NQO1 (NADPH:Quinone Oxidoreductase)1
GR (Glutathione Reductase)
GCLc (Glutamate Cysteine Ligase Catalytic Unit)
GPx (Glutathione Peroxidase)

Pulicaria jaubertii E. Gamal-Eldin reduces triacylglyceride content and modifies cellular antioxidant pathways in 3T3-L1 adipocytes

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Funding was provided through USDA-ARS Project 5450-51000-048-00D. Dr. Al-Naqeb was the recipient of a Fulbright Scholarship that provided funding for this research.

Abstract