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# Effects of Xanthohumol On Biomarkers of Metabolic Syndrome In Obese Rats


**Library Undergraduate Research Award**

**May 9, 2014**

Effects of Xanthohumol on Biomarkers of Metabolic Syndrome in Obese Rats.

by

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A PROJECT

submitted to

Oregon State University

University Honors College

In partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Bioresource Research (Honors Associate)

Presented August 27, 2013
Commencement June 2014

## ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to Dr. Jan Frederik Stevens, for his mentorship and for the great opportunity to work in his lab on the xanthohumol project. Also, I am deeply indebted to Dr. LeeCole Legette and Dr. Cristobal Miranda for their help, advice and patience over the past two years. I extend my appreciation to Katharine G. Field, Director of the Bioresource Research Interdisciplinary Program and Wanda Crannell, my major advisor, for their guidance and encouragement for me to pursue research training in my undergraduate studies. Thank you, Ana, Maria and all of my friends for your friendship and moral support. Lastly, I am very grateful to my mother, Pablina, and my sister, Ayeza, who have been there to support me and encourage me throughout my college studies at Oregon State University.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| Xanthohumol | XN |
| Cardiovascular Disease | CVD |
| National Cholesterol Education Program | NCEP |
| World Health Organization | WHO |
| American Heart Association | AHA |
| National Hearth Lung and Blood Institute | NHLBI |
| Non-Communicable Disease | NCD |
| Zucker obese *fa/fa* | ZOF |
| Body Weight | BW |
| Mass Spectrometry | MS |
| Mass-to-Charge Ratio | *m/z* |
| Isoxanthohumol | IX |
| 8-Prenylnaringenin | 8PN |
| 6-Prenylnaringenin | 6PN |
| Liquid Chromatography-Tandem Mass Spectrometry | LC-MS/MS |
| Sodium Dodecyl sulfate-Polyacrylamide Gel Electrophoresis | SDS-PAGE |
| 5' Adenosine Monophosphate-Activated Protein Kinase | AMPK |
| A Serine/Threonine Protein Kinase or Protein Kinase | Akt |
| Short heterodimer partner | SHP |
| Phosphoenolpyruvate carboxykinase | PEPCK |
| Glucose-6-phosphatase | G6Pase |
| Enzyme-Linked Immunosorbent Assay | ELISA |

## LIST OF ABBREVIATIONS (CONTINUED)

| | |
|---|---|
| Sodium-glucose transporter-1 | SGLT-1 |
| Sodium-glucose transporter-2 | SGLT-2 |
| Glucagon-like peptide 1 | GLP-1 |
| Carnitine palmitoyltransferase 1 | CPT1 |

Con mucho cariño esta tesis es dedicada para mis Padres

"With love, this thesis is dedicated to my Parents"

Effects of Xanthohumol on Biomarkers of Metabolic Syndrome in Obese Rats

# Introduction

## 1.1 Metabolic Syndrome

Worldwide, there has been an epidemic of obesity and diabetes. It is estimated in the U.S. that 24% of adults (20-70 years) have metabolic syndrome. The prevalence of metabolic syndrome increases with age and is over 40% for subjects over 60 years old. In addition, there is increase in the frequency of metabolic syndrome heterogeneity by sex and ethnicity [1].

Metabolic syndrome is clinically defined as having three or more of the following conditions: obesity, type II diabetes, hypertension, hyperinsulinemia, and cardiovascular disease [2]. The term, metabolic syndrome, has been used for over 80 years. Kylin, a Swedish physician, first described it in 1920s and defined it as the clustering of hypertension, hyperglycemia, and gout. In 1947, Vague correlated upper body adiposity with obesity, which was associated with metabolic abnormalities related to type II diabetes and cardiovascular disease [2].

Metabolic syndrome is also known as: the insulin resistance syndrome, syndrome X, and the deadly quartet. Some of the abnormalities of the syndrome are: type II diabetes (glucose intolerance), insulin resistance, dyslipidemia, central obesity and hypertension. Dr. Lemieux and colleagues have suggested a central component of metabolic syndrome is abdominal obesity, also known as hyper-triglyceride waist phenomenon [2].

The National Cholesterol Education Program (NCEP) and the World Health Organization (WHO) propose the most frequent definitions used in most of the research for metabolic syndrome, which include obesity, hypertension, hyperglycemia and dyslipidemia [2]. WHO's definition requires evidence of insulin resistance and the measurement of fasting insulin or its surrogates [2].

Previous studies done in Finland by Ilanne-Parikka *et al.* in 2004 found that the prevalence of the metabolic syndrome using the modified WHO criteria was 14.4% in men and 10.1% in women aged 45-65 [3] Dr. Mattsson *et al.* in 2007 also found that metabolic syndrome was higher amongst Finnish young adult men compared with women [4]. From 1986 (0.1%) to 2001 (7.5%), the prevalence of the metabolic syndrome has increased markedly in 24-year-old adults. Overall, prevalence of the metabolic syndrome has increased amongst U.S. adults from 23.1% in 1988 to 26.7% in 1994 [2] .

### 1.2 Obesity and Type II Diabetes

Definition of abdominal obesity varies according to population. Having an effective weight reduction improves all risk factors associated with the metabolic syndrome [2] . One of the most effective methods of weight reduction is to reduce the energy intake and increase physical activity to enhance energy expenditure. It is suggested to have a caloric intake of 500-1000 calories per day to produce a weight loss of 0.5-1.0 kg per week [2]. Currently, weight loss drugs have not been effectively tested for treatment of obesity. Bariatric surgery in the USA has increased to treat patients with morbid obesity. The effectiveness of this surgery

is 95%; most patients are no longer obese for one year after operation. However, a lot of risk factors arise post surgery such as: heart failure, atherosclerosis, heart rhythm disorders, pulmonary embolism, sleep apnea, etc. In addition, a surgery treatment only cures for fat removal and does not help to treat other diseases. Alternative treatments need to be discovered that can prevent the patient from all the described problems and have a more holistic approach. For instance, dietary flavonoids, present in fruits and vegetables, can help treat several conditions associated with metabolic syndrome including obesity and type II diabetes.

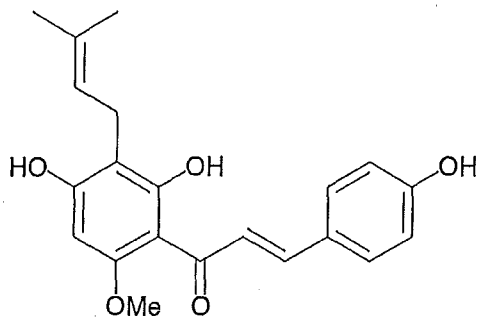
There are two main forms of diabetes; it is usually due to an autoimmune-mediated destruction of pancreatic β –cell islets, creating an absolute insulin deficiency. People with type I diabetes must take insulin to function properly and to prevent the development of ketoacidosis [5]. Type II diabetes, also known as non-insulin dependent diabetes mellitus, is characterized by insulin resistance and/or an abnormal production of insulin secretion [3]. Globally, 90% of cases are type II diabetes. People affected by type II diabetes are not dependent on exogenous insulin; however, they may require it for control of blood glucose levels if not supplied by their diet or hypoglycemic agents. A type II diabetes epidemic is taking place in both developing and developed nations. Currently, the global figures are estimated at 150 million to 220 million in 2010, and 300 million are estimated for 2025, where most cases are type II diabetes. Type II diabetes is associated with the lifestyle and obesity [5].

Currently, there are drugs in the market that can treat obesity and type II

diabetes. However most of current drugs that are synthetically made have side effects. Research needs to move toward natural components such as flavonoids to discover a possible remedy for metabolic syndrome.

### 1.3 Flavonoids:

Current research indicates that dietary flavonoids show promise for mitigating and/or preventing chronic conditions associated with metabolic syndrome. Flavonoids come from a large family of compounds, synthesized by plants that have a common chemical structure. Scientists have become interested in the health benefits of fruits and vegetables due to dietary flavonoids content [1]. The metabolic syndrome shows a chronic inflammatory condition, which has been involved in the onset and development of several pathological disturbances. Previous investigators have showed that various types of flavonoids have anti-inflammatory effects [6]; the impact of flavonoids on inflammation occurs via direct effects and indirect through flavonoid metabolites [7]. Xanthohumol, a prenylflavonoid, has been shown to have an anti-inflammatory effect and other health benefits from its consumption [8].




**Xanthohumol (XN)**

**Figure 1.** Structure of xanthohumol [11]

### 1.4 Xanthohumol:

Xanthohumol (XN) is the major prenylated flavonoid of the female inflorescences (cones) of the hop plant (*Humulus lupulus)* [8]. Hops are used in the brewing industry to give beer its special characteristics such as flavor and aroma. XN is the principal flavonoid present in the hop cone extracts and has a prenylated chalcone structure. Milligan et al. [9] identified 8-prenylnaringenin (8PN) as a phytoestrogen in hops. The hop flowers in the lupulin glands contain the 8-prenylnaringenin along with other prenylflavonoids and the hop acids that are the key for brewing [9]. Lupulin may reach a content of about 10% of the dry weight of the hop cone [10]. The molecular formula for XN is $C_{21}H_{22}O_5$, and molecular weight is 354.40 g/mol (Figure 1). XN is known to exert several beneficial health effects, however only few studies evaluated the safety characteristics of this natural compound.

### 1.4.1 Health benefits:

In Germany, hop baths were used for the treatment of gynecological disorders. Hop extracts have been reported to reduce hot flashes in menopausal women [11]. XN is reported to have anti-inflammatory, anti-oxidant, and anti-angiogenic effects. It also exhibits anti-infective activity against bacteria, viruses, fungi, and plasmodia [12]. Research showed that of the five hop chalcones tested, XN was the most effective anti-proliferative agent in human breast cancer cells (MCF-7), colon cancer (HT-29), and ovarian cancer cells (A-2780) [13]. Moreover, XN has the ability to induce apoptosis in cancer cells and to limit tumor cell invasiveness, suggesting suppression of cancer development post-initiation as well [14]. In addition, other studies suggest that inhibition of NF-kB and induction of apoptosis may be a critical mechanism by which XN acts as anti-proliferative agent in prostate hyperplasia [14].

### 1.4.2 Xanthohumol and Metabolic Syndrome:

Currently, there is an increase of research in functional foods to treat obesity and its complications. Hop components have been described to inhibit the metabolic activation of xenobiotics, prostaglandins, and NO production [15]. It has been shown that XN reduces lipid accumulation in 3T3-L1 adipocytes [16]. Even though there is some *in* vitro information about the effects of XN on lipid metabolism, there is not enough data *in vivo* of the effects of XN. It is important to understand the effect of oral administration of XN, using different doses and see how it affects metabolic syndrome.

# MATERIALS AND METHODS

## 2.1 Study Design

We tested the effects of a chronic feeding of XN on metabolic syndrome using a genetic model of obesity, the Zucker obese *fa/fa* (ZOF) rat. In 1962, the ZOF rat was found to have an autosomal recessive mutation that prohibits the production of leptin [17]. Leptin is a hormone involved in the regulation of energy intake and energy expenditure, which includes appetite and metabolism. The basic phenomenon in the *fa/fa* is an error in lipid metabolism. Due to a greater excess of lipids (probably low-density lipoproteins) circulating in the blood, an excessive amount of lipids is deposited in tissues; thus, overall *ZOF* rats become noticeably obese at 5 weeks of age [17]. Four-week old male and female ZOF rats were purchased from Harlan (Livermore, CA, USA). Animals were housed in individual cages in temperature and humidity controlled rooms with a 12:12 on-off light cycle. All animals handling was approved by and in accordance with ethical standards of Oregon State University's Institutional Animal Use and Care Committee (Protocol # 3689). After a two-day acclimation period, male and female animals were divided into four treatment groups (n=6/group) according to their body weight to ensure similar average body weight across groups. For three weeks of the study, all rats were maintained on a high fat (60% kcal fat) AIN-93G to induce severe obesity, then placed on a normal AIN-93G (15% kcal fat) diet for the remainder of the study. Body weight and food intake was recorded weekly.

### 2.2 Treatments

Four different doses of XN were administrated daily to ZOF female and male rats and were determined from previous work [18]. It was calculated using allometric scaling and an interspecies scaling factor [19, 20] for an individual weighing 64 kg (141 lb). Rats were placed into four treatment groups: control (0 mg XN /kg BW), low (1.86 mg XN/kg BW), medium (5.64 mg XN/ KG BW) and high (16.9 mg XN/kg BW).

### 2.3 Source Material

Animals received daily XN doses via a test meal as described [21]. XN powder (>99% pure by HPLC-UV and NMR; Anheruser Busch, St. Louis, MO, USA) was dissolved in a self-emulsifying isotropic mixture (oleic acid, Tween 80, and propylene glycol) to create various dosing solutions (0, 1.86, 5.64, and 16.9 mg XN/kg BW). The XN solutions were then added to a modified AIN-93G [22] powdered diet (corn oil replacing soybean oil) and homogeneously mixed with mortar and pestle to result in individual test meal doses (3 g) . The test meal was administered after holding food for three hours to ensure complete XN consumption within 30 min. Test meals were made weekly for each treatment group based on average body weight.

### 2.4 Xanthohumol Analysis

Currently, mass spectrometry is an indispensible analytical tool in chemistry, biochemistry, pharmacy, and medicine. Mass spectrometry (MS) is used to

identify a specific compound from the molecular mass of its constituents. MS elucidates the connectivity of atoms within smaller molecules, identifies functional groups, determine the (average) number and eventually the sequence of constituents of macromolecules, and at times it can yield their three-dimensional structure [23]. The basic principle of MS is to generate ions from either inorganic or organic compounds by any suitable method, to separate these ions by their *mass-to-charge ratio (m/z)* and detect them qualitatively and quantitatively by their respective *m/z* and abundance.

Plasma levels of XN, 8PN, isoxanthohumol (IX), and 6-prenylnaringenin (6PN) were determined using the procedures and conditions detailed in earlier work [18]. Samples were analyzed with a structure elucidation to study mass-selected ions in liquid chromatography-tandem mass spectrometry (LC-MS/MS). The term *tandem mass spectrometry* encompasses the numerous techniques where mass-selected ions are subjected to a second mass spectrometric analysis. Samples were prepared with and without enzymatic hydrolysis to be able to determine the amount of free and conjugated XN, IX, 8PN, and 6PN in plasma. A homogenized tissue preparation was done under these conditions: while resting on ice, liver tissue (0.500g) was homogenized in extraction solvent (MeOH-H2O, 9:1 v/v, 5ml). Samples were homogenized (30 sec) with an Omni Tissue Homogenizer (Omni TH, Omni International, Marietta, GA, USA), followed by sonication (1min Model F60 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA, USA), and centrifuged at 4000 x *g* for 15 min (Allegra X15R centrifuge, Beckman Coulter, Brea, CA USA). The supernatant was removed and stored as

tissue extract. The tissues extracted were prepared in duplicate. Tissue extracts underwent similar sample preparation and analysis as previously described for plasma samples. Test meals (400 mg) were analyzed by HPLC in triplicate using conditions described in [18].

**2.5 Biochemical Markers**

Before sacrifice, animals underwent overnight fasting. Via a cardio-puncture blood was collected and centrifuged (International Clinical Centrifuge setting 7, 10 min). Plasma was stored at -80°C until analysis. Livers were collected, flash-frozen in liquid nitrogen and stored at -80°C prior to analysis. Commercial assay kits were used to determine plasma glucose (Wako Pure Chemicals, Richmond, VA), triglycerides, cholesterol (Thermo Scientific, Middletown, VA). Insulin was assayed by ELISA (Alpco Insulin RAT ELISA, Alpco Diagnostics, Salem, NH) according to the manufacturer's directions.

In the glucose assay, α–D-glucose found in the plasma sample is first converted to the β-isomer by mutarotase, and is then oxidized by glucose oxidase to produce hydrogen peroxide ($H_2O_2$) as one of the products. $H_2O_2$ reacts with 4-aminoantipyrine and phenol in the presence of peroxidase to produce a red pigment. This red pigment present in the sample is read on a 96 well plate reader at 505 nm. Quantification was done with a standard curve of increasing concentrations of glucose that was also measured along with the samples.

In the triglycerides assay, triglycerides present in the sample are hydrolyzed by a lipase to free fatty acids and glycerol. The glycerol is phosphorylated by glycerol

kinase to produce glycerol-3-phosphate, which is then oxidized by glycerolphosphate oxidase forming $H_2O_2$ as one of the products. $H_2O_2$ reacts with 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzene sulfonate to produce a red-colored dye, which is then read at 550 nm.

In the cholesterol assay, the cholesterol esters are hydrolyzed by cholesterol esterase to free cholesterol. Free cholesterol is then oxidized to cholest-4-en-3-one and $H_2O_2$ by cholesterol oxidase. $H_2O_2$ reacts with hydroxybenzoic acid and 4-aminoantipyrine to produce the quinoneimine dye, which is read at 550 nm.

