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St. John's Wort and Artemisia Extracts Modulate Adipocyte Development and Function

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St. John's Wort and *Artemisia* Extracts Modulate Adipocyte Development and Function

by

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Background

Adipocytes have long been known for their ability to store fat, but only recently have their myriad of roles pertaining to endocrine function and insulin sensitivity emerged. There are three hormones that are solely excreted from fat cells: adiponectin (ADN), leptin, and resistin. Both adiponectin and leptin increase energy expenditure and insulin sensitivity, while resistin interferes with insulin signaling and is associated with insulin resistance. Furthermore, adipose tissue secretes molecules that contribute to blood vessel formation as well as inflammatory signaling. It is clear that the previous definition of fat as a mere lipid depository has become obsolete.

Given that adipose tissue plays multiple roles in metabolism and energy expenditure, adipose dysfunction is associated with metabolic diseases, like type 2 diabetes mellitus (T2DM). Obesity is a disease state characterized by excess fat and is a growing epidemic with over 400 million people who are obese worldwide and about 1.6 million obese Americans (1). As fat accumulates and expands, going from a lean to obese state, modulations occur in the adipocyte in response to these changes (2). Changes in the extracellular matrix have been associated with impaired adipocyte differentiation and it is thought that this can cause ectopic lipid accumulation and subsequent lipotoxicity. Moreover, expanding fat pads experience hypoxia. Hypoxic adipose tissue secretes high levels of pro-angiogenic molecules as well as inducing inflammatory signals such as Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα). Both signals are elevated in cardiovascular disease and diabetes (3). These problems are compounded by the fact that obese insulin resistant tissue also has increased macrophage levels which contribute to inflammation and vascularization (4).

Low expression of ADN is associated with fat accumulation in insulin resistant tissue –especially visceral tissue—and contributes to type 2 diabetes while healthy fat is associated with elevated levels of ADN and is protective against chronic diseases (5).In addition, chronic inflammation of adipocytes can lead to

localized insulin resistance (6). Of course, insulin resistance is characteristic of metabolic diseases like T2DM. As adipose dysfunction can cause metabolic disease, understanding how adipose tissue functions has lead to therapies for metabolic diseases. For example, a specific group of drugs called thiazolidinediones (TZDs) promote insulin sensitivity by activating peroxisome proliferator-activator receptor gamma (PPAR γ), a transcription factor that is required for adipogenesis and is a well-known marker of adipogenesis. While using thiazolidinediones will cause weight gain, the adipocytes that accumulate are insulin sensitive and contribute to glucose homeostasis (7).

Insulin sensitivity is largely determined by the ability to activate the insulin receptor and its subsequent signaling cascade in both adipocytes and skeletal muscle. As insulin circulates through the bloodstream, it binds to insulin receptors on the surface of cells which phosphorylates insulin receptor substrate-1 (IRS-1). IRS-1 activates phosphatidylinositol 3-kinase (PI3K) which phosphorylates Akt, ultimately resulting in the release of glucose transporter 4 (GLUT4) from the nucleus to the cell surface. Akt is a protein kinase whose inhibition is linked with the failure of the GLUT4 vesicle to translocate to the membrane. The name Akt is a reference to its discovery in the retrovirus AKT8 and is not indicative of its function in adipocytes. GLUT 4 allows glucose to be taken up from the bloodstream and into skeletal muscle and adipocytes. If any of these steps are inhibited, then insulin resistance can follow in these tissues. For example, many of the inflammatory molecules mentioned can inhibit IRS-1 resulting in insulin resistance. We use 3T3-L1 murine adipocytes which are a classic cell line to use for adipocytes studies as they can be cultured, in theory, for an indefinite amount of times, but most importantly because they show the same three major characteristics of *in vivo* adipocytes: they accumulate lipid, are insulin sensitive , and express cytokines and hormones in an endocrine manner. Because of these properties, 3T3-L1 studies can correctly discern molecular pathways and mechanisms that are applicable to *in vivo* adipocytes (8).

