secretion was measured over a 1 h period in response to 2, 6, 8, 10, or 16 mM glucose (a) or 2 mM glucose and 30 mM KCl (b). Results are expressed as ng of insulin per well per hour. Intracellular insulin content was measured in cells under basal (2 mM glucose) condition (c). Results are expressed as ng insulin per well. Mean ± SEM for n=4. * = significantly different ($p \leq 0.05$) from the corresponding control (DMSO).

Figure 2: The effect of NS extract on pancreatic β cell proliferation was assessed in βTC-tet cells. Replicating cells were treated with 200 μg/ml of NS extract or with vehicle only (0.1%DMSO) for 18 h, while growth-arrested cells were treated with vehicle only. During the last 6 h of treatment, cells were exposed to ^{3}H-thymidine. Cells were washed and lysed and incorporated radioactivity was then assessed. Result are expressed as counts per minute (cpm) per well per 6 h. Mean ± SEM for n=4. Different letters denote significant differences ($p \leq 0.05$) between conditions.

Figure 3: Basal and insulin-stimulated ^{3}H-deoxyglucose uptake were assessed in C2C12 myotubes treated with 200 μg/ml of NS extract or vehicle (0.1% DMSO) for 18 h (a) or 1 h (b). Data is expressed normalized to basal uptake in vehicle control

condition. Mean ± SEM for n=5-6. * = significantly different ($p \leq 0.05$) from vehicle control corresponding to same insulin condition.

Figure 4: Basal and insulin-stimulated ^{3}H -deoxyglucose uptake were assessed in 3T3-L1 adipocytes treated with 200 μg/ml of NS extract or vehicle (0.1% DMSO) for 18 h (a) or 1 h (b). Data is expressed normalized to basal uptake in vehicle control condition. Mean ± SEM for n = 6-8. * = significantly different ($p \leq 0.05$) from vehicle control corresponding to same insulin condition.

Figure 5: The rate of accumulation of triglycerides was assessed in differentiating 3T3 preadipocytes treated for 6 days with 200 μg/ml of NS extract, vehicle (0.1% DMSO), or 10 μM rosiglitazone in differentiation medium. At the end of the treatment period, triglyceride content was assessed by AdipoRed fluorescent dye. Results are expressed normalized to vehicle control in differentiation minus vehicle control in proliferation medium. Mean ± SEM for n = 4. * = significantly different ($p \leq 0.05$) from vehicle control.

3. Article 2

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Anti-diabetic effects of *Nigella sativa* seed extract are mediated through both the insulin signaling pathway and AMPK in muscle and liver cells

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ABSTRACT

Nigella sativa (N. sativa) is a plant widely used in traditional medicine of North African countries. During the last decade, several studies have shown that extracts from the seeds of *N. sativa* have antidiabetic effects. Our group has recently demonstrated that *N. sativa* seed ethanol extract (NSE) induces an important insulin-like stimulation of glucose uptake in C2C12 skeletal muscle cells and 3T3-L1 adipocytes following an 18 h treatment. The purpose of the present study was to elucidate the pathways mediating this insulin-like effect and the mechanisms through which these pathways are activated. Results from western immunoblot experiments indicate that in C2C12 cells as well as in H4IIE hepatocytes, but not in 3T3-L1 cells, NSE increases activity of Akt, a key mediator of the effects of insulin, and activity of AMP-activated protein kinase (AMPK), a master metabolic regulating enzyme. To test whether the activation of AMPK resulted from a disruption of mitochondrial function, the effects of NSE on oxygen consumption were assessed in isolated liver mitochondria. NSE was found to exhibit potent uncoupling activity. Finally, to provide an explanation for the effects of NSE in adipocytes, PPARγ stimulating activity was tested using a reporter gene assay. Results indicate that NSE behaves as an agonist of PPARγ. Our data support the ethnobotanical use of *N. sativa* seed oil as a treatment for diabetes, as well as potential uses of this product, or compounds derived thereof, against obesity and the metabolic syndrome.

INTRODUCTION

Type II diabetes is a serious health problem in most developed and developing countries (WHO 2006). The World Health Organization estimates that 180 million people worldwide are afflicted and that this number will double by 2030 (WHO 2006). These figures greatly underestimate the epidemic as they do not take into consideration insulin resistance / glucose intolerance, the pathophysiological state that precedes by several years pancreatic β-cell exhaustion and overt diabetes (Golay et Felber 1994), and that is also a precursor to the other diseases that comprise the metabolic syndrome (Lien et Guyton 2008).

Rates of hepatic glucose output and skeletal muscle glucose uptake are the major determinants of glycemia and are under the control of insulin (Gerich 1993). When liver and muscle become insulin resistant as a consequence of a sedentary lifestyle and excess caloric intake (Turner et Clapham 1998), postprandial glycemia necessarily becomes difficult to normalize. Pharmaceutical interventions are limited to the sulfonureas, the biguanides, and the thiazolinediones. The sulfonureas address the problem indirectly by stimulating additional secretion of insulin (Del Prato *et al.* 2007). The biguanides target liver, and to a lesser extent skeletal muscle, where they stimulate the AMP-activated protein kinase (AMPK) pathway (Leverve *et al.* 2003), an insulin-independent signaling cascade with several insulin-like effects including the inhibition of hepatic glucose output and the stimulation of muscle glucose uptake (Misra et Chakrabarti 2007). Finally, the thiazolinediones are thought to exert their effect primarily in another insulin-sensitive tissue, the adipose tissue, where they

activate the peroxisome proliferator-activated receptor (PPAR) γ nuclear receptors (Gelman *et al.* 2007) and upregulate the expression of effectors of insulin.

Medicinal plants with anti-hyperglycemic activity are found in the traditional medicine of numerous cultures throughout the world. This tradition is frequently rooted in centuries of use for the treatment of diabetes (Alarcon-Aguilara *et al.* 1998; Yeh *et al.* 2003). Today, such remedies remain important in many cultures, even when availability of modern pharmaceuticals is not an issue. These facts are testimonies to the efficacy of these products, which for the most part have not been the object of scientific study. Of the plant products that have been studied in pre-clinical and clinical settings, most have been shown to be efficacious (Marles et Farnsworth 1995). While the understanding of the mechanisms of action of these plant products is generally lacking, the AMPK pathway is emerging as a common mediator of their activity (Cheng *et al.* 2006; Tan *et al.* 2008; Yin *et al.* 2008).

N. sativa is one such medicinal plant that has stood the test of time, the seeds of this herb having been used extensively in Middle Eastern and Northern African countries for over 2 millennia to treat diabetes and several other ailments (Bellakhdar *et al.* 1991; Ziyyat *et al.* 1997; Haddad *et al.* 2001). Its anti-hyperglycemic activity has been validated by several studies (Al-Awadi *et al.* 1985; Al-Awadi et Gumaa 1987; Al-Awadi *et al.* 1991; Al-Hader *et al.* 1993; Labhal *et al.* 1999; Meral *et al.* 2001; El-Dakhakhny *et al.* 2002; Fararh *et al.* 2002; Zaoui *et al.* 2002; Kanter *et al.* 2003; Fararh *et al.* 2004; Kanter *et al.* 2004; Le *et al.* 2004; Rchid *et al.* 2004;

Haddad *et al.* 2006; Benhaddou-Andaloussi *et al.* 2008). In a recent study to identify the cellular targets through which this activity is mediated, our group has demonstrated that *N. sativa* seed extract (NSE) exhibits the remarkable combined ability to increase insulin secretion, induce proliferation of pancreatic β-cells, and stimulate glucose uptake in skeletal muscle cells and adipocytes (Benhaddou-Andaloussi *et al.* 2008). The goal of the present study was to elucidate the mechanisms of action mediating the extra-pancreatic effects of NSE. Our results demonstrate that this plant product possesses the unique property of activating the AMPK pathway, the insulin signaling pathway, as well as PPARγ.

MATERIAL AND METHODS

N. sativa plant material and constituent compounds

Seeds of *N. sativa* were obtained from an herbalist in Rabat, Morocco in August 2005 and were authenticated by an experienced botanist (Prof. A. Oulyahya, Institut Scientifique, Rabat, Morocco). A voucher specimen was deposited in the herbarium of the Institut Scientifique of Rabat (voucher 10359). Seeds were washed, dried, and then powdered with an electric microniser. Powder was extracted three times with 80% ethanol and the solvent was evaporated at 40 °C under reduced pressure. This procedure resulted in a crude extract characterized by an oily phase and a solid phase. Fractions were also prepared from *Nigella sativa* seed powder by sequential extraction with organic solvents of increasing polarity, namely hexane, ethyl acetate, methanol, and 70% methanol / 30% water, and with evaporation as above. The crude extract and the fractions were conserved at 4°C and protected from light and humidity. The crude extract and the fractions were solubilized freshly in DMSO prior to treatment of cells (below). The oily and solid phases of the crude extract were solubilized at a proportion of 70 % and 30 % (w/vol), respectively, in accordance with their yield; this product is henceforth referred to as *Nigella* seed extract (NSE).

Some constituent compounds of *N. sativa* seeds were tested for biological activity. These included thymol (CAS # 89-83-8) (Basha *et al.* 1995), carvacrol (CAS # 499-75-2) (Michelitsch *et al.* 2004), and thymoquinone (CAS # 490-91-5) (Basha *et al.* 1995), purchased from Sigma-Aldrich (St. Louis, MO), hederagenin (CAS #

465-99-6) (Mustafa et Soliman 1943), purchased from Enquce Biochemicals (Kamsack, SK), and nigellimine (CAS # 4594-02-9) (Atta-ur-Rahman et Zaman 1992), a gift from Dr. Tony Durst (University of Ottawa, Ottawa, ON, Canada).

Reagents, cell-lines and antibodies

Reagents were purchased from Sigma-Aldrich, unless otherwise noted. Cell culture medium was purchased from Wisent (Saint Bruno, QC). Fetal bovine serum (FBS), antibiotics, and trypsin were purchased from Invitrogen Life Technologies (Carlsbad, CA). Culture plates were purchased from Corning Inc. (New York, NY). C2C12 murine myoblasts, H4IIE hepatocytes, and 3T3-L1 murine pre-adipocytes were obtained from American Type Culture Colection (Manassas, VA). Antibodies directed against phosphorylated Akt (Ser 473), ERK 1/2 (Thr 202/Tyr 204), AMPKα (Thr 172) and ACC (Ser 79), as well as corresponding pan-specific antibodies were purchased from Cell Signaling Technology (Danvers, MA). Secondary horseradish peroxidase (HRP)-conjugated antibodies were purchased from Jackson Immunoresearch (West Grove, PA). Protein assay reagents were purchased from Pierce Protein Research Products (Thermo Scientific, Rockford, IL).

Cell culture

C2C12 myoblasts were cultured as previously described (Spoor *et al.* 2006; Benhaddou-Andaloussi *et al.* 2008) in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % FBS, 10 % horse serum (HS), and antibiotics (penicillin 100 UI/ml, streptomycin 100 μg/ml) at 37 °C in a 5% CO_2 atmosphere. Upon

reaching 80 % confluence, serum content was reduced to 2 % HS in order to induce differentiation into myotubes. Experiments were performed on well-differentiated multinucleated cells 6 to 7 days later. 3T3-L1 preadipocytes were grown as previously described (Spoor *et al.* 2006; Benhaddou-Andaloussi *et al.* 2008) in DMEM supplemented with 10% FBS and antibiotics until 80% confluent. Differentiation into adipocytes was then induced by adding 500 µM 3-isobutyl-1-methylxanthine, 10 µM dexamethasone, and 500 nM insulin to the medium for 2 days. Experiments were performed when over 90 % of cells exhibited lipid droplets visible by phase-contrast microscopy, typically after 10 to 14 additional days in insulin-supplemented medium. H4IIE hepatocytes were grown in DMEM containing 10 % HS until fully confluent and experiments were performed 1 to 3 days later (Chaung *et al.* 2008). All cells were treated with 200 µg/ml of NSE, the maximal soluble concentration in DMSO. This concentration was observed to not induce any morphological changes in the cell lines used following a 24 h treatment.

Western immunoblot

Differentiated C2C12 myotubes, differentiated 3T3-L1 adipocytes, and postconfluent H4IIE hepatocytes were cultured in 6-well plates and treated with NSE for 18 h in conditions similar to those used previously to assess effects of NSE on glucose uptake (Benhaddou-Andaloussi *et al.* 2008), with the following modifications. For immunoblot analysis of phospho-Akt and ERK1/2, the last three hours of treatment were performed in serum-free medium containing fresh extract. Cells treated with extract or vehicle (DMSO 0.1 %) were incubated 5 min at 37 ºC

with 0, 1 or 100 nM insulin (dissolved in 0.01 M HCl and prepared fresh from a stock solution of 0.5 mM and kept on ice). For immunoblot analysis of phospho-AMPK and ACC, cells were treated with NSE or 0.1 % DMSO in complete medium with no change of medium prior to lyse. 5-Aminoimidazole-4-carboxamide-1-b-D-rubofuranozide (AICAR) and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) were used as positive controls for the activation of AMPK. AICAR (Toronto Research Chemicals, North York, ON) solubilized in water was added to DMSO-treated cells for 30 min at 2 mM. FCCP was used for 1 h at 5 μM. For insulin time-course experiments, parallel plates of cells were washed free of insulin in warm serum-free growth medium and incubated for an additional 10, 25, 55, or 85 minutes. Following treatment plates were placed on ice and immediately washed three times in ice-cold PBS (8.1 mM $NaHPO_4$, 1.5 mM KH_2PO_4, pH 7.4, 137 mM NaCl and 2.7 mM KCl) and lysed in 250 μl of lysis buffer (25 mM Tris-HCl pH 7.4, 25 mM NaCl, 0.5 mM EDTA, 1% Triton-X-100, 0.1% SDS) containing a commercial cocktail of protease inhibitors (Complete Mini, Roche, Mannheim, Germany) supplemented with 1 mM phenylmethanesulfonyl fluoride, as well as a cocktail of phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride). Cells were allowed to lyse for 15 min on ice, and were then scraped into microcentrifuge tubes, vortexed, and centrifuged at 12 000 x g for 10 min at 4 ^{o}C. Supernatants were decanted and stored at -80 C until further analysis. The lipid layer of 3T3-L1 cells was aspirated prior to decanting of supernatant. Protein content was assayed by the bicinchoninic acid method standardized to bovine serum albumin.

Lysates were diluted to a concentration of 1.25 mg/ml total protein and boiled for 5 min in reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue). 20 µg of protein of each sample was separated on 10 % polyacrylamide mini-gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked for 2 h at room temperature with 5 % skim milk in Tris-buferred saline (20 mM Tris-HCl, pH 7.6 and 137 mM NaCl) containing 0.1 % Tween 20 (TBST). Membranes were then incubated overnight at 4 ºC in blocking buffer with primary antibodies at a concentration of 1:1000 in TBST plus 5 % milk. Membranes were washed 5 times with TBST and incubated 90 min at ambient temperature in TBST with appropriate HRP-conjugated secondary antibodies at 1:50 000 to 100 000. Revelation was performed using the enhanced chemiluminescence method (Amersham Biosciences, Buckinghanshire, England) and blue-light-sensitive film (Amersham Biosciences). Experiments were repeated on 3 different passages of cells, each passage containing all conditions in parallel. All samples from a given passage were separated and transferred simultaneously to a single membrane. Densitometric analysis of films was performed using a flatbed scanner (Hewlett Packard, ScanJet 6100C/T) and NIH Image software (National Institutes of Health, Bethesda, MD).

Isolation of mitochondria

Mitochondria were isolated from the liver of Sprague-Dawley rats (Charles River, St-Constant, QC) weighing between 225 and 250 g. Rats were anesthetized

with an intraperitoneal injection of sodium pentobarbital (50 mg/kg B.W.) and underwent laparotomy. All experimental procedures were approved by the Université de Montréal Animal Experimentation Ethics Committee and animals were treated in accordance with guidelines of the Canadian Council on Animal Care. The portal vein was cannulated while the hepatic artery and the infrahepatic inferior vena cava were legated. The livers were flushed with 100 ml of Krebs-Henseleit buffer (25 mM NaHCO3, 1.2 mM, KH2PO4, pH 7.4, 137 mM NaCl, 4.8 mM KCl, 2.1 mM CaCl2, 1.2 mM MgSO4) at ambient temperature and livers were removed and placed on ice**.** Mitochondria were isolated from 1 g of liver as described by Johnson and Lardy (Johnson et Lardy 1967). Briefly, tissue was homogenized on ice using a Teflon potter homogenizer in ice-cold isolation buffer (10 mM Tris, pH 7.2, 250 mM sucrose, 1 mM EGTA) cooled to 4 °C. The homogenate was centrifuged at 600 g for 10 min at 4 °C in order to remove cellular fragments. The supernatant was recovered and centrifuged at 12 000 g for 6 min at 4 °C. The supernatant was discarded and the pellet was washed in ice-cold isolation buffer and recentrifuged. The pellet was then washed in isolation buffer without EGTA, and recentrifuged. The final pellet containing viable mitochondria was resuspended in ice-cold isolation buffer without EGTA and this homogenate was kept on ice until respiration experiments. Protein content of the homogenate was determined according to the Lowry method.

Mitochondrial respiration

Oxygen concentration was measured at 25°C at a frequency of 1 Hz using a Clark-type oxygen microelectrode system with a 1 ml temperature-controlled

chamber (Oxygraph System, Hansatech Instruments, Norfolk, England) as previously described (Ligeret *et al.* 2008)(Morin *et al.* 2001). Briefly, 1 mg of mitochondrial protein was added to 990 µl of respiration buffer (5 mM KH_2PO_4, pH 7.2, 250 mM ultra-pure sucrose, 5 mM $MgCl_2$, 1 mM EGTA). Mitochondrial respiration was initiated by addition of the complex II substrate succinate (5 mM final concentration). After reaching a stable rate of state-4 respiration, 2 µl of NSE or vehicle solution (0.1% DMSO) were injected for final concentrations of 25 to 200 µg/ml of NSE. Six fractions and a number of purified constituents of *N. sativa* seed were also tested. State-4 respiration was allowed to proceed for at least 30 additional seconds before the induction of oxidative phosphorylation and state-3 respiration by the addition of ADP (250 µM final concentration)**.** In some experiments, mitochondria were pretreated with 2 µM of atractyloside potassium salt (AK), an inhibitor of the adenine nucleotide translocase (ANT) to explore the implication of this transporter in the action of NSE. The increase in the rate of basal O_2 consumption, an index of uncoupling, was calculated from each experiment by subtracting the rate measured in the presence of extract from the rate measured in the absence of extract and dividing the difference by the later rate. Decrease in the rate of ADP-stimulated O_2 consumption, an index of inhibition of oxidative phosphorylation (i.e. ATP synthase), was calculated by subtracting the rate measured in the extract experiment by the average rate measured in the vehicle control experiments of the corresponding experimental session, and dividing the difference by this average control rate.

PPARγ activation experiments

The following experiments were performed by Indigo Biosciences (State College, PA). HEK 293-T fibroblasts (ATCC, Manassas, VA) were cultured in high-glucose DMEM supplemented with 10 % FBS and antibiotics. Cells were transiently transfected with Gal4-human PPARγ and Gal4-luciferase plasmid DNA. The ligand-binding domain of human PPARγ was fused to the DNA-binding domain of the yeast transcription factor Gal4 under the control of the SV40 promoter. This plasmid also encoded the UAS-firefly luciferase reporter under the control of the Gal4 DNA response element. Plasmid verification was done by sequencing and through examination of positive controls. Cells were also co-transfected with pRL (renilla) to allow correction for transfection efficiency. Cells at 80 % confluence in 100 mm culture dishes were transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 6 h, the DNA-Lipofectamine complex was removed and the cells were maintained overnight in culture medium. Following overnight culture, the media was replaced 4 h after replating with fresh media containing NSE, or positive control in DMSO (0.1 % final concentration). Fourteen hours after treatment, the cells were lysed with passive lysis buffer (Promega, Madison, WI) for 30 min; luciferase and renilla activity were measured with the Luciferase dual reporter assay kit (Promega, Madison, WI) following the manufacturer's instructions and using a Tecan GeniosPro plate reader (Research Triangle Park, NC). The fold induction of normalized luciferase activity was calculated relative to DMSO vehicle-treated cells, and represents the mean of three independent samples per treatment group. For agonism experiments, cells were

treated with NSE (200, 50, 12.5, 3.13, 0.78 μg /ml), or with positive control (PPARγ agonist Rosiglitazone; 20, 2, 0.2, 0.02, 0.002 μM).

Glucose transport assay

Differentiated C2C12 cells grown in 12-well culture plates were treated with 200 μg/ml of NSE, thymoquinone, or vehicle control (0.1 % DMSO) for 18 h as previously described (Benhaddou-Andaloussi *et al.* 2008). Three hours prior to the experiment, the medium was replaced with serum-free DMEM containing NSE to establish a baseline insulin-free state. Following this, cells were incubated for 30 min in Krebs-phosphate buffer (KPB; 20 mM HEPES, 4.05 mM Na_2HPO_4, 0.95 mM NaH_2PO_4, pH 7.4, 120 mM NaCl, 4.7 mM KCl, 1mM $CaCl_2$ and 1 mM $MgSO_4$) containing 5 mM glucose and different treatments at 37 ^oC. Cells were then washed twice in warm glucose-free KPB before incubation for exactly 10 min at 37 ^oC in glucose-free KPB containing 0.5 μCi/ml 2-deoxy-D-[1-^{3}H]glucose (TRK383, Amersham Biosciences, Buckinghamshire, UK). Cells were then placed on ice and immediately washed three times with ice-cold KPB. Cells were lysed and scraped in 0.1 N NaOH. Lysates were transferred to 4 ml of Ready-Gel (Beckman Coulter Inc, Fullerton, CA) and radioactivity was counted in a liquid scintillation counter (1219 RackBeta; Perkin-Elmer, Waltham, MA). Four replicates were performed for each experimental condition. Co-treatment experiments of NSE with 2 μg/ml cycloheximide (inhibitor of protein translation) (Purintrapiban *et al.* 2006) were also performed.

Rate of acidification of extracellular medium

A spectrophotometric assay of change in cell culture medium pH over time, quantitative between pH 7.2 and 6.4, was developed based on similar assays (Schornack et Gillies 2003). The assay medium consisted of Dulbecco's PBS containing Phenol Red as a pH indicator and modified to contain a total of 2 mM phosphate (for approx. 20 % of the buffering capacity of Dulbecco's original recipe) while keeping other ion concentrations within the physiological range of extracellular fluid. The composition of this modified Dulbecco's PBS (mD-PBS) was: 1.5 mM Na2HPO4, 0.5 mM KH2PO4, 137 mM NaCl, 25 mM glucose, 4 mM KCl, 2 mM CaCl2, 2 mM MgCl2, Phenol Red 0.1 mM (Sigma-Aldrich Phenol Red 0.5 % solution), and deionized ultra-filtered water (Fisher Scientific, Ottawa, ON). This formulation results in a pH of 7.1, which was adjusted to 7.20 at ambient temperature with NaOH immediately prior to the assay using an Accumet pH meter with calomel electrode (Fisher Scientific). Absorbance of 100 µl samples transferred to 96-well plates (Sarstedt Inc., Montreal, QC) was measured at ambient temperature at 530 and 450 nm using a Wallac Victor 2 plate reader (Perkin-Elmer, Waltham, MA) and the ratio of abs 530 / abs 450 was calculated. The relationship between pH and the log of this ratio was observed to be linear over the range of pH of 6.4 to 7.4, in agreement with a pKa of 6.9 for balanced-salt phosphate buffers. The following function was used to model the relationship between pH and absorbance over the pH range of 6.4 to 7.2: pH = 0.765 * ln (abs 530 / abs 450) + 7.61 (r^2 = 0.99). A titration experiment was performed to determine the buffering capacity of mD-PBS. This capacity was

observed to be nearly linear between pH 6.2 and 7.2 and was calculated to be 1.075 mM equivalents per pH units between 6.3 and 7.1.