**2.6 Preparation of Liver Protein Extracts for Western Blotting**

Liver tissue samples (100-150 mg) were homogenized in homogenization buffer (HB) (1-1.5mL) using an OMNI homogenizer. HB was prepared as described in [24] and consisted of 20 mM Hepes, 50 mM disodium glycerol 2-phosphate, 2 mM Na EDTA, 1 mM sodium orthovanadate, 1 % Triton X-100, 10% glycerol, 2 mM DTT, and protease inhibitors (Protease Inhibitor Cocktail (Cat no P8340), Sigma-Aldrich, St. Louis, MO, USA). Liver homogenates were incubated (30 min, 4°C) to solubilize proteins. After incubation, homogenates were centrifuged (12,000 x g, 20 min, 4°C) and supernatant was removed. Samples were analyzed for total protein content using Coomassie Plus Protein Assay Reagent (Pierce cat. no.23236, Rockford, IL) according to the manufacturer's directions.

### 2.6 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Liver extracts were mixed with Laemmli sample buffer (Bio-Rad cat. no. 161-0737, Hercules, CA) with 2-mercaptoethanol (5%) and heated (100°C, 5 min) before cooling to room temperature. The protein samples were loaded onto a 10-well 10% Tris-glycine precast gel (Bio-Rad cat. no. 456-1033) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes by electroblotting. The membranes were blocked by incubating in 5% non-fat dry milk dissolved in Tris buffered saline with 0.1% Tween 20 (TBS-T). After washing with TBS-T, the membranes were incubated with a primary antibody and a secondary antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase, Bio-Rad). The primary antibodies (Cell Signaling Technology, Danvers, MA) used were raised in rabbits against AMPK, phosphorylated AMPK (pAMPK), Akt, phosphorylated Akt (pAkt), and β-actin. Protein bands were detected by incubating the nitrocellulose membranes with a chemiluminescence substrate (SuperSignal West Pico Chemiluminescent substrate, Thermo cat. no. 34080) and exposing the membranes to X-ray film.

### 2.7 Statistics

The data was analyzed using Statistical Analysis Software (SAS) (Version 9.1, SAS Institute, Cary, NC) or GraphPad InStat (GraphPad Software, La Jolla, CA). A data point for insulin that was more than four standard deviations from the mean of the group average was excluded from further statistical analysis. The treatment effects on body weight were assessed using repeated measure analysis over time (PROC MIXED procedure). Effects on plasma biochemical markers were determined by Student *t*-test or one-way ANOVA and post-hoc test using LSD or Dunnett's test. The significance level was set at $p<0.05$.

## Results and Discussion

### 3.1 Body Weight

In our study, we analyzed the effects of XN consumption on body weight. A total of 48 ZOF rats were observed for a total of six weeks. In our study, we discovered that the supplementation of XN reduced body weight in male rats. This effect was seen in male high XN group. Comparing the high treatment to control group there was a significant decrease ($p<0.05$) in their body weight. We also observed lower body weight among the other XN treatment groups both in males and females; to the control group, but this was not significant (Figure 2). Previous research has demonstrated that consumption of dietary soy isoflavones decreases body weight and abdominal white adipose tissue, similar to our findings with XN [25]. Also, a trend was observed in female rats for a dose dependent effect of XN on body weight (Figure 2). Perhaps, this observation of lower body weight in female rats can become statistically significant if we increase the number of the rats per treatment group or duration of XN supplementation.

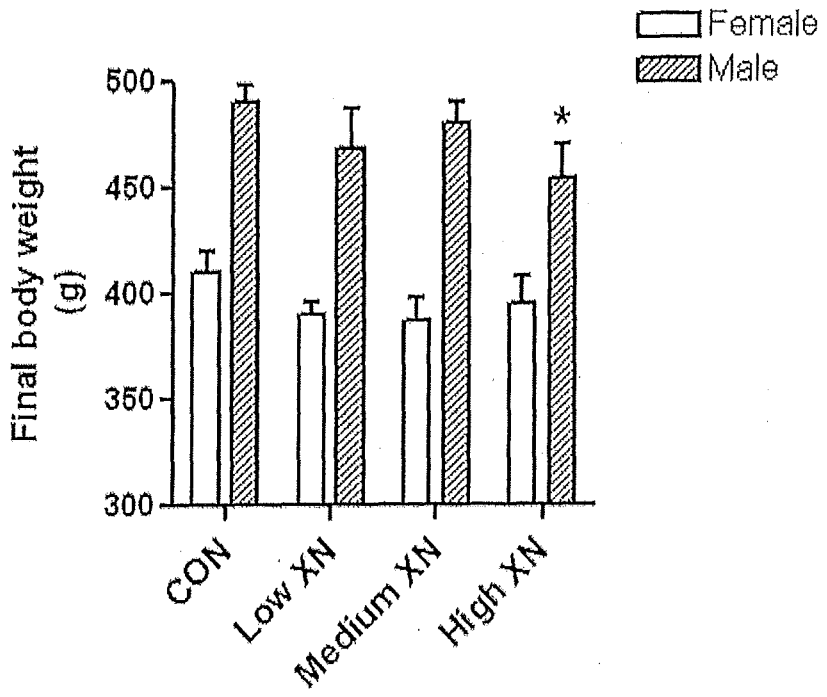



**Figure 2.** Mean body weight of male and female obese Zucker *fa/fa* rats (n=6/group) receiving various daily doses (0, 1.86, 5.64, 16.9 mg/kg BW) of xanthohumol for 6 weeks. Statistical difference from the control group is denoted by the * ($p < 0.05$). All values are expressed as mean ± SE.

### 3.2 Plasma Glucose

There are several classes of medications to lower plasma glucose in metabolic syndrome or in Type 2 diabetes. They include sulfonylureas (increases insulin secretion by the pancreas), acarbose (inhibitor of intestinal alpha-glucosidase), metformin (inhibitor of hepatic gluconeogenesis), and rosiglitazone (activator of PPAR-γ in fat and muscle). Inhibitors of SGLT1 and SGLT2 such as LX4211 lowers plasma glucose by inhibiting the reabsorption of glucose by the kidneys [26]. Glucagon-like peptide-1 (GLP-1 secreted by intestinal cells) agonists (or incretin mimetics) such as Exenatide also lower plasma glucose by stimulating insulin secretion while suppressing glucagon secretion [27]. Glucagon, as opposed to insulin, increases plasma glucose by stimulating the conversion of liver glycogen into glucose. However, these drugs have certain undesirable side effects prompting us to investigate natural products such as XN to supplement or replace existing therapies for lowering blood glucose in metabolic syndrome or in Type 2 diabetes.

Low and medium XN treatment did not exhibit effects on plasma glucose. However, when comparing the high XN treatment group to the control male group, there was a significant decrease ($p<0.05$) in plasma glucose levels (Figure 3) but not in female rats. This also corresponds to other research, with the consumption of soy or soy isoflavones decreasing plasma LDL levels in males but not in female hamsters [28]. Past research has shown that XN feeding for four weeks caused a decrease in plasma glucose in obese male mice [29]. A plasma glucose level is the amount of glucose (sugar) present in the blood.

Naturally each organism regulates blood glucose levels as part of the metabolic homeostasis. Primary glucose is the source of energy for the body's cells, and blood lipids. Glucose is transported from the intestines or liver to body cells via bloodstream and is available for cell absorption mediated by insulin produced primarily in the pancreas. Future research needs to be done to examine the mechanisms why females do not have the same response as males (Figure 3).

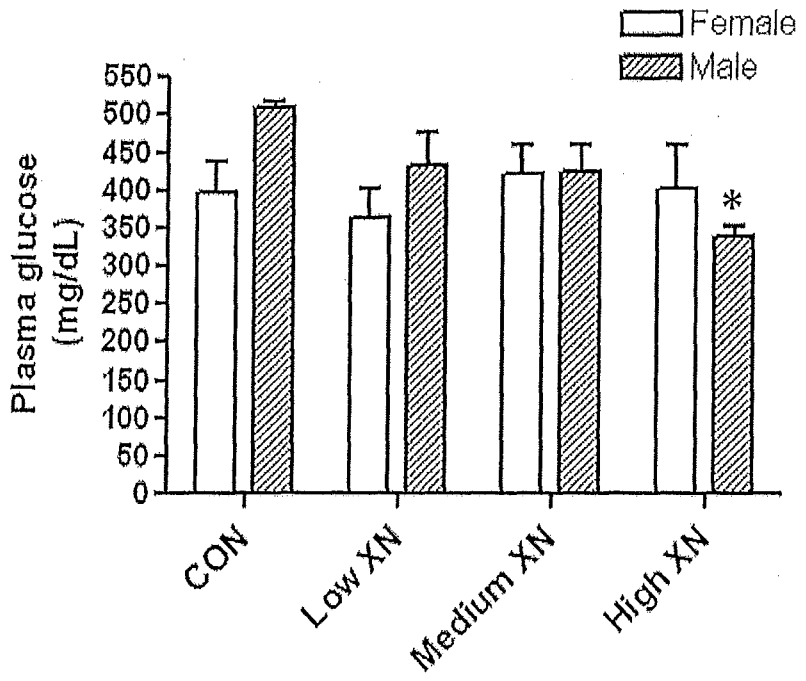


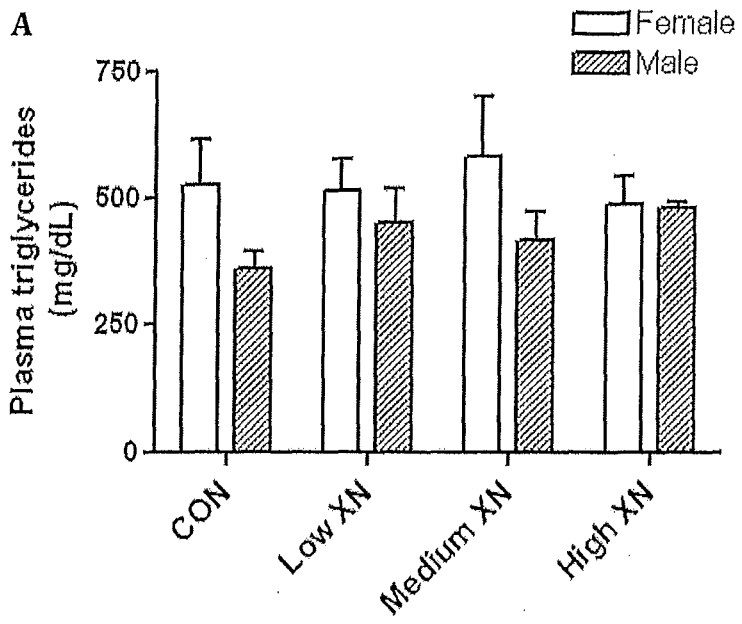

**Figure 3.** Mean plasma glucose levels of male and female Zucker *fa/fa* rats (n=6/group) receiving various daily doses (0, 1.86, 5.64, 16.9 mg/kg BW) of xanthohumol for 6 weeks. Statistical difference from the control group is denoted by ($p < 0.05$). All values are expressed as mean ± SE.

### 3.3 Plasma Insulin, Triglycerides and Cholesterol

Insulin is a peptide hormone produced in the pancreas by beta cells and is a regulator of carbohydrate and fat metabolism in the body. Insulin in the body causes cells of the liver, skeletal muscles and fat tissues to absorb glucose from the blood. Once in the liver and skeletal muscles, glucose is stored as glycogen, and it is stored as triglycerides in fat cells. Cholesterol is considered within the class of lipid molecules. As biomarkers of metabolic syndrome, we analyzed insulin, triglycerides and total cholesterol levels in the plasma of control and XN-treated rats. Plasma triglycerides (Figure 4A) and total cholesterol (Figure 4B) were not affected by XN treatment. However, plasma insulin levels were significantly decreased in male rats treated with medium- and high-dose XN but not in females (Figure 4C). The reduction in plasma glucose in the high-dose XN male group may be explained in part by alleviating the hyperinsulinemia or insulin resistance in these obese male rats. The control male rats had much higher levels of plasma insulin as compared to the control female rats (Figure 4C). Plasma cholesterol was also significantly higher in control males than in control females (Figure 4B).

Although there was no effect of high XN on the triglycerides levels in male rats in our study, Kirkwood et.al. [30] found that XN decreased fasting plasma dicarboxylic acids and medium-chain acylcarnitines in these male rats, suggesting that XN alleviates dysfunctional lipid oxidation. These metabolites are considered markers of dysfunctional lipid metabolism or dysfunctional mitochondria. Fatty acids are transported across the mitochondrial membrane as

acylcarnitines, which are produced by the reaction of fatty acids with carnitine mediated by carnitine acyltransferases such as carnitine palmitoyltransferase 1 (CPT1). Acylcarnitines become elevated in plasma when there is incomplete fatty acid oxidation because of mitochondrial dysfunction. Therefore, using total plasma triglyceride levels alone may not be a satisfactory biomarker for examining the beneficial effects of XN on dysfunctional lipid metabolism.



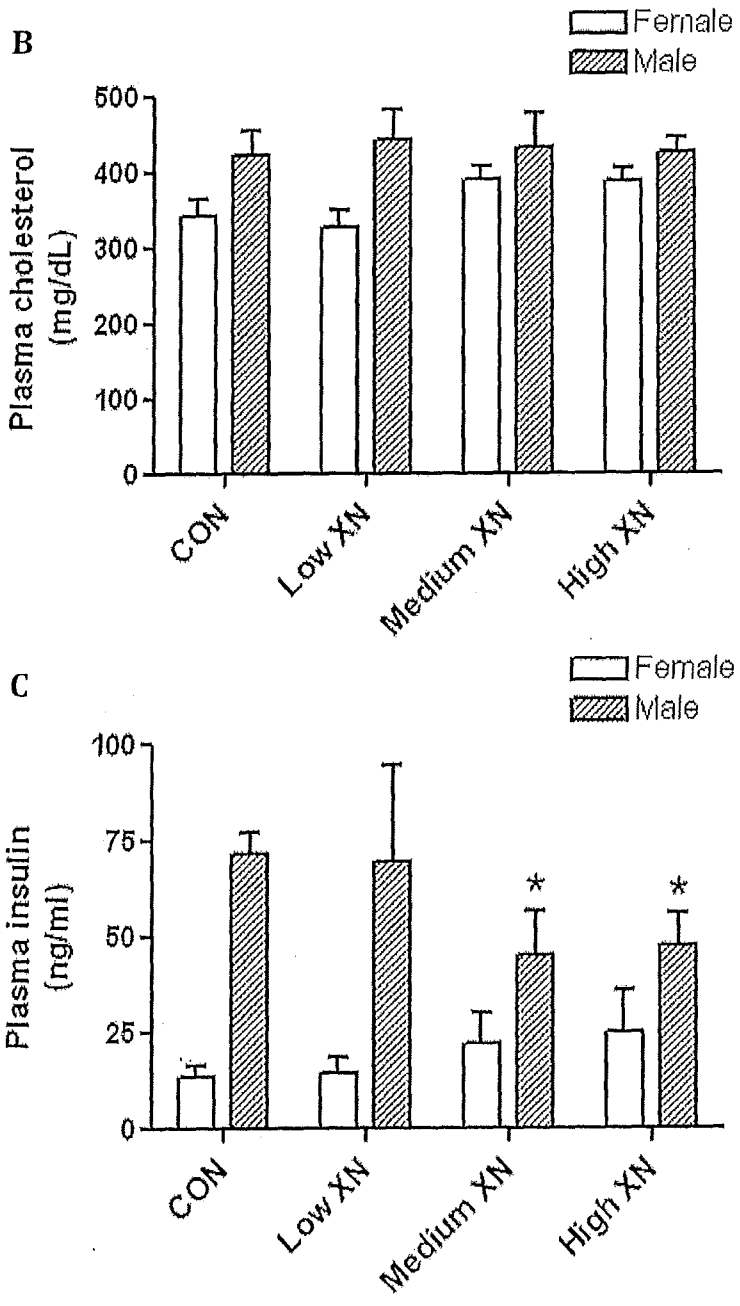



**Figure 4.** Mean plasma triglyceride (A), cholesterol (B) and insulin (C) levels of male and female obese Zucker *fa/fa* rats receiving various doses (0, 1.86, 5.64, 16.9 mg/kg BW) of xanthohumol 3 for 6 weeks. A data point for insulin that was more than four standard deviations from the mean of the group average was excluded from further statistical analysis. All values are expressed as mean ± SE.