Botanical Background:

Botanicals have historically been used to treat a wide variety of disorders. Many common medicines were first isolated from botanicals, such as aspirin, morphine and digitalis. Botanicals can be found from plant, algal or fungal sources, and in the USA, botanicals are classified in three groups, as foods, botanical supplements and botanical drugs. Botanicals are considered to be food when they are used for purely culinary purposes, as with herbs. If a botanical is used for diagnosis or treatment of a disease it is considered a botanical drug and is held to the same standards as other drugs. A botanical supplement may make claims to remedy a nutrient deficiency, but may not make claims to treat or prevent a specific disease (9). As such, botanical supplement distribution is poorly controlled in the USA. Some countries have stricter regulations. In Germany a comprehensive review of botanical extracts was performed by Commission E which approved 250 out of 1400 medicinal plants using "significant experimental studies [to support] traditional use," and many of these botanical extracts are controlled, available only by prescription. (10)

The use of botanical extracts is on the rise around the world with an estimated $12 billion spent each year on alternative medicine –supplements being the most popular. However, there is insufficient data to prove the efficacy of botanical products, and often anecdotal evidence is a consumer's only recommendation. Patients have the perception that natural remedies are harmless and are lulled into a sense of security, many taking botanicals without informing their healthcare provider. As aforementioned, botanical supplements are poorly regulated in the USA so not only are botanicals not screened for efficacy, but the compounds that are sold may contain contaminants and some may not even contain the botanical. Furthermore, there is little uniformity on dosage or administration guidelines, which yields inconsistent results. Due to the widespread popularity of botanical remedies, funding for botanical research has increased. Some botanicals, like cinnamon, have had their purported uses substantiated by empirical evidence. It is now known that cinnamon used in tandem with traditional diabetes care lowers glycemia better than traditional care alone (11).

Likewise, garlic—a virtual panacea of homeopathic remedies— has had its hypotensive properties tested and confirmed (12). In addition, botanical extracts have demonstrated new potentially therapeutic uses. Some extracts exhibit the ability to overcome drug and antibiotic resistance giving way to new treatments for infections like *Staphylococcus aureus.* More relevant to adipocyte research, recent screenings of botanical extracts have identified multiple extracts that may be antidiabetic, improving one's metabolic profile (13; 14; 15).

In the Stephens Lab, we have explored the effects of St. John's Wort (SJW) extracts and *Artemisia* extracts on preadipocytes differentiation and function. SJW earliest use was to ward off evil spirits, and according to tradition its potency was due to its flowering on St. John's Day. In more recent history SJW has been used as a mood stabilizer and has had success in treating mild to moderate depression. Since SJW is readily available and consumed by millions of people and both obesity and diabetes are world-wide epidemics; understanding the effects of SJW on adipocyte development and function is worthwhile but has been largely unstudied to date.

Artemisia species have a history of use in Chinese medicinal tradition to treat a wide variety of dieases. *A. santolinifolia* has been used in Eastern medicine as a diuretic and to treat jaundice, hepatitis, gall bladder inflammation, and ear-aches. *A. scoparia* is also known as Redstem Wormwood and has been historically used to treat gastrointestinal diseases and skin diseases like eczema and dermatitis (16). *A. dracunculus*, or Russian tarragon, has an antiquated use as a snake bite treatment and in modern times is a known analgesic (17). Recent studies performed at Pennington Botanical Biomedical Research Center have shown that *A.dracunculus*, and more specifically a fraction PMI 5011, have insulin sensitizing effects on skeletal muscle and induce the expression of glycogen synthase in hepatocytes (18).

Materials & Methods

Materials. Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen. Bovine and fetal bovine (FBS) sera were purchased from Hyclone. Both the PPARγ monoclonal antibody and the STAT 5A

polyclonal antibody were purchased from Santa Cruz. The adiponectin antibody was a rabbit polyclonal obtained from Affinity Bioreagents. HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch. Enhanced chemiluminescence (ECL) kit was purchased from Pierce. Nitrocellulose was purchased from BioRad. St. John's Wort extracts were prepared by the Rutgers University Botanical Research Center. Briefly, plant samples were air dried and extracted with 80% ethanol (1:5 w/v) three times, infused each time for 24 h, and evaporated in a rotary evaporator to thick aqueous suspension. The extracts were further dried under vacuum and stored in amber glass vials at -20 °C. Prior to use, extracts were re-suspended in DMSO.