Experiments were performed on 7-day differentiated C2C12 muscle cells and on 1-day post-confluent H4IIE liver cells grown in 12 well plates. On the day of the experiment, cells were gently rinsed twice with mD-PBS, and then allowed to equilibrate in exactly 1.0 ml of mD-PBS for 30 min at 37 ^{o}C in a humidified air atmosphere. The assay was started by gently mixing pre-warmed 3 x concentrated treatments in a 500 µl volume of mD-PBS to the 1.0 ml volume of mD-PBS already present, for a final volume of exactly 1.5 ml and treatments at their final working concentration. After the rapid addition of treatments to all the wells of a single plate, an initial 100 µl sample of medium, corresponding to time 0, was transferred to microtiter plate for spectrophotometric analysis. Cells were then incubated at 37 ^{o}C in a humidified air atmosphere for the duration of the experiment. At times 20, 40, 60, 120, 180, and 240 min, plates were stirred and a 100 µl sample of medium was transferred to a microtiter plate for analysis. Calculations of rate of acidification and cumulative secretion of acid equivalents over time accounted for the decreasing experimental volume with each sampling. Because DMSO was observed to stimulate acidification, as noted by others (Gerber *et al.* 1996), extracts were solubilized in 80 % ethanol at 1000 x their final concentration, for a final ethanol concentration of 0.08 %. The addition of extracts or controls affected the pH of mD-PBS and therefore all treatments were adjusted to pH 7.2 separately immediately prior to the assay. FCCP solubilized in ethanol was used at 5 µM as a positive control. Results were expressed

as cumulative secretion of acid equivalents (micromoles) for two experiments of 4 to 5 replicates per condition per time point.

Cytosolic ATP assays

Total cytosolic ATP was measured in cell lysates by luminescence using the ATPlite assay kit (Perkin-Elmer, Waltham, MA), as per the manufacturer's protocol. Briefly, C2C12 myotubes in 24-well plates or H4IIE hepatocytes in 96-well plates were treated in parallel for 1, 3, or 6 h with extract or 0.1 % DMSO. FCCP was used at 5 μM as a positive control. Two separate experiments were performed, each with 3-4 replicates per condition per time point. Results were expressed as % ATP content of vehicle-treated wells.

Statistical analysis

Data are reported as the mean ± SEM of the indicated number of experiments. Results were analysed by one-way analysis of variance (ANOVA) using StatView software (SAS Institute Inc, Cary, NC), with post-hoc analysis as appropriate (Fisher). Statistical significance was set at $p \leq 0.05$.

RESULTS

NSE treatment activates the insulin signaling pathway in muscle and liver cells

We have previously shown that NSE potently stimulates glucose uptake in C2C12 skeletal muscle cells and 3T3-L1 adipocytes following an 18 h treatment (Benhaddou-Andaloussi *et al.* 2008). To elucidate the signaling pathways through which the extra-pancreatic effects of NSE are mediated, we first tested whether the insulin-signaling pathway was activated. This was performed by assessing phosphorylation of Akt and ERK 1/2, two major components of the insulin receptor signaling cascade (Tirosh *et al.* 2000), by western immunoblot with phospho-specific antibodies. C2C12 cells treated with NSE for 18 h showed increased contents of basal and of insulin-stimulated (1 nM; 5 min) phospho-Akt and -ERK 1/2. The increased insulin-stimulated contents of phospho-Akt and -ERK were found to persist as far as 60 min after insulin stimulation (Figure 1A). Plotting phospho-Akt content over time indicated that NSE treatment induces a proportional upward shift of content at every time point. Contents of total Akt and ERK were not affected by the treatment (Figure 1A).

In 3T3-L1 cells, NSE treatment had no effect on phospho-Akt or phospho-ERK contents. No effect was seen on basal or insulin-stimulated states (Figure 2B, 2C). Likewise total adipocyte protein content in Akt or ERK 1/2 was not modified by treatment with NSE (Figure 2A).

The effect of NSE on the activation of Akt and ERK1/2 after 18 h of treatment in the H4IIE hepatocytes was next tested. Akt and ERK1/2 activities were measured by densitometry of immunoblots. Moreover, NSE increased Akt phosphorylation by 1.5 fold (Figure 3B). However, NSE did not increase phosphorylation of ERK 1/2 (Figure 2C). In H4IIE cells, NSE did not change the total content in unphosphorylated Akt and ERK1/2 (Figure 3A, 3C).

NSE treatment activates the AMPK signaling pathway in muscle and liver cells

We next tested whether NSE treatment activated the AMPK pathway, an alternate and insulin-independent pathway through which glucose uptake can be stimulated (Winder et Hardie 1999). The content of phosphorylated AMPKα (catalytic subunit of AMPK) and its downstream substrate acetyl coenzym A carboxylase (ACC) were assessed by western immunoblot. AICAR, a cell-permeable AMP mimetic, was used as a positive control (2 mM; 30 min). NSE treatment increased the content of phospho-AMPK and -ACC in C2C12 cells to levels comparable to that of AICAR (Figure 4). Total AMPK was not altered. Similar results were observed following NSE treatment in H4IIE cells (Figure 5). In 3T3-L1 adipocytes, however, NSE treatment did not increase phosphorylation of AMPK or ACC (Figure 6).

Treatment with NSE uncouples oxidative phosphorylation

AMPK is responsive to perturbations of energy homeostasis. To elucidate the mechanism through which AMPK was activated, we tested whether mitochondrial

oxidative phosphorylation was disrupted by NSE treatment; uncoupling or inhibition of oxidative phosphorylation are common activities of plant defensive metabolites (Polya 2003). NSE treatment was observed to uncouple oxidative phosphorylation of isolated liver mitochondria, as demonstrated by an increase in basal (state-4) succinate-supported O_2 consumption immediately upon addition of extract. This effect was dose-dependent and uncoupling was nearly complete at 200 μg/ml. The extract also exhibited a mild inhibitory activity, as demonstrated by a slight decrease in ADP-stimulated (state-3) O_2 consumption (Figure 7A, Table1). The activity of NSE was unaffected by pretreatment of mitochondria with the adenine nucleotide translocase inhibitor potassium atractyloside (AK), despite complete inhibition of state-3 respiration by AK (Figure 7B). The activity of sequential solvent fractions as well as that of the oily and solid phases of NSE were also tested. The hexanolic and ethyl-acetate fractions, as well as the oily phase of the NSE, presumably containing the least polar constituents of *N. sativa* seed, exhibited conserved uncoupling activity (Table 1). Known constituents thymol, carvacrol, hederagenin, nigellimine and thymoquinone, exhibited little or no uncoupling activity at 100 μM (Table 1).

NSE treatment activates PPARγ signaling pathway

As the effects of NSE on glucose uptake in 3T3-L1 adipocytes previously observed (Benhaddou-Andaloussi *et al.* 2008) cannot be explained by signaling through the insulin receptor pathway or through AMPK, we next tested whether NSE activated PPARγ. Using a luciferase gene reporter assay in HEK 293-T cells, NSE was observed to stimulate PPARγ activity in a logarithmic dose-response fashion

(Figure 8). Effects of NSE were significantly different from the vehicle control at 50 and 200, reaching a maximal enhancement of PPARγ-dependent luciferase production of 50% (Figure 8).

Inhibition of protein translation abolishes the effect of NSE on muscle cell glucose uptake

To determine whether NSE-induced stimulation of glucose uptake is dependent on protein translation, co-treatment experiments with cycloheximide, an inhibitor of translation, were performed. Cycloheximide decreased uptake to comparable levels in vehicle-treated and NSE-treated cells (Figure 9A).

Thymoquinone stimulates muscle cell glucose uptake

Thymoquinone, a small phenolic constituent compound of *N. sativa* seeds, has been proposed by others to be an active principle. This compound was tested for a glucose-uptake stimulating effect under the same conditions used to demonstrate the effect of NSE (18 h treatment). Thymoquinone increased uptake by a maximum of 29 % at a concentration of 30 μM, an effect smaller than that of NSE (Figure 9B).

Rate of acidification of extracellular medium is not increased by NSE

To assess whether the uncoupling effect of NSE causes a compensatory increase in the anaerobic metabolism of cells, an indication of the potential for causing systemic lactic acidosis, H4IIE hepatocytes and C2C12 muscle cells were treated with NSE for 4 h and the pH of the culture medium was assayed as various

time points over this period. In both cell types, NSE treatment did not increase rate of acidification as compared to vehicle control (Figure 10).

Cytosolic ATP concentration is not depressed by NSE

To assess whether the uncoupling effect of NSE produces a decrease in ATP content, C2C12 muscle cells and H4IIE hepatocytes were treated for 0.5, 1, 3, and 6 h and cytosolic ATP content was assayed. NSE treatment did not induce a decrease in ATP content in muscle cells (Figure 11B). Rather, ATP content was slightly increased at 1 h and this increase was sustained up to 6 h. The reference uncoupler FCCP (5 μM) caused a decrease in ATP content of approximately 20 % at 0.5 h, which was normalized by 1 h. Furthermore, like NSE, FCCP induced an overshoot effect observable at 3h. In hepatocytes, effects were generally more important (Figure 11A). FCCP caused ATP content to fall by approximately one third, an effect that lasted up to 6 h. NSE also caused a decrease in ATP but this was smaller and shorter-lived than that of FCCP.

DISCUSSION

N. sativa seeds are used in the traditional medicine of numerous Middle Eastern and North African countries (Bellakhdar *et al.* 1991; Ziyyat *et al.* 1997; Haddad *et al.* 2001) for their anti-hyperglycemic activity (Al-Awadi *et al.* 1985; Al-Awadi et Gumaa 1987; Al-Awadi *et al.* 1991; Al-Hader *et al.* 1993; Labhal *et al.* 1999; Meral *et al.* 2001; El-Dakhakhny *et al.* 2002; Fararh *et al.* 2002; Zaoui *et al.* 2002; Kanter *et al.* 2003; Fararh *et al.* 2004; Kanter *et al.* 2004; Rchid *et al.* 2004; Benhaddou-Andaloussi *et al.* 2008). This action has been attributed to insulinotropic effects, as observed with NSE in isolated pancreatic islets (Rchid *et al.* 2004) and in two pancreatic β cell lines (Benhaddou-Andaloussi *et al.* 2008), as well as extra-pancreatic effects, as observed in-vivo (Al-Hader *et al.* 1993; Labhal *et al.* 1999; Meral *et al.* 2001; El-Dakhakhny *et al.* 2002; Fararh *et al.* 2002; Zaoui *et al.* 2002; Kanter *et al.* 2003; Fararh *et al.* 2004; Kanter *et al.* 2004) and in cultured skeletal muscle cells and adipocytes (Benhaddou-Andaloussi *et al.* 2008). The present study foccussed on extra-pancreatic effects and aimed to elucidate the mechanisms of action of NSE in C2C12 skeletal muscle cells, 3T3-L1 adipocytes and H4IIE hepatocytes.

We have previously demonstrated (Benhaddou-Andaloussi *et al.* 2008) that glucose uptake in the absence of insulin is greatly enhanced by an 18 h treatment with NSE in muscle cells and adipocytes; in both cell lines, this effect on basal uptake was equal to or greater than the effect of stimulation with 100 nM of insulin for 30 min. In adipocytes, the effect of insulin is potentiated. The goal of the present

study was to identify the signaling pathway through which NSE induced these enhancements in glucose uptake. We first tested whether the insulin-like effect of NSE was mediated through the insulin signaling pathway. Insulin acts on its target tissues by way of its cell-surface receptor and the well-characterized signaling cascade downstream of this receptor (Tirosh *et al.* 2000). In skeletal muscle cells and adipocytes, insulin signals the translocation of Glut4 glucose transporters to the plasma membrane to increase glucose uptake (Tirosh *et al.* 2000). In hepatocytes, insulin signals the inhibition of hepatic glucose production (Wu *et al.* 2005). In the three target tissues, we assessed the degree of phosphorylation of Akt, a signaling protein involved in the regulation of Glut4 translocation, several steps removed from the insulin receptor, and generally acknowledged as a reliable marker of activity through the insulin signaling pathway. We also assessed the phosphorylation of ERK, a signaling protein involved in the anabolic effects of insulin. Our results demonstrate that in skeletal muscle and liver cells, but not in adipocytes, NSE increases basal phosphorylation of Akt in the absence of insulin and that this increase is additive to the acute effect of insulin. Phosphorylation of ERK mirrored these results in skeletal muscle. However, ERK was not activated by NSE in hepatocytes or adipocytes.

We next tested whether activation of the AMPK pathway also contributed to the insulin-like effects of NSE in muscle and liver cells, and whether this pathway could explain effects in adipocytes. The function of AMPK is to detect and transduce perturbations in cellular energy homeostasis, triggering cytoprotective responses to

counter metabolic stress (Sanz 2008). The cytoprotective responses include profound effects on cellular metabolism, and some of these, including the stimulation of glucose uptake in skeletal muscle cells and adipocytes and the inhibition of hepatic glucose output (Hayashi *et al.* 1998; Bergeron *et al.* 2001), are very similar to those of insulin. Because these effects translate into the normalization of hyperglycemia, AMPK is considered a key therapeutic target for insulin resistance and diabetes (Sanz 2008). Our findings indicate that the AMPK pathway is activated by NSE treatment in C2C12 and H4IIE, as assessed by increased phsophorylation of AMPK and of ACC, one of its key effectors. However, this pathway was not activated in 3T3-L1 adipocytes. This tissue-specificity of AMPK activation by NSE is interesting and potentially highly desirable, as an absence of AMPK activation in adipocytes can prevent unwanted increases in adipocity through this pathway (Rossmeisl *et al.* 2004).

In light of the fact that the AMPK pathway is responsive to perturbations in energy homeostasis, we tested the hypothesis that treatment with NSE indirectly activates AMPK by disrupting mitochondrial energy transduction and producing metabolic stress. Indeed many natural products and naturally-occurring compounds are known to inhibit or uncouple mitochondrial oxidative phosphorylation (Wheeler et Camp 1971; Toyomizu *et al.* 2000; Polya 2003). We treated isolated liver mitochondria with NSE and observed uncoupling activity, evidenced by an increased rate of basal oxygen consumption. This effect was dose-dependent and almost complete at the NSE concentration used to demonstrate activation of AMPK and

enhancement of glucose uptake in cells. Such an uncoupling effect is expected to decrease the maximal capacity for mitochondrial ATP production by dissipating the protomotive force necessary for ATP synthesis from ADP. While cellular ATP can be maintained at normal concentrations if demand does not exceed capacity, more work is obviously required to meet this demand. This results in increased AMP production through the adenylate kinase reaction (Noda 1970), and therefore activation of AMPK (Sanz 2008). In addition to resulting in the activation of AMPK and anti-hyperglycemic effect, uncoupling is also a potential anti-obesity activity since the additional work necessary to make up for decreased metabolic efficiency at the cellular level translates to an increase in basal metabolic rate at the organism level.

Targeting mitochondrial function is inherently dangerous as aerobic energy production capacity can easily fall below cellular energy demand. This can be compensated to some degree by anaerobic glycolysis (Alberti 1977). However, the end product of this pathway is lactic acid which can potentially produce systemic acidosis (Alberti 1977). Indeed the potential for lactic acidosis is the reason why the more powerful members of the biguanides were withdrawn from most markets (Dykens *et al.* 2008). To evaluate the potential danger of NSE, we tested its effects on the rate of acidification of the culture medium of muscle and liver cells. We observed that in both cell types, the rate of acidification was not different from vehicle and was significantly lower than that of the classic uncoupler FCCP tested at 5 μM. We also assessed the cytosolic concentration of ATP one hour after onset of

treatment, at a time when ATP is significantly depressed by FCCP, and found it to be unchanged. These findings suggest that the uncoupling activity of NSE is not threatening and that treatment with *N. sativa* seeds is unlikely to cause systemic acidosis. Powerful uncoupling activity producing only mild metabolic stress may be explained by rapidly metabolized active principles.

While *N. sativa* seeds and seed oil are phytochemically well-characterized natural products (Khan 1999), their active principle(s) have yet to be determined. The elucidation of molecular mechanisms of action, such as the finding that AMPK is activated in response to metabolic stress provoked by uncoupling of oxidative phosphorylation, can provide insight into the nature of the active compounds. For example, knowing that the common mechanism of uncoupling involves the protonophoric activity of lipophilic weak acids (Terada 1990), one approach is to narrow the search for actives to compounds that exist as both neutral and ionized species at mitochondrial pH (acid-dissociation constant (pKa1) on the order of 6.5 to 8.5) and that exhibit moderate to high lipophilicity (water-octonol partition coefficient (logP) on the order of 2 and above). The soundness of this strategy was in part supported when we fractionated *N. sativa* seeds using the classical approach of sequential extraction with solvents of increasing polarity, and observed that the most lipophilic fractions demonstrated the highest uncoupling activity in isolated mitochondria. Unfortunately, no heretofore reported constituents of *N. sativa* seeds were predicted to exhibit the desired physicochemical properties. *N. sativa* seeds are particularly rich in triterpenes but most of these can be ruled out as weak acid

uncouplers based simply on the fact that they are not ionizable at physiological pH. These include β-amyrin, avenasterol, butyrospermol, campestanol, campesterol, citrostadienol, cycloartanol, cycloartenol, cycloeucalenol, lophenol, obtusifoliol, β-sitosterol, α-spinasterol, stigmastanol, stigmasterol, taraxerol, tirucallol (Kudryashova et Kolobkova 1953; Salama 1973; Sener *et al.* 1985; Menounos *et al.* 1986; Merfort *et al.* 1997). Hederagenin (logP=5.3; pKa1=4.7) is an exception to this since, by virtue of its carboxyl group, it is almost completely ionized at cytosolic pH. However, its low pKa1 is also incompatible with this mechanism and the compound was demonstrated to have no uncoupling activity. A number of small phenolics can also ruled out due to absence of ionizable groups or clearly incompatible pKa1. This includes cystine, damascenine, dithymoquinone, nigellicine, and thymoquinone. Thymoquinone was nevertheless tested as it has been proposed by others to be an active principle (Badary *et al.* 2000; El-Saleh *et al.* 2004; Khattab et Nagi 2007). However, it was found to have no uncoupling activity. Similarly, carvacrol (logP=3.4; pKa1=10.4) and thymol (logP=3.4; pKa1=10.6) were tested and found to have little or no activity. Finally, nigellimine (logP=1.6; pKa1=7.0) was also found to be inactive, presumably due to low lipophilicity.

This suggests that the powerful uncoupling activity of NSE may 1) result from the additive effect of several mild uncouplers; 2) may be attributable to compounds other than the ones tested; or 3) may be attributable to another mechanism such as the interaction with a transmembrane protein of the inner mitochondrial membrane (Belzacq *et al.* 2002), such as the adenine nucleotide translocase (ANT). Interaction

with the ANT was directly ruled out when the ANT inhibitor atractyloside (2 μM) had no effect on the activity of NSE. Finally, the compound thymoquinone, suggested by several groups to be the active principle of *N. sativa* (Salem 2005), was shown to have no effect on basal mitochondrial respiration, concordant with its lack of ionizable groups. This compound did, however, stimulate glucose uptake in muscle cells up to a maximum of approximately 30%. While this is less than the effect of NSE, this compound could nevertheless be responsible for the activation of the insulin signaling pathway rather than the activation of AMPK.

Finally to address the mechanism of action through which NSE enhances glucose uptake in adipocytes, apparently distinct from that of skeletal muscle, we tested whether NSE activated the PPARγ receptor. Stimulation of this nuclear receptor, the target of the thiazolinediones (Girard 2001), upregulates the expression of effectors of the insulin signaling pathway (Olefsky et Saltiel 2000). Using a reporter-gene assay, we observed that NSE behaves like an agonist of PPARγ, increasing its activity by more than 50% at 200 μg/ml. While this is a relatively small effect when compared to that of rosiglitazone, it must be considered that the active principles responsible may be very minor components of NSE. Nevertheless this increase in PPARγ activity is likely sufficient to result in some upregulation of proteins such as PI3-kinase and Glut4 transporters, and likely also accounts for the stimulation of adipogenesis previously observed (Benhaddou-Andaloussi *et al.* 2008). Interestingly, *N. Sativa* has recently been shown to contain a unique family of compounds, the nigellamines, that activate PPARα (Morikawa *et al.* 2004).

The finding that distinct pathways are responsible for enhanced glucose uptake in muscle and adipocytes helps reconcile the previous observation that in adipocytes, but not in skeletal muscle, NSE potentiated the effect of insulin (Benhaddou-Andaloussi *et al.* 2008). As the AMPK and insulin signaling pathways regulating the translocation of vesicle-bound Glut4 transporters to the plasma membrane converge at AS160 (Cartee et Wojtaszewski 2007), potentiation is not likely. Therefore it can be proposed that in adipocytes, where there is no AMPK activation by NSE, insulin exerts its full acute effect on a pool of transporters whose content, both cytoplasmic and plasma membrane-bound, has been increased through the stimulation of PPARγ. Conversely, in skeletal muscle cells where AMPK is activated and transporters are mostly at the plasma membrane, insulin can have little additional effect. However, it must be noted that in addition to its acute effect, AMPK activation also causes an increase in upregulation of its effectors, including Glut transporters (Winder 2000). Therefore the effect of NSE in skeletal muscle likely involves an increase in the pool of transporters in addition to short-lived translocation of preexisting transporters. This is supported by co-treatment of cells with NSE and the translation inhibitor cycloheximide resulting in the abolishment of enhanced glucose uptake at the end of the 18h treatment.

In summary, NSE exerts insulin-like actions on skeletal muscle cells, hepatocytes, and adipocytes through three major pathways involved in glucose homeostasis: the insulin signaling pathway, the AMPK pathway, and the PPARγ pathway. The trigger for the AMPK pathway appears to be the metabolic stress that

results from the uncoupling of oxidative phosphorylation by NSE. However, the stress appears to be mild and short-lived suggesting that uncoupler active principles are easily metabolized and that *N. sativa* seeds have low potential for inducing dangerous systemic acidosis. The identification of these effector pathways of the biological activity of NSE explain in large part the well-documented anti-hyperglycemic effects *N. sativa* and the use of this plant in the traditional medicine of several cultures. The results presented here support the notion that *N. sativa* has exceptional potential as a complementary or alternative approach to currently available anti-diabetic medications.