### 3.4 Western Blotting AMPK and Akt

To understand the mechanisms by which XN decreases plasma glucose levels in male rats, we tested for protein levels of AMPK, pAMPK, Akt, and pAkt in male rat livers by Western blotting. The enzyme 5' adenosine monophosphate-activated protein kinase (AMPK) plays a role in cellular energy homeostasis [31]. It is expressed in different tissues such as the liver, brain, and skeletal muscle. The main role of AMPK activation is the stimulation of hepatic fatty acid oxidation and ketogenesis, improved binding of insulin to insulin receptors, inhibition of cholesterol synthesis, lipogenesis and triglycerides synthesis, inhibition of adipocyte lipolysis and lipogenesis, inhibition of skeletal muscle fatty acid oxidation and muscle glucose uptake, and modulation of insulin secretion by pancreatic beta-cells. Activation of AMPK (phosphorylation of AMPK) leads to suppression of glucose production by the liver (gluconeogenesis) through increased expression of SHP which in turn inhibits the expression of the hepatic gluconeogenic genes PEPCK and G6Pase [32]. A serine/threonine protein kinase (Akt) that is a member of the AGC kinase family (PKA, PKC, PKG), also known as Protein Kinase B (PKB), is a protein kinase that plays a role in multiple cellular processes like glucose metabolism, and apoptosis [33]. The phosphorylation of Akt can affect numerous downstream targets such as insulin activation, survival, protein synthesis and proliferation. Additional research needed to find the possible link between AMPK activation by XN supplementation. In the ApoE- deficient mouse model, supplementation of XN increases the activation hepatic AMPK which could be due to inhibition of Akt

[33]. In our present study, XN treatment on male rats did not produce a consistent reduction in the levels of liver Akt (Figure 5B). However, XN treatment of male rats unexpectedly decreased the levels of liver AMPK (Figure 5A). Unfortunately, the effects of XN on the levels of pAMPK and pAkt could not be evaluated because the Western blots did not detect any bands for these protein targets. This could have been due to the antibodies used, or that in our rat model, phosphorylated forms of AMPK and Akt are undetectable. In the Apo-E mouse model, pAMPK was also not detected by Western blotting in controls but were markedly increased by XN treatment [33]. Further work needs to be done to confirm the effects of XN on the activation of AMPK and Akt in the rat obese model using mass spectrometry to detect phosphorylated AMPK and phosphorylated Akt.

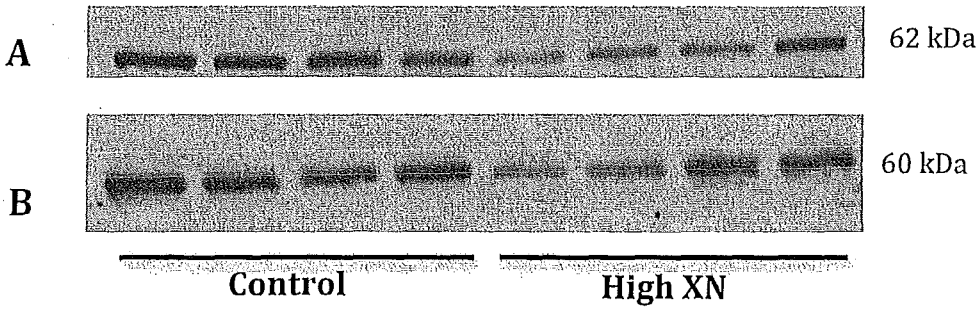



**Figure 5**. AMPK (A) and Akt (B) levels in liver samples of obese male rats as determined by Western blotting.

## CONCLUSIONS

In this study, we analyzed the effects of dietary XN supplementation with on components of metabolic syndrome using an obese rodent model Zucker *fa/fa* rats. XN was found to reduce body weight, plasma glucose and plasma insulin levels in male, but not in females rats. There was no effect of XN on plasma triglyceride and total cholesterol levels in both males and females. Overall, our study has shown that ingestion of XN through the diet for six weeks in obese male rats showed a beneficial effect on metabolic syndrome. Further research needs to be done with human subjects to test for any side effects. Also, further research is needed to understand the mechanism behind XN affecting male rats but not female rats.

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# Xanthohumol Effects on Biomarkers of Cardiovascular Disease, Obesity, and Type 2 Diabetes in Obese Rats

Arlyn Y. Moreno Luna[1], LeeCole Legette[2,3], Ralph Reed[2,3], Cristobal Miranda[2,3], Rosita Rodriquez-Proteau[3], and J. Fred Stevens[2,3]

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## Introduction

Obesity is one of the leading causes of preventable death. Worldwide, there is an epidemic of obesity and diabetes associated with an increase in the development of cardiovascular disease (CVD) and metabolic syndrome. CVD is the leading cause of death globally causing an estimated 17.1 million deaths per year – approximately 29% of all deaths.[1] Recent findings suggest that dietary botanicals may act as a preventive and/or mitigating agent in the progression of several chronic diseases. Specifically, flavonoid consumption has been associated with numerous health benefits including decreases in blood pressure, body weight, and cholesterol levels.[2] Xanthohumol (XN), a prenylated flavonoid, has been shown to lower plasma triglycerides and glucose levels. However, there is no information on the effects of chronic supplementation with XN.

The purpose of this study is to determine the effect of dietary supplementation of XN at various doses on metabolic syndrome and associated conditions utilizing an obese rodent model.



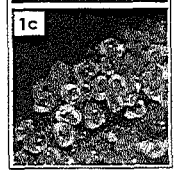
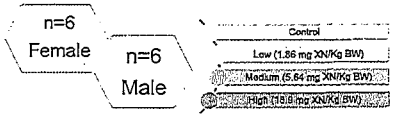



**Figure 1A.** *Humulus lupulus* (Hops plant)
**Figure 1B.** Female hops flower
**Figure 1C.** Lupulin glands

## Study Design

A chronic feeding study was conducted in four-week old male and female obese Zucker *fa/fa* rats. Body weight and food intake were recorded weekly. Plasma glucose, triglycerides, and insulin levels were assay using a commercial kit.

**Treatment Groups**




**Diet**


## Results

**Statistics.** Data were analyzed using SAS (Version 9.1, SAS Institute, Cary, NC). One-way ANOVA was used in the analysis. Treatment effects were determined by ANOVA and post-hoc multiple comparison testing using Dunnett's test. Significance was set at $p<0.05$.

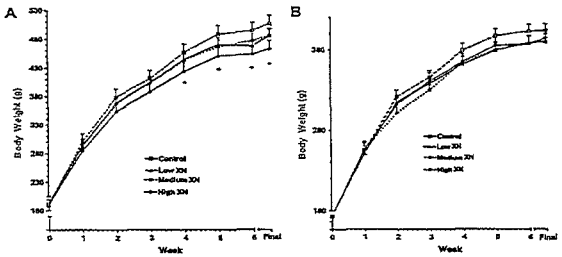



**Figure 2.** Mean body weight levels of (A) male and (B) female obese Zucker *fa/fa* rats (n=6/group) receiving various daily doses (0, 1.86, 5.64, 16.9 mg/kg BW) of Xanthohumol (XN) for 6 weeks. Statistical trends are denoted by + and * ($0.05 < p < 0.10$) and difference ($p < 0.05$) from the control group, respectively. All values are expressed as mean ± SE (Legette, 2012).

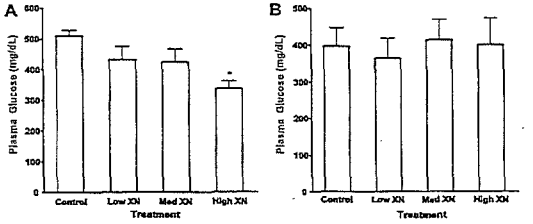
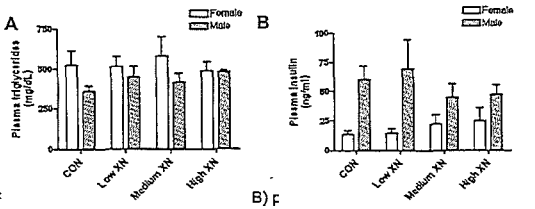
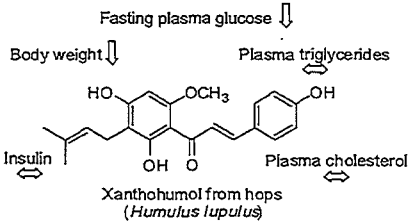



Figure 3. Mean plasma glucose levels of (A) male and (B) female Zucker *fa/fa* rats (n=6/group) receiving various daily doses (0, 1.86, 5.64, 16.9 mg/kg BW) of Xanthohumol (XN) for 6 weeks. Statistical difference from the control group is denoted by *. All values are expressed as mean ± SE (Legette, 2012).




F B) F
female obese Zucker *fa/fa* rats receiving various doses (0, 1.86, 5.64, 16.9 mg/kg BW) of Xanthohumol (XN) for 6 weeks. Error bars indicate standard deviation.

## Conclusion




- There was no difference in food intake among treatment groups
- A high dose of xanthohumol lower body weight in obese male rats
- Plasma glucose levels were also significantly ($p < 0.05$) decreased in the high dose group compared to control group

## Acknowledgements

- National Institute of Health (R21AT005294, S10RR022589, and P30ES000210
- A sincere thank you to: LeeCole L. Legette, Cristobal L. Miranda, and J. Fred Stevens

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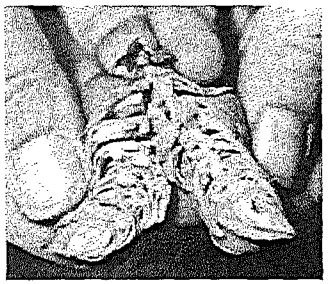
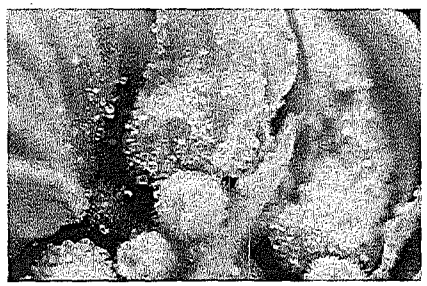
Oregon State University

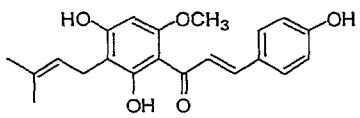
The Linus Pauling Institute

Nobel Peace 1962 Nobel Chemistry 1954

# Effects of Xanthohumol on Biomarkers of Metabolic Syndrome in Obese Rats



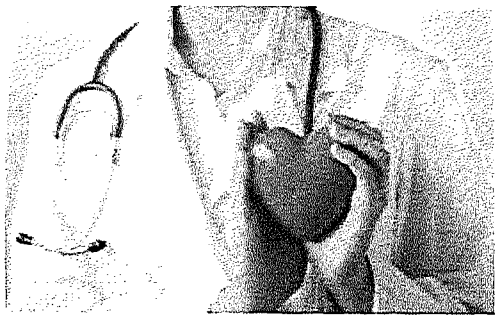





xanthohumol (XN) (1)

Arlyn Y. Moreno Luna
Bioresource Research
PI: Fred Stevens

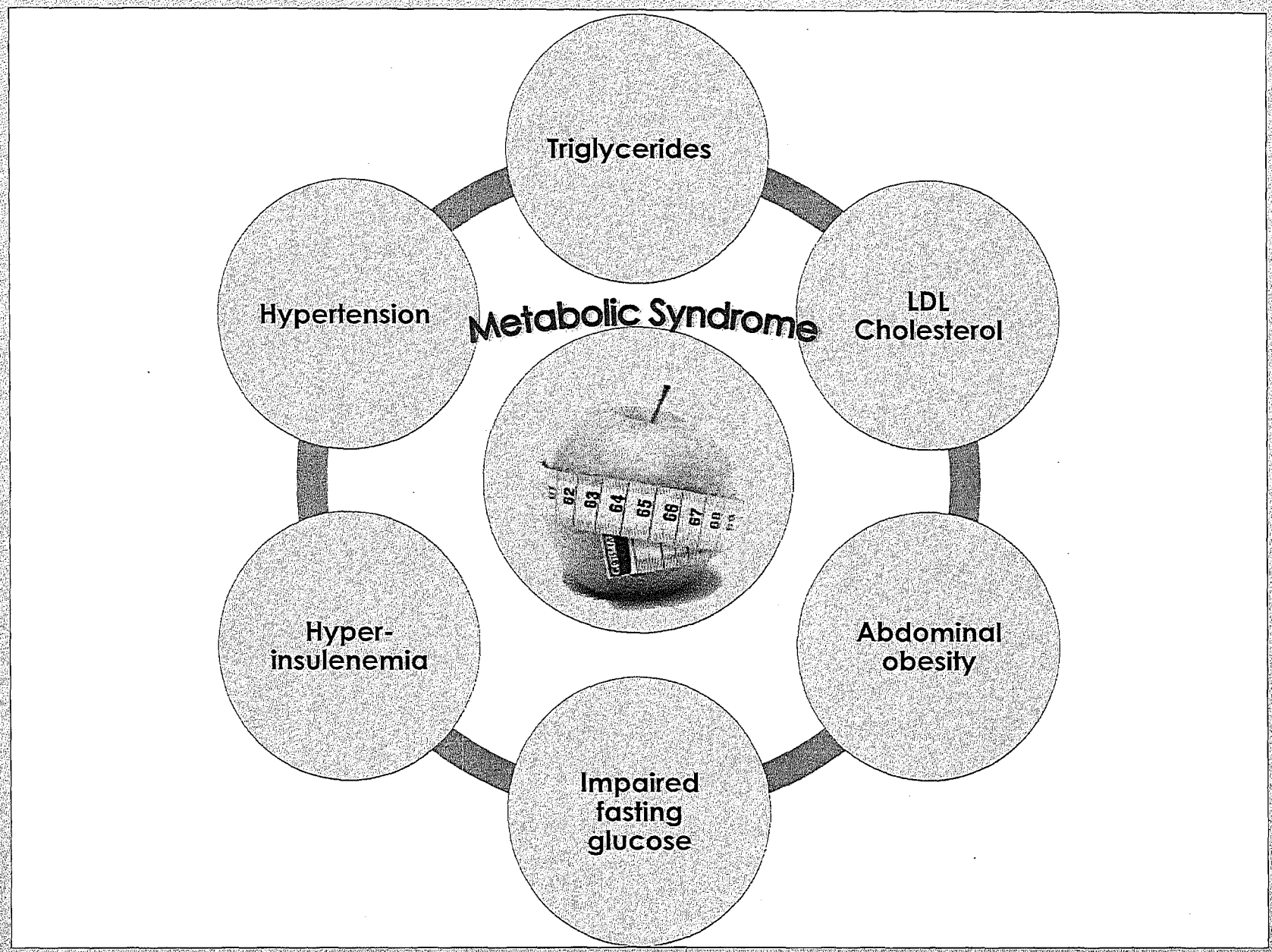
# Health Concern



- Worldwide, there is an epidemic of obesity and type II diabetes associated with an increase in the development of metabolic syndrome (MS)
- About 12 million (16.9%) of U.S. children ages 2-19 are obese
- Over one-third (33.7%) of U.S adults are obese (nearly 75 million adults)
- Type II diabetes is the most common type of diabetes

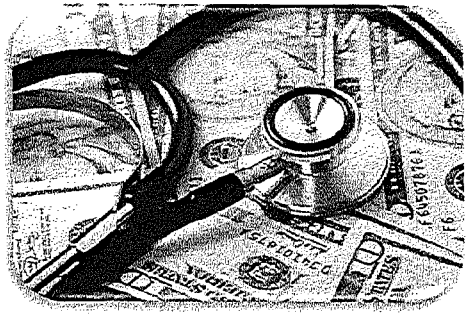
# Metabolic Syndrome

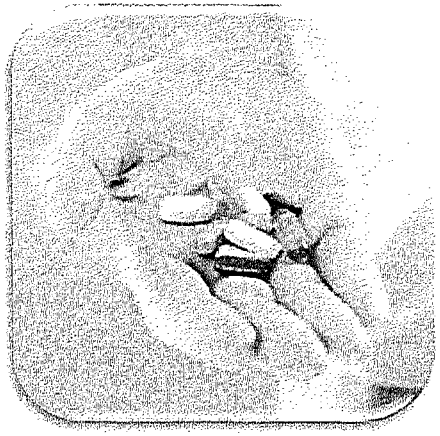
- **Metabolic Syndrome (MS)**- is a name for a group of risk factors that occur together and increase the risk for coronary artery disease, stroke, obesity, and type 2 diabetes
- Almost 40% of U.S. adults were classified as having the metabolic syndrome


Triglycerides
Hypertension
Metabolic Syndrome
LDL Cholesterol
Hyper-insulenemia
Abdominal obesity
Impaired fasting glucose

# Obesity

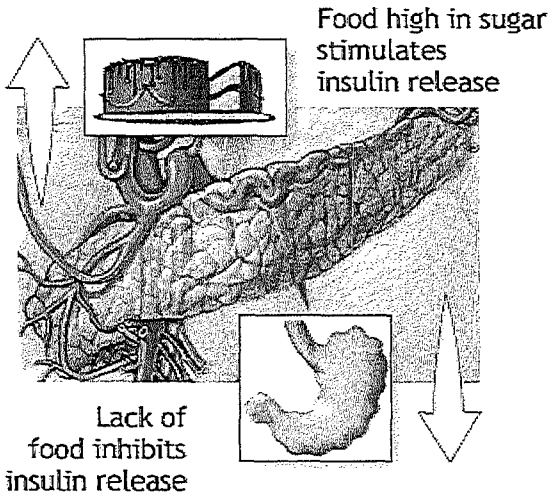
- It is considered to be the main cause of metabolic syndrome
- Is one of the leading causes of preventable death
- Direct health-care costs from obesity and/or related disorders are estimated to be 7-10% of all US health care expenditures annually (Trongdon et.al. 2012)