Cell culture. Murine 3T3-L1 preadipocytes were plated and grown to 2 days post confluence in DMEM with 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone, and 1.7 µM insulin. At this time, cells were treated with a 1000x stock of the botanical extracts suspended in DMSO. Cells were also treated with DMSO alone. After 48 h this medium was replaced with DMEM supplemented with 10% FBS and treated again with botanical extracts or vehicle (DMSO).

Preparation of whole cell extracts. Monolayers of 3T3-L1 preadipocytes or adipocytes were rinsed with phosphate-buffered saline (PBS) and then harvested in a non-denaturing buffer containing 150 mMNaCl, 10 mMTris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 0.5% Igepal CA-630 (Nonidet P-40), 1 µM PMSF, 1 µM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, and 10 µM leupeptin, and 2 mM sodium vanadate. Samples were extracted for 30 min on ice and centrifuged at 10,000g at 4 °C for 15 min. Supernatants containing whole cell extracts were analyzed for protein content using a BCA kit (Pierce) according to the manufacturer's instructions. *Gel electrophoresis and Western blot analysis.* Proteins were separated in 7.5% polyacrylamide (acrylamide from National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli and transferred to nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol (19). Following transfer, the membrane was blocked in 4% fat-free milk for 1 h at room temperature. Results

were visualized with HRP-conjugated secondary antibodies and enhanced chemiluminescence. *Determination of 3H-labeled 2-deoxyglucose uptake*. The assay of 2-[3H] deoxyglucose was performed as previously described (20). Prior to the assay, mature 3T3-L1 adipocytes were serum deprived for 2 h and treated with various doses of botanical extracts for different periods of time. Next, the cells were incubated in the cells were incubated in the presence or absence of insulin (10 nM) for 10 min. Glucose uptake was initiated by addition of 2-[3H] deoxyglucose at a concentration of 0.1 mM 2-deoxyglucose in 1 µCi 2-[3H] deoxyglucose in Krebs– Ringer–Hepes buffer and incubated for 3 min at room temperature. Glucose uptake is reported as [3H] radioactivity, corrected for nonspecific diffusion (5 µM cytochalasin B) and normalized to total protein content as determined by BCA analysis. Nonspecific uptake and absorption was always less than 10% of the total uptake. Uptake measurements were performed in triplicate under conditions where hexose uptake was linear.

St. John's Wort Results

In a blinded study, we screened 425 botanical extracts to examine their ability to modulate adipocyte differentiation of 3T3-L1 cells. We observed that less than 2% of the extracts had substantial effects on adipocyte differentiation. After the screening was complete, and identification of the extracts was unblinded, we noted that two of the botanical extracts that inhibited adipogenesis were extracts from SJW. As shown in Fig. 1, three extracts from SJW were examined for their ability to inhibit the induction of adipocyte specific proteins such as PPARγ and adiponectin. Extracts from the root (R) of SJW had no effect on adipocyte