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TABLEAUX AND FIGURES

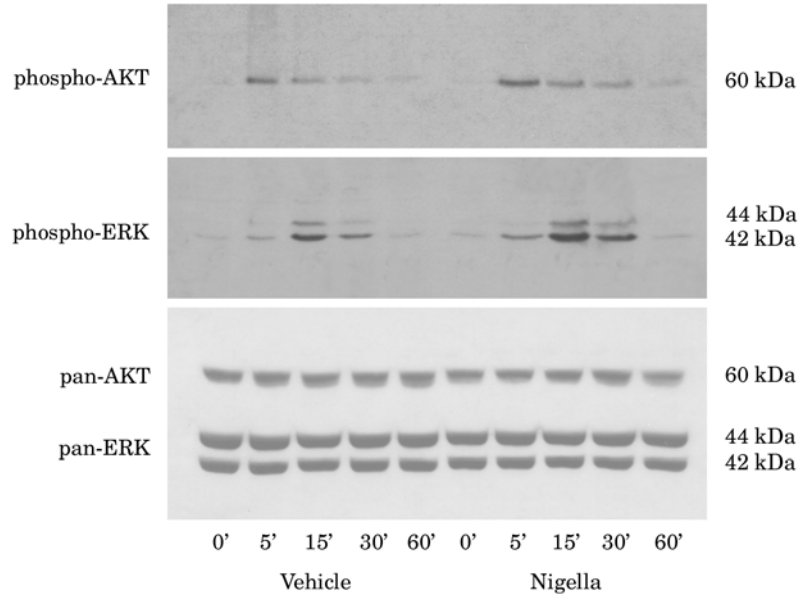
Table 1: Calculated effects of ethanol extract, fractions, and constituent compounds of *N. sativa* seeds on oxidative phosphorylation in isolated mitochondria.

***N. sativa* seed product**	**Decrease in ATP synthetic capacity attributable to uncoupling effect**	**Decrease in ATP synthetic capacity attributable to inhibitory effect**
ethanol extract (NSE) (200 μg/ml)	80.9 % ± 7.3	23.9 % ± 6.6
NSE liquid phase only (140 μg/ml)	92.5 % ± 9.1	17.4 % ± 6.2
NSE solid phase only (60 μg/ml)	23.3 % ± 5.6	27.8 % ± 6.5
hexane fraction (200 μg/ml)	68.4 % ± 6.8	48.5 % ± 10.2
ethyl acetate fraction (200 μg/ml)	34.1 % ± 6.0	63.1 % ± 3.3
methanol fraction (200 μg/ml)	2.3 %	37.7 %
methanol/water fraction (200 μg/ml)	3.6 % ± 1.6	19.9 % ± 3.8
carvacrol (100 μM)	9.2 % ± 0.8	8.8 % ± 1.9
hederagenin (100 μM)	4.9 % ± 0.4	9.1 % ± 2.6
nigellimine (100 μM)	3.1 % ± 0.0	6.1 % ± 1.1
thymol (100 μM)	6.8 % ± 0.1	18.9 % ± 1.0
thymoquinone (100 μM)	0.3 % ± 0.1	7.3 % ± 0.8

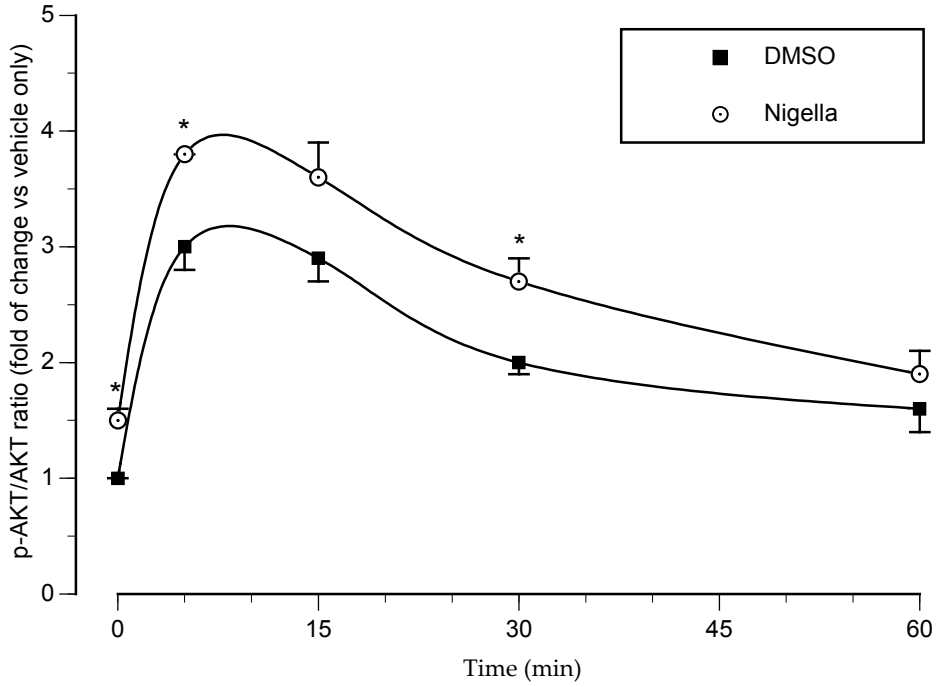
The ADP-stimulated rate of O2 consumption per mg of mitochondrial protein is taken to be the maximal respiration rate. The basal rate of O2 consumption is taken to be the respiration rate required to compensate for proton leakage. Capacity is calculated as the difference between maximal respiration rate and basal rate, both measured in the absence of treatments. Reduction in capacity due to uncoupling is a calculation of the increase in basal rate induced by the treatment. Reduction in capacity due to inhibition is a calculation of the decrease in ADP-stimulated rate induced by the treatment and applies to the residual capacity after subtraction of the uncoupling effect.

Figure 1

A:


phospho-AKT
60 kDa
phospho-ERK
44 kDa
42 kDa
pan-AKT
60 kDa
pan-ERK
44 kDa
42 kDa
0' 5' 15' 30' 60' 0' 5' 15' 30' 60'
Vehicle
Nigella

B:


DMSO
Nigella
p-AKT/AKT ratio (fold of change vs vehicle only)
Time (min)
0
1
2
3
4
5
0
15
30
45
60
*
*
*

C:

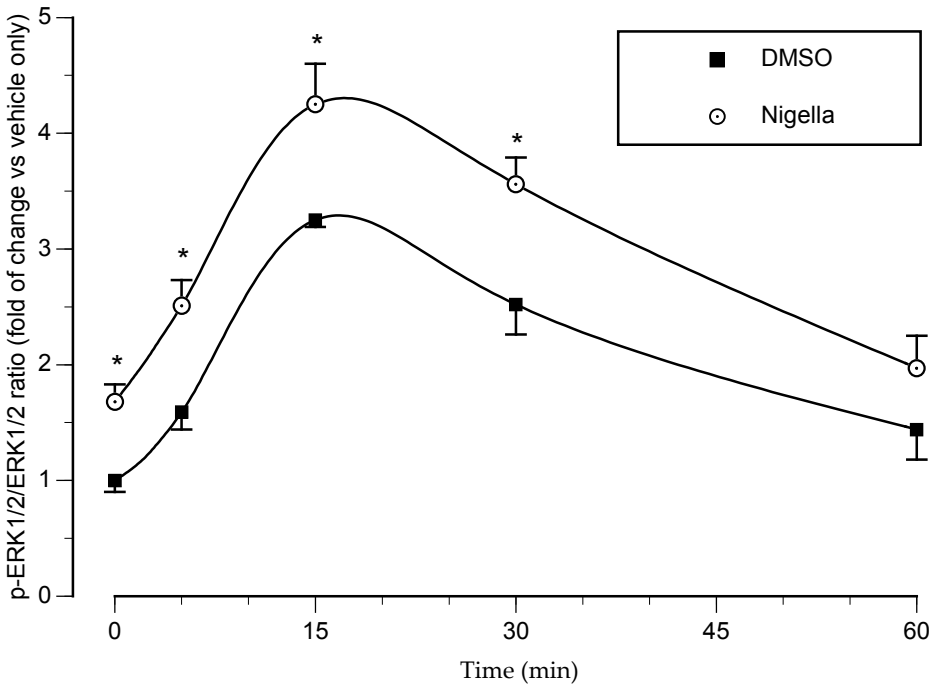
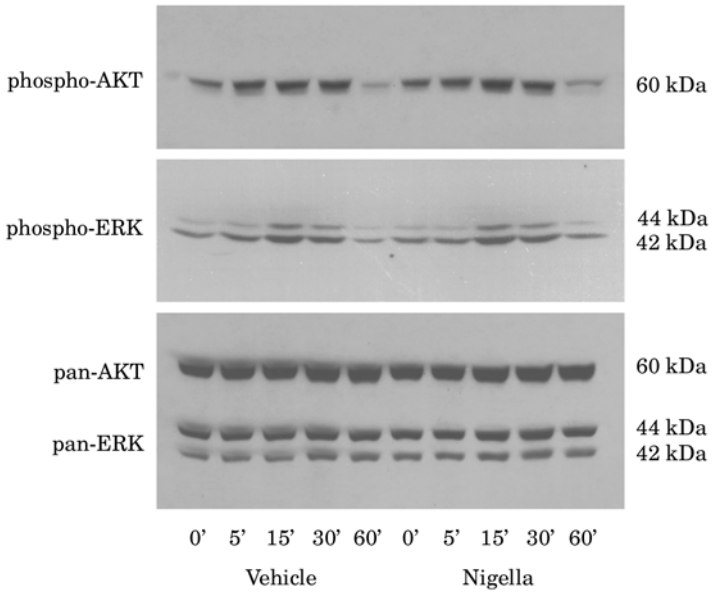
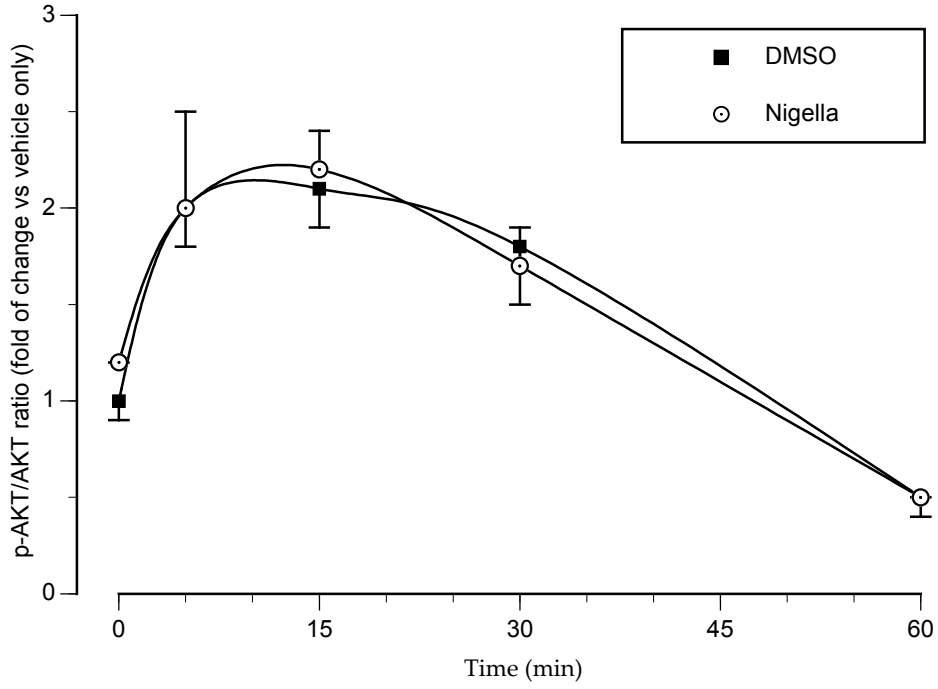

p-ERK1/2/ERK1/2 ratio (fold of change vs vehicle only)
0
1
2
3
4
5
0
15
30
45
60
Time (min)
DMSO
Nigella
*
*
*
*

Figure 2

A:


phospho-AKT
60 kDa
phospho-ERK
44 kDa
42 kDa
pan-AKT
60 kDa
pan-ERK
44 kDa
42 kDa
0' 5' 15' 30' 60' 0' 5' 15' 30' 60'
Vehicle
Nigella

B:


DMSO
Nigella
p-AKT/AKT ratio (fold of change vs vehicle only)
0
1
2
3
0
15
30
45
60
Time (min)

C:

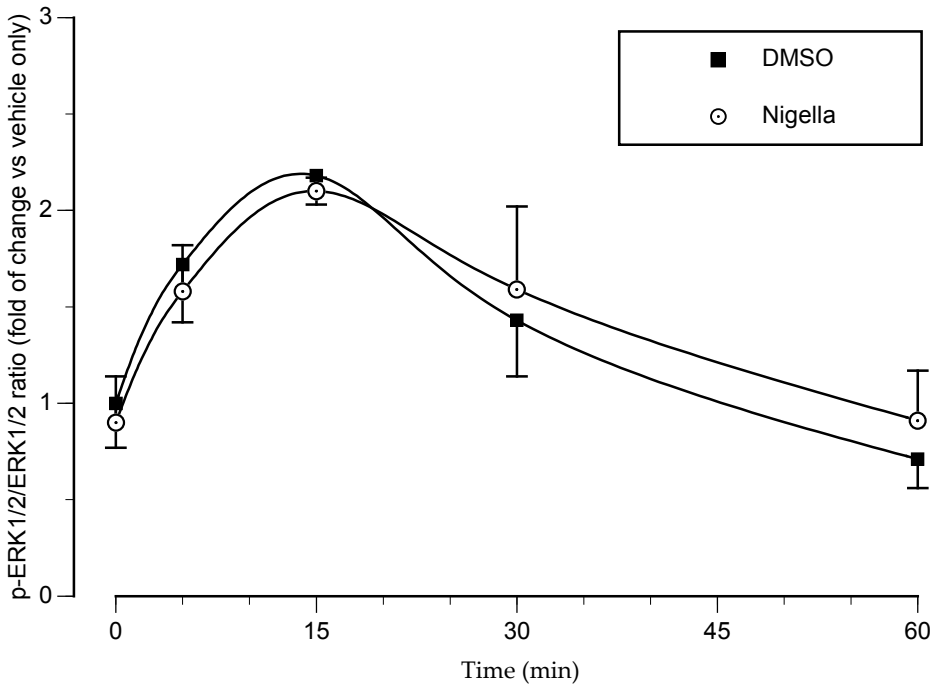
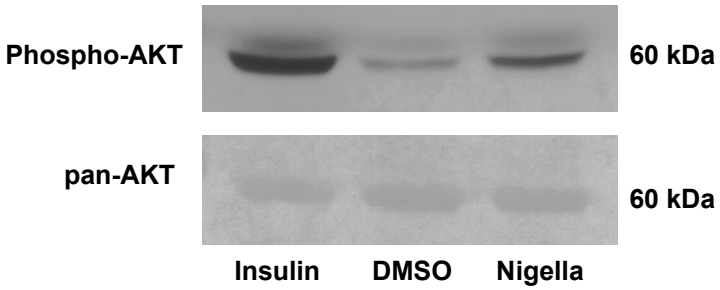
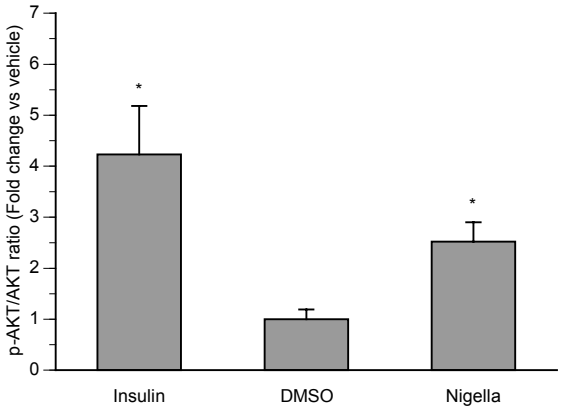

p-ERK1/2/ERK1/2 ratio (fold of change vs vehicle only)
3
2
1
0
DMSO
Nigella
0
15
30
45
60
Time (min)

Figure 3

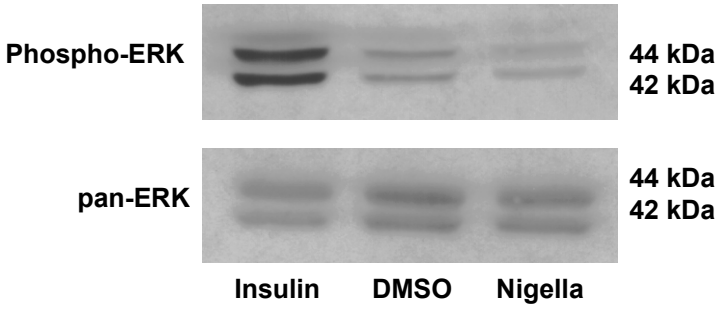
A:


Phospho-AKT
60 kDa
pan-AKT
60 kDa
Insulin
DMSO
Nigella

B:


p-AKT/AKT ratio (Fold change vs vehicle)
0
1
2
3
4
5
6
7
*
*
Insulin
DMSO
Nigella

C:


Phospho-ERK
44 kDa
42 kDa
pan-ERK
44 kDa
42 kDa
Insulin
DMSO
Nigella

D:

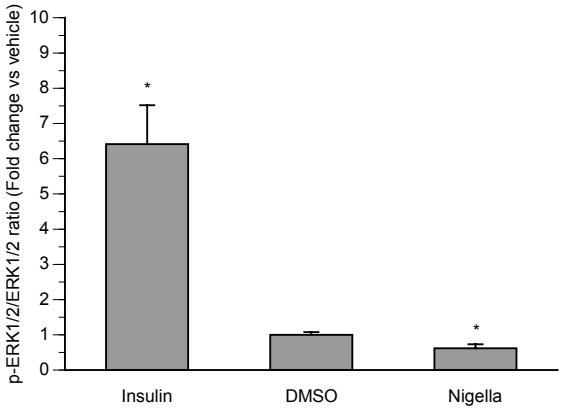
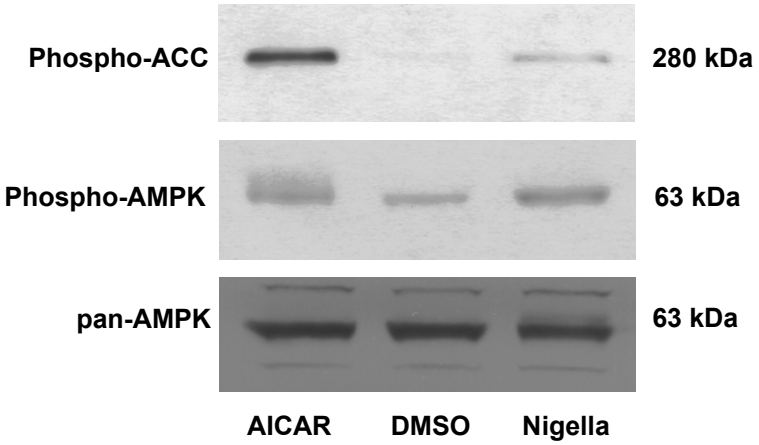

p-ERK1/2/ERK1/2 ratio (Fold change vs vehicle)
10
9
8
7
6
5
4
3
2
1
0
*
*
Insulin
DMSO
Nigella

Figure 4

A:


Phospho-ACC
280 kDa
Phospho-AMPK
63 kDa
pan-AMPK
63 kDa
AICAR
DMSO
Nigella

B:

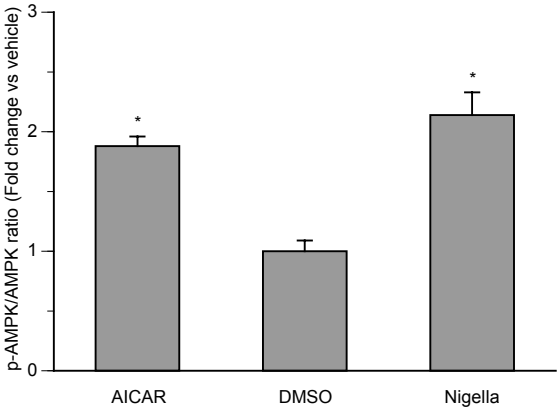
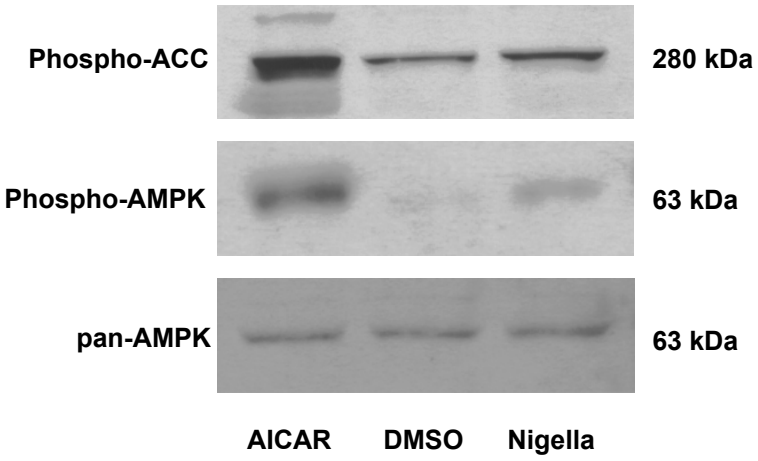

p-AMPK/AMPK ratio (Fold change vs vehicle)
3
2
1
0
*
*
AICAR
DMSO
Nigella

Figure 5

A:


Phospho-ACC
280 kDa
Phospho-AMPK
63 kDa
pan-AMPK
63 kDa
AICAR
DMSO
Nigella

B:

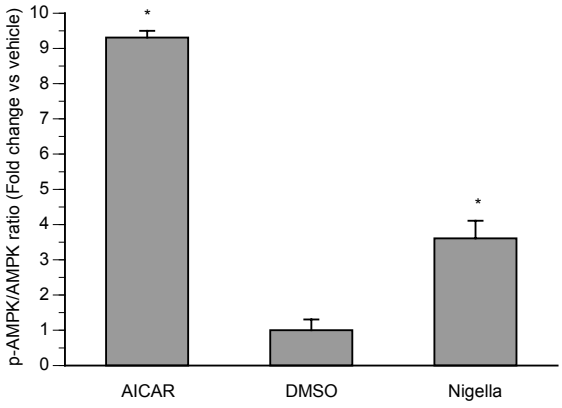

p-AMPK/AMPK ratio (Fold change vs vehicle)
0
1
2
3
4
5
6
7
8
9
10
*
*
AICAR
DMSO
Nigella

Figure 6

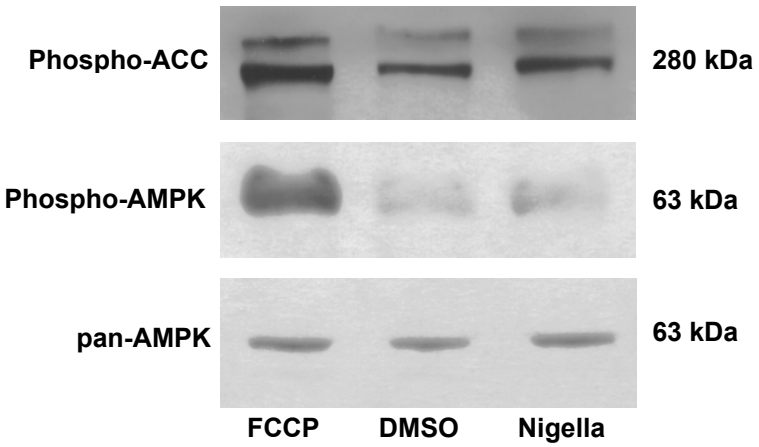
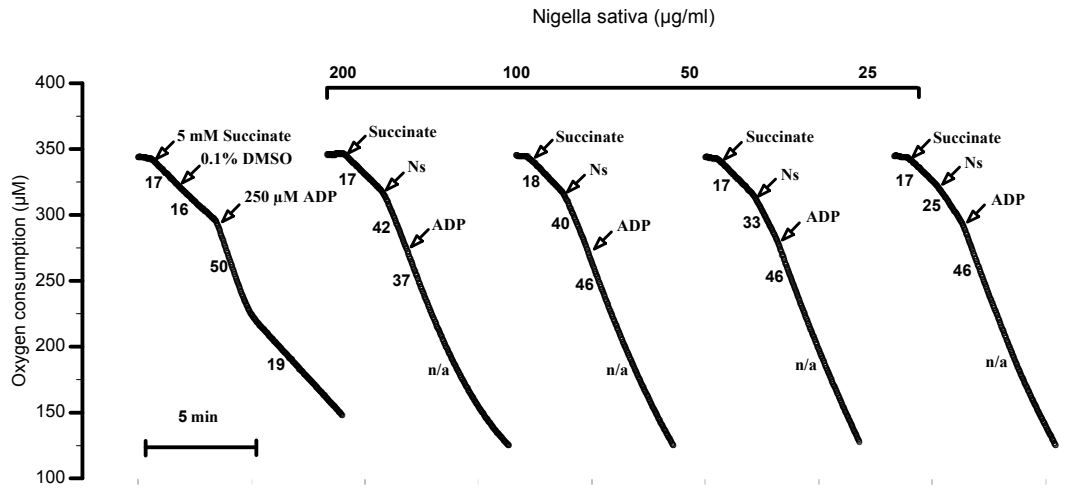