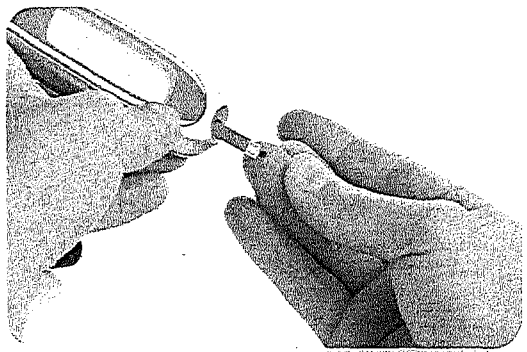




# Type II Diabetes

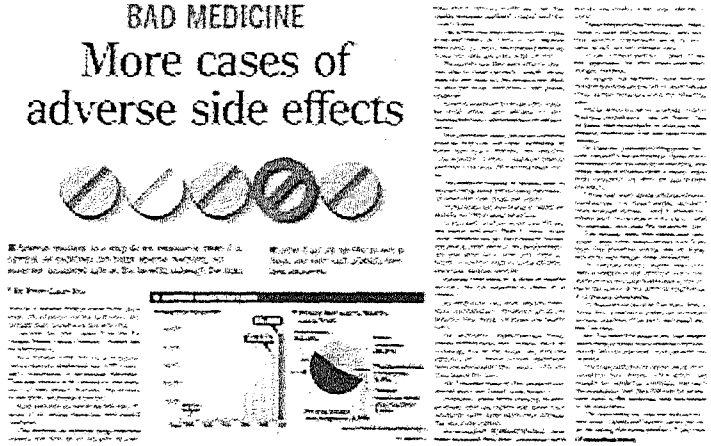
- Also known as non-insulin dependent diabetes mellitus, and is characterized by insulin resistance and/or abnormal production of insulin secretion resulting in hyperinsulinemia



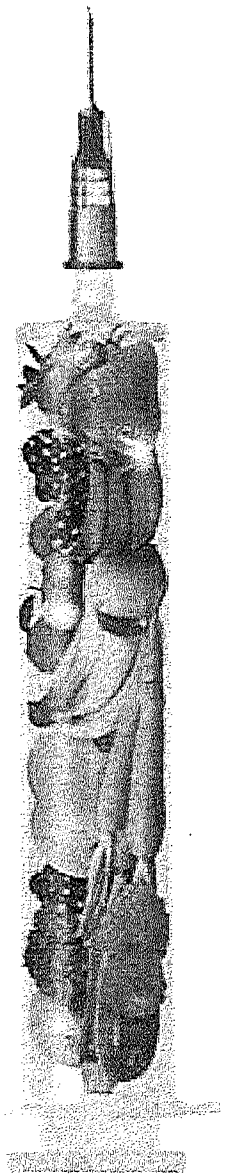
# Current Treatments

- Exercise, lose weight, and healthy diet
- Drugs in the market that can treat conditions contributing to MS: acarbose (Precose), metformin (Glucophage) and rosiglitazone (Avandia)
- Most of current drugs have several side effects



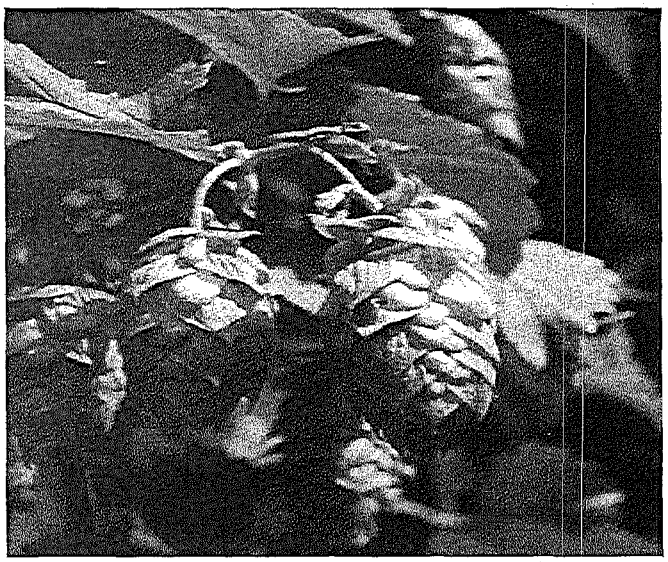
# Past Research

- **Dietary bioactives** may act as preventive and/or mitigating agents in the progression of atherosclerosis and cardiovascular health (Ferrari, 2004)
- **Flavonoids** has been shown show promise for mitigating and/or preventing chronic conditions associated with metabolic syndrome, in rats and mice
- **Flavonoids** come from large family of compounds synthesized by plants
  - ✓ Vegetables
  - ✓ Fruits
  - ✓ Chocolate
  - ✓ Teas
  - ✓ Soybeans

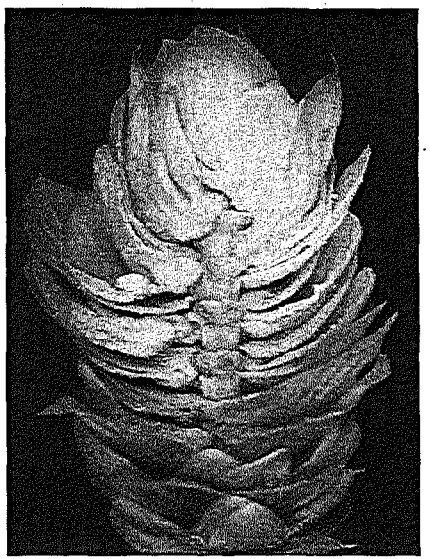


# Xanthohumol

- **Xanthohumol (XN)** is the major prenylated flavonoid of the hops plant
- **Hops** are used in the brewery industry to give beer its special characteristics such as flavor and aroma



Hop plant

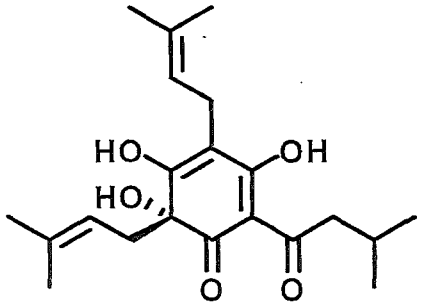


Female Hop flower

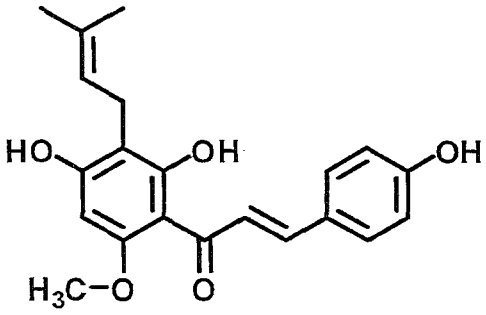
Glandular trichomes covering flower bracts

# Isomerization

- Chemical changes of hop compounds during the brewing process

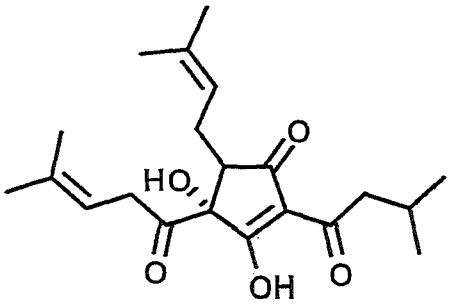



Humulone (α-bitter acid)

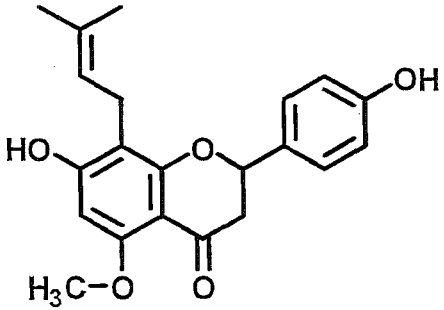



Xanthohumol

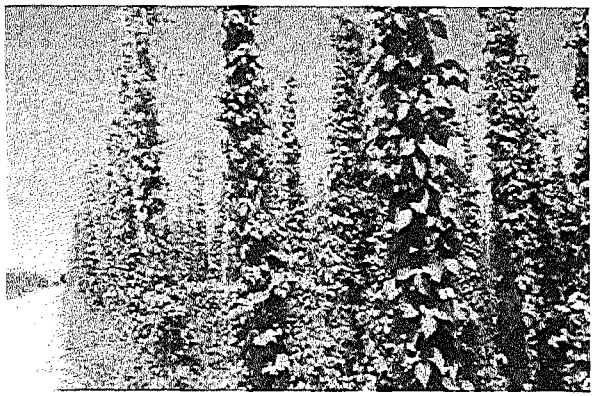
*Isomerization in the brew kettle*



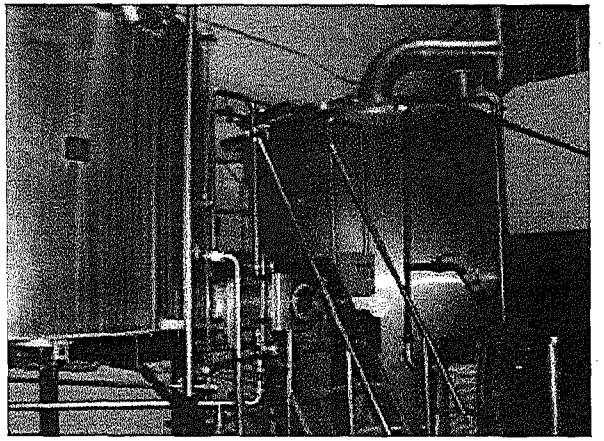

Isohumulone (iso-α-acid)




Isoxanthohumol

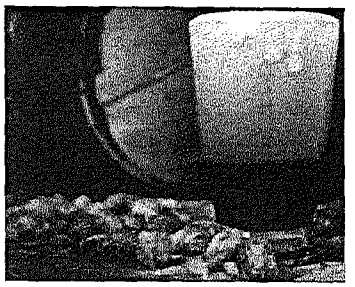


*Courtesy of Hopsteiner*



*Courtesy of Hopsteiner*

# XN in Beer



Prenylflavonoid contents in hops and beer measured by LC–MS/MS [adapted from (Stevens et al., 1999b)]

| | Xanthohumol | Isoxanthohumol | 8-Prenylnaringenin | Desmethylxanthohumol |
|---|---|---|---|---|
| Hop cones (% dry wt): | 0.48[a] | 0.008 | 0.002 | 0.12 |
| Beer (μg/L)[b]: | Xanthohumol | Isoxanthohumol | 8-Prenylnaringenin[d] | Total[c] |
| US major brand | | | | |
| Lager/pilsner | 34 | 500 | 13 | 590 |
| Lager/pilsner | 9 | 680 | 14 | 750 |
| Lager/pilsner | 14 | 400 | 17 | 460 |
| Lager/pilsner | – | – | – | – |
| Northwest/US microbrews | | | | |
| American porter | 690 | 1330 | 240 | 2900 |
| American hefeweizen | 5 | 300 | 8 | 330 |
| Strong ale | 240 | 3440 | 110 | 4000 |
| India pale ale | 160 | 800 | 39 | 1160 |
| Imported beers | | | | |
| European stout | 340 | 2100 | 69 | 2680 |
| European lager | 2 | 40 | 1 | 43 |
| European pilsner | 28 | 570 | 21 | 680 |
| European pilsner | 12 | 1060 | 8 | 1100 |
| Other | | | | |
| Non-alcohol beer | 3 | 110 | 3 | 120 |

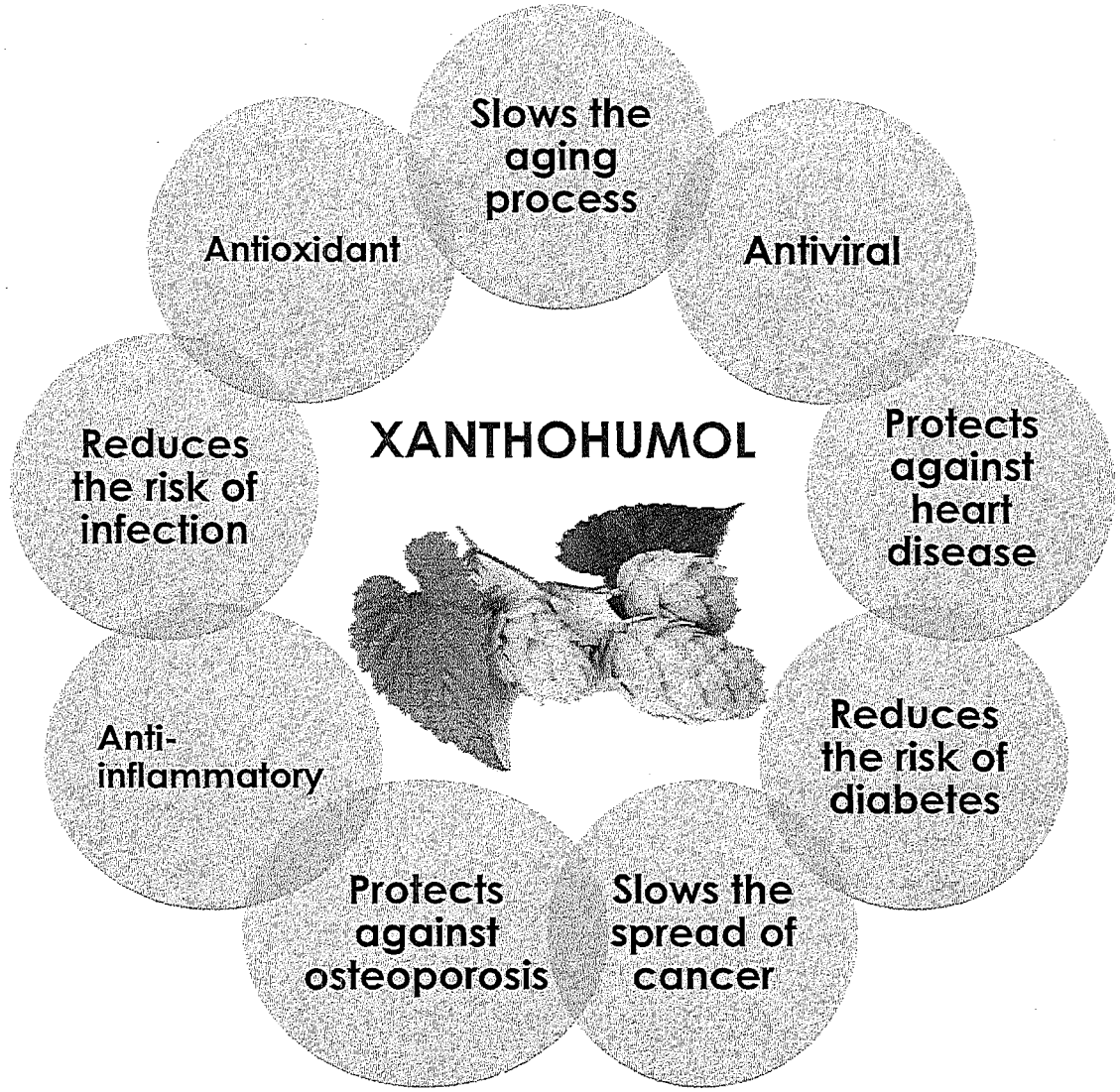
[a] The content of xanthohumol in hops can vary from 0.1% or less for aged hops to over 1% for high xanthohumol-producing varieties.

[b] Most beers contain no desmethylxanthohumol due to thermal isomerization in the brew kettle.

[c] Minor prenylflavonoids contributing to the total include 6-prenylnaringenin and 6-/8-geranylnaringenin.

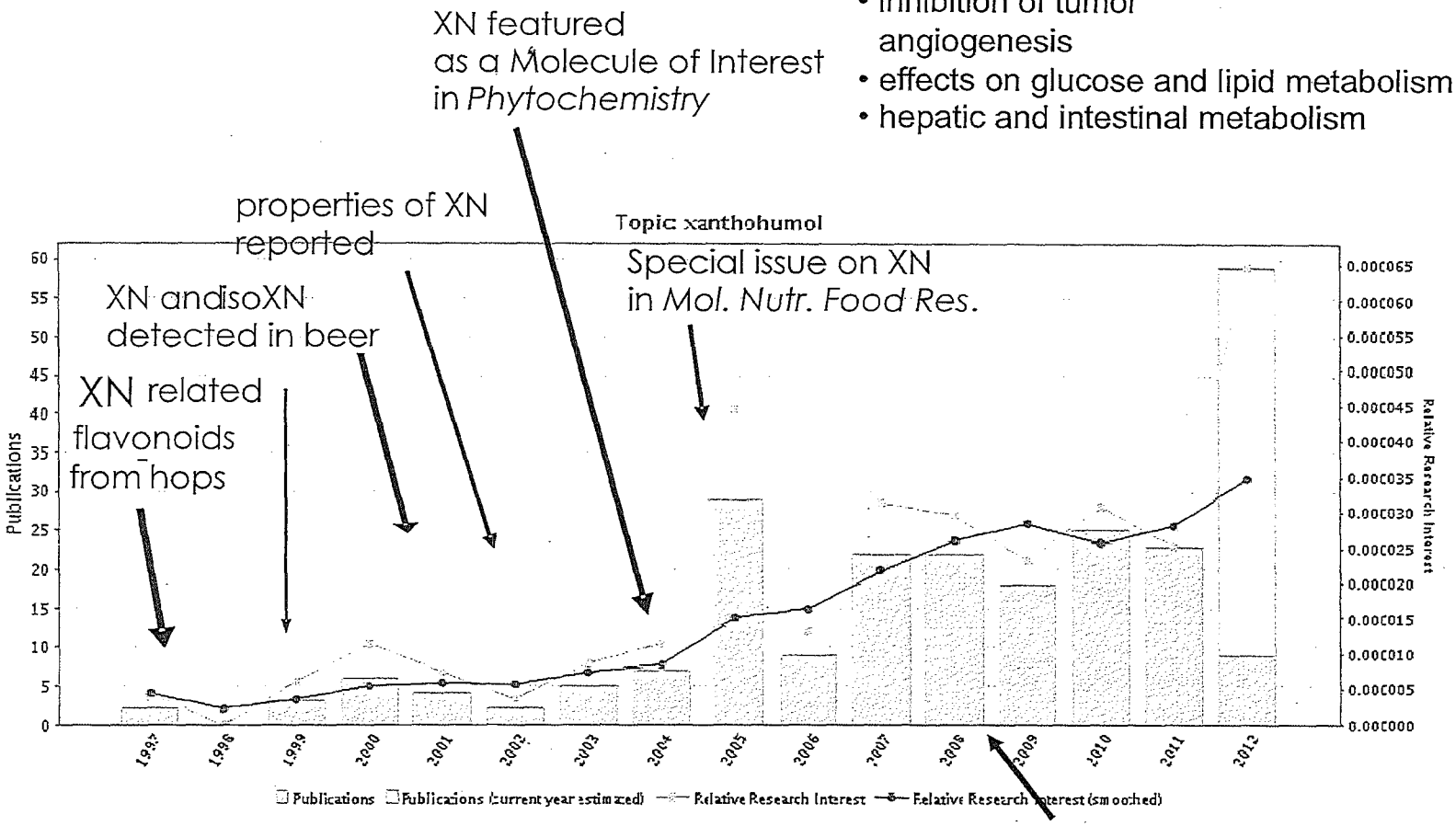
[d] Tekel and co-workers developed a GC–MS method for analysis of 8-prenylnaringenin in beer and found concentrations ranging from 5 (limit of quantitation) to 19.8 μg/L in Belgian and other beers (Tekel et al., 1999).