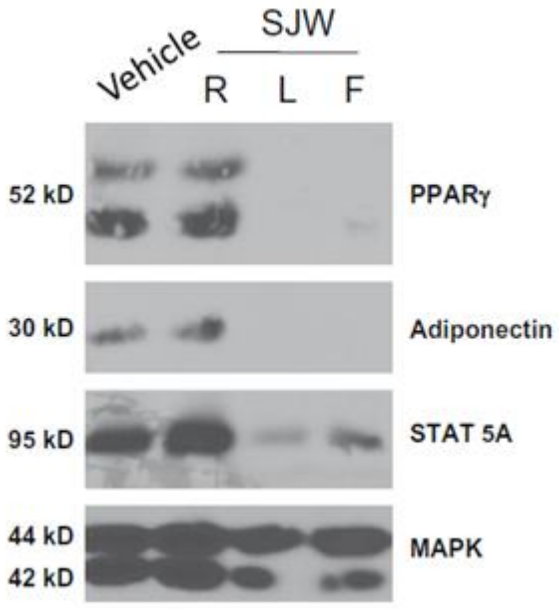

Fig. 1. Leaf and flower extracts from SJW inhibit adipocyte differentiation of 3T3-L1 cells. Whole cell extracts were prepared from 3T3-L1 cells one week after they were induced to differentiate with the normal induction cocktail in the presence or absence of 50 µg/ml of SJW extracts obtained from SJW. Cells were also treated with a 1000x stock of DMSO (vehicle). One hundred micrograms of each extract were separated by SDS–PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. The detection system was horse-radish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Pierce). This is a representative experiment independently performed three times.

development, but extracts from the leaves (L) and flowers (F) blocked the induction of PPARγ and adiponectin expression. In addition, the normal induction in STAT5A expression which accompanies differentiation was also inhibited (21). Botanical extracts obtained from SJW were re-suspended in DMSO and diluted 1000-fold into the cell culture media. Vehicle treatment of DMSO had no effect on adipocyte development. Furthermore, there was no observable cell death (data not shown) and MAPK expression was examined to indicate equivalent levels of protein in each sample.

To assess the specificity of this inhibitory effect, we examined the ability of various doses of the flower extract to modulate adipocyte differentiation. As shown in Fig. 2, treatment with a flower extract of SJW resulted in a dose dependent inhibition of adipogenesis as indicated by an inhibition in the induction of PPARγ and adiponectin expression. As indicated in Fig. 1, a dose of 50 µg/ml completely blocked the induction of PPARγ and adiponectin. In addition, a dose of 25 µg/ml significantly attenuated the induction of PPARγ and blocked the induction of adiponectin. Treatment with12 µg/ml did not inhibit the induction of PPARγ, but substantially reduced the induction of adiponectin expression. Treatment with the lowest dose of flower extract (6 µg/ml) or treatment with DMSO (V) had no effect on the induction of PPARγ and adiponectin as compared to untreated (CTL) cells. MAPK expression does not change during the differentiation of 3T3-L1 cells and is shown to indicate equivalent levels of protein in each sample.

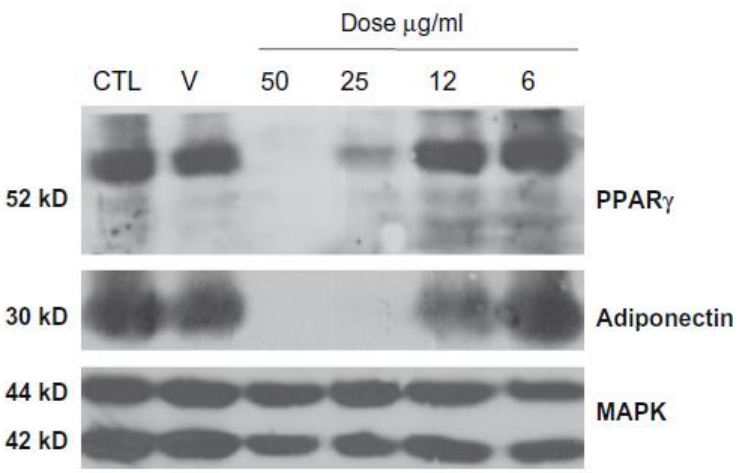

Fig. 2. A flower extract from SJW results in a dose dependent inhibition of adipogenesis in 3T3-L1 cells. Whole cell extracts were prepared from 3T3-L1 cells one week after they were induced to differentiate with the normal induction cocktail in the presence of various doses of a SJW flower extract. Cells were also treated with a 1000x stock of DMSO (V). One hundred micrograms of each extract were separated by SDS–PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed and results were visualized as described in the legend of Fig. 1. This is a representative experiment independently performed three times.