Phospho-ACC
280 kDa
Phospho-AMPK
63 kDa
pan-AMPK
63 kDa
FCCP
DMSO
Nigella

Figure 7

A:


Nigella sativa (µg/ml)
200
100
50
25
Oxygen consumption (µM)
400
350
300
250
200
150
100
5 mM Succinate
0.1% DMSO
250 µM ADP
Succinate
Ns
ADP
5 min
17
16
50
19
42
37
n/a
18
40
46
33
25

B:

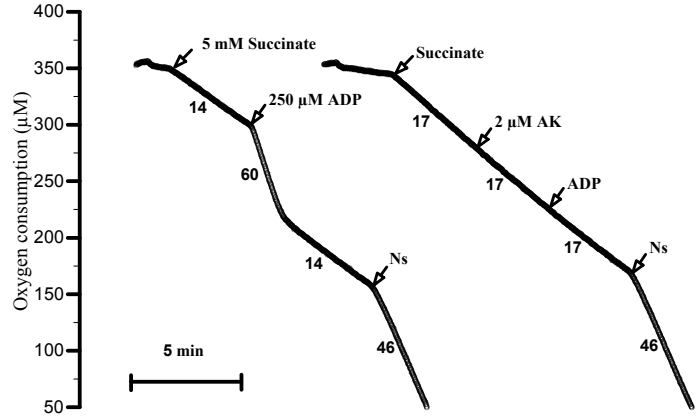

Oxygen consumption (µM)
400
350
300
250
200
150
100
50
5 mM Succinate
250 µM ADP
Succinate
2 µM AK
ADP
Ns
5 min
14
60
17
46

Figure 8:

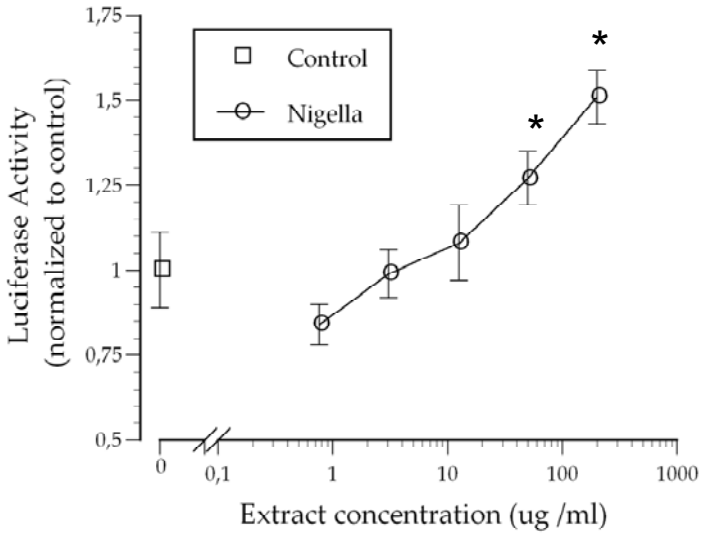
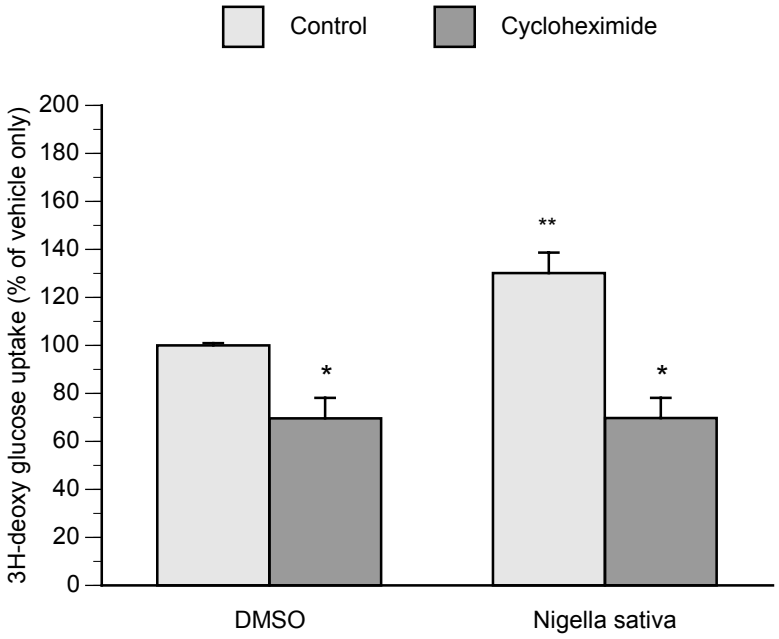

1,75
1,5
1,25
1
0,75
0,5
Luciferase Activity
(normalized to control)
Control
Nigella
*
*
0
0,1
1
10
100
1000
Extract concentration (ug /ml)

Figure 9

A:


Control
Cycloheximide
3H-deoxy glucose uptake (% of vehicle only)
200
180
160
140
120
100
80
60
40
20
0
**
*
*
DMSO
Nigella sativa

C:

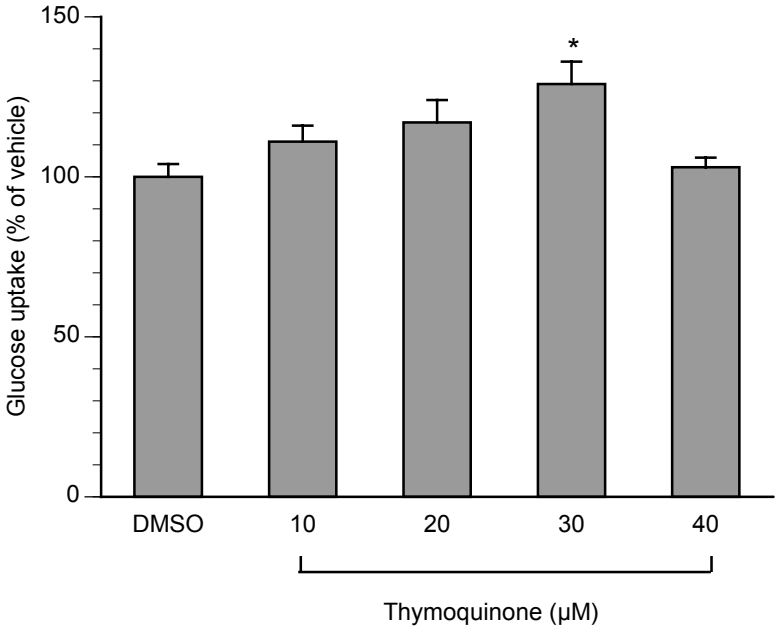

Glucose uptake (% of vehicle)
150
100
50
0
*
DMSO
10
20
30
40
Thymoquinone (μM)

Figure 10:

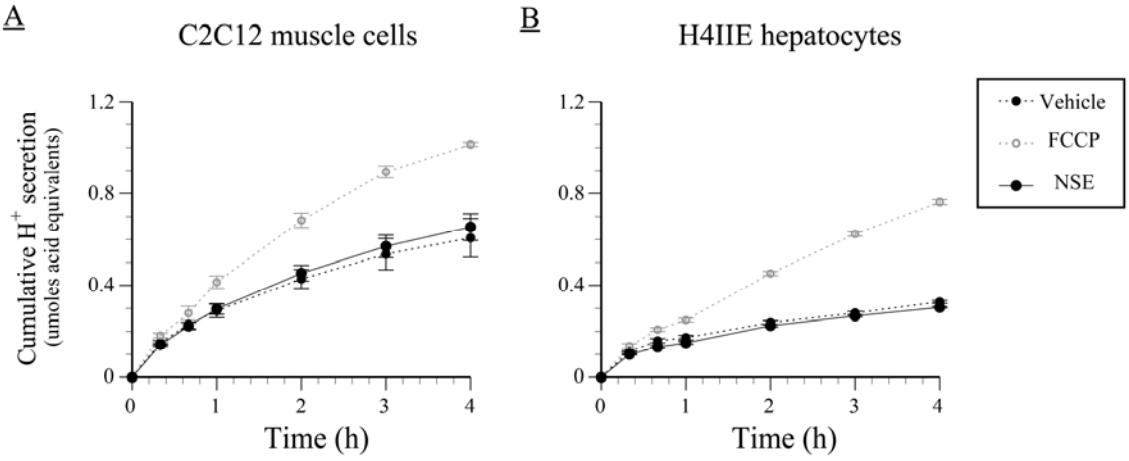
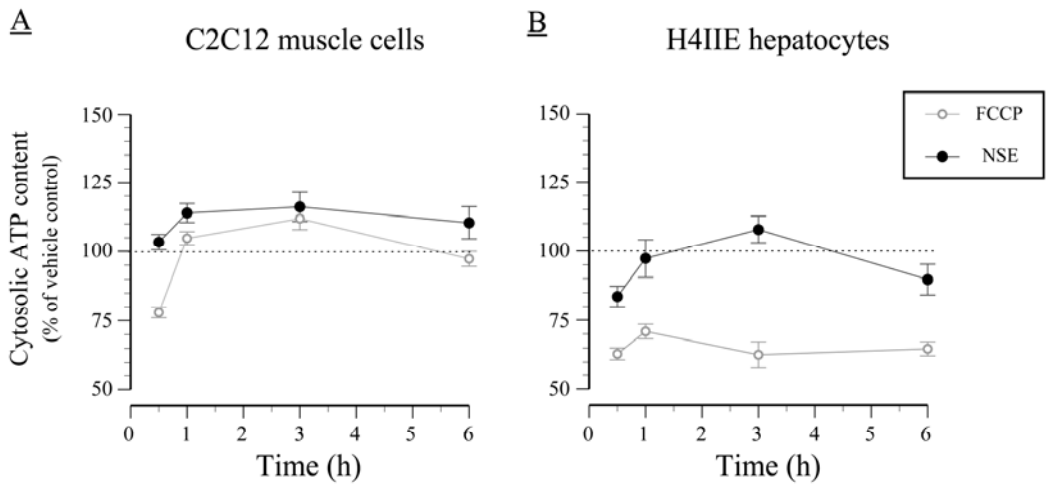

A
C2C12 muscle cells
B
H4IIE hepatocytes
Cumulative H+ secretion
(umoles acid equivalents)
1.2
0.8
0.4
0
0
1
2
3
4
Time (h)
Vehicle
FCCP
NSE

Figure 11


A
C2C12 muscle cells
B
H4IIE hepatocytes
FCCP
NSE
Cytosolic ATP content
(% of vehicle control)
150
125
100
75
50
0
1
2
3
4
5
6
Time (h)

LEGEND OF FIGURES

Figure 1: NSE increases phosophorylation of Akt in C2C12 muscle cells. Differentiated C2C12 myotubes were treated with 200 µg/ml of NSE for 18 h. Cells were then treated for 5 min with 0 or 1 nM of insulin, followed by an additional incubation in DMEM for 5 to 55 min prior to lysis and immunoblot analysis for phosphorylated and pan Akt and ERK. A) Representive immunoblots. B) Quantitation of Akt immunoblots. C) Quantitation of ERK immunoblots. For B and C, data are integrated densities (arbitrary units) expressed as normalized ratios of phospho to total content. Data are mean ± SEM for n=3 passages of cells. * indicates significantly different (p 0.05) from vehicle control.

Figure 2: NSE not affect phosophorylation of Akt in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with 200 µg/ml of NSE for 18 h. Cells were then treated for 5 min with 0 or 1 nM of insulin, followed by an additional incubation in DMEM for 5 to 55 min prior to lysis and immunoblot analysis for phosphorylated and pan Akt and ERK. A) Representive immunoblots. B) Quantitation of Akt immunoblots. C) Quantitation of ERK immunoblots. For B and C, data are integrated densities (arbitrary units) expressed as normalized ratios of phospho to total content. Data are mean ± SEM for n=3 passages of cells. * indicates significantly different (p 0.05) from vehicle control.

Figure 3: NSE increases phosophorylation of Akt in H4IIE hepatocytes. H4IIE hepatocytes were treated with 200 µg/ml of NSE for 18 h. Cells were then lysed and

immunoblot analysis for phosphorylated and pan Akt and ERK. 100 nM of insulin (100 nM; 15 min) served as positive control. A) Representive Akt immunoblots. B) Quantitation of Akt immunoblots. C) Representative ERK immunoblots. D) Quantitation of ERK immunoblots. For B and D, data are integrated densities (arbitrary units) expressed as normalized ratios of phospho to total content. Data are mean ± SEM for n=3 passages of cells. * indicates significantly different (p 0.05) from vehicle control.

Figure 4: NSE increases phosphorylation of AMPK and ACC in C2C12 myotubes. C2C12 myotubes were treated 18 h with 200 μg/ml of NSE or 0.1 % DMSO lysed, and analysed by immunoblotting for content of phospho-AMPK and phospho-ACC, as well as total AMPK. 2mM of AICAR (2 mM; 30 min) served as positive control. A) Representative immunoblots. B) Quantitation of results of p-AMPK immunoblots where data are integrated densities (arbitrary units) expressed as normalized ratios of phospho to total content. Data are mean ± SEM for n=3 passages of cells. * indicates significantly different (p 0.05) from vehicle control.

Figure 5: NSE increases phosphorylation of AMPK and ACC in H4IIE hepatocytes. H4IIE hepatocytes were treated 18 h with 200 μg/ml of NSE or 0.1 % DMSO lysed, and analysed by immunoblotting for content of phospho-AMPK and phospho-ACC, as well as total AMPK. 2mM of AICAR (2 mM; 30 min) served as positive control. A) Representative immunoblots. B) Quantitation of results of p-AMPK immunoblots where data are integrated densities (arbitrary units) expressed as normalized ratios of

phospho to total content. Data are mean ± SEM for n=3 passages of cells. * indicates significantly different (p 0.05) from vehicle control.

Figure 6: NSE increases phosphorylation of AMPK and ACC in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated 18 h with 200 μg/ml of NSE or 0.1 % DMSO, lysed, and analysed by immunoblotting for content of phospho-AMPK and phospho-ACC, as well as total AMPK. 5 μM of FCCP (5 μM; 60 min) served as positive control.

Figure 7: NSE uncouples oxidative phosphorylation in isolated rat liver mitochondria.

The effect of NSE on succinate-supported oxygen consumption of isolated mitochondria was tested using a Clark-type oxygraph system. Rates of basal and ADP-stimulated oxygen consumption were measured. A) Representative tracings of the effects of vehicle (0.1 % DMSO) or NSE (25 to 200 μg/ml). NSE significantly increases the rate of basal oxygen consumption (i.e. uncoupling effect) in a dose-dependent manner. Also, the rate of ADP-stimulated oxygen consumption is slightly decreased (i.e. inhibitory effect). At 200 μg/ml, these two effects are additive in completely abolishing the ATP-synthetic capacity of NSE-treated mitochondria. B) Representative tracings of the effects of vehicle or NSE in the presence of atractyloside potassium salt (AK), an inhibitor of the adenine nucleotide translocase (ANT). Pre-treatment with AK does not block the uncoupling effect of NSE.

Figure 8: NSE stimulates PPAR activity. The effect NSE on the activation of PPARγ was assessed using a reporter-gene assay. NSE increased luciferase activity in a

dose-dependent manner by up to 50% at 200 μg/ml. This effect, while significant, is much smaller than that of the PPARγ agonist, rosiglitazone, used as a positive control (800 nM; not illustrated). * indicates significantly different (p 0.05) from vehicle control.

Figure 9: Translation inhibition inhibits NSE stimulation of glucose uptake in C2C12 myotubes. C2C12 myotubes were treated 18 h with 200 μg/ml of NSE or 0.1% DMSO. A) Basal ^{3}H-deoxyglucose uptake were assessed in presence or absence 15 μg/ml cycloheximide. B) We essayed thymoquinone with different concentration. Data are expressed normalized to basal uptake in vehicle control condition. Mean ± SEM for n=4-6 passages of cells. * indicates significantly different (p 0.05) fromcorresponding control.

Figure 10: NSE does not affect the rate of acidification of the cell medium. To assess whether anaerobic glycolysis was upregulated as a consequence of an NSE-induced disruption of mitochondrial function, the pH of the culture medium of H4IIE hepatocytes and C2C12 muscle cells was assessed at several time points over a 4 h treatment with NSE. pH was assessed with a Phenol-red based spectrophotometric assay. Change in pH was expressed as the cumulative secretion of acid equivalents. FCCP (5 μM) was used as a positive control. Data are mean ± SEM for 2 experiments of 4 to 5 replicates per condition per time point. NSE treatment was not significantly different from vehicle (0.08 % ethanol) in either cell lines.

Figure 11: Cytosolic ATP concentration is normal or supra-normal 1 h after onset of treatment with NSE.To assess whether NSE-induced disruption of mitochondrial function tramslates into decreased content of ATP, cytosolic ATP content was measured in H4IIE hepatocytes and C2C12 muscle cells using a luminescent ATP assay. FCCP (5 μM) was used a positive control. Data are mean ± SEM of two experements of 4 to 5 replicates per condition per time point.

4. Article 3

Article publié dans Journal of Ethnopharmacology 94 (2004) 251-9

The petroleum ether extract of *N. sativa* exerts lipid-lowering and insulin-sentitizing actions in the rat

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* *These authors contributed equally to this work*

Affiliation

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Keywords: Diabetes; Insulin; MAP kinase

ABSTRACT

We studied the effect of a 4-week intragastric gavage with a petroleum ether extract of *Nigella sativa* seeds on blood glucose, insulin and lipids in the normal rat. Petroleum ether extract caused a 25% reduction in food intake that translated into a transient weight loss. No sign of toxicity of the plant could be seen in vivo or in vitro. Fasting plasma glucose remained stable throughout *Nigella sativa* treatment. At the end of the 4-week treatment, *Nigella sativa*-treated rats had lower fasting plasma levels of insulin and triglycerides, and higher HDL-cholesterol as compared to pair-fed controls. Response to insulin was evaluated in hepatocytes isolated from animals of all groups by Western blot analysis of phosphorylated MAPK p44/42erk and PKB. In vivo *Nigella sativa* treatment resulted in greater dose-dependent activation of MAPK and PKB in response to insulin. These results suggest that the petroleum ether extract of *Nigella sativa* has a slight anorexic effect, and that it contains the hypolipidemic activity previously obtained with the plant. More significantly, our data demonstrate that in vivo treatment with the petroleum ether extract exerts an insulin-sensitizing action by enhancing the activity of the two major intracellular signal transduction pathways of the hormone's receptor.

INTRODUCTION

Also known as “black seed” or “black cumin”, *Nigella sativa* is a spicy plant widely used in North Africa and the Middle East. It is known for its hypotensive (Tahir et al., 1993 and Zaoui et al., 2000), immunomodulatory (Chakravarty, 1993; Haq et al., 1999 and Swamy and Tan, 2000), and hepatoprotective (Daba and Abdel-Rahman, 1998; El-Dakhakhny et al., 2000 and Mahmoud et al., 2002) effects. In a recent survey of Moroccan herbalists, *Nigella sativa* was also found to rank high among the most recommended plants against diabetes (Haddad et al., 2003).

In Kuweit, a plant mixture comprised of *Nigella sativa*, myrrh, gum olibanum, gum asafetida, and aloe has been commonly used against diabetes. This mixture has glucose-lowering effects in normal and streptozotocin-treated rats without affecting plasma insulin or intestinal glucose absorption (Al-Awadi et al., 1985). A later study found that this mixture was as efficient as the biguanide drug phenformin to lower plasma glucose in the streptozotocin model (Al-Awadi et al., 1991). Unlike phenformin, however, the plant mixture could reduce glucose production from lactate, alanine, or glycerol in hepatocytes isolated from diabetic animals (Al-Awadi et al., 1991).

The Kuweiti group found that no one extract from an individual plant of the mixture could account for the improvement of glucose tolerance in the streptozotocin diabetic rat (Al-Awadi and Gumaa, 1987). In contrast, Moroccan researchers have more recently found that a crude aqueous extract of *Nigella sativa* alone was very effective at restoring glucose homeostasis in the sand rat model (Labhal et al., 1997).

In a similar model, the desert gerbil *Meriones shawi*, they showed that a *Nigella sativa* decoction given by intragastric gavage for 9 months was able to correct diabetes and obesity (Labhal et al., 1999). The seed decoction reduced blood glucose within 1 month and also insulin within two, as well as plasma cholesterol and triglycerides thereafter. The volatile oil of *Nigella sativa* seeds demonstrated hypoglycemic activity in normal and alloxan-diabetic rabbits (Al-Hader et al., 1993). It was later found to be as effective a cholesterol-lowering agent as the drug simvastatin in the obese pre-diabetic sand rat (Settaf et al., 2000). The Moroccan group also recently showed that a daily gavage with a hexane extract of *Nigella sativa* seeds in Wistar-Kyoto rats for 12 weeks reduced serum cholesterol, triglycerides, and glucose (Zaoui et al., 2002a). Finally, El-Dakhakhny et al. recently presented evidence for a hypoglycemic action of the volatile oil in streptozotocin-treated rats, but neither the oil nor constituents, such as thymoquinone or nigellone, affected insulin secretion from isolated pancreatic islets (El-Dakhakhny et al., 2002). This suggests that the plant may be acting to increase peripheral insulin sensitivity or glucose utilization, or to decrease intestinal glucose absorption.

The aim of the present study was to determine the effect of a non-polar petroleum ether extract of *Nigella sativa* seeds in normal rats in vivo and on insulin sensitivity in vitro in order to better delineate the mechanism of action. Such evidence would lend support to the ethnopharmacological use of *Nigella sativa* as an anti-diabetic plant.

MATERIALS AND METHODS

Chemicals

Insulin radioimmunoassay kit was purchased from Linco Research (St.-Charles, MO). Bovine serum albumin, fraction V, was obtained from Sigma–Aldrich (Mississauga, ON). Type 2 collagenase (318 units/mg) was purchased from Worthington (Lakewood, NJ). Cell culture medium and antibiotics were obtained from Life Technologies (Burlington, ON). The antibodies against the phosphorylated form of p44/42erk MAP kinase (Thr202/Tyr204) and against the phosphorylated form of Akt/PKB (Ser 473) were purchased from New England Biolabs (Beverly, MA).