# Potential Health Benefits of XN



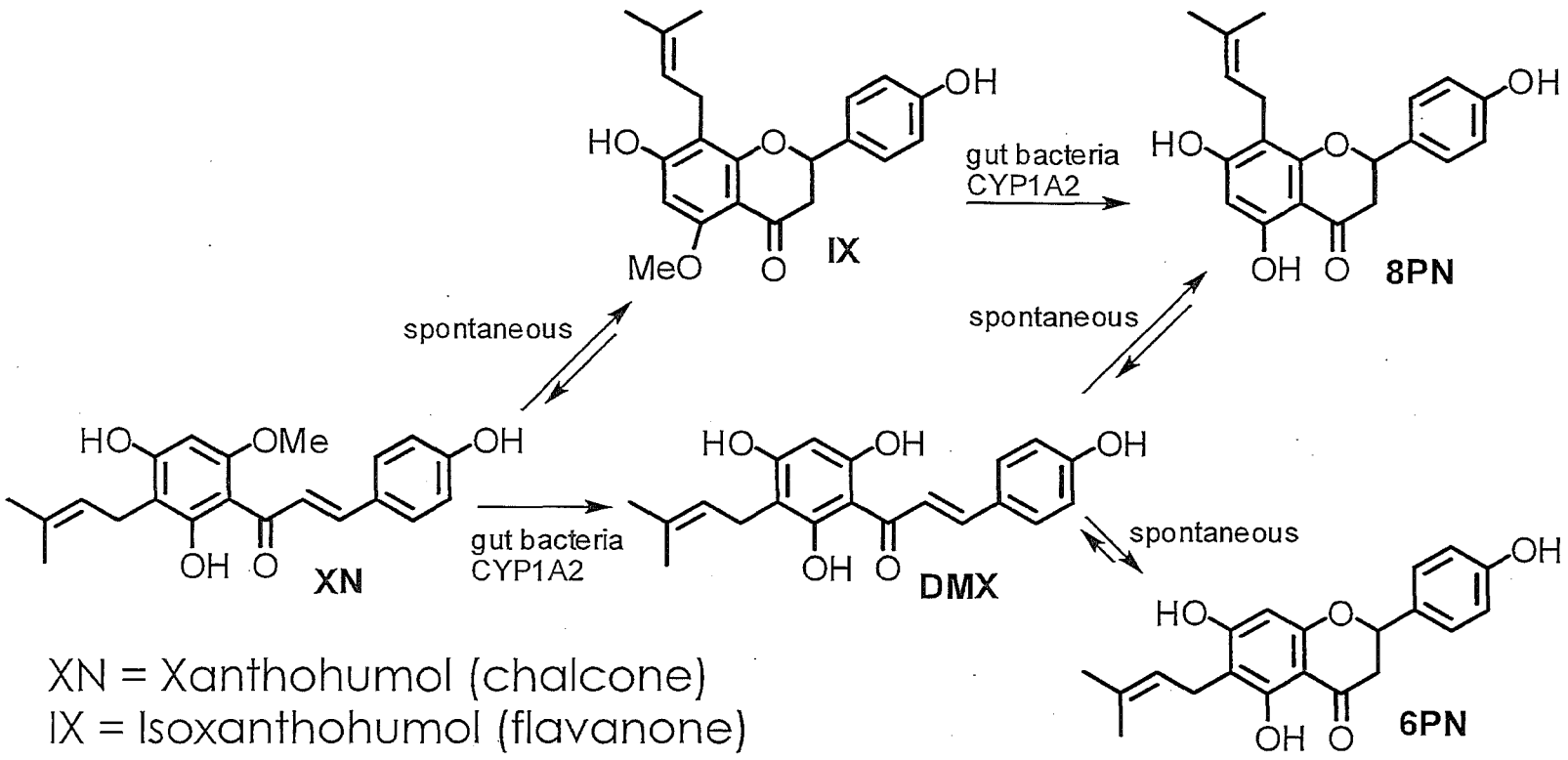
# XN Publications Over Time

**XN bioactivity studies**
- estrogenic effects (HRT)
- cancer chemoprevention
- inhibition of tumor angiogenesis
- effects on glucose and lipid metabolism
- hepatic and intestinal metabolism




(Source: gopubmed.org)

# Metabolism of XN




XN = Xanthohumol (chalcone)
IX = Isoxanthohumol (flavanone)
8PN = 8-Prenylnaringenin (flavanone)
6PN = 6-Prenylnaringenin (flavanone)
DMX = Desmethylxanthohumol (chalcone)

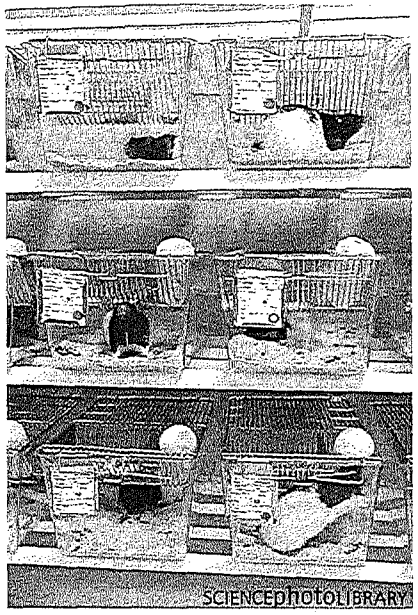
# Hypothesis

- **XN consumption** has positive effects on metabolic syndrome

- **XN consumption** should lower body weight, plasma glucose, plasma insulin, plasma cholesterol and triglyceride levels

# Study Design

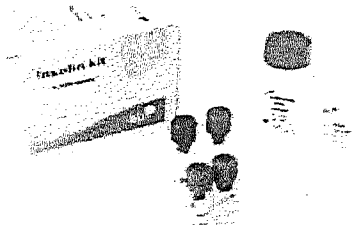
- Six week administration of XN at 3 different doses in Zucker fa/fa rats.
- Animals were housed in individual cages in temperature and humidity-controlled rooms with a 12:12 light:dark cycle ratio
- Food intake and body weight were recorded weekly



# Biomarkers

- Plasma glucose, insulin, triglyceride, and cholesterol levels were determined using commercial assay kits

- Western blotting for rat liver AMPK, Akt, phosphorylated AMPK (pAMPK) and phosphorylated Akt (pAkt) and $\beta$–actin for normalization

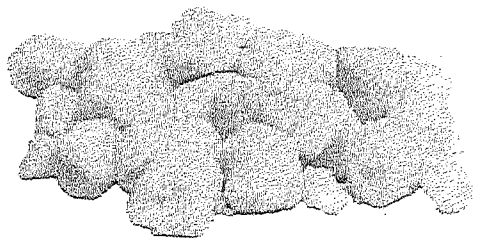


# Feeding Protocol

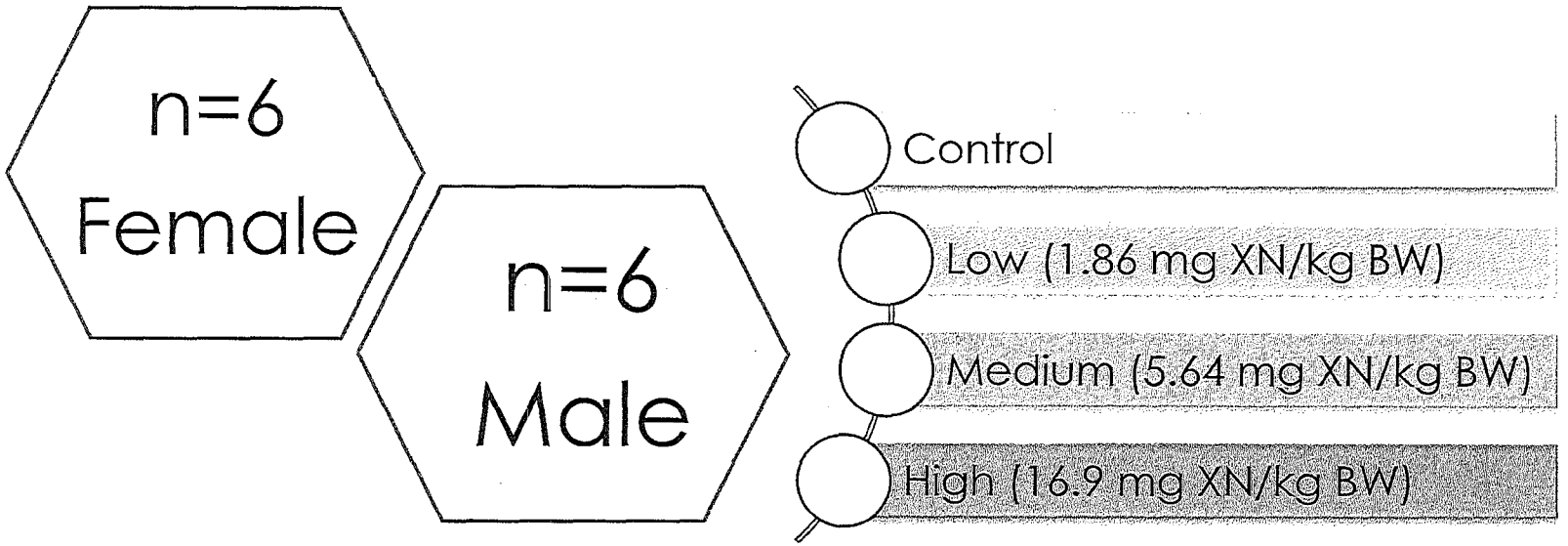
2-Day: Acclimation Period

3 Weeks: High-fat Diet (60% kcal)

3 Weeks: Normal Diet (15% kcal)



# Treatment Groups



# Work Flow

XN Treatment

- 6 weeks
- Food Intake
- Body Weight

Sacrifice

Analysis

# Work Flow

XN Treatment

- 6 weeks
- Food Intake
- Body Weight

**Sacrifice**

- **Body Weight**
- **Blood and Tissue Collection**

Analysis

# Work Flow

XN Treatment

- 6 weeks
- Food Intake
- Body Weight

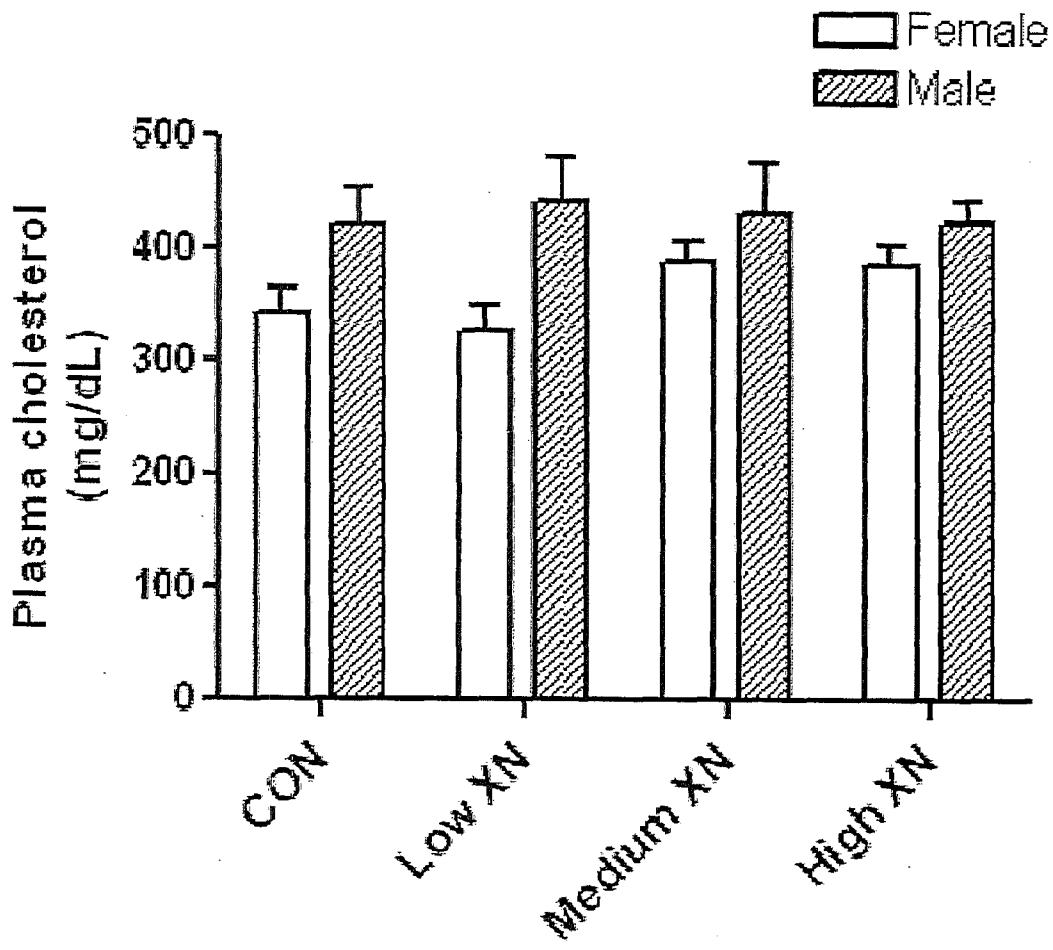
Sacrifice

- Body Weight
- Blood and Tissue Collection

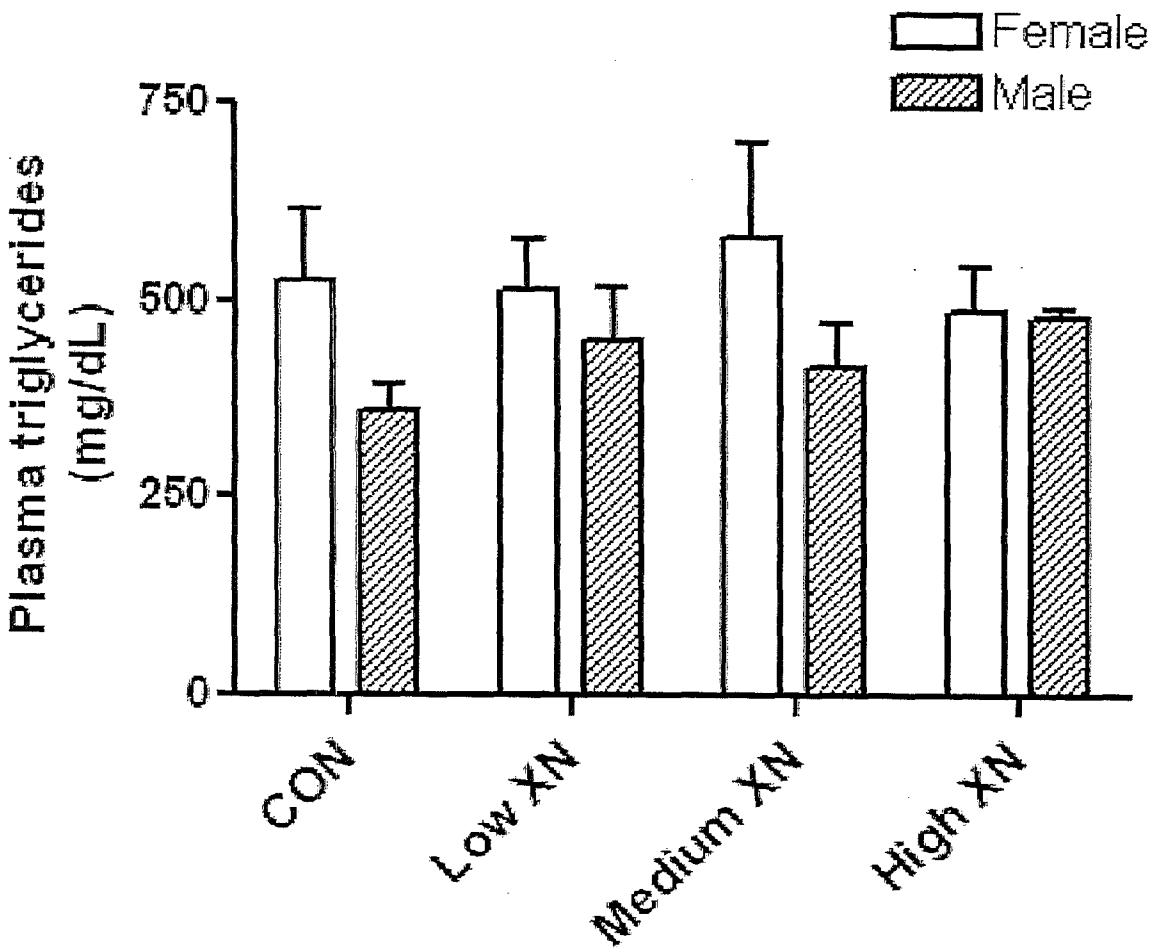
**Analysis**

- **Fasting blood glucose, insulin, cholesterol and triglycerides**
- **Liver- Akt, AMPK, PAkt, PAMPK and $\beta$ -actin**