Because two of the SJW extracts affected adipocyte development, we examined the ability of these SJW extracts to modulate adipocyte function. Fully differentiated 3T3-L1 adipocytes were treated with SJW extracts for 90 min and then glucose uptake was examined in the presence and absence of an acute stimulation with insulin. As shown in Fig. 3, vehicle (control) treated cells were highly responsive to insulin treatment which resulted in a 4.5-fold increase in glucose uptake. None of the SJW extracts had any effect on glucose uptake in the absence of insulin. However, treatment with a leaf or flower extract resulted in a significant inhibition of insulin stimulated glucose uptake. To assess the specificity of these effects, mature 3T3-L1 adipocytes were pretreated overnight with the various doses of SJW flower extract indicated in Fig. 4A. These studies revealed a dose dependent inhibition in insulin sensitive glucose uptake.

Mature adipocytes were also pretreated for various times with 25 µg/ml flower extract of SJW. After a 1 or 2 h treatment, there was a substantial inhibition of insulin sensitive glucose uptake. An 18 h pretreatment consistently

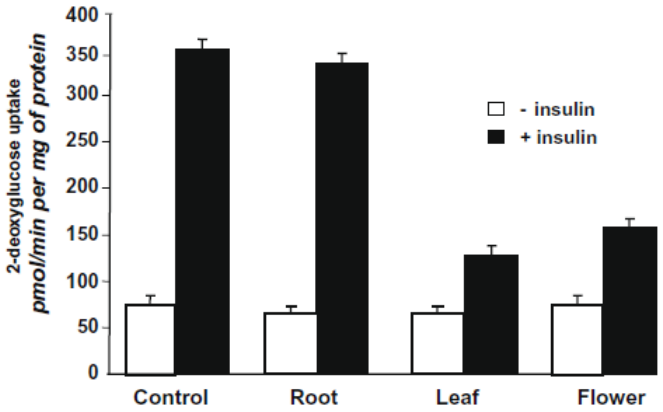

Fig. 3. Leaf and flower, but not root, extracts from SJW induce insulin resistance in fully differentiated 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were serum deprived and treated overnight (20 h) with 25 µg/ml of the indicated SJW extract. After the pretreatment, cells were stimulated with insulin (10 nM) for 10 min. Glucose uptake was initiated by addition of 2-[3H] deoxyglucose. The glucose uptake values shown represent the mean ± SE of triplicate determinations from four independent experiments.

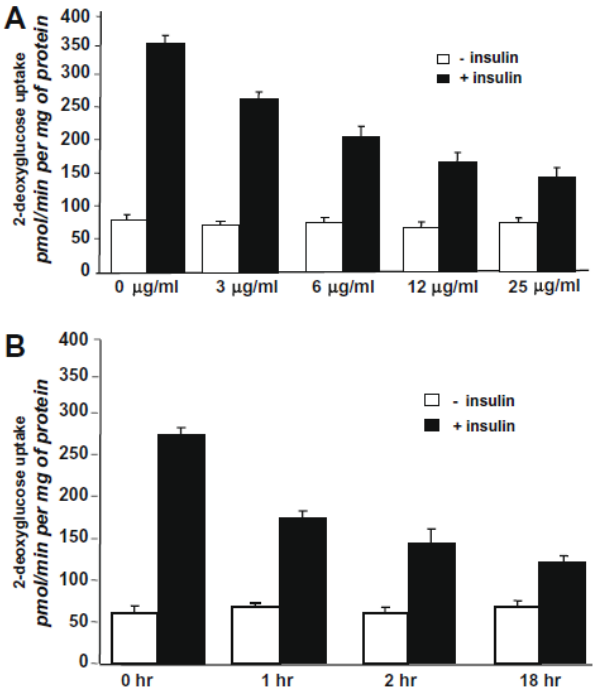

Fig. 4. A flower extract of SJW induces insulin resistance in cultured murine adipocytes in a dose and time dependent manner. **(A)** Fully differentiated 3T3-L1 adipocytes were serum deprived and pretreated for 90 min with various doses of a SJW flower extract. **(B)** Fully differentiated 3T3-L1 adipocytes were serum deprived and pretreated for the indicated times with 25 µg/ml of a SJW flower extract. After the pretreatment, cells were stimulated with insulin (10 nM) for 10 min. Glucose uptake was performed as described in Fig.3.