Plant material

Seeds of *Nigella sativa* were obtained from a local herbalist in Rabat, Morocco, in August 2000. Identification was provided by Prof. Oulyahya, an experienced botanist from the Scientific Institute of Rabat, Morocco. A voucher specimen was deposited in the Herbarium of the Rabat Institutes, Morocco. Briefly, 800 g of the powdered seeds of *Nigella sativa* was extracted in a Soxhlet apparatus with petroleum ether, Et_2O. The extract obtained was evaporated to a viscous liquid at 40 °C under reduced pressure (yield of 300 ml, 310.7 g, 39.2%). Gas chromatographic analysis of the final extract failed to detect significant amounts of *n*-hexane and *n*-heptane (0.1%), which are the major markers for this distillate.

Animals

Male Sprague–Dawley rats, aged 7 weeks and weighing approximately 250 g, were obtained from Charles River, Canada (Montreal, QC). They were individually housed in plastic cages with wire-mesh tops in a room with a 12/12-h light/dark cycle and an ambient temperature of 22–25 °C. Animals had free access to standard rat chow and tap water, and were treated in accordance with the guidelines of the Canadian Council for the Care and Protection of Animals. The Institutional Ethics Committee of the Université de Montréal approved all experimental protocols.

Experimental protocol

Animals were kept for 3 weeks on the normal diet and tap water before being divided into two groups of seven animals each. The first group of animals received the petroleum ether extract (equivalent to 2 g/kg/day of the original seed powder as used previously (Labhal et al., 1999) by daily intragastric gavage for a period of four consecutive weeks. The second group served as control and received a volume of tap water equivalent to that of the Nigella sativa extract by daily intragastric gavage. Control animals were pair-fed with their Nigella sativa-treated counterparts in order to avoid effects due to altered appetite. Pair-feeding was initiated 10 days prior to the onset of Nigella sativa treatment.

Body weight was measured weekly, whereas food and liquid intake was evaluated every day. Blood samples (200 μl) were collected weekly from the tail veins of fasting animals in EDTA-containing vessels. Fasting blood glucose was determined using a standard glucometer (Elite model, Bayer Inc.). After

centrifugation at 4 °C, 5 min, 2500 rpm, plasma was stored at −20 °C until assayed for other fasting blood parameters (insulin, cholesterol, triglycerides, HDL). On the day of sacrifice, a maximal amount of blood (600 µl) was sampled from the portal vein prior to liver perfusion (see below) and treated as described above to determine plasma parameters. Plasma insulin was determined using the radioimmunoassay technique. Cholesterol, HDL, and triglycerides were measured by standard enzymatic methods with an automatic analyzer (Clinical Biochemistry Department, Sainte-Justine Hospital Research Center).

Isolation and culture of hepatocytes

On the day of sacrifice, rats were anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg). Hepatocytes were isolated by collagenase perfusion of the liver as described previously (Haimovici et al., 1994). Cells were purified by centrifugation on a Percoll gradient for 12 min at 3750 rpm and 4 °C. Cell viability was assessed by Trypan blue (0.4% solution) exclusion and was always greater than 85% prior to each experiment. Freshly isolated hepatocytes were suspended in Williams' E medium supplemented with 25 mM sodium bicarbonate, 1% bovine serum albumin, and penicillin/streptomycin (pH 7.4 at 37 °C). Cells were plated at a density 7–9 million cells/ml onto rat tail collagen-coated 35-mm plastic Petri dishes and incubated for 60 min at 37 °C in a humidified atmosphere of 5% CO_2–95% O_2. The medium was changed to remove unattached hepatocytes, and incubation was prolonged for an addition 30 min.

Insulin sensitivity bioassay

Isolated hepatocytes were cultured as described above. Insulin was dissolved in 0.01 M HCl and added to the culture medium to give final concentrations of 0.0, 0.1, 1.0, 10.0, and 100.0 nM. Separate Petri dishes were challenged with one of these doses of insulin for 5 min at 37 °C, and the stimulation stopped by two rapid washes with ice-cold phosphate-buffered saline (PBS). Cells were harvested immediately with a rubber policeman using 200 μl of ice-cold lysis buffer containing 25 mM Tris–HCl, 25 mM NaCl, 1 mM Na-orthovanadate, 10 mM Na-fluoride, 10 mM Na-pyrophosphate, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 1% Triton X-100, and 0.1% SDS (pH 7.4 at 4 °C). Cell lysates were clarified by centrifugation at 4 °C, 12,000 rpm for 12 min, and were stored at −80 °C until assayed by Western blot analysis.

Cell lysates were thawed by the addition of Laemmli buffer and their protein concentration was determined by the method of Bradford (Bio–Rad protein kit) using BSA as a standard. Cell lysates containing 25 μg protein were boiled for 3 min, subjected to electrophoresis on a 10% SDS–polyacrylamide gel, and transferred onto nitrocellulose membranes. The membranes were treated overnight at 4 °C in blocking solution (NaCl/Tris, 0.1% Tween-20 (v/v), 5% powdered milk (w/v)) and then blotted for 60 min at room temperature with primary antibodies anti-phospho-p44/42erk MAPK (1:2000 dilution) and anti-phospho-Akt/PKB (1:2000 dilution) in PBST (0.1% Tween-20 in PBS, pH 7.4). After three washes for 5 min with PBST, the membranes were incubated with secondary antibodies antimouse IgG (1:4000) for 60 min. After three washes with PBST, immunoreactive proteins were revealed by an enhanced chemiluminescence (ECL) reaction.

Cytotoxicity studies

Petroleum ether extract of Nigella sativa seeds was dissolved in DMSO and added to the culture medium to give a final concentration of 500 μg/ml. The DMSO concentration in culture medium never exceeded 0.1% (v/v). Control cultures were treated with the same concentration of solvent alone. Hepatocytes (700,000–800,000 cells/ml) were incubated with the petroleum ether extract for 2, 4, and 6 h after plating in the conditions described above. At the end of the incubation period, the cells were loosened with trypsin. Cell viability was determined by Trypan blue exclusion in a standard hemocytometer. In parallel experiments, release of LDH into the medium was measured by a colorimetric enzymatic assay (NADH production) using a cytotoxicity kit from Roche Diagnostics (Laval, QC).

Statistical analysis

Data are expressed as the mean ± S.E.M. of the indicated number of experiments. One-way analysis of variance (ANOVA) was performed. When the F-ratio value was significant ($P < 0.05$), pairwise comparisons between treatment group means were carried out using the Bonferroni test, and considered significant at $P < 0.05$.

RESULTS

Body weight and food intake

Body weight of all rats at the beginning of the experiment period averaged 220 ± 2 g. (n = 14). Fig. 1 shows that the increase of body weight in the two groups was similar for the initial 3-week baseline period. After 1 week of treatment with Nigella sativa extract, body weight decreased from 349 ± 9 to 327± 9 g ($P < 0.05$, n = 7), whereas it continued to rise in vehicle-treated rats. Thereafter, body weight in both groups increased but at a rate slower than during the baseline period. By the end of the 4-week gavage period, body weight reached values of 368 ± 20 and 350 ± 10 g (N.S., n = 7 per group) in control and extract-treated groups, respectively.

Onset of *Nigella sativa* petroleum ether extract treatment was also accompanied by a significant and sustained reduction in food intake, which dropped on average from 27 ± 2 to 20 ± 3 g per day (Fig. 2; $P < 0.05$, $n = 7$). As mentioned, control animals received the same amount of food as their *Nigella sativa*-treated counterparts (pair-feeding). In contrast, water intake remained stable throughout the experimental period and averaged 40–42 ml per day (data not illustrated).

Blood parameters

The fasting plasma glucose concentration of all rats during the initial 3-week baseline period averaged 5.0 ± 0.4 mmol/l ($n = 14$) and did not differ between the two groups. As shown in Fig. 3, fasting plasma glucose remained stable during the oral intake of the petroleum ether extract of *Nigella sativa* for 4 weeks. Moreover, it did not differ significantly from that of pair-fed control animals. However, on the eve

of sacrifice, fasting plasma insulin levels were found to be lower in *Nigella sativa*-treated animals (0.80 ± 0.22 ng/ml, $n = 7$) than in pair-fed controls (1.90 ± 0.4 ng/ml, $P < 0.05$, $n = 7$), as illustrated in Fig. 4(A).

Nigella sativa treatment was also found to induce a significant decrease of plasma triglyceride levels when compared with the control group (0.89 ± 0.37 versus 1.52 ± 0.36 mM, $P < 0.05$, $n = 7$/group; Fig. 4B). In contrast, the concentration of total cholesterol at sacrifice was similar in both groups (1.91 ± 0.21 mM in *Nigella sativa*-treated animals versus 1.65 ± 0.16 mM in pair-fed controls; N.S., $n = 7$/group; Fig. 4C). However, HDL-cholesterol was significantly increased in rats treated with the petroleum ether extract as compared with the control group (1.20 ± 0.33 versus 0.83 ± 0.25 mM, $P < 0.05$, $n = 7$/group; Fig. 4D).

Acute toxicity study

In vivo, the rats receiving the petroleum ether extract of *Nigella sativa* seeds at a dose equivalent to 2 g/kg body weight of the original seed powder for 4 weeks did not show any physical or behavioral signs of toxicity, such as lethargy, hyperactivity, restlessness, respiratory distress, or convulsions. Similarly, when added at a dose of 500 μg/ml to isolated hepatocytes in vitro for 2, 4, or 6 h, the petroleum ether extract of *Nigella sativa* did not significantly affect the viability of the cells as compared to DMSO-treated controls (Fig. 5A). Similar results were obtained by measuring LDH release (Fig. 5B).

Insulin sensitivity bioassay

As shown in Fig. 6, insulin administered in vitro to hepatocytes isolated from control animals (treated in vivo with distilled water) stimulated both p44/42erk MAPK and Akt/PKB activity in a dose-dependent manner, as expected. Most interestingly, hepatocytes isolated from *Nigella sativa*-treated animals displayed a more pronounced baseline level of the phosphorylated (hence activated) form of p44/42erk MAPK and responded to insulin in a much stronger manner than hepatocytes isolated from pair-fed control animals. In the case of the kinase Akt/PKB, the expression of its phosphorylated (hence activated) form was also increased in hepatocytes from *Nigella sativa*-treated animals as compared to cells isolated from pair-fed animals receiving distilled water by daily intragastric gavage. In both groups, Akt/PKB response to insulin was dose-dependent as expected.

DISCUSSION

The results of the present study show that treatment of normal rats with the petroleum ether extract of *Nigella sativa* for 4 weeks induces a transient initial weight loss and a sustained reduction in food but not water intake. The effect of the *Nigella sativa* extract did not seem to result from a toxic effect, since no physical or behavioural signs of toxicity could be observed. Moreover, our animals resumed a near-normal growth curve for the last 3 weeks of *Nigella sativa* treatment and the petroleum ether extract was non-toxic for isolated hepatocytes in vitro. Recent studies also failed to show any in vivo toxicity of *Nigella sativa* fixed oil in mice (Zaoui et al., 2002b). This suggests that the petroleum ether extract of *Nigella sativa* has a slight anorexic effect, and more detailed studies will be necessary to confirm and identify the presence of appetite-reducing components. Nonetheless, our results are in line with our previous studies showing that *Nigella sativa* aqueous extract normalized body weight in the obese diabetic models of *Psammomys obesus* (Labhal et al., 1997) or *Meriones shawi* (Labhal et al., 1999) sand rats. Similarly, the fixed oil of *Nigella sativa* was recently found to slow the growth rate of normal rats (Zaoui et al., 2002a).

Despite this apparent decrease in appetite and body weight, *Nigella sativa*-treated animals displayed stable and normal fasting glucose levels throughout the 4-week treatment period with the petroleum ether extract. These results contrast with the hypoglycemia obtained with a *Nigella sativa*-containing plant mixture (Al-Awadi et al., 1985) or with the fixed oil of *Nigella sativa* (Zaoui et al., 2002a) in normal

rats. Similarly, the volatile oil of *Nigella sativa* had a hypoglycemic effect in normal New Zealand rabbits (Al-Hader et al., 1993). It is possible that petroleum ether extraction removed hypoglycemic principles present in the volatile or fixed oil. Alternatively, our 4-week treatment period may not have been long enough for the hypoglycemic effect to manifest itself, hypoglycemia being apparent after a 12-week treatment with the fixed oil of *Nigella sativa* in normal rats (Zaoui et al., 2002a).

On the other hand, our study showed that the petroleum ether extract of *Nigella sativa* significantly reduced plasma triglycerides while increasing HDL-cholesterol. These studies confirm our previous studies with the aqueous extract of *Nigella sativa* seeds (Labhal et al., 1997 and Labhal et al., 1999) or with their fixed oil (Zaoui et al., 2002a). We have also previously reported that the volatile oil of *Nigella sativa* was as efficient as the cholesterol-reducing drug simvastatin (Settaf et al., 2000). Thus, our study suggests that petroleum ether extracts contain the cholesterol-lowering components of *Nigella sativa* seeds.

Most interesting are the results on the in vitro response to insulin of hepatocytes isolated from rats treated in vivo with *Nigella sativa* petroleum ether extract. We analyzed the expression of two insulin-responsive kinases by Western blot analysis, namely the MAPK p42/44erk as well as PKB/Akt. The antibodies used specifically recognized the phosphorylated form of each enzyme, which is known to reliably reflect their activated state. The MAPK p44/42erk is well known to be stimulated by insulin and to participate in the stimulation of cell proliferation and protein synthesis induced by the hormone (Virkamäki et al., 1999). On the other

hand, PKB/Akt is a kinase that lies downstream of phosphatidylinositol-3-kinase (PI3K), another important signal transduction pathway stimulated by insulin and responsible for its metabolic effects, notably for enhanced glucose transport in skeletal muscle (Tirosh et al., 2000). In the liver, both the MAPK p44/42erk and PI3K pathways have been implicated in the activation of glycogen synthesis by insulin (Carlsen et al., 1997).

Hence, the results of the present study pertaining to these kinases are most significant. Dose-dependent activation of both MAPK p44/42erk and PKB/Akt was observed after in vitro insulin stimulation of hepatocytes isolated from control animals. This confirms results obtained previously with this model (Benzeroual et al., 1997 and Benzeroual et al., 2000). Most significant, however, is the fact that in vivo treatment of normal rats with the petroleum ether extract of *Nigella sativa* for 4 weeks was translated by a greater response of both MAPK p44/42erk and PKB/Akt to in vitro insulin stimulation. These data strongly suggest that the *Nigella sativa* extract sensitized rat hepatocytes to the action of insulin. This is important since the resistance of peripheral tissues to insulin is well known to be the basis for the development of type 2 diabetes (Shulman, 1999).

Our results support the previous observation by members of our group that *Nigella sativa* aqueous extract restored fasting plasma glucose levels in obese *Meriones shawi* gerbils prior to reducing fasting plasma insulin (Labhal et al., 1999). This indicated that *Nigella sativa* could sensitize the animals to the action of insulin or alternatively could stimulate peripheral glucose utilization. The present data show

that two major components of the insulin signaling pathway are amplified by in vivo treatment with the plant extract, and provide an explanation for those previous observations. Our results also agree with the recent conclusion of El-Dakhakhny and collaborators that *Nigella sativa* oil exerts its hypoglycemic action by extrapancreatic actions in streptozotocin-induced diabetic rats (El-Dakhakhny et al., 2002). To our knowledge, however, this is the first demonstration of the insulin-sensitizing action of *Nigella sativa* at the molecular level.

This insulin sensitization could also explain the reduction in fasting plasma insulin observed herein in *Nigella sativa*-treated animals as compared to their pair-fed normal controls. Our results, along with those of El-Dakhakhny and collaborators (El-Dakhakhny et al., 2002), thus disagree with the reported insulinotropic action of *Nigella sativa* oil in hamsters with a diabetic state induced by streptozotocin plus nicotinamide (Fararh et al., 2002). Species differences may be implicated in this discrepancy.

Interestingly, the baseline activity of both MAPK p44/42erk and PKB/Akt also appeared to be stimulated by in vivo treatment with *Nigella sativa* extract. Indeed, even without insulin stimulation, the signal of the phosphorylated form of both proteins was greater in cells from *Nigella sativa*-treated animals than from pair-fed normal controls. This suggests that *Nigella sativa* contains pharmacological agents that can increase the tonic stimulation of these crucial components of insulin signaling pathways responsible for the metabolic effects of the hormone. Further

studies will be necessary to identify these agents and to determine their mode(s) of action.

In summary, the present study clearly shows that the petroleum ether extract of *Nigella sativa* seeds possesses significant anti-diabetic potential that is unrelated to any toxic effect of the plant. Indeed, the extract had a slight anorexic effect, exerted a beneficial action on serum lipids and insulin, and, most importantly, rendered liver cell intracellular signaling pathways more sensitive to the action of insulin. Our work thus provides strong evidence in support of the ethnopharmacological use of *Nigella sativa* seeds in the treatment of type 2 diabetes.

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Figure 1:

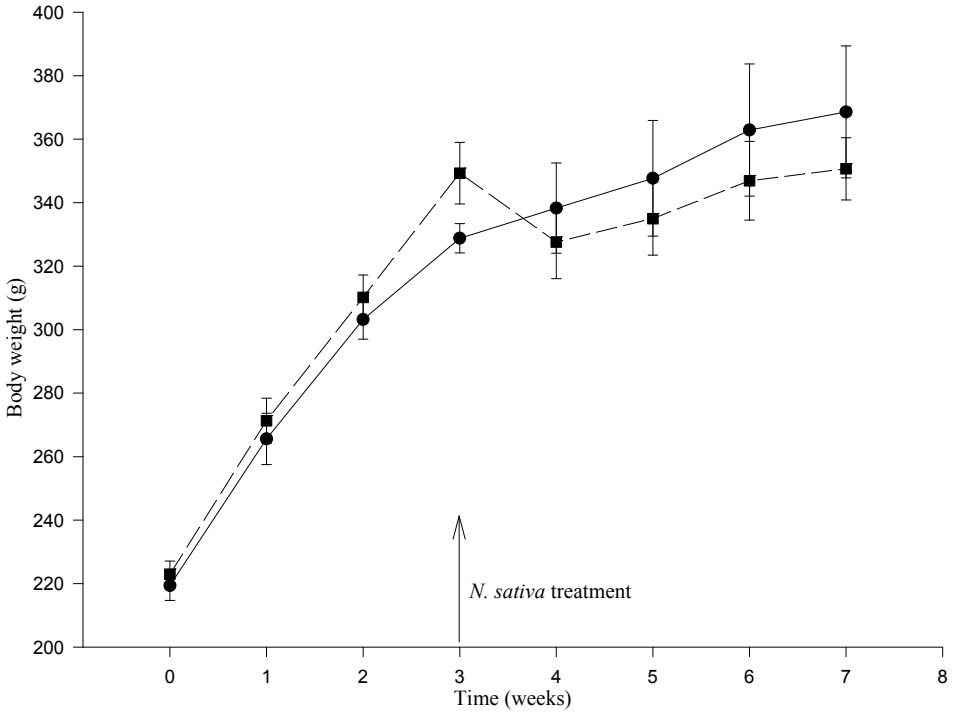

400
380
360
340
320
300
280
260
240
220
200
Body weight (g)
0
1
2
3
4
5
6
7
8
Time (weeks)
N. sativa treatment

Figure 2:

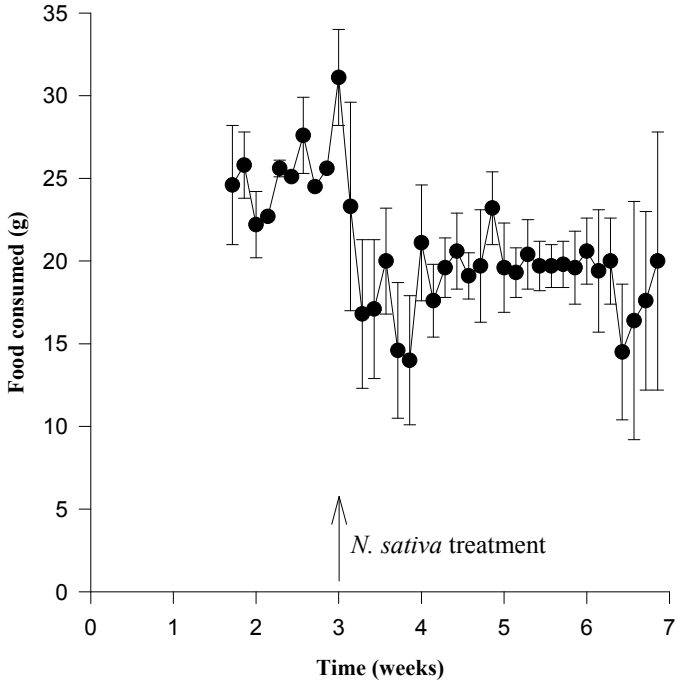

35
30
25
20
15
10
5
0
Food consumed (g)
0
1
2
3
4
5
6
7
Time (weeks)
N. sativa treatment

Figure 3:

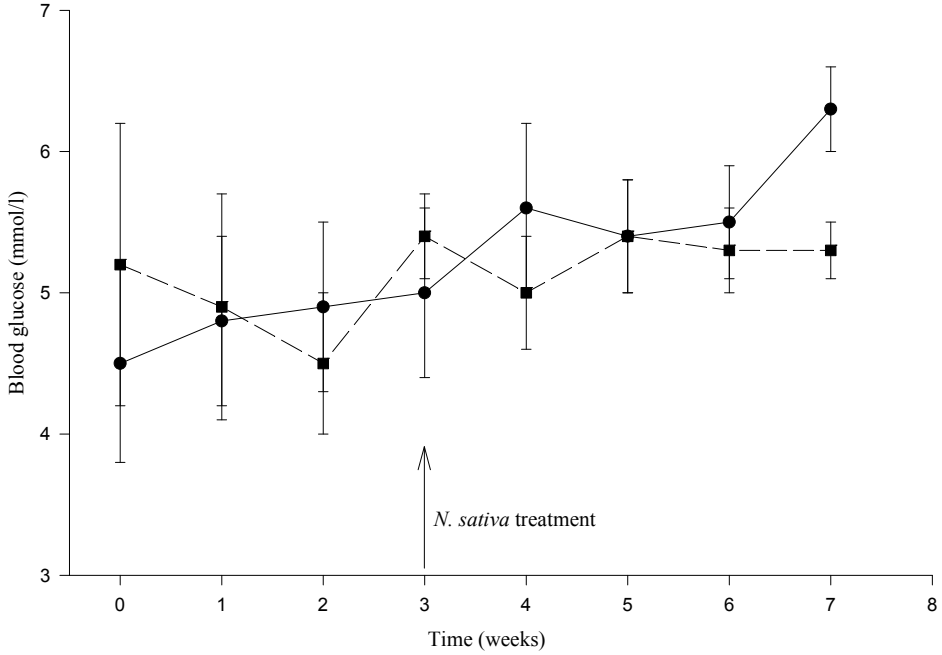

7
6
5
4
3
Blood glucose (mmol/l)
N. sativa treatment
0
1
2
3
4
5
6
7
8
Time (weeks)

Figure 4:

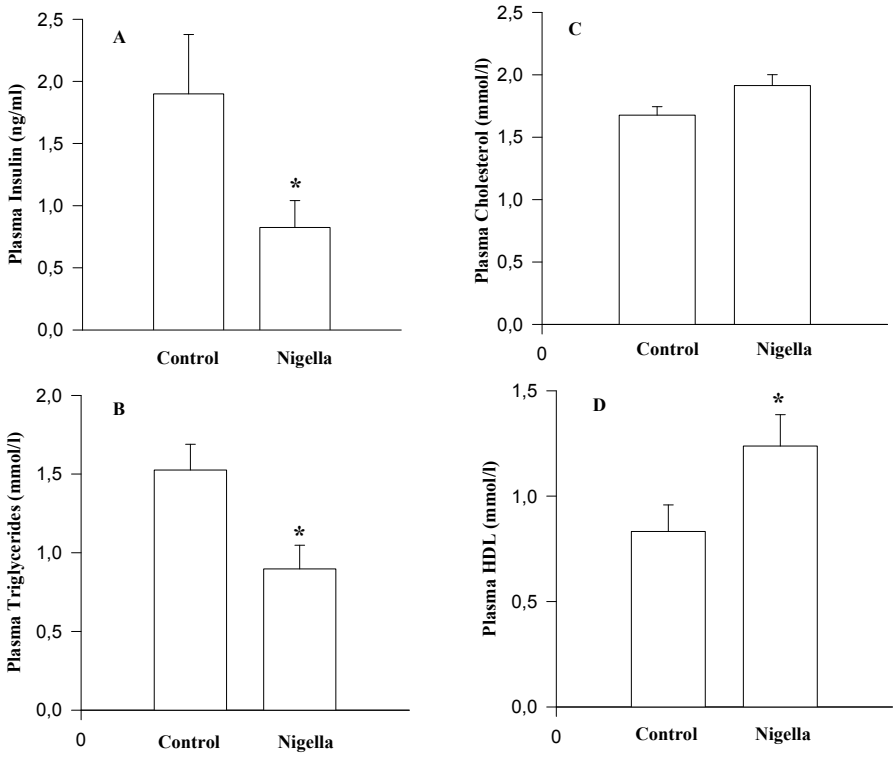
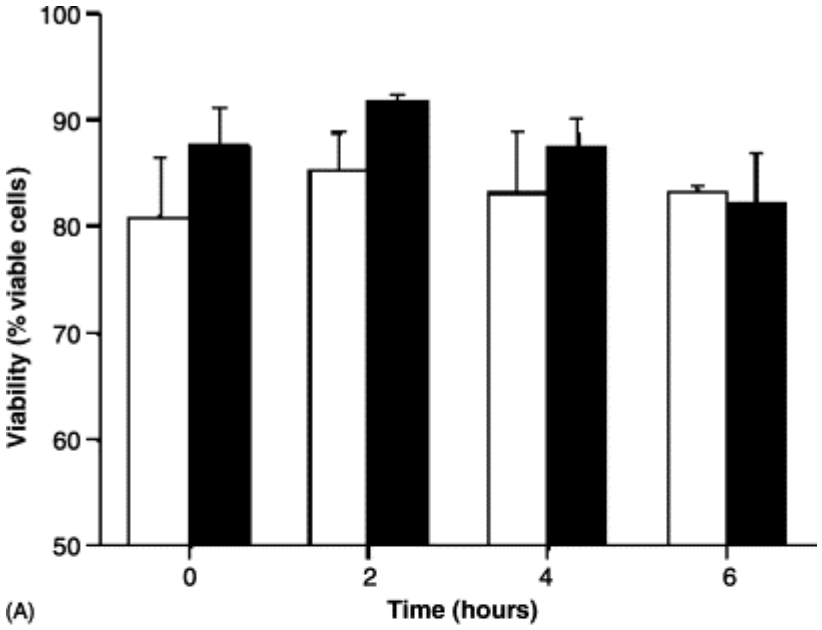

A
Plasma Insulin (ng/ml)
2,5
2,0
1,5
1,0
0,5
0,0
*
Control
Nigella
C
Plasma Cholesterol (mmol/l)
2,5
2,0
1,5
1,0
0,5
0,0
0
Control
Nigella
B
Plasma Triglycerides (mmol/l)
2,0
1,5
1,0
0,5
0,0
0
*
Control
Nigella
D
Plasma HDL (mmol/l)
1,5
1,0
0,5
0,0
0
*
Control
Nigella

Figure 5:


100
90
80
70
60
50
Viability (% viable cells)
0
2
4
6
Time (hours)
(A)

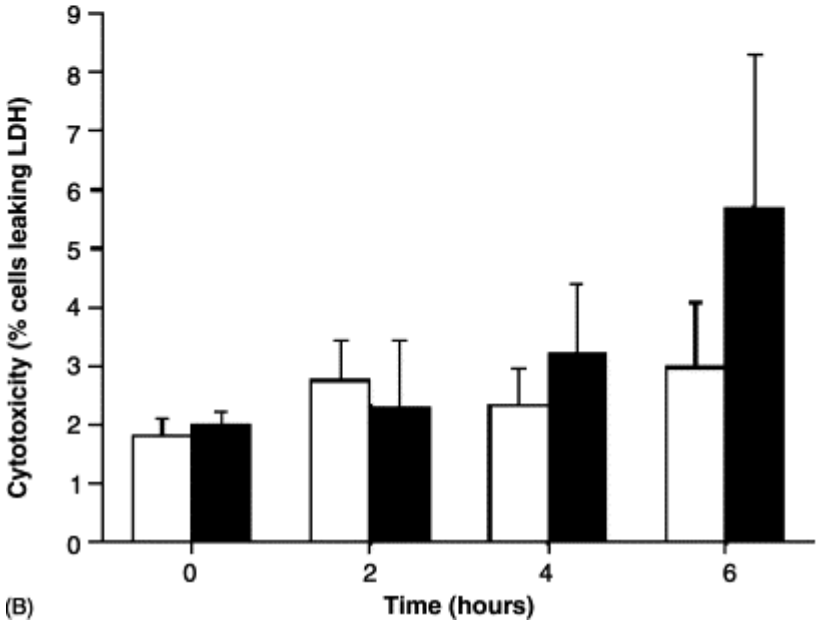

9
8
7
6
5
4
3
2
1
0
Cytotoxicity (% cells leaking LDH)
0
2
4
6
Time (hours)
(B)

Figure 6:

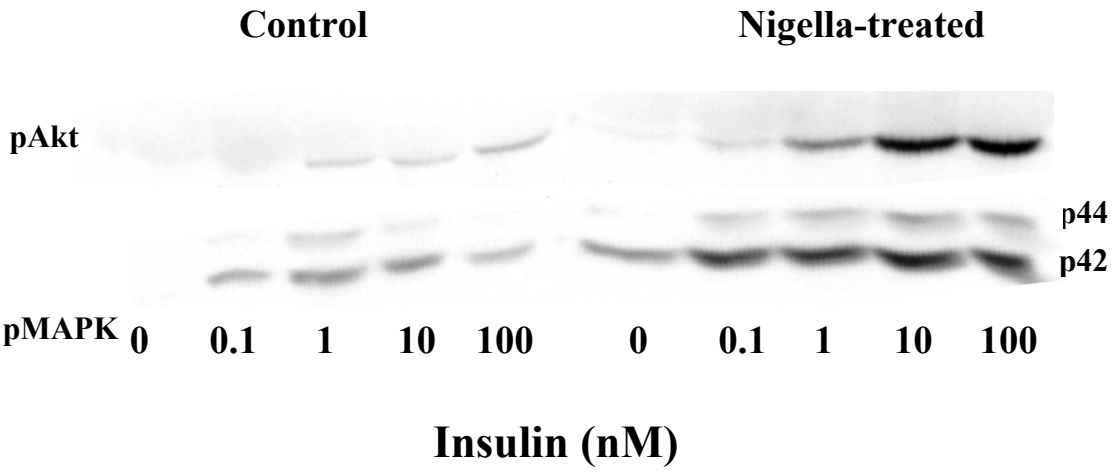

Control
Nigella-treated
pAkt
p44
p42
pMAPK
0
0.1
1
10
100
0
0.1
1
10
100
Insulin (nM)

FIGURE LEGENDS

Figure 1: Progression of body weight during *in vivo* treatment with *N. sativa* extract. Body weight was measured weekly during a three-week baseline period and during a four-week gavage with petroleum ether extract of *N. sativa* (square symbols, dotted line) or with an equivalent volume of distilled water (round symbols, solid line) as described in Materials and Methods. It must be noted that control animals receiving distilled water were pair-fed with their *N. sativa*-treated counterparts.

Figure 2: Food consumption during in vivo treatment with N. sativa extract.
The amount of standard rat chow consumed by *N. sativa*-treated animals was measured every two days as described in Materials and Methods. Again, note that control animals receiving distilled water by gavage were pair-fed with extract-treated counterparts.

Figure 3: Progression of fasting plasma glucose during *in vivo* treatment with *N. sativa* extract.Fasting plasma glucose was measured weekly during a three-week baseline period and during a four-week gavage with petroleum ether extract of N. sativa (square symbols, dotted line) or with an equivalent volume of distilled water (round symbols, solid line) as described in Materials and Methods. It must be noted that control animals receiving distilled water were pair-fed with their N. sativa-treated counterparts.

Figure 4: Fasting blood parameters after a four-week *in vivo* treatment with *N. sativa* extract. Fasting plasma insulin (panel A), triglycerides (panel B), total cholesterol (panel C) and HDL-cholesterol (panel D) were measured at the end of a four-week

treatment with the petroleum ether extract of *N. sativa* (striped bars) or with an equivalent volume of distilled water (open bars) as described in Materials and Methods. * Significantly different from control, $p<0.05$.

Figure 5: *In vitro* cytotoxicity of petroleum ether extract of *N. sativa* on isolated rat liver cells.Isolated rat hepatocytes in short term culture were incubated with 500 µg/ml of *N. sativa* extract dissolved in DMSO or with DMSO (0.1%) alone for 2, 4 or 6h. Cell viability was then assessed by Trypan blue exclusion after suspension of the cells by gentle trypsin treatment as described in Materials and Methods.

Figure 6: Response of MAPK p44/42erk and PKB to insulin *in vitro* in hepatocytes isolated from control and *N. sativa*-treated rats.Cells isolated from the liver of control or *N. sativa*-treated animals were stimulated *in vitro* with the indicated concentrations of insulin. Cells were lysed and proteins subjected to SDS-PAGE. Proteins immunoreactive to antibodies to the phosphorylated forms of MAPK p44/42erk and PKB were revealed by enhanced chemiluminescence as described in Materials and Methods. Results are representative of three separate determinations obtained from four or five different cell preparations.

5. Article 4

Article en préparation

Anti-diabetic effects of *Nigella sativa* seed extract on diabetic *Meriones shawi* are mediated through activation of ACC and increase Glut4 quantity

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Short Title: the anti-diabetic properties of *Nigella sativa* in vivo

Keywords: type 2 diabetes mellitus, *Nigella sativa, Meriones shawi*, OGTT, HDL, Glycaemia, Insulinemia, ACC, and Glut4.

ABSTRACT

The present study was undertaken to evaluate the antidiabetic effect of *N. sativa* ethanol extract (NSE) in *Meriones shawi* after development of diabetes by hypercaloric diet and physical inactivity. *Meriones shawi* were divided randomly into different groups: normal control, diabetic control, diabetic NSE and diabetic treated by metformin positive control. NSE (2g eq plant/kg) and metformin (300 mg/kg) were administrated by daily intragastric gavage for 4 weeks. Glycaemia and body weight were evaluated every week. At the end of the study, an Oral Glucose Tolerance Test (OGTT) was performed to estimate insulin sensitivity. Upon euthanasia, plasma lipid profile as well as insulin, leptin and adiponectin levels were assessed, whereas liver and skeletal muscle tissue samples were used for the determination of Acetyl-coenzyme A carboxylase (ACC) activation and Glut4 protein content. *Meriones shawi* receiving NSE showed a progressive normalization of glycaemia that was slower than that of metformin controls, but of similar amplitude. Moreover, insulinemia and HDL-cholesterol were increased after 4 weeks of NSE treatment as compared to diabetic controls, whereas leptin and adiponectin were unchanged. Treatment of *Meriones shawi* with NSE decreased the area under curve (AUC) of OGTT and had a tendency to decrease intra-hepatic and muscular triglyceride content, both parameters being elevated in diabetic controls, confirming their insulin resistant state. NSE stimulated activation of ACC in muscle and liver and increase Glut4 in muscle. These results confirm that NSE exhibits the hypoglycaemic and hypolipidemic activity previously reported with other preparations of the plant. More significantly, our data demonstrate that *in vivo*

treatment with NSE exerts an insulin-sensitizing action by enhancing the activity of ACC, a major component of the insulin-independent AMPK intracellular signaling pathway, and by enhancing the quantity of Glut4 protein.

INTRODUCTION

Diabetes is a chronic disease that occurs when the pancreas does not produce enough insulin, and/or when the body cannot effectively use the insulin it produces. Hyperglycaemia, or high blood sugar, is a common effect of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially the nerves, kidney and blood vessels (WHO, Fact sheet N°312, September 2006). The World Health Organization (WHO) estimates that more than 180 million people worldwide have diabetes. This number is likely to more than double by 2030 (WHO, Fact sheet N°312, September 2006)

N. Sativa is an herbaceous plant growing to about 20 - 30 cm in height, known as black seed because of the small triangular black seeds it generates. The plant is also known as Blessed Seed (Arab: Habbat ul Baraka, or Habbat ul Sauda). It has been consumed for more than 2000 years, is used extensively in the traditional medicine of many southern Mediterranean and Middle Eastern countries, and has been shown to produce multi-systemic beneficial actions (Ali and Blunden, 2003), including hypocholesterolemic (Labhel et al.,1997), antioxidant (Meral et al., 2001; Kanter et al., 2004), and anti-inflammatory (Houghton et al., 1995) effects.

The hypoglycaemic and anti-diabetic effect of *N. Sativa* has been reported by numerous *in vivo* and *in vitro* scientific studies (Al-Awadi et al., 1991, 1987, 1985; Al Hader et al., 1993; Labhal et al., 1999; Meral et al., 2001; El-Dakhakhny et al., 2002; Farah et al., 2002, 2004; Kanter et al., 2003; Rchid et al., 2004; Le et al., 2004;

Haddad et al., 2006; Benhaddou-Andaloussi et al., 2008). In a recent study, we have demonstrated that *N. Sativa* seed ethanol extract (NSE) exhibits the remarkable ability *in vitro* to concomitantly increase insulin secretion, induce proliferation of pancreatic β cells, and stimulate glucose uptake in muscle skeletal and fat cells (Benhaddou-Andaloussi et al., 2008). On the other hand, most of the *in vivo* studies of the antidiabetic effect of *N. Sativa* were done using models of type I diabetes. We therefore investigated the effects of NSE on the diabetic *Meriones shawi* that represents a model of type II diabetes associated with hyper-insulinemia and dyslipidemia. We have also attempted to determine some of the mechanisms of action through which NSE may exert its antidiabetic effect, notably adipokines, AMP-kinase (AMPK) dependent signaling and Glut4 protein content.

MATERIAL AND METHODS

Reagents and antibodies

Antibodies against pan-specific and phosphorylated ACC (Ser 79), as well as Glut4 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Secondary HRP-conjugated antibodies were purchased from Jackson Immunoresearch (Cedarlane Laboratories, Hornby, ON). Protein assay kit was purchased from Pierce (Brockville, ON). Rat Insulin-specific RIA kit, Rat Leptin RIA kit and Mouse Adiponectin RIA kit-125T were purchased from Linco Research Inc (Saint Charles, MO). Sodium pentobarbital, triglyceride and free glycerol reagents, as well as D-glucose were purchased from Sigma-Aldrich (Saint Louis, MO).

Plant material

Seeds of *N. Sativa* were obtained from a herbalist in Rabat, Morocco in August 2005 and were authenticated by an experienced botanist (Prof. A. Oulyahya, Institut Scientifique, Rabat, Morocco). A voucher specimen has been deposited in the herbarium of the Institut Scientifique of Rabat (Number 10359). Seeds were washed, dried, and then powdered with an electric microniser. Powder was extracted three times with 80% ethanol and the solvent was evaporated at 40 °C under reduced pressure. This procedure resulted in a two-phased extract. The oily and the solid phases were recombined in proportion to their yield (typically 70% and 30%, respectively). The extract was conserved at 4 °C and protected from light and humidity.

Animals

One hundred *Meriones shawi* of both sexes were caotured in the semi-arid area in the of Boulmane in the Middle Atlas region of Morocco. Appropriate traps were used for catching the animals. They were then transported to Faculty of Medicine and Pharmacy of Mohamed V University in Rabat, Morocco. Animals were allowed to adapt to the laboratory environment, numbered and placed in individual cages. All animals received a standard laboratory diet, ad libitum, which represents a hypercaloric food source for them. After three months of such diet, *Meriones shawi* having blood glucose greater than 8 mmol/l and having developed insulin resistance (AUC greater than 1500), were considered diabetic. This selection has enabled us to have a yield of 25%.

Experimental procedure

Twenty-four diabetic and eight normal *Meriones shawi* were divided into four groups of 8 animals each as follows: Normal control animals, diabetic control animals, diabetic NSE treated animals, and diabetic metformin treated animals. Treatment was given by daily intragastric gavage at a dose of 2g eq of plant/kg/day of *N. sativa* extract and 300 mg/kg/day metformin for four weeks in one milliliter of 0.5% methyl cellulose suspension. Control animals received equal volume of vehicle (1ml). Glycaemia and body weight were measured every week. At the end of the experimental period (4 weeks), the animals were fasted overnight, anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and euthanazed befor obtaining blood, and tissues samples (liver, soleus muscle). The study was conducted

in accordance with the accepted principles outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health and all efforts were made to minimize animal suffering and the number of animals used. Ethics approval was obtained from Mohammed V University.

Oral glucose tolerance test (OGTT)

One day before the beginning and the end of the experiment, an Oral Glucose Tolerance Test (OGTT) was performed to assess glucose tolerance. For this purpose, overnight fasted rats were fed D-glucose (3 g/kg body weight) by intragastric gavage, and then blood was collected After 0, 15, 30, 60, and 120 min intervals from the tail vein. Plasma glucose concentrations were determined by the glucose oxydase method using a glucometer (One Touch Ultra, LifeScan Inc, Milpitas, CA). The areas under the curve (AUC) of changes in the blood glucose were calculated by using Origin software.

Plasma lipid assays

Fasting blood samples were also taken to measure the lipid profile (cholesterol, HDL-C, LDL-C and triglyceride). After 4 weeks of the treatment, *Meriones shawi* were sacrificed and blood samples were collected for plasma chemical analysis. Total amount of cholesterol, LDL-C, HDL-C, serum triglyceride (TG) and blood glucose were measured by an automated analyzer (Cobas-Mira Plus,Hoffman-LaRoche Diagnostics, Germany).

Radioimmunoassay

Plasma insulin, leptin, and adiponectin levels were determined by radioimmunoassay (RIA). Rat Insulin-specific RIA kit, Rat Leptin RIA kit or Mouse Adiponectin RIA kit-125T were used. Generally, the samples were incubated in 12 x 75 mm polypropylene RIA tubes with rat ^{125}I-insulin, ^{125}I-leptin or ^{125}I-adiponectin and a primary antibody against rat insulin, rat leptin or mouse adiponectin, respectively, at 4 ºC overnight in the dark. The tubes were then incubated with the precipitating reagent for 20 min at 4 ºC, and tubes were then centrifuged at 5350 g for 15 min. Radioactivity in the pellet was measured by gamma counter (Wallac Wizard 1470, Perkin Elmer, Waltham, MA). Human insulin, rat leptin or mouse adiponectin were used as respective standards.

Triglyceride assay in liver and skeletal muscle tissues

Tissue was homogenized and extracted with a 2:1 chloroform-methanol mixture and washed by addition of 50 mM NaCl solution, resulting into two phases. The lower phase contained the total lipid extract. A fixed volume of this extract was dried, resuspended in isopropanol and an aliquot was used for triglyceride measurement using triglyceride and free glycerol reagents. Absorbance was measured at ambient temperature at 540 nm using a Wallac Victor 2 plate reader (Perkin-Elmer, Waltham, MA) Triglyceride content of the tissue was expressed as mg/g of wet weight of tissue.

Western blot for proteins involved in glucose homeostasis

Samples of liver and muscle tissues were ground in liquid nitrogen and subsequently lysed. For ACC western blot analysis, 1 ml of RIPA lysis buffer was used (25 mM Tris-HCl pH 7.4, 25 mM NaCl, 0.5 mM EDTA, 1% Triton-X-100, 0.1% SDS), whereas sucrose lysis buffer (20 mM Tris-HCl pH 7.4, 255 mM sucrose, 1 mM EDTA) was used for Glut4 . For all samples, a protease inhibitor cocktail was added (Roche, Mannheim, Germany) as well as 1 mM phenylmethanesulfonyl fluoride and phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride). Cells were allowed to lyse for 30 min on ice and were then centrifuged at 12000 x g for 10 min. Supernatants were then stored at -80^{o}C until analysis. Protein content was assayed by the bicinchoninic acid method standardized to bovine serum albumin (Roche, Laval, QC).

Lysates were diluted to a concentration of 1.25 mg/ml total protein and boiled for 5 min in reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue). 20µl of each sample was separated on 10% polyacrylamide mini-gels and transferred to nitrocellulose membrane (Millipore, Bedford, MA). Membranes were blocked for 2 h at room temperature with Tween-20 and 5% skim milk in TBS (20 mM Tris-HCl, pH 7.6 and 137 mM NaCl). Membranes were then incubated overnight at 4ºC in blocking buffer with appropriate phospho-specific or pan-specific antibodies against ACC and Glut4 at 1:1000. Membranes were washed 5 times and incubated 1.5 h at room temperature

in TBS plus Tween 20 with anti-rabbit HRP-conjugated secondary antibodies at 1:100000 to 1:50000. Revelation was performed using the enhanced chemiluminescence method and luminescence captured to blue-light-sensitive film (Amersham Biosciences, Buckinghanshire, England). Lysates from all experimental conditions were separated and transferred simultaneously to a single membrane.

Statistical analysis

Data are reported as the mean ± SEM of the indicated number of experiments. All experiments in cells were performed at least in triplicate. Results were analysed by one-way analysis of variance (ANOVA) using StatView software (SAS Institute Inc, Cary, NC), with post-hoc analysis as appropriate (Fisher). Statistical significance was set at $p \leq 0.05$.

RESULTS

Effect of NSE on body weight

The results of body weight are presented in Table 1. At the beginning of the trial period (day 0), the diabetic *Meriones shawi* weighed between 177g - 187g as compared to approximately 158g for non-diabetic controls. At the end of four weeks of treatment, an increment of weight of about 6 to 10g for each *Meriones shawi* group was observed but no significant difference was apparent among them, albeit the weight gain of NSE treated animals had a tendency to be lower.

NSE improves blood glucose of diabetic *Meriones shawi*

Daily NSE treatment for four weeks resulted in a gradual decrease in glycemia that reached values similar to normal non-diabetic controls animals by the end of the treatment period (reduction from 8.2 mmol/l to 6.4 mmol/l, Figure 2). By comparison, metformin reduced blood glucose from 9.2 mmol/l to 5.4 mmol/l within the first week and glycemia remained stable until the end of the study. In contrast, both diabetic and non-diabetic control animals displayed stable glycaemia during the four weeks of treatment; normal animals demonstrated normal blood sugar levels and control diabetic animals were hyperglycaemic throughout the study period (Figure 1).