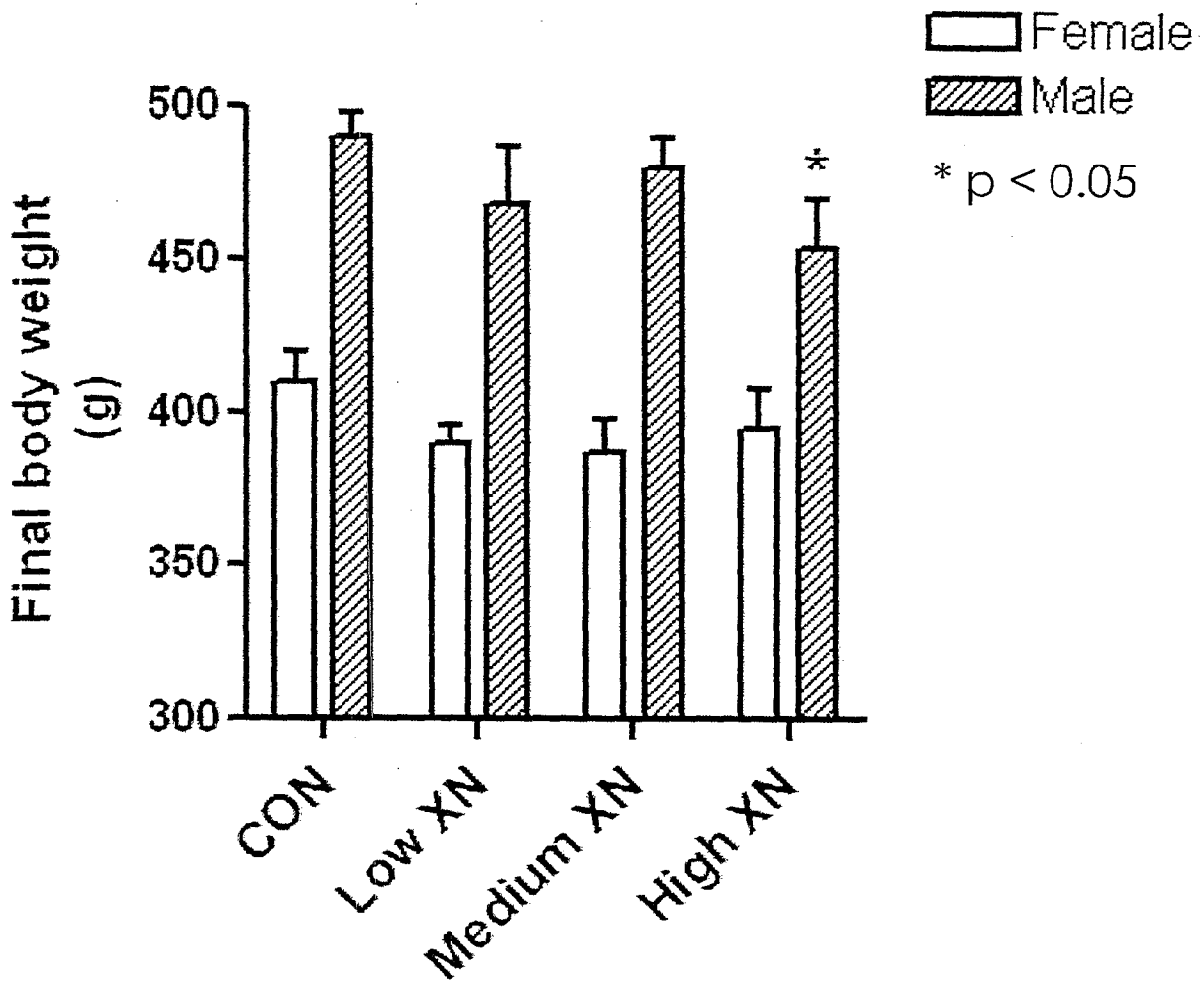
# Plasma Cholesterol



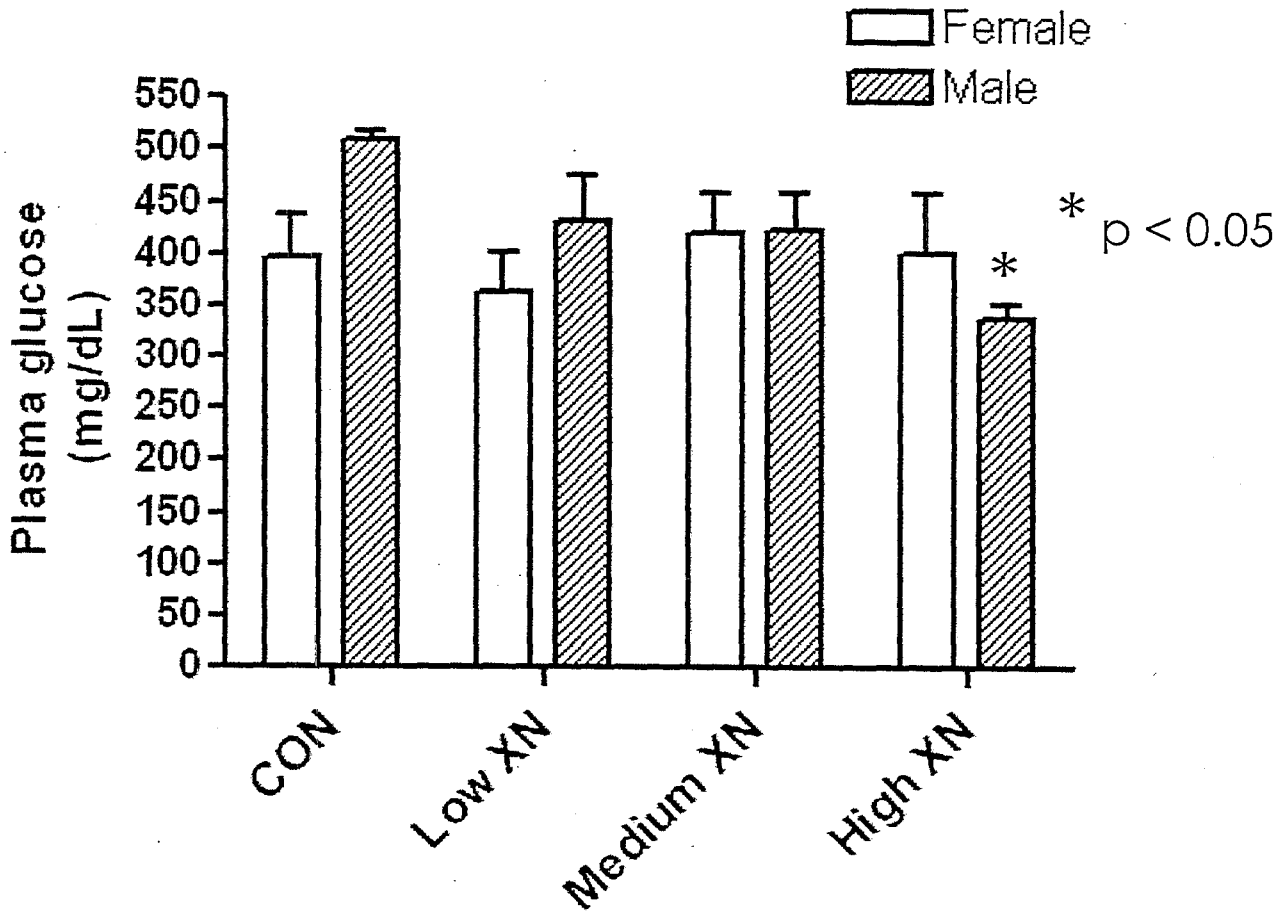
# Plasma Triglycerides



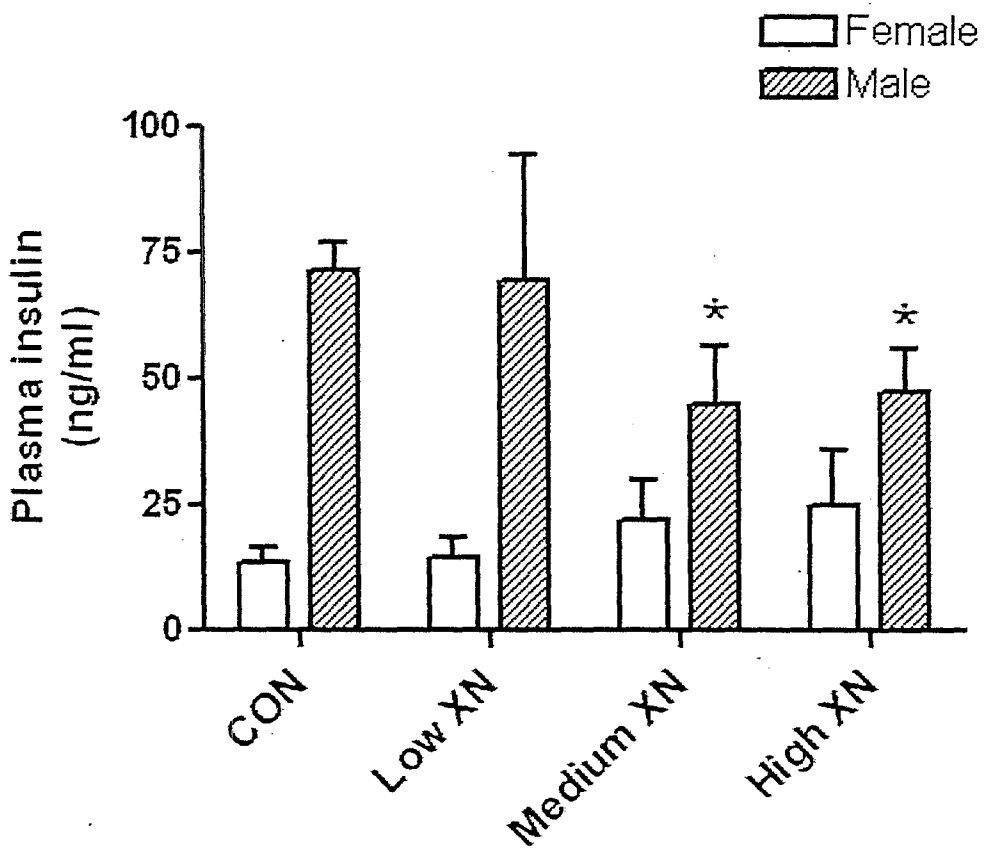
# XN lowers body weight in male rats



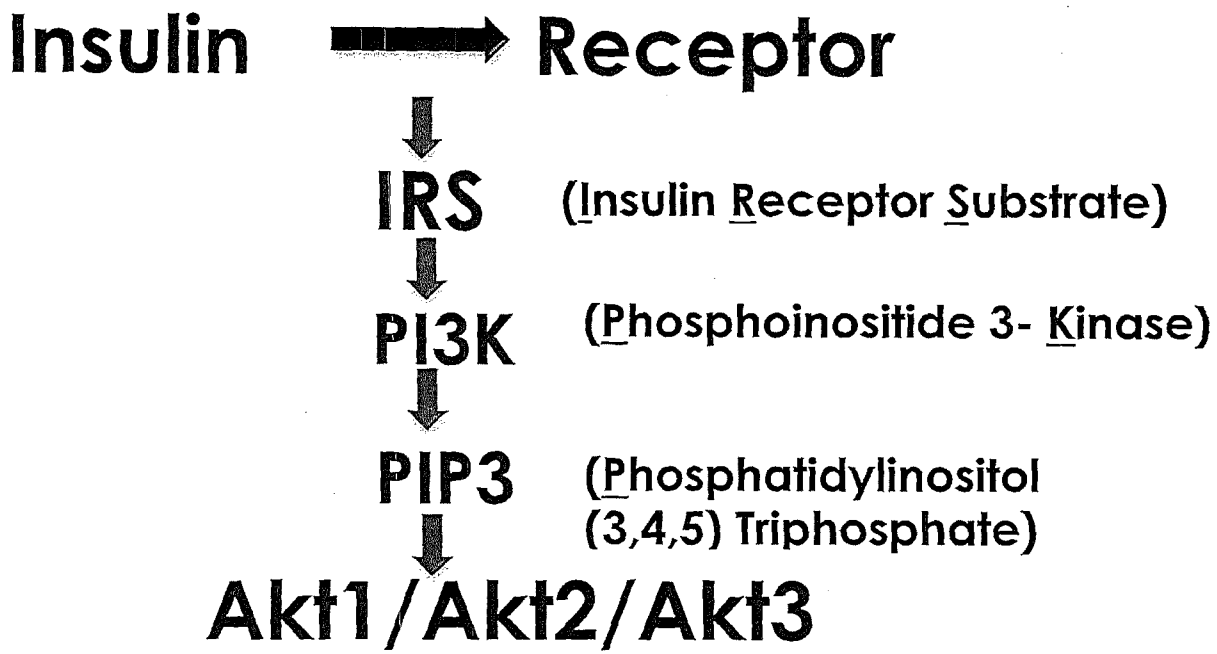
# XN lowers plasma glucose in male rats



# XN lowers plasma insulin in male rats




A data point for insulin that was more than four standard deviations from the mean of the group average was excluded from further statistical analysis.

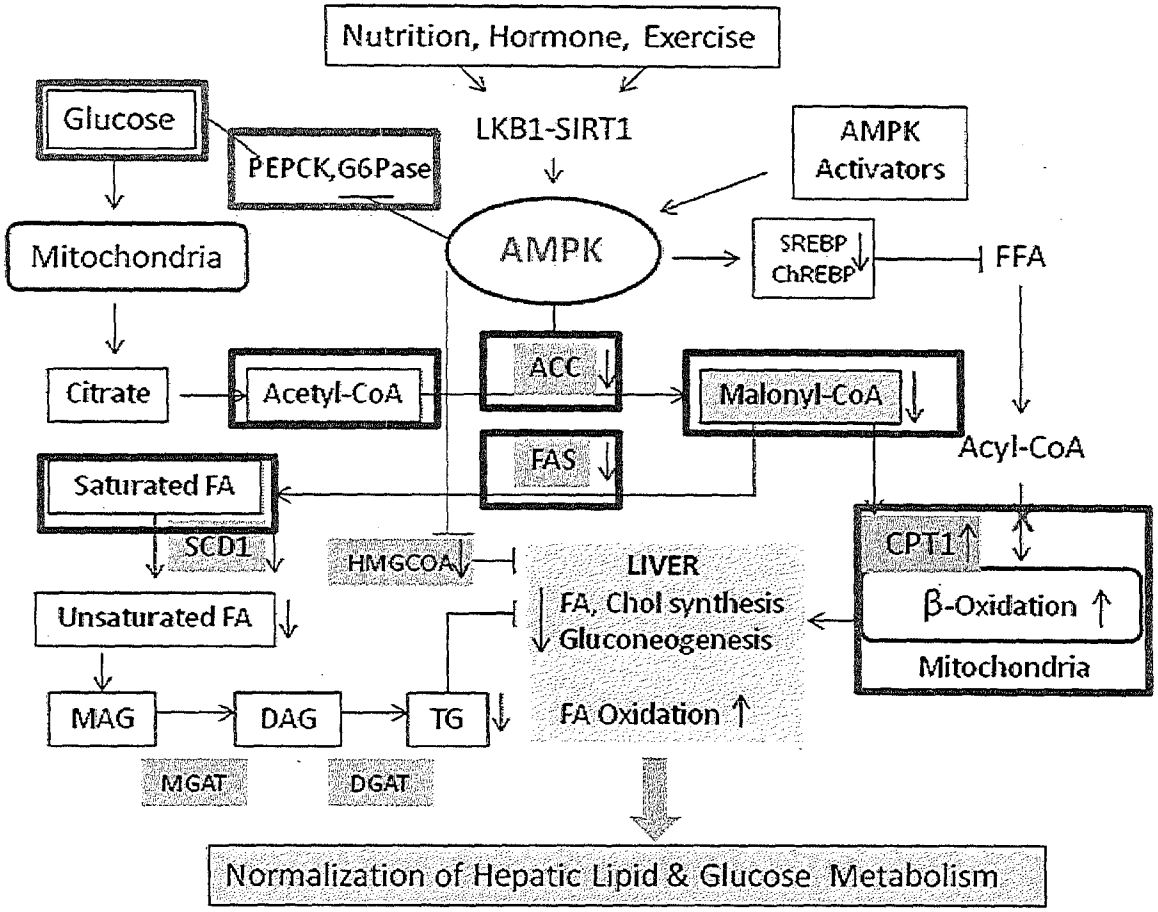



- Insulin binds to alpha subunits of the insulin receptor.
- This causes a conformational change in the insulin receptor that activates the kinase domain.
- The activated kinase domain autophosphorylates tyrosine residues on the C-terminus of the receptor as well as tyrosine residues in the IRS-1 protein.