resulted in an even greater inhibition of insulin stimulated glucose uptake. As the SJW flower extract stimulates insulin resistance, IRS-1 (a protein of the insulin signaling cascade) was tested to see if it was incapacitated by the botanical. As shown in Fig. 5, an overnight pretreatment with SJW extracts obtained from leaf or flowers resulted in a substantial inhibition of IRS-1 tyrosine phosphorylation following an acute insulin treatment. However, treatment with vehicle or root extract had no effect on the ability of insulin to induce IRS-1 tyrosine phosphorylation. To determine if this effect occurred in human adipocytes, we pretreated fully differentiated subcutaneous human adipocytes (Zenbio) for various times with SJW flower extracts. Following the pretreatment, cells were exposed to a 5 nM insulin treatment for 2 minutes and whole cell extracts were isolated and subject to Western blot analysis. As shown in Fig. 6, a 30 min, 6 hour, and overnight treatment of SJW flower extract blocked the ability of insulin to induce IRS-1 tyrosine 896 phosphorylation. For the previous results, STAT5A expression does not change with insulin treatment and is shown to indicate equivalent levels of protein in each sample.

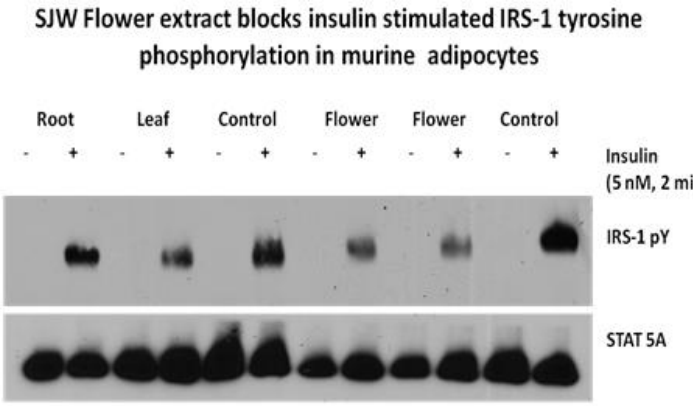

Figure 5. Ten days after the induction of differentiation, mature 3T3-L1 adipocytes were serum deprived and then treated for 20 hours with 25 ug/ml of flower, leaf, or root extracts. Cells were also treated with a 1000 X stock of DMSO (control). Next, cells were treated for 2 minutes with 5 nM insulin and whole cell extracts were isolated. 100 ug of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed and results were visualized as described in Figure Legend 1.

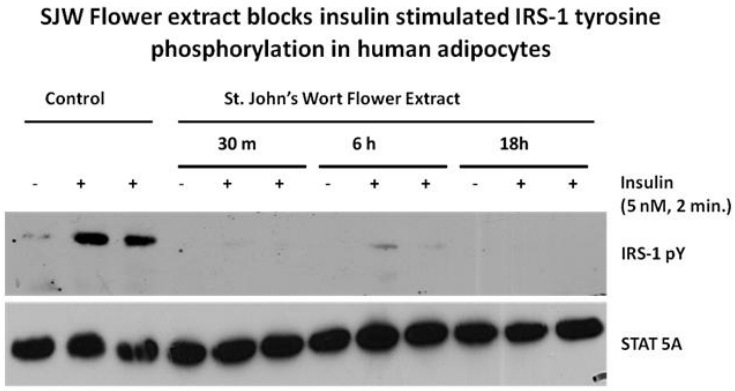

Figure 6. Subcutaneous human adipocytes were derived from subcutaneous human preadipocytes from a lean individual and fully differentiated. These cells were purchased from Zenbio and microscopic analyses revealed that all the cells had substantial lipid accumulation. The cells in a twelve well plate were serum deprived and then treated with 25 ug/ml of SJW flower extract for the indicated times. Next, cells were treated for 2 minutes with 5 nM insulin and whole cell extracts were isolated. 75 ug of each extract was separated by SDS-PAGE. Samples were processed and results were visualized as described in Figure Legend 1.