NSE reduces insulin resistance in the diabetic *Meriones shawi*

An oral glucose tolerance test (OGTT) was performed at the beginning of the study to identify the *Meriones shawi* that developed diabetes after three months of

relatively hypercaloric diet. Another OGTT was also carried out on all selected study animals at the end of the treatment to determine the effect of NSE and metformin positive control on insulin resistance. Figure 2 presents the changes in blood sugar observed during the two hours following administration of the glucose load. As can be seen, normal (non-diabetic) control animals had a small response to the glucose load as compared to control diabetic *Meriones*, indicating glucose intolerance/insulin resistance in the latter group. Treatment of diabetic animals for four weeks with the positive control metformin succeeded in completely restoring the OGTT response to that of non-diabetic congeners. In contrast, NSE treated animals displayed a significant improvement in insulin sensitivity but the glyceamic response to the OGTT was intermediate between normal and diabetic *Meriones* controls. The AUC of the glyceamic response to the glucose load over time confirmed and quantified the interpretation of Figure 2. Indeed, all diabetic animals showed similarly elevated AUC values at the beginning of experimental protocol as compared to non-diabetic controls (Table 2). In *Meriones shawi* treated for four weeks with metformin, the values of the AUC fell significantly to reach values seen in non-diabetic controls. By comparison, NSE treatment for the same period significantly reduced the levels of AUC to values slightly greater than those of non-diabetic animals.

Effect of NSE on plasma lipid profile

In the diabetic *Meriones shawi*, treatment for 4 weeks with 2g eq plant/kg/day NSE or 300 mg/kg/day metformin increased total plasma cholesterol concentration by 49% and 38%, respectively, and the HDL-cholesterol concentration by 142% and 92%, respectively, as compared to the diabetic control group (Figure 3, Panles A and

C). These increases brought levels of total cholesterol and HDL-cholesterol close to values observed in the normal control group, although HDL-cholesterol in NSE treated animals had a tendency to be higher. Plasma LDL-cholesterol concentration for all groups of *Meriones shawi* did not show any significant difference (figure 3B), whereas metformin induced a significant rise in plasma triglycerides, as compared to control diabetic animals (Figure 3D).

Effect of NSE on liver and skeletal muscle triglyceride content

As illustrated in Figure 4, liver and skeletal muscle triglyceride content appeared to be elevated in control diabetic versus control non-diabetic *Meriones shawi* and metformin treatment did not change this tendency. In contrast, NSE had a tendency to decrease the triglyceride content of both tissues (Figure 4). Because of the large data variability, however, none of these changes reached statistical significance.

NSE increases insulinemia but does not affect circulating leptin or adiponectin

Untreated diabetic *Meriones shawi* exhibited a significant increase in plasma insulin as compared to their non-diabetic congeners (Figure 5). Treatment for four weeks with metformin reduced insulinemia back to levels observed in normal controls. In contrast, NSE treatment significantly increased plasma insulin levels beyond those observed in the diabetic control group.

The determination of plasma leptin in different groups of diabetics *Meriones shawi* yielded very similar values irrespective of NSE or metformin treatment. However, plasma leptin in normal *Meriones shawi* was found to be significantly higher than values observed in control diabetic animals (Figure 6A). In contrast, the results of plasma adiponectin showed no significant differences between groups, although NSE and metformin treated animals had a tendency to have lower adiponectin values (Figure 6B).

Effect of NSE on Glut4 and ACC

In order to begin understanding the mechanisms through which the effects of NSE are mediated, we assessed some key intracellular components involved in glucose homeostasis. We first evaluated the activation of ACC, a major component of the AMPK pathway. As shown in Figure 7, NSE treatment significantly increased the phosphorylation of ACC in liver (Panel A) and skeletal muscle (Panel B) in comparison with control diabetic *Meriones shawi* . Secondly, we probed the soleus muscle tissue of control and NSE treated diabetic animals for their total content in Glut4 protein content. As illustrated in figure 7C, NSE significantly increased the amount of Glut4 present in the soleus muscle of diabetic *Meriones shawi* animals.

Discussion

N. sativa seeds are used in the traditional medicine of numerous Middle Eastern and North African countries for their anti-hyperglycemic activity (Bellakhdar et al., 1991; Ziyyat et al., 1997; Haddad et al., 2001). Most studies to date reported results from either normal animals or models of Type I diabetes (Al Awadi et al., 1991, 1987, 1985; Al Hader et al., 1993; Meral et al., 2001; Fararh et al., 2002, 2004; El-Dakhakhny, 2002; Kanter et al., 2003, 2004), or from cells in culture (Rchid et al., 2004; Benhaddou-Andaloussi et al., 2008). Only a single study presented limited evidence for an antidiabetic effect of *N. sativa* in an animal model of Type II diabetes (Labhal et al., 1999). The present study focussed on antidiabetic and hypolipidimic effects in the *Meriones shawi* model of Type II diabetes and aimed to elucidate the mechanisms of action of NSE in skeletal muscle and liver tissues.

Meriones shawi are rodents from semi-arid regions of Morocco that can gain weight and become insulin resistant and diabetic if kept in captivity (reduced physical activity) and fed normal laboratory chow (hypercaloric relative to their natural diet composed mainly of salty bushes (Labhal et al., 1999). This was confirmed in our study by the greater body weight of the *Meriones shawi* animals that demonstrated insulin resistance as assessed by an OGTT, as compared to animals maintaining normal glucose tolerance. Treatment with NSE or metformin for four weeks failed to significantly affect body weight, although a tendency for smaller weight gain was seen in NSE treated animals as compared to untreated diabetic controls. This contrasts with the results of Labhal and collaborators who reported a

decrease in body weight among *Meriones shawi* treated with an aqueous extract of *N. sativa* (Labhal et al., 1999); similar results having been obtained with the sand rat *Psammomys obesus* (Labhal et al., 1997). Aside from the difference in *N. sativa* extract type, the treatment period of these studies lasted three months. It is therefore possible that our shorter treatment regimen and use of an ethanol extract may explain the lack of an effect in body weight in the present studies.

In contrast, our studies confirmed the oral hypoglycemic action of *N. sativa* in an animal model of Type II diabetes. As mentioned, such a decrease in blood glucose was reported in several studies using animal models of type I (Al Hader et al., 1993; Meral et al., 2001; Fararh et al., 2002, 2004; El-Dakhakhny, 2002; Kanter et al., 2003, 2004) and on similar models of type II diabetes (Labhal et al., 1997, 1999). In our experimental conditions, NSE decreased blood sugar starting from the 3rd week of treatment whereas metformin normalized glycaemia within first week. It is known that thiazolidinediones (TZD) also decrease blood sugar after 3 or 4 weeks of treatment (Ducobu, 2003). *In vitro* studies in our laboratory have confirmed that NSE stimulates PPAR-γ in cultured adipocytes as do TZD (Benhaddou-Andaloussi et al., 2009 – observations submitted for publication). Part of the action of NSE on the regulation of blood glucose *in vivo* may therefore be similar to TZDs.

Moreover, the decrease of blood glucose by NSE was associated with a significant reduction in insulinresistance, as revealed by the pattern of the OGTT response in diabetic animals andthe resulting significant decrease in AUC that clearly

indicate an improvement in glucose tolerance. This improvement in insulin sensitivity is completely in accordance with our previous results showing increases of basal and insulin stimulated phospho-Akt in hepatocytes isolated from normal rats treated with the petroleum ether extract of *N. sativa* (Le et al., 2004).

NSE treatment also modulated the lipid profile of diabetic *Meriones shawi*, most notably bysignificantly increasing HDL-cholesterol, an effect that we have previously observed in normal rats with a different *N. sativa* extract (Le et al., 2004). We also observed a tendency towards a decrease in triglycerides in the NSE treated group. Labhal (1999) observed a significant decrease in blood triglycerides in the same animal model using an aqueous extract of *N. sativa* administered for a period of three months. Once again, differences in extract type and treatment period may explain discrepancies with our results at the level of blood lipid profile.

However, our studies went further by assessing triglyceride content in insulin-sensitive tissues, notably skeletal muscle and liver. Although the NSE induced decreasein these parameters failed to reach statistical significance, such an action could participate in the improvement of systemic insulin sensitivity observed in our studies. Indeed, it is known that high levels of intracellular triglycerides can increase some lipid metabolites such as ceramides, diacylglycerol and long chain acyl-coenzyme A (Ferré, 2007). The latter play a key role in attenuating insulin signaling by increasing intracellular serine-threonine phosphorylation of IRS protein, with a

resultant reduction in insulin signal transduction that underlies insulin resistance (Ferré, 2007).

More importantly, the NSE treated *Meriones shawi* also showed large increase in insulinemia after 4 weeks of treatment that can contribute to the anti-hyperglycemic effect of NSE. Such an increase was previously observed by our group in normal rats (Le et al., 2004) and is also observed in streptozotocin-nicotinamide hamters (Fararh et al., 2002). In addition, treatment of pancreatic β cells in culture with *N. sativa* was found to increase insulin secretion as a result of an improved secretagogue capacity of these cells (Rchid et al., 2004). On the other hand, *N. sativa* increases regeneration of pancreatic β cells (Kanter et al., 2003) and protects the same from streptozotocin (Kanter et al., 2004). Our own recent *in vitro* studies clearly demonstrate that NSE can enhance the proliferation of β cells and increase glucose stimulated insulin secretion (Benhaddou-Andaloussi et al., 2009 – observations submitted for publication). Taken together, these actions can explain the important increase in insulin levels observed in diabetic NSE treated *Meriones shawi*. They further support the notion that *N. sativa* products can help maintain pancreatic β cell mass and hence mitigate the progression of diabetes.

Our studies also attempted to examine certain key intracellular components involved in glucose homeostasis. Western blot analysis showed that NSE treatment *in vivo* can significantly increase the total amount of Glut4 glucose transporters, which play a major role in controlling hyperglycemia in skeletal muscle (Wallberg-

Henriksson and Zierath, 2001). Moreover, Glut4 proteins are known to be subject to transcriptional regulation that allows for its increased synthesis (Zorzano et al., 2005), with a resultant contribution in reducing hyperglycemia. Further studies will be necessary to determine the mechanisms underlying the action of NSE to increase skeletal muscle Glut4, but this action is likely very relevant to the overall glucose-lowering activity of the plant.

Finally, we assessed ACC, a key component of the insulin-independent, metabolic sensing, AMPK pathway (Hardie and Pan, 2002). We found that NSE treatment *in vivo* can increase the phosphorylation of ACC in liver and skeletal muscle tissues. The phosphorylation of ACC reduces its activity and results in a decrease of lipogenesis in the liver and an increase of fatty acid oxidation in skeletal muscle (Munday et al., 1988). These effects on lipid metabolism can underlie the tendency for NSE to reduce plasma and tissue triglycerides observed herein. Lastly, it is known that the activation of the AMPK pathway can lead to increased synthesis of Glut4 (Ojuka, 2004) and this is in accordance with our results.

In conclusion, NSE greatly improves systemic glucose homeostasis and HDL-cholesterol in diabetic *Meriones shawi* by acting through severalmechanisms. Most importantly, *N. sativa* increases circulating insulin and enhances the sensitivity of peripheral tissues to the hormone. The latter effect can be attributed in part to an activation of the AMPK pathway in skeletal muscle and liver and to an increased content of Glut4 in skeletal muscle. Such pleiotropic actions provide strong evidence

in support of the traditional use of *N. sativa* seeds for the treatment of diabetes. They further call for high quality clinical studies to determine the optimal conditions for complementary or alternative treatment in diabetic patients.

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TABLES AND FIGURES

Table 1: Evolution of Body Weight over the course of the 4-week treatment

Time (week)	*N. sativa* (g±SEM)	Metformin (g±SEM)	Diabetic control (g±SEM)	Normal control (g±SEM)
0	178 ±17	177 ±11	187 ±15	158 ±14
1	172 ±16	166 ±11	178 ±15	153 ±12
2	183 ±19	180 ±10	186 ±15	161 ±13
3	185 ±18	183 ± 9	192 ±16	166 ±13
4	184 ±16	184 ± 9	197 ±15	170 ±12

Tableau 2: Area-under-the-curve (glyceamic (mmol/L)*time (min)) for OGTT before and after 4-week treatment

	Treatment			
Time (week)	*N. sativa*	Metformin	Diabetic control	Normal control
0	1648 ± 107	1685 ± 84	1666 ± 82	956 ± 61
4	1163 ± 81*§	819 ± 54*§	1514 ± 83	941 ± 41

*Significantly different from week 0; $p<0.05$; §Significantly different from Diabetic controls; $p<0.05$.

Figure 1:

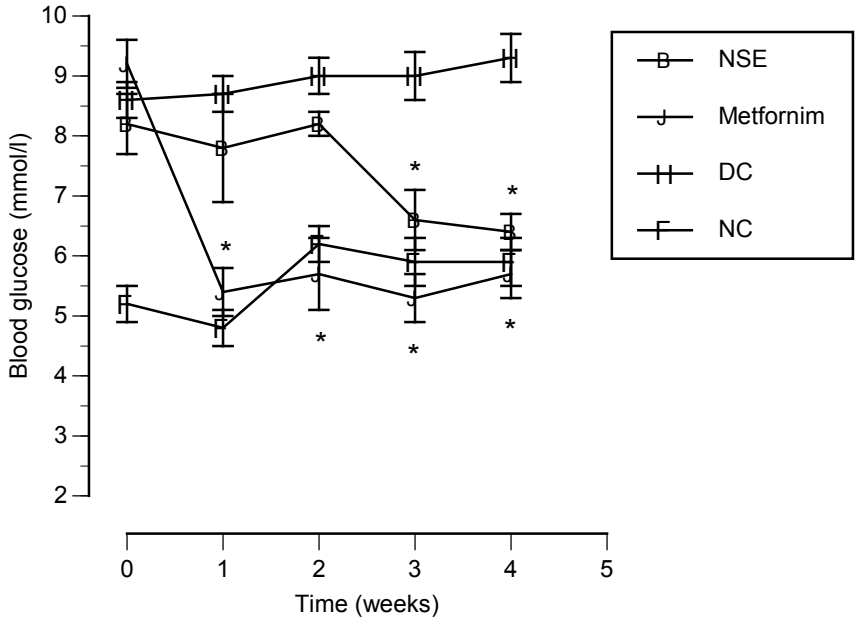

Blood glucose (mmol/l)
10
9
8
7
6
5
4
3
2
0
1
2
3
4
5
Time (weeks)
NSE
Metfornim
DC
NC
*
*
*
*
*

Figure 2:

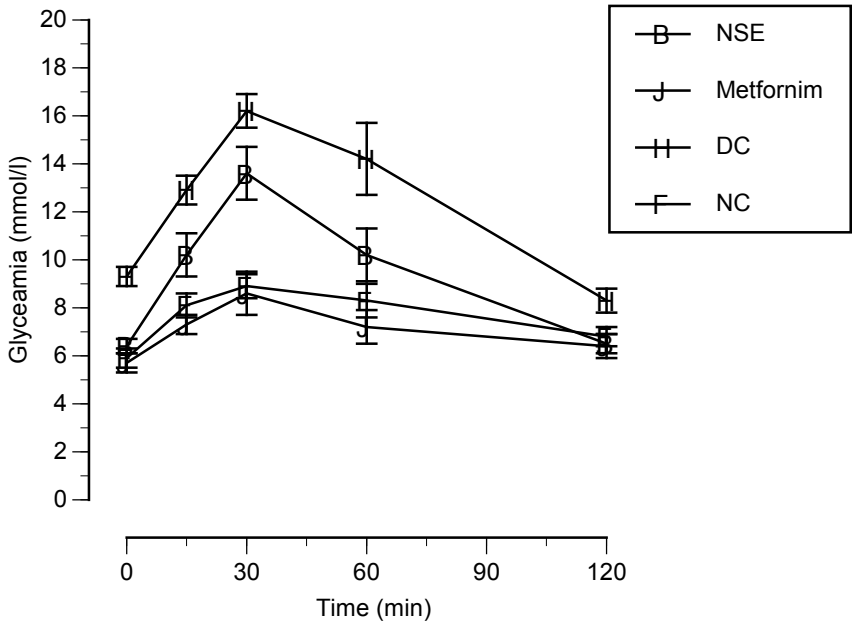
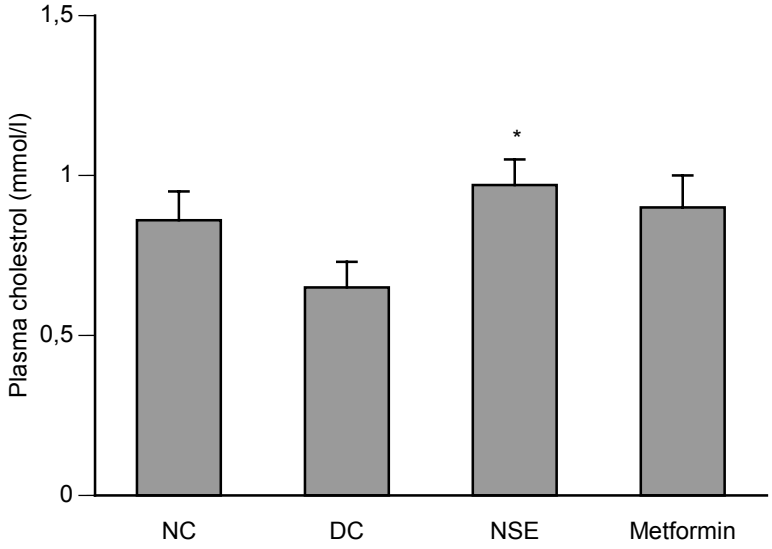
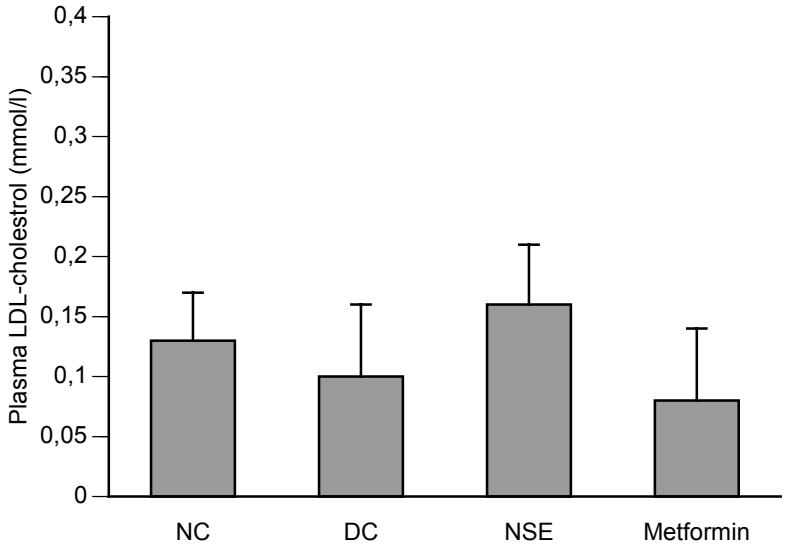

NSE
Metfornim
DC
NC
Glyceamia (mmol/l)
0
2
4
6
8
10
12
14
16
18
20
0
30
60
90
120
Time (min)

Figure 3

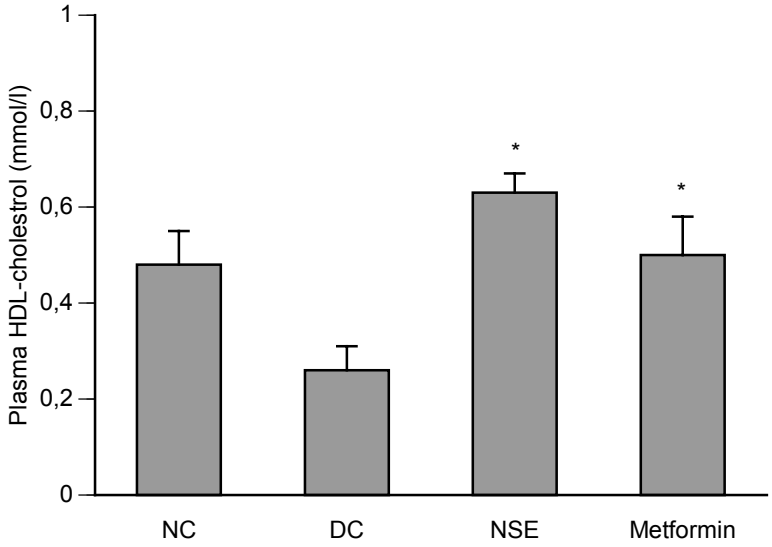
A:


1,5
1
0,5
0
Plasma cholestrol (mmol/l)
*
NC
DC
NSE
Metformin

B:


0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
Plasma LDL-cholestrol (mmol/l)
NC
DC
NSE
Metformin

C:


Plasma HDL-cholestrol (mmol/l)
1
0,8
0,6
0,4
0,2
0
*
*
NC
DC
NSE
Metformin

D:

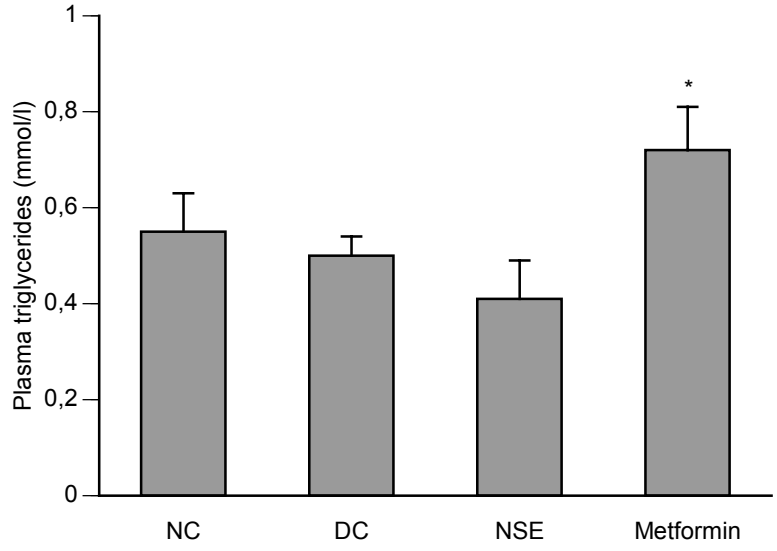
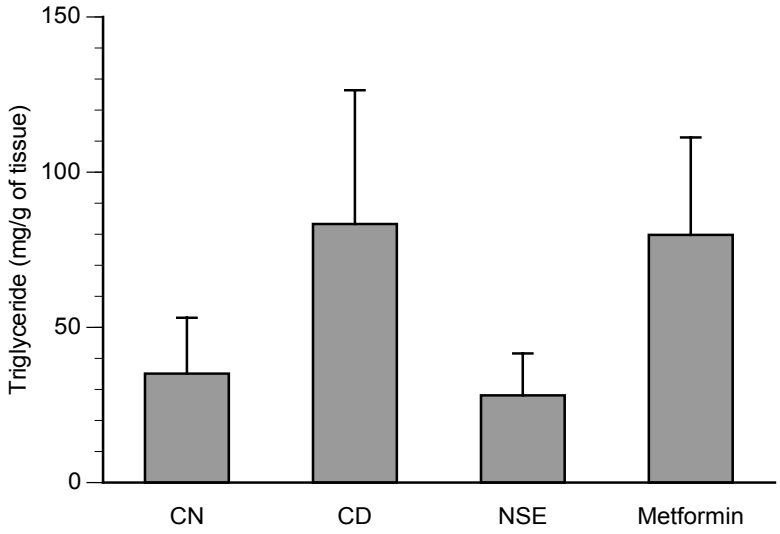

Plasma triglycerides (mmol/l)
1
0,8
0,6
0,4
0,2
0
*
NC
DC
NSE
Metformin

Figure 4:

A:


150
100
50
0
Triglyceride (mg/g of tissue)
CN
CD
NSE
Metformin

B:

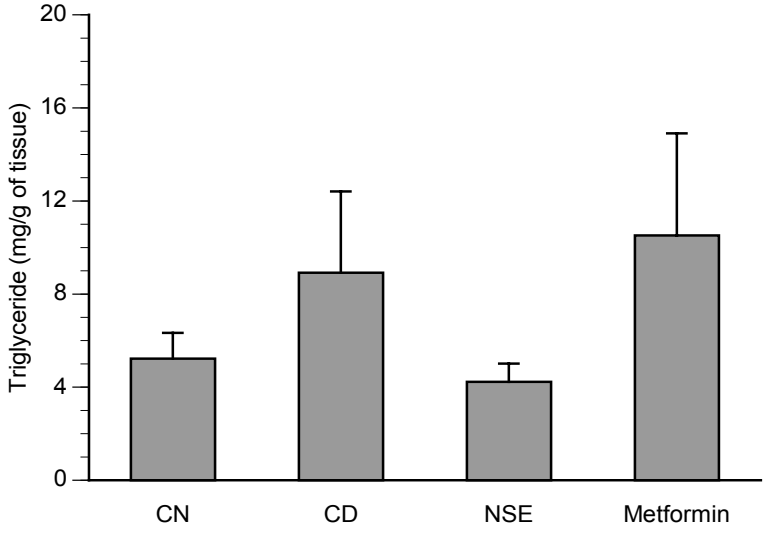

20
16
12
8
4
0
Triglyceride (mg/g of tissue)
CN
CD
NSE
Metformin

Figure 5:

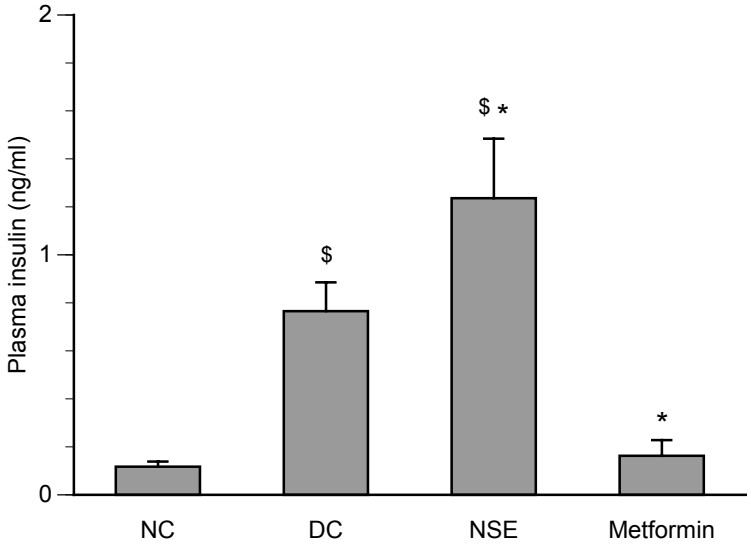
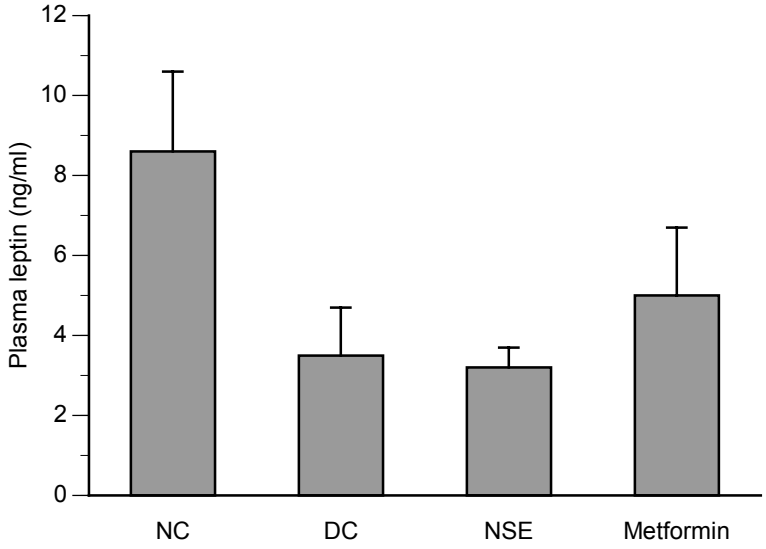


Figure 6

A:


Plasma leptin (ng/ml)
0
2
4
6
8
10
12
NC
DC
NSE
Metformin

B:

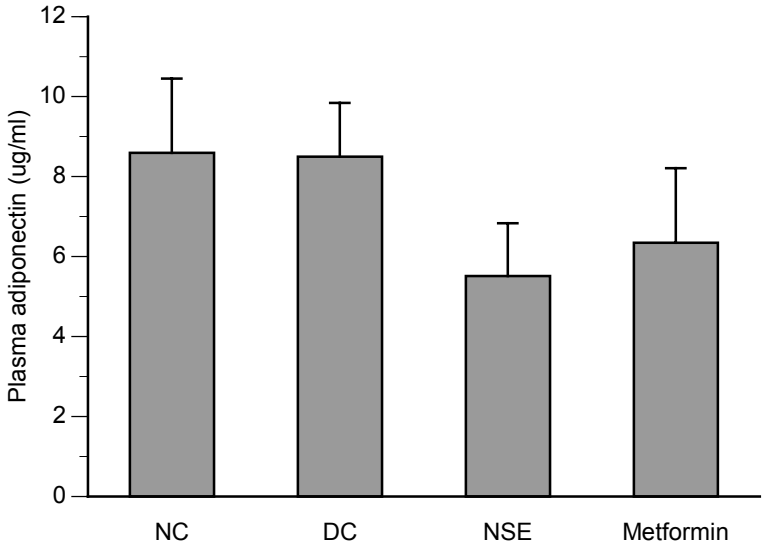
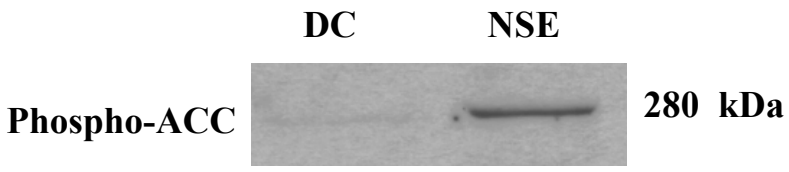
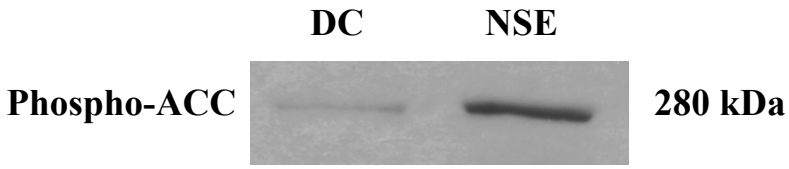

Plasma adiponectin (ug/ml)
0
2
4
6
8
10
12
NC
DC
NSE
Metformin

Figure 7

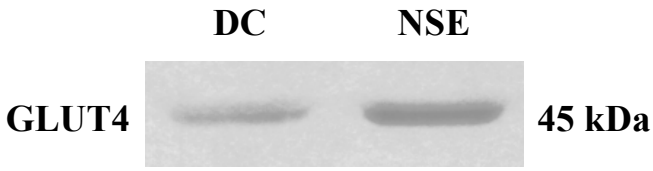
A :


DC
NSE
Phospho-ACC
280 kDa

B :


DC
NSE
Phospho-ACC
280 kDa

C :


DC
NSE
GLUT4
45 kDa

LEGEND OF FIGURES

Figure 1. Changes of plasma glucose in diabetic *Meriones shawi* treated with NSE.

Meriones shawi received a daily oral administration of NSE at 2 g eq plant/kg and 300 mg/kg metformin (positive control) whereas normal control group (NC) and diabetic control (DC) groups received the methyl-cellulose vehicle. Values (mean ±SEM) were obtained from each group of eight animals. * Significantly different from DC group at the same time point, $p < 0.05$.

Figure 2. Effect of NSE on oral glucose tolerance test (OGTT) in diabetic *Meriones shawi*.

OGTT (glucose 2 g/kg) was performed on fasted animals and blood glucose measured at the onset of glucose challenge (0 min) and at various time points afterwards (15–120 min). Results are expressed as mean±SEM (n=8). Significantly different from diabetic control *$p < 0.05$.

Figure 3. Effect of NSE on blood lipid profile.

Fasting total cholesterol (panel A), LDL-cholesterol (panel B), HDL-cholesterol (panel C), and triglycerides (panel D) were measured in plasma at the end of a 4-week treatment with 2 g/kg *N. sativa* ethanolic extract (NSE), or 300 mg/kg of the reference oral antidiabetic drug metformin or with an equivalent volume of vehicle (methyl-cellulose) in normal (NC) or diabetic (DC) *Meriones shawi* as described in Material and Methods section. Results were expressed as mean±SEM (n=8). *Significantly different from diabetic control ($P < 0.05$).

Figure 4. Effect of NSE on triglyceride content in skeletal muscle and liver tissues.

Triglyceride content in soleus muscle (A) and liver (B) tissues were measured in diabetic control group (DC), normal control group (NC), 2 g/kg *N. sativa* ethanolic extract (NSE), or 300 mg/kg of the reference oral antidiabetic drug metformin after 4 weeks of treatment. Each value is the mean ± SEM for 8 *Meriones shawi* in each group.

Figure 5. Effect of NSE on plasma insulin levels in *Meriones shawi*.

The plasma insulin was measured in diabetic control group (DC), normal control group (NC), 2 g/kg *N. sativa* ethanolic extract (NSE), or 300 mg/kg of the reference oral antidiabetic drug metformin after 4 weeks of treatment. Each value is the mean ± SEM for 8 *Meriones shawi* in each group. *$p < 0.05$ significantly different from diabetic control animals (DC). $^{\$}$ $p < 0.05$ significantly different from normal (non-diabetic) control animals (NC).

Figure 6. Effect of NSE on plasma leptin and adiponectin levels in *Meriones shawi*.

The plasma leptin (A) and adiponectin (B) were measured in diabetic control group (DC), normal control group (NC), 2 g/kg *N. sativa* ethanolic extract (NSE), or 300 mg/kg of the reference oral antidiabetic drug metformin after 4 weeks of treatment. Each value is the mean ± SEM for 8 *Meriones shawi* in each group.

Figure 7. Effect of NSE on the phosphorylation of ACC in skeletal muscle and liver tissues and on Glut4 in skeletal muscle tissue.

Samples of soleus muscle (A, C) and liver (B) tissues were obtained from diabetic *Meriones shawi* treated with NSE or vehicle (DC) and analysed by immunoblotting with antibody specific to phospho-ACC (Panels A and B) and Glut4 (Panel C). Immunoblots are representative of results obtained from four animals of each group.

6. Discussion générale

La cytotoxicité de l'extrait éthanolique de *N. sativa* (NSE) a été évaluée par deux méthodes, soit le test d'exclusion du marqueur vital bleu de trypan et le dosage de la relâche de l'enzyme lactate déshydrogénase (LDH). En fait, les résultats de cytotoxicité obtenus nous montrent que la concentration maximale de solubilisation de NSE dans le DMSO utilisé dans les expériences in vitro (200 μg/mL) ne présente aucune différence significative en comparaison avec les résultats du témoin DMSO. Dans le même sens, cette concentration ne présente aucun effet négatif sur la morphologie de la plupart des lignées cellulaires utilisées dans ces expériences. La dose de NSE utilisée pour les études in vivo chez les rats normaux et les *Mériones shawi* diabétiques (2g (équivalent de plante)/kg de poids corporel de l'animal) n'engendre pas des signes de toxicité chez ces animaux tout le long de la durée des études. La même concentration a été adoptée par plusieurs groupes de recherche surtout celui de Labhal (Labhal et coll., 1997, 1999). Zaoui et collaborateurs ont aussi rapporté que 2 mL/kg des huiles fixes de *N. sativa* montrent une absence de fuite d'enzymes hépatiques (ALAT, des GGT et des ASAT) ainsi qu'une absence de modifications histopathologiques après 12 semaines de traitement chez des rats normaux (Zaoui et coll., 2002). L'absence d'altérations histopathologiques suite à un traitement avec *N. sativa* a été suggéré par plusieurs groupes de chercheurs (Meddah et coll., 2009; Kanter et coll., 2003, 2004; Meral et coll., 2001; Tenekoon et coll., 1991). Par contre, il a été montré qu'un extrait aqueux de graines de *N. sativa*, entraîne une augmentation de la concentration plasmatique des ALAT et des GGT (Tenekoon et coll., 1991). Au contraire, la thymoquinone, composé principal des

huiles de *N. sativa* (Ali et Blunden, 2003), exerçait une action hépatoprotectrice en réduisant la fuite enzymatique des ALAT et des ASAT observée lors d'un traitement des hépatocytes par l'hydropéroxide tert-butyl in vitro (Daba et Abdel-Rahman, 1998). Ainsi, la grande majorité des études s'entendent sur la grande innocuité des produits dérivés de *N. sativa*.

Par ailleurs, le traitement des rats normaux avec un extrait d'éther de pétrole de *N. sativa* nous a permet d'observer une diminution de la prise pondérale (Chapitre 4). Les mêmes résultats ont été observés chez les *Mériones shawi* après un traitement avec NSE de 4 semaines (Chapitre 5). Dans le même sens, des études chez des rats normaux (Zaoui et coll., 2002; Meddah et coll., 2009), chez des *Mériones shawi* diabétiques (Labhal et coll., 1999) et chez des *Psammomys obesus* diabétiques (Labhal et coll., 1997) montrent que *N. sativa* diminue la prise de poids corporel. Notre étude chez les rats normaux (Chapitre 4) montre que cette diminution de poids corporel s'accompagne d'une diminution de la prise alimentaire. Cela nous a permis de suggérer que *N. sativa* peut avoir un effet sur le contrôle de l'appétit et de la satiété. Cette hypothèse nécessite des expériences plus poussées pour être confirmée.
Une des actions principales que nous avons identifiée pour expliquer la normalisation de la glycémie élevée par *N. sativa* se situe au niveau de la stimulation de la sécrétion de l'insuline chez les cellules β pancréatiques. En effet, nous avons démontré que NSE augmente la sécrétion de l'insuline chez les lignées cellulaires β pancréatiques INS832/13 et βTC-tet. Ces résultats sont en parfaite concordance avec ceux trouvés par l'équipe de Rchid qui ont montré que la nigelle stimule la sécrétion d'insuline

chez les cellules β pancréatiques isolées de rat (Rchid et coll., 2004). D'autres études devront être effectuées afin de bien comprendre l'effet insulinotropique de *N. sativa* et de déterminer les voies de signalisations intracellulaires sous-jacentes.

Nous avons aussi trouvé que *N. sativa* augmente la prolifération des cellules β, déterminé chez les cellules βTC-tet. Ceci concorde avec l'effet régénérateur de la plante observé par Kanter et coll., 2003 au niveau du même tissu. *N. sativa* possède aussi un effet antioxydant observé chez les cellules β des rats streptozotocine (Kanter et coll., 2004). Tous les effets cités ci-haut expliquent parfaitement les différentes voies via lesquelles la nigelle peut augmenter l'insulinémie chez les *Mériones shawi* diabétiques traités au NSE (Chapitre 5).

Par ailleurs, *N. sativa* améliore la sensibilité à l'insuline chez tous les modèles étudiés. En effet, au niveau des cellules de muscle squelettique C2C12, la nigelle améliore la sensibilité à l'insuline en augmentant l'activation de l'Akt, une protéine clef de la voie de signalisation de l'insuline. Cette amélioration se traduit aussi par une augmentation du transport de glucose chez ce type cellulaire. Les hépatocytes H4IIE traitées au NSE ou ceux isolées de rats traités à l'extrait d'éther de pétrole de *N. sativa* présentent aussi une activation de la voie de signalisation de l'insuline traduit par une potentialisation de l'activation de l'Akt. Chez les *Mériones shawi*, *N. sativa* permet aussi d'améliorer la sensibilisation à l'insuline puisque les AUC des OGTT diminue de façon significative après 4 semaines de traitement au NSE. De même NSE augmente le contenu des cellules musculaires en Glut4, principal

transporteur de glucose responsable de la régularisation des états hyperglycémiques. La diminution des triglycérides au niveau des tissus musculaire et hépatique peut aussi jouer un rôle primordial dans cette amélioration de la sensibilité à l'insuline. En effet, les concentrations élevées des triglycérides intramusculaire ou intra-hépatique permettent d'augmenter certains métabolites lipidiques comme les céramides, le diacylglycérol et les acyl-coenzyme A à chaîne longue. Ceux-ci jouent un rôle principal dans l'atténuation du signal insulinique intracellulaire en augmentant la phosphorylation serine-thréonine au niveau des protéines IRS (Viollet et coll., 2007). La nigelle contrecarre donc ces effets.

Comme les biguanides, nous avons trouvé que le NSE active la voie de l'AMPK chez les myotubes C2C12 et les hépatocytes H4IIE. Ces résultats montrent une autre voie qu'emprunte NSE pour avoir son effet antidiabétique. Cette activation de l'AMPK est due en grande partie à une augmentation intracellulaire du rapport AMP/ATP. Cette augmentation est à son tour reliée à un effet de découplage au niveau de la chaine de respiration mitochondriale, tel que démontré par l'augmentation de la consommation d'oxygène induite par à un traitement au NSE chez des mitochondries isolées. Finalement, il est connu que l'AMPK augmente l'expression des gènes codants pour la protéine Glut4 (Viollet et coll., 2007). Cela concorde nos résultats obtenus chez le muscle squelettique des *Meriones shawi* traités au NSE.

Chez les adipocytes, nous avons trouvé que la nigelle stimule le transport de glucose. Cette stimulation peut être attribuée à une activation des PPARγ, tel que démontré par une augmentation de l'adipogenèse chez des cellules 3T3-L1 et par une augmentation de l'activation du gène luciférase qui est sous contrôle d'un promoteur qui contient une séquence PPAR-RE (essai de gène rapporteur). *N. sativa* possède aussi un effet anti-lipidémiant chez les rats normaux et chez les *Meriones shawi* diabétiques. En fait, le traitement avec la nigelle *in vivo* permet de diminuer les triglycérides plasmatiques ainsi que d'augmenter le cholestérol-HDL. Une étude antérieure a montré que l'extrait aqueux des graines de *N. sativa* aurait une action hypocholestérolémiante et hypotriglycéridémiante chez le rat des sables (Labhel et coll., 1997). Ces effets ont été également soulignés par d'autres auteurs chez le rat diabétique (Al-Awadi et Shoukry, 1988; Eskander et coll., 1995). Chez le rat Wistar, la nigelle diminue significativement les valeurs de cholestérol et des triglycérides au niveau plasmatique (Zaoui et coll., 2002). L'activation de l'AMPK que nous avons identifiée aux niveaux musculaire et hépatique permet d'amener une explication pour l'activité hypotriglycéridémiante de *N. sativa*. En effet, l'activation de l'AMPK permet d'augmenter la β oxydation des acides gras au niveau musculaire (Lin et coll., 2000). Cette activation permet aussi une augmentation de la β oxydation des acides gras et une diminution de leur biosynthèse chez les hépatocytes (Yamauchi et coll., 2002). Ainsi, nos travaux confirment que, comme les fibrates et les thiazolidinediones, *N. sativa* fait baisser la concentration plasmatique en triglycérides et améliore la concentration plasmatique en HDL-cholestérol en agissant par une activation des récepteurs nucléaires PPARγ et PPARα (Morikawa et coll., 2004).

7. Conclusions et perspective

Les résultats de nos analyses fonctionnelles cellulaires et moléculaires ont pu identifier avec succès les mécanismes expliquant les effets anti-hyperglycémiques des graines de *N. sativa*. En effet, nous avons confirmé l'action insulinotropique de *N. sativa* au niveau des cellules β pancréatiques et présenté l'évidence d'un effet prolifératif de NSE chez ces cellules. Ceci démontre que *N. sativa* pourra avoir un effet bénéfique sur la régénération de la masse des cellules β et par conséquent un ralentissement de la progression du diabète de type II. Notre étude a mis en évidence pour la première fois que *N. sativa* exerce son activité antidiabétique par des effets insulino-mimétique et insulino-sensibilisateur directs permettant ainsi d'augmenter le transport de glucose au niveau du muscle squelettique et du tissu adipeux. Cette action de NSE est liée à une stimulation des voies de signalisation intracellulaires insulinodépendants et -indépendantes (AMPK) chez le muscle squelettique et le foie alors qu'elle passe par la voie des PPARγ au niveau de tissu adipeux. Finalement, l'étude *in vivo* vient confirmer l'effet antidiabétique de *N. sativa* déjà rapportés par plusieurs auteurs chez différents modèles animaux. Notre apport novateur se situe au niveau de la démonstration que l'activité antidiabétique de NSE chez le mérione diabétique est la résultante des mêmes activités que celles déterminées par l'étude *in vitro*. En effet, NSE active la voie de l'AMPK, améliore la sensibilité à l'insuline et augmente l'insulinémie chez les *Meriones shawi* diabétiques. Les études *in vivo* effectuées montrent aussi que NSE possède une activité antilipidémiante non seulement chez les *Meriones shawi* diabétiques mais aussi chez le rat normoglycémique. En général, *N. sativa* agit de façon semblable aux sulfonylurées et

aux glitinides en favorisant la sécrétion de l'insuline, possède les mêmes propriétés que les biguanides en améliorant la sensibilité à l'insuline et en activant l'AMPK, puis stimule les PPARγ comme les thiazolidinediones. Ces données sont impressionnantes et soutiennent l'utilisation ethnopharmacologique de cette plante comme traitement du diabète. Des études cliniques de grande qualité (à double insu et avec placebo) devraient ainsi être effectuées afin de confirmer et d'optimiser l'usage thérapeutique de la nigelle; un grand soin au niveau de la qualité des produits dérivés de la nigelle étant une condition essentielle et incontestable.

Outre ces études cliniques, les résultats très satisfaisants de cette thèse amèneront sans doute à effectuer d'autres travaux pour élucider les cibles moléculaires de cette plante au niveau des cellules bêta pancréatique. Il serait aussi important de déterminer l'action de NSE sur les transporteurs de glucose constitutifs (Glut1) et ceux insulinodépendants (Glut4) afin de bien comprendre les modalités par lesquelles NSE potentialise le transport de glucose chez les cellules de muscle squelettique et les adipocytes.

L'extrait de *N. sativa* devra également être fractionné par des techniques de chromatographie sur colonne et de chromatographie liquide à haute performance afin de séparer les composés phytochimiques les uns des autres. De la même façon que l'extrait brut, chaque fraction semi-purifiée ou chaque molécule pure ainsi obtenue devra être testée sur des modèles cellulaires et des modèles animaux de diabète, en

vue de déterminer la ou les molécules actives responsables de l'effet euglycémiant de *N. sativa*.

Il serait aussi très intéressant d'essayer d'évaluer l'effet de la nigelle sur le métabolisme lipidique ainsi que son effet anti-obésité chez un modèle animal d'obésité. Par ailleurs, les effets potentiellement bénéfiques de *N. sativa* sur les différents mécanismes menant à des complications de diabète restent à déterminer. Dans ce contexte, il convient de penser à évaluer l'augmentation intracellulaire de sorbitol induite par la voie de l'aldose réductase, la formation des produits terminaux de glucosylation avancé (*advanced glycation endproducts*), l'activation de certaines isoformes de la protéine kinase C et la stimulation de la voie d'hexosamine.

8. Références

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