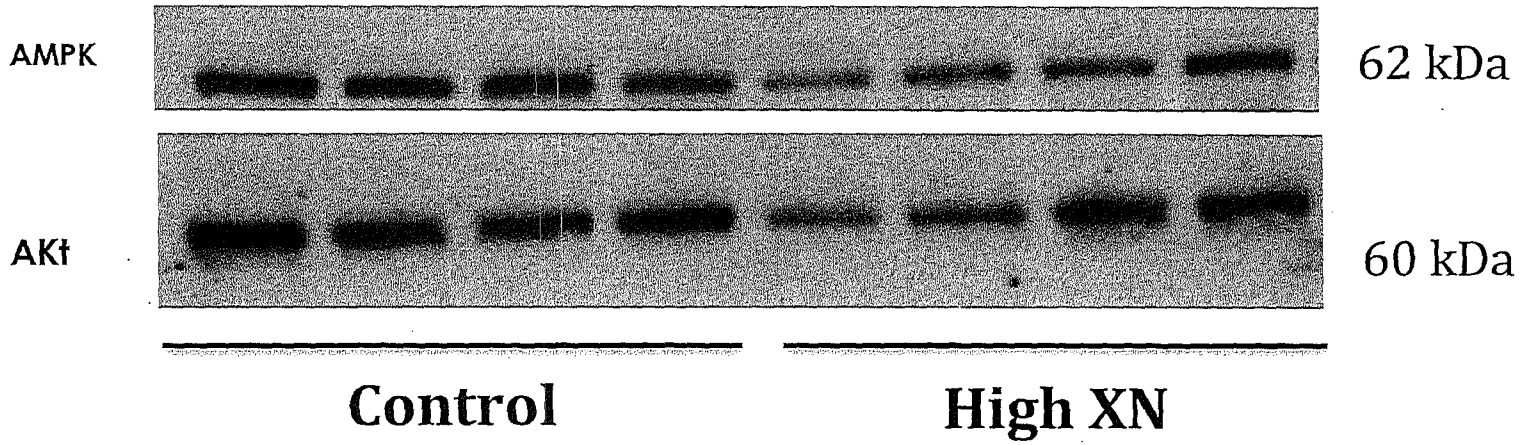
# Role of Akt in glucose homeostasis

- Insulin promotes the activation of Akt, resulting in the phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3) and concomitant activation of glycogen synthase and increased glycogen synthesis. Phosphorylation of glycogen synthase by GSK-3 decreases its activity.
- Akt regulates pancreatic beta-cell mass by stimulating proliferation and survival of $\beta$ cells
- Activated Akt promotes the translocation of glucose transporter GLUT-4 to the plasma membrane leading to increased uptake of glucose in adipose and muscle tissues

# AMPK- Glucose and Lipid Metabolism



# Western Blotting AMPK and Akt



# Conclusion

- XN was found to reduce body weight and plasma glucose in male rats, but not females
- No effect of XN on plasma triglyceride and total cholesterol levels in both males and females.
- XN lowers plasma insulin levels in males, but not on females
- Overall beneficial effect on metabolic syndrome
- We were not able to see the activation of AMPK and Akt by XN

# Further Research

- Needs to be done with human subjects to test for efficacy and safety
- Understand the mechanisms behind XN affecting male rats but not female rats
- Understand the mechanisms by which XN reduces plasma glucose levels in male rats

# Acknowledgements


- Members of Stevens Lab:
  - Dr. Fred Stevens
  - Dr. Cristobal Miranda
  - Dr. LeeCole Legette
- BRR Director Kate Field
- Wanda Crannell
- Friends and family
- Rebekah Lancelin

- **Funding Source:** National Institutes of Health (R21AT005294, S10 RR022589 and P30 ES000210)
- Linus Pauling Institute





# Questions?

Phytochemistry 91 (2013) 236–241

Contents lists available at SciVerse ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

# Xanthohumol lowers body weight and fasting plasma glucose in obese male Zucker fa/fa rats

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ARTICLE INFO

*Article history:*
Available online 27 May 2012

This manuscript is dedicated to memory of late Professor Meinhart H. Zenk (Zenk Memorial issue of Phytochemistry).

*Keywords:*
Hops
*Humulus lupulus*
Cannabaceae
Xanthohumol
Metabolic syndrome
Obesity
Rats
Type 2 diabetes

ABSTRACT

Obesity contributes to increased risk for several chronic diseases including cardiovascular disease and type 2 diabetes. Xanthohumol, a prenylated flavonoid from hops (*Humulus lupulus*), was tested for efficacy on biomarkers of metabolic syndrome in 4 week old Zucker fa/fa rats, a rodent model of obesity. Rats received daily oral doses of xanthohumol at 0, 1.86, 5.64, and 16.9 mg/kg BW for 6 weeks. All rats were maintained on a high fat (60% kcal) AIN-93G diet for 3 weeks to induce severe obesity followed by a normal AIN-93G (15% kcal fat) diet for the last 3 weeks of the study. Weekly food intake and body weight were recorded. Plasma cholesterol, glucose, insulin, triglyceride, and monocyte chemoattractant protein-1 (MCP-1) levels were assessed using commercial assay kits. Plasma and liver tissue levels of XN and its metabolites were determined by liquid–chromatography tandem mass spectrometry. Plasma and liver tissue levels of xanthohumol were similar between low and medium dose groups and significantly ($p < 0.05$) elevated in the highest dose group. There was a dose-dependent effect on body weight and plasma glucose levels. The highest dose group ($n = 6$) had significantly lower plasma glucose levels compared to the control group ($n = 6$) in male but not female rats. There was also a significant decrease in body weight for male rats in the highest dose group (16.9 mg/kg BW) compared to rats that received no xanthohumol, which was also not seen for female rats. Plasma cholesterol, insulin, triglycerides, and MCP-1 as well as food intake were not affected by treatment. The findings suggest that xanthohumol has beneficial effects on markers of metabolic syndrome.



## 1. Introduction

Metabolic syndrome is a condition defined by clinical diagnosis of three or more of the following conditions: abdominal obesity, atherogenic dyslipidemia, insulin resistance and/or impaired glucose tolerance, hypertension, pro-inflammatory state, and prothrombotic state (Grundy et al., 2004). The 2003–2006 National Health and Nutrition Examination Survey (NHANES) established that over 34% of US adults have metabolic syndrome and are at increased risk for cardiovascular disease and type 2 diabetes (Ervin, 2009). Direct health care costs arising from obesity and/or related disorders are estimated to be 7–10% of all US health care expenditures annually (Trogdon et al., 2012).

Dietary bioactives may act as preventive and/or mitigating agents in the progression of several chronic diseases (Ferrari, 2004; Kris-Etherton et al., 2002; Milner, 2004). In particular, xanthohumol (XN) (**1**), the principal prenylated flavonoid from hops (Stevens et al., 2000), has been shown to have broad biological activity and may have several health benefits (Stevens and Page, 2004). Studies have demonstrated that XN (**1**) may exert beneficial health effects as an antioxidant (Miranda et al., 2000), anti-inflammatory (Lupinacci et al., 2009; Peluso et al., 2010), anti-microbial (Gerhauser, 2005), cancer chemopreventive agent (Colgate et al., 2007; Miranda et al., 1999), and an immune system modulator (Xuan et al., 2010). With growing interest in health promoting abilities of XN (**1**), several researchers have examined aspects of its metabolism. *In vitro* and *in vivo* animal studies have established key steps of XN (**1**) metabolism which are depicted in Fig 1, and have been described previously (Legette et al., 2012).

Emerging evidence on the role of XN (**1**) in cholesterol regulation has led to speculation that it may reduce risk factors associated with metabolic syndrome including hypercholesterolemia

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0031-9422/$ - see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.phytochem.2012.04.018

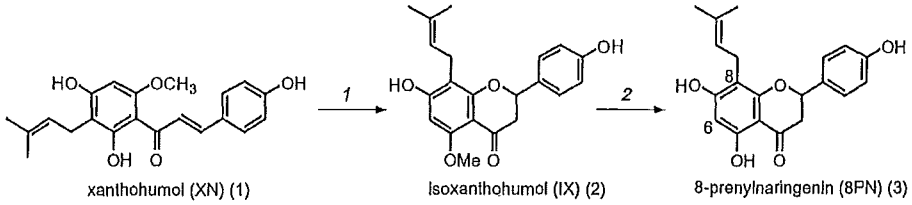


Fig. 1. Metabolic conversion of xanthohumol (XN) (1) into isoxanthohumol (IX) (2) and 8-prenylnaringenin (8PN) (3). Step 1, non-enzymatic cyclization. Step 2, cytochrome P450-mediated demethylation. The plasma metabolite profile showed no support for the alternative metabolic pathway from XN (1) to 6-prenylnaringenin by sequential demethylation and cyclization.

and dyslipidemia. Nozawa identified XN (1) as an agonist of the farnesoid X receptor (FXR), which is involved in cholesterol and bile acid metabolism (Nozawa, 2005). Nozawa also observed that feeding XN (1) for 4 weeks led to lower plasma glucose and triglyceride levels in KK-$A^y$ mice, a model for obesity and type 2 diabetes (Nozawa, 2005). Additional *in vitro* work has shown that it may have anti-obesity effects through its actions on adipocytes. XN (1) inhibited differentiation, reduced proliferation and increased apoptosis in 3T3-L1 (murine preadipocytes) cells (Mendes et al., 2008; Rayalam et al., 2009; Yang et al., 2007).

Dietary flavonoids may have potential as therapeutic agents for metabolic syndrome by influencing factors associated with obesity and type 2 diabetes (Xia and Weng, 2010). Most of the promising results of XN (1) effects on lipid metabolism were observed *in vitro* (Mendes et al., 2008; Rayalam et al., 2009; Yang et al., 2007) with only one *in vivo* study assessing the effects of feeding it via the diet (Nozawa, 2005). The aim of the present study was to determine the effect of oral administration of XN (1) at various doses on biomarkers of metabolic syndrome utilizing an obese rodent model. To our knowledge, this is the first investigation to determine relationships between circulating plasma levels, tissue levels, and effects of XN (1) on biomarkers of metabolic syndrome following chronic administration.

## 2. Results and discussion

### 2.1. Study design

Disadvantages of administering a potential therapeutic agent in the diet include variation in dose, potential negative effect of the test agent on food intake, and effect of the diet on bioavailability of the test agent, all of which may result in difficulties in accurately assessing its biological activities *in vivo*. Based on available safety data on oral doses of xanthohumol (1) in rodents (Vanhoecke et al., 2005), a dose range was selected equivalent to daily oral doses of 20, 60, and 180 mg in humans. Findings from our recent pharmacokinetic study of XN (1) (Legette et al., 2012) predict that the selected doses lead to steady-state plasma levels of XN (1) and metabolites in the high nanomolar to low micromolar concentration range. Using allometric scaling and an interspecies scaling factor (Food and Drug Administration, 2005) for an individual weighing ca. 64 kg (141 lb), this results in equivalent doses of 1.86, 5.64 and 16.9 mg/kg BW for rats (here referred to as low, medium and high doses, respectively). In this study, test meals were utilized to ensure accurate administration of defined XN (1) doses. A test meal is a defined dose incorporated in a compatible matrix for oral consumption. In addition to using test meals, the effect of XN (1) on metabolic syndrome was examined with the use of a genetic model of obesity, the Zucker obese fa/fa (ZOF) rat. ZOF rats have an autosomal recessive mutation that prohibits the production of leptin, a regulatory hormone involved in appetite control and energy metabolism. This mutation in ZOF rats leads to hypercholesterolemia, hyperinsulinemia, and obesity (Zucker and Zucker, 1962).

### 2.2. Body weight and food intake

A dose-dependent effect of XN (1) treatment on body weight was observed in male rats (Fig. 2A). During the last 3 weeks of the study, a significant trend ($0.05 < p < 0.10$) emerged, with male rats on high XN (1) having a lower body weight compared to rats receiving no XN (1). The difference in body weight between

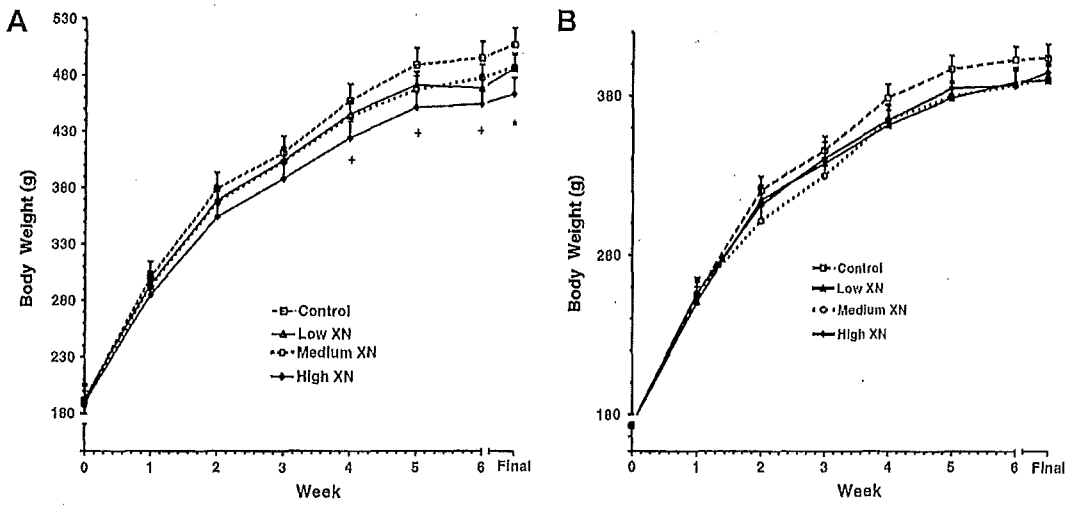


Fig. 2. Mean body weight levels of (A) male and (B) female obese Zucker fa/fa rats ($n = 6$/group) receiving various daily doses (0, 1.86, 5.64, 16.9 mg/kg BW) of xanthohumol (XN) (1) for 6 weeks. + and * denotes a statistical trend ($0.05 < p < 0.10$) and difference ($p < 0.05$) from the control group, respectively. All values are expressed as mean ± SE.

treatments only became significant ($p < 0.05$) at sacrifice indicating that longer treatment duration may be needed to fully evaluate health effects of XN (**1**). Although lower body weight was observed in female rats with XN (**1**) treatment (Fig. 2B), it was not significant suggesting that higher treatment dose of XN (**1**) may be needed to induce significant change in body weight of female rats. Differences in body weight cannot be attributed to changes in food intake, which was not affected by treatment (data not shown). Similar to our findings, others have shown that soy isoflavonoid consumption decreases body weight (Blake et al., 2011; Guo et al., 2009).

### 2.3. Xanthohumol analysis

Plasma levels of XN (**1**) and its metabolites (**2**) and (**3**) for low, medium, and high dose groups are shown in Table 1. There were no detectable plasma levels of XN (**1**) or its metabolites (isoxanthohumol, IX (**2**); 8-prenylnaringenin, 8PN; (**3**) 6-prenylnaringenin, 6PN) in control animals. As observed previously (Legette et al., 2012), the only major metabolites in plasma after XN (**1**) treatment were IX (**2**) and 8PN (**3**) and they were largely present in their conjugated forms. Steady-state plasma XN (**1**) levels observed in this study correspond to predicted values from earlier research. (Legette et al., 2012). As seen in Table 2, projected steady-state plasma levels calculated from the single-dose pharmacokinetics (PK) study are in good agreement with the observed experimental steady-state concentrations measured in this chronic treatment study.