Artemisia Results

As a continuation of our previous screening efforts, many more botanical extracts were tested for potential therapeutic effects on adipocytes. These experiments indicated that *Artemisia santolinifolia* and *Artemisia scoparia* induced adipogenesis as demonstrated by the increased expression of adipocyte gene markers PPARγ and adiponectin (ADN) when compared to the control (Fig. 7).To examine specificity of the extracts on adipogenesis, isolated fractions from the *Artemisia* extracts were isolated and used to treat adipocytes. In Fig. 8 we can see that *A.santolinifolia* fraction 2 has increased expression of adipocyte specific proteins, aP2 and ADN when compared to the control. Fraction 4 also has elevated aP2 levels, and a marginal increase of ADN. The elevated expression of these proteins was seen in adipocytes treated with *A.scoparia* fractions 1-3 when compared to the control. Fraction 4 had elevated aP2 levels and no observable effect on ADN expression.

As we are working with the Pennington Botanical Biomedical Research Center, we also screened *A.dracunculus* which they had found to

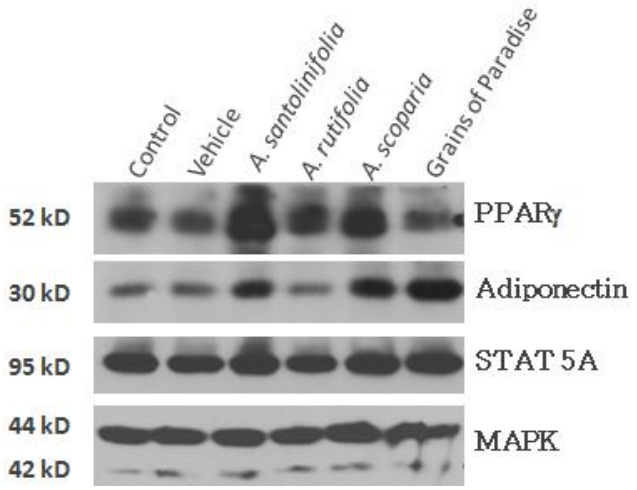

Figure 7. Cell were treated with 50 µg/mL of different botanical extracts. Cells were also treated with a 1000 X stock of DMSO (V). 100 mg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis**.** Samples were processed and results were visualized as described in Figure Legend 1.

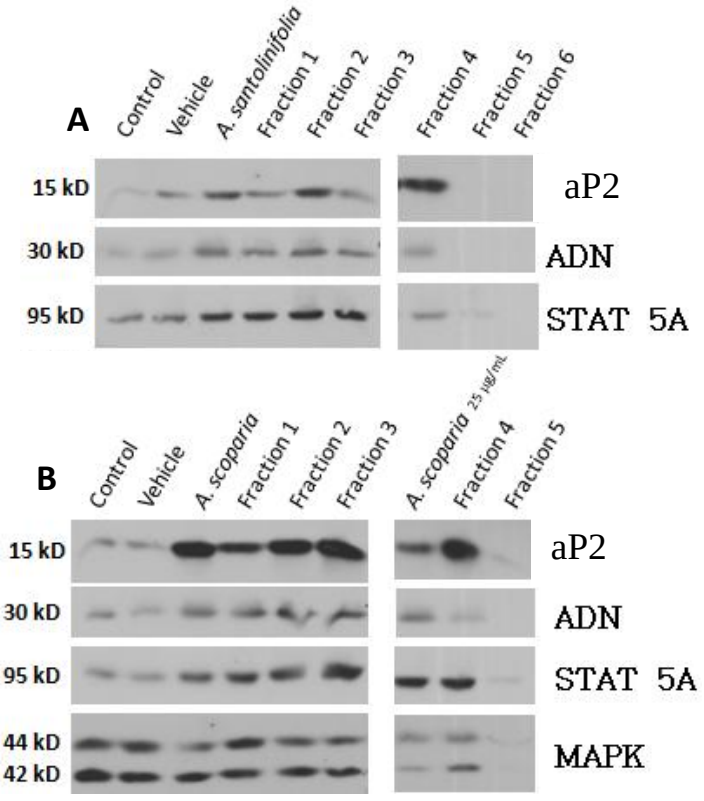

Figure 8. Cell were treated with 50 µg /mL of different botanical extracts. Cells were also treated with a 1000 X stock of DMSO (V). 100 µg of each extract was separated by SDS-PAGE, Samples were processed and results were visualized as described in Fig.1. **(A)** Cells were treated with different fractions of *A.santilinifolia. (***B).** Cells were treated with different fractions of *A.scoparia.*