Liver tissue concentrations of XN (**1**), IX (**2**) and 8PN (**3**) are displayed in Table 3, and follow the same trends observed with plasma levels including the presence of only these three metabolites. Plasma and liver concentrations of XN (**1**) and its metabolites (**2**) and (**3**) were higher in female rats compared to males across all treatment groups. This is attributed to the fact that XN (**1**) formulations were based on average body weight of treatment groups including both male and female rats. Since females tended to weigh less than males, they received slightly higher doses at each XN (**1**) level. As expected, both male and female rats consuming high XN (**1**) had significantly higher plasma and liver concentrations of XN (**1**) than all other dose groups. There were no detectable liver tissue levels of 8PN (**3**) for animals receiving low XN (**1**) dose. There was also no detectable liver concentration of IX (**2**) in male rats on the low dose whereas female rats had small amounts of IX (**2**) in liver. Although plasma and liver tissue levels were higher for rats receiving medium XN (**1**) compared to those on low XN (**1**), the differences were not significant, probably due to considerable variation across animals within treatment groups. This suggests that perhaps a larger population size is needed in subsequent studies to thoroughly evaluate the effects of low and medium doses of XN (**1**). Additional investigations are also needed to determine which XN (**1**) metabolite is contributing to health effects of XN (**1**) supplementation. Perhaps the biological effects observed at high XN (**1**) dose are due to increasing levels of 8PN (**3**), which has been shown to have greater estrogenic activity (Bovee et al., 2004; Coldham and Sauer, 2001; Milligan et al., 2002, 1999, 2000) than most flavonoids including its parent compound, XN.

### 2.4. Plasma biochemical markers

Plasma glucose levels were significantly ($p < 0.05$) decreased in the high dose group compared to control but not in the other dose groups. Nozawa and others also observed a decrease in plasma glu-

**Table 1**
Mean plasma concentrations[1] of xanthohumol (XN) and its metabolites (isoxanthohumol, IX, and 8-prenylnarigenin, 8PN) of male ($n$ = 4–6/group) and female ($n$ = 5–6/group) obese Zucker fa/fa rats who received a daily supplementation of xanthohumol (XN) at four dose levels (0, 1.86, 5.64, and 16.9 mg/kg BW) for 6 weeks.

| Treatment | Control (0 mg/kg BW) | Low XN (1.86 mg/kg BW) | Medium XN (5.69 mg/kg BW) | High XN (16.9 mg/kg BW) |
|---|---|---|---|---|
| XN (**1**) (nM) | | | | |
| Male rats | ND | $50 \pm 14^{a}$ | $107 \pm 46^{a}$ | $389 \pm 153^{b}$ |
| Female rats | ND | $75 \pm 28^{a}$ | $240 \pm 83^{a}$ | $555 \pm 228^{b}$ |
| IX (**2**) (nM) | | | | |
| Male rats | ND | $10 \pm 3^{a}$ | $32 \pm 16^{a}$ | $100 \pm 24^{b}$ |
| Female rats | ND | $21 \pm 8^{a}$ | $56 \pm 11^{a}$ | $181 \pm 50^{b}$ |
| 8PN (**3**) (nM) | | | | |
| Male rats | ND | $35 \pm 5^{a}$ | $82 \pm 17^{a}$ | $364 \pm 82^{b}$ |
| Female rats | ND | $40 \pm 7^{a}$ | $111 \pm 31^{a+}$ | $211 \pm 52^{b}$ |
| Total (nM) | | | | |
| Male rats | ND | $95 \pm 21^{a}$ | $221 \pm 68^{a}$ | $853 \pm 178^{b}$ |
| Female rats | ND | $136 \pm 37^{a}$ | $407 \pm 125^{b}$ | $947 \pm 322^{b}$ |

All values are expressed as means ± SE. ND indicates levels were not detectable. Different letters denote statistical differences ($p < 0.05$) and $^{+}$ denotes a significant trend ($0.05 < p < 0.10$) from low XN group.
[1] Plasma concentrations of XN (**1**) and metabolites (**2**) and (**3**) include both free and conjugated forms as determined by LC-MS/MS following sample preparation with enzymatic hydrolysis.

**Table 2**
Mean plasma total concentrations (nM) of xanthohumol (XN) (**1**) and its metabolites (isoxanthohumol, IX (**2**), and 8-prenylnarigenin, 8PN (**3**)) of male rats from a single dose pharmacokinetic and a chronic supplementation study with the following doses of XN (**1**): (0, 1.86, 5.64, and 16.9 mg/kg BW).

| Studies | Low XN (**1**) (1.86 mg/kg BW) | Medium XN (**1**) (5.69 mg/kg BW) | High XN (**1**) (16.9 mg/kg BW) |
|---|---|---|---|
| Single dose pharmacokinetic (PK) study[1] maximum concentration, $C_{max}$ | 191 ± 20 | 434 ± 18 | 1510 ± 70 |
| Projected steady state levels from PK study[1] | 94 | 129 | 213 |
| Chronic supplementation study | 50 ± 14 | 107 ± 46 | 389 ± 153 |

All values are expressed as mean ± SE.
[1] Results from a single XN (**1**) dose pharmacokinetics study discussed in detail elsewhere (Legette et al., 2012).

**Table 3**
Mean liver tissue concentrations[1] of xanthohumol (XN) (1) and its metabolites (isoxanthohumol, IX (2), and 8-prenylnarigenin, 8PN (3)) of male ($n$ = 4–6/group) and female ($n$ = 5–6/group) obese Zucker fa/fa rats who received a daily supplementation of xanthohumol (XN) (1) at four dose levels (0, 1.86, 5.64, and 16.9 mg/kg BW) for 6 weeks.

| Treatment | Control (0 mg/kg BW) | Low XN (1.86 mg/kg BW) | Medium XN (5.69 mg/kg BW) | High XN (16.9 mg/kg BW) |
|---|---|---|---|---|
| XN (1) (nmol/g tissue) | | | | |
| Male rats | ND | 0.15 ± 0.04[a] | 0.35 ± 0.07[a] | 1.1 ± 0.35[b] |
| Female rats | ND | 0.35 ± 0.06[a] | 0.97 ± 0.20[a] | 1.9 ± 0.50[b] |
| IX (2) (nmol/g tissue) | | | | |
| Male rats | ND | ND | 0.06 ± 0.01 | 0.21 ± 0.03 |
| Female rats | ND | 0.04 ± 0.00[a] | 0.06 ± 0.02[a] | 0.39 ± 0.11[b] |
| 8PN (3) (nmol/g tissue) | | | | |
| Male rats | ND | ND | 0.05 ± 0.01 | 0.22 ± 0.04 |
| Female rats | ND | ND | 0.06 ± 0.01 | 0.11 ± 0.03 |
| Total (nmol/g tissue) | | | | |
| Male rats | ND | 0.15 ± 0.04[a] | 0.46 ± 0.08[a] | 1.5 ± 0.38[b] |
| Female rats | ND | 0.39 ± 0.05[a] | 1.1 ± 0.20[a] | 2.4 ± 0.53[b] |

All values are expressed as mean ± SE. ND indicates levels were not detectable. Different letters denote statistical differences ($p < 0.05$) from low XN group.
[1] Tissue concentrations of XN (1) and metabolites include both free and conjugated forms as determined by LC-MS/MS following sample preparation with enzymatic hydrolysis.

cose levels after 4 weeks of feeding XN (**1**) (1000 mg/kg BW) to obese mice (Nozawa, 2005). Our findings showed that XN (**1**) exerted an effect on glucose metabolism at a much lower dose (16.9 mg/kg BW). The difference in dose effect could be due to form and/or mode of XN (**1**) administration in the studies. Our study used a XN (**1**) formulation composed of a self-emulsifying mixture which allows for high absorption as detailed previously (Legette et al., 2012) whereas Nozawa provided XN (**1**) in a basal diet. The effect of the highest XN (**1**) dose on plasma glucose, as with body weight, was seen in male (Fig. 3A) but not in female (Fig. 3B) rats.

The gender differences we found in response to flavonoid treatment have also been observed for other flavonoids (Blair et al., 2002; Camper-Kirby et al., 2001; Guo et al., 2005). Blair and others demonstrated that dietary consumption of soy and soy isoflavones for 16 weeks affects LDL plasma concentrations in male but not female hamsters (Blair et al., 2002). Additional research is needed to determine what factors are influencing differential responses. Possible causes include treatment dose and/or treatment duration. Previous study findings also illustrate an effect of chronic flavonoid consumption (16–20 weeks) on plasma cholesterol in obese animals (Ali et al., 2004; Blair et al., 2002). Guo et al. (2009) examined the effects of administration of daidzein, a soy isoflavone, via oral gavage for 4 weeks at doses of 25, 50 and 100 mg/kg BW in male obese mice and found no effect on plasma triglyceride levels but did observed a decrease in plasma cholesterol and free fatty acid concentrations. However, in our study plasma cholesterol, insulin and triglyceride levels were not affected by treatment (data not shown), which may be attributed to a variety of factors including base diet, sample size, and treatment duration. Our high fat AIN-93G base diet contained 60% kcal fat diet whereas Guo's diet (Guo et al., 2009) only had 45% kcal fat to induce obesity in male mice. Additionally, a small sample size ($n$ = 6/group), large variation, short exposure period (6 weeks), and relatively low doses of XN used in the treatment could have impaired our ability to detect an effect of XN on plasma biochemical markers.

Plasma MCP-1 levels, a biomarker of inflammation, were not altered by XN (**1**) treatment (data not shown) which was unexpected since XN (**1**) has been shown to inhibit MCP-1 formation in LPS-activated monocytes (Peluso et al., 2010) and RAW 264.7 mouse macrophages (Lupinacci et al., 2009) in culture.

### 2.5. Dose/plasma-effect relationships

The significantly higher steady-state XN (**1**) plasma and liver tissue levels in the high-dose animals compared to the medium and low dose groups parallel the effects of treatment on body weight and plasma glucose in the high-dose males compared to the control group. This finding suggests that the low and medium doses fail to generate high enough plasma and especially tissue levels required to exert effects on the endpoints used in this study.

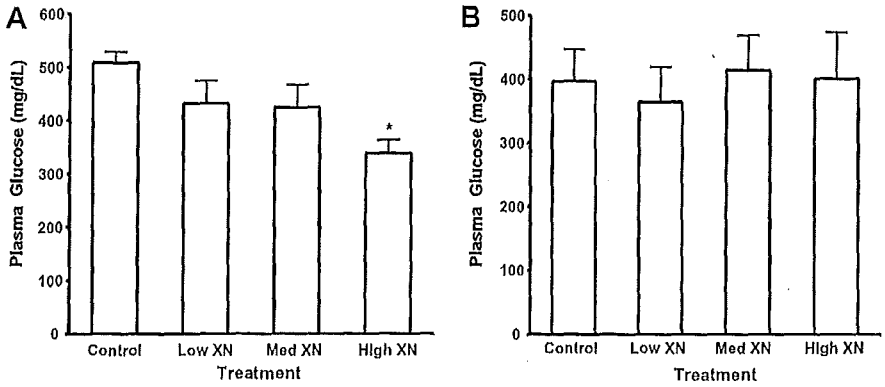


Fig. 3. Mean plasma glucose levels of (A) male and (B) female obese Zucker fa/fa rats ($n$ = 4–6/group) receiving various daily doses (0, 1.86, 5.64, 16.9 mg/kg BW) of xanthohumol (XN) (1) for 6 weeks. *denotes statistical difference from control group. All values are expressed as mean ± SE.

## 3. Concluding remarks

Our exploratory study on chronic XN (**1**) exposure showed that the highest XN (**1**) dose (16.9 mg/kg BW) exerted beneficial effects on body weight and glucose metabolism in obese male rats. This suggests that XN (**1**) holds promise as a therapeutic agent for treating obesity and dysregulation of glucose metabolism, conditions associated with metabolic syndrome. Our findings indicate that chronic administration of XN (**1**) in a self-emulsifying matrix produces effects at doses low enough to be feasible as a supplement (16.9 mg/kg BW). The potential of XN (**1**) as an effective preventive for metabolic syndrome warrants further investigation. Additional research is also needed to explore the mechanisms of action of XN (**1**) and its metabolites, including factors related to pharmacologic and pharmacokinetic effects.

## 4. Experimental

### 4.1. General information

#### 4.1.1. Animals

Four-week old male and female Zucker fa/fa rats were purchased from Harlan (Livermore, CA, USA). Animals were housed in individual cages in temperature and humidity controlled rooms with a 12:12 on–off light cycle. All procedures were approved by and in accordance with ethical standards of Oregon State University's Institutional Animal Use and Care Committee (Protocol # 3689).

#### 4.1.2. Diet

All rats were maintained on a high fat (60% kcal) AIN-93G diet for 3 weeks to induce severe obesity followed by a normal AIN-93G (15% kcal fat) diet for the last 3 weeks of the study. Weekly food intake and body weight were recorded.

#### 4.1.3. Treatment groups

After a 2 days acclimation period, 48 animals (24 males and 24 females) were divided into four treatment groups ($n = 6$/gender group): control (0 mg XN (**1**)/kg BW), low (1.86 mg XN (**1**)/kg BW), medium (5.64 mg XN (**1**)/kg BW), and high (16.9 mg XN (**1**)/kg BW). Animals were selected to ensure similar body weight average across treatment groups and received daily doses of XN (**1**) for 6 weeks before undergoing sacrifice. Animals were euthanized with an overdose of $CO_2$. The sacrifice period occurred over 4 days with a total of 12 animals being euthanized daily. Three animals were randomly selected from each treatment group every day of the sacrifice period.

#### 4.1.4. Statistics

Data were analyzed using Statistical Analysis Software (SAS) (Version 9.1, SAS Institute, Cary, NC). All values are expressed as mean ± SE. Treatment effects on body weight were assessed using repeated measure analysis over time (PROC MIXED procedure). Effects on plasma endpoints were determined by one-way ANOVA (PROC GLM procedure) and post hoc multiple comparison testing using LSD test. Significance was set at $p < 0.05$.

### 4.2. Source material

Animals received daily XN (**1**) doses via a test meal. XN (**1**) powder (>99% pure by HPLC-UV and NMR; Anheuser Busch, St. Louis, MO, USA) was dissolved in a self-emulsifying isotropic mixture (oleic acid, propylene glycol, and Tween 80; 0.9:1:1 by weight) to create various dosing solutions (0, 1.86, 5.64, and 16.9 mg/kg BW). XN (**1**) solutions were added to a modified AIN-93G powdered diet (corn oil replacing soybean oil) and homogeneously mixed with mortar and pestle to result in a 3 g test meal for each dose. Test meals were administered after withholding food for 3 h to ensure complete XN (**1**) consumption within 30 min. Test meals were made weekly for each treatment group based on average body weight of male and female rats. All test meals used for XN (**1**) treatment were analyzed and found on average to be within 10% of their nominal doses.

### 4.3. Xanthohumol (1) analysis

Plasma levels of XN (**1**), IX (**2**), 8PN (**3**), and 6PN were determined using procedures and conditions reported previously (Legette et al., 2012). In brief, samples were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) after sample preparation with and without enzymatic hydrolysis to determine amount of free and conjugated XN (**1**), IX (**2**), 8PN (**3**), and 6PN present in plasma. Tissue extracts were prepared in duplicate and on ice by homogenizing 0.500 g of liver tissue in 5 ml of extraction solvent (MeOH–$H_2O$, 9:1, v/v) using an Omni Tissue Homogenizer (Omni TH, Omni International, Marietta, GA, USA) for 30 s. Tissue homogenates underwent sonication for 1 min (Model F60 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA, USA) prior to being centrifuged at 4000 g for 15 min (Allegra X15R centrifuge, Beckman Coulter, Brea, CA, USA). The resulting supernatant was removed and stored as tissue extract. After obtaining tissue extracts, sample preparation and analysis were conducted as described for plasma samples. Test meals were analyzed by HPLC. Triplicate samples (400 mg) of test meals were extracted twice with MeOH 10 ml using vortexing (20 s) and sonication (5 min). The extracts were centrifuged for 5 min at setting = 6 using a clinical centrifuge (International Model CL, International Equipment Co., Needham, MA, USA) and the resulting supernatants were centrifuged for 5 min at 13000g. The two centrifuged extracts were analyzed separately using an HPLC system (Waters Corp., Milford, MA) consisting of a model 600 pump/controller, a model 717 Plus autosampler, and a 2996 photodiode array detector. Analytes were separated on a 4 × 250 mm Luna 5 μm C18 reversed phase column (Phenomenex, Torrance, CA), eluted with a linear gradient of 40–100% solvent B ($CH_3CN$) in solvent A ($H_2O$ containing 0.1% $CF_3CO_2H$) in 15 min at a flow rate of 1 ml/min. XN (**1**) was detected at 368 nm and quantified using the external standard method.

### 4.4. Biochemical analysis

Animals underwent overnight fasting before sacrifice. Blood was collected via a cardio-puncture and centrifuged for 10 min at setting = 7 using an International clinical centrifuge. Plasma was stored at −80 °C until analysis. Commercial assay kits were used to determine plasma cholesterol (Infinity™ Cholesterol Liquid Stable Reagent, Thermo Scientific, Middletown, VA, USA), glucose (Autokit Glucose, Wako Chemicals, Richmond, VA, USA), insulin (Alpco Insulin RAT ELISA, Alpco Diagnostics, Salem, NH, USA), triglyceride (Infinity™ Triglycerides Liquid Stable Reagent, Thermo Scientific, Middletown, VA, USA), and MCP-1 (Alpco MCP-1 rat ELISA, Alpco Diagnostics, Salem, NH, USA) levels.

## Acknowledgements

The authors wish to acknowledge Mr. Jeffrey Morré for his technical assistance. This study was supported by the National Institutes of Health (R21AT005294, S10 RR022589 and P30 ES000210).

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