have insulin sensitizing effects on skeletal muscle (13; 18). However our data (not shown), while not indicating any ill effects of the extract on adipocytes, did not show any protective properties either.

Discussion

Botanical supplements have very poor regulation in the United States and are readily available to consumers. In a blinded screening study, we identified extracts with therapeutic potential and injurious potential to metabolic processes. We observed that extracts obtained from the flower and leaf of SJW, but not the roots, were capable of inhibiting the adipogenesis of murine preadipocytes while *Artemisia scoparia* and *Artemisia santolinifolia* induced adipogenesis of murine preadipocytes. Of note, a current hypothesis is that Type 2 diabetes can be viewed as a failure to appropriately expand fat mass in the context of a positive energy balance (2). In light of this notion, the ability of SJW to inhibit adipogenesis may not be metabolically favorable and *A.scoparia* and *A.santolinifolia's* ability to induce adipogenesis may have beneficial whole body effects.

Several studies suggest that inhibiting adipocyte development can be a causative factor in the development of insulin resistance (3). In addition to its ability to inhibit adipocyte development, SJW extracts can also have negative effects on mature fat cells. We observed that SJW flower extracts resulted in a time and dose dependent inhibition of insulin stimulated glucose uptake in mature mouse fat cells. Although less insulin sensitive, we also observed that these extracts could inhibit insulin stimulated glucose uptake in cultured human fat cells. Based on our in vitro studies, our results suggest that SJW may have some negative effects on adipocyte development and function. There is a substantial body of literature indicating that SJW can interfere with the action of numerous drugs, and it is well established that SJW lowers the circulating concentrations and pharmacological effects of a number of drugs and oral contraceptives (22). Although our studies on SJW are limited to in vitro observations, there is some data to support a role of SJW in insulin resistance. A recent paper demonstrated that a protein found in SJW can increase monocyte chemotactic protein-1 (MCP-1) expression which is an inflammatory protein (23). As previously mentioned, both obese and

insulin resistant tissue are associated with high levels of inflammatory markers (3), so a person who is metabolically compromised and is also taking SJW could exacerbate their inflammation.

Artemisia has TZD-like characteristics in that it induces expression of PPARγ and PPARγ target proteins aP2 and ADN. TZDs are widely used to treat T2DM and are known to improve one's metabolic profile by reducing plasma glucose and improving insulin sensitivity in adipose tissue (24) (25). Furthermore, we performed a lipid accumulation experiment (not shown) that demonstrated that our two *Artemisia* species were as effective as the TZD rosiglitazone in protecting preadipocytes from a TNFα treatment that would have attenuated differentiation. This data indicates that *A.scoparia* and *A.santolinifolia* may have insulin sensitizing properties similar to that of thiazolidinediones.

In summary, a screening process to identify botanical extracts that regulated adipocyte development resulted in the observation that extracts from leaf or flower of SJW inhibit adipogenesis and insulin sensitive glucose uptake in both human and murine fat cells and that *A.scoparia* and *A.santolinifolia* induce adipogenesis. Obesity and diabetes are world-wide epidemics and botanical extracts are readily available over the counter and consumed by millions of people wide. Overall, our studies suggest that SJW may have a negative impact on adipocyte related diseases by limiting differentiation of preadipocytes and significantly inducing insulin resistance in mature fat cells. However *A.scoparia* and *A.santolinifolia* may be protective against metabolic diseases by promoting the transcription of PPARγ and PPARγ target proteins.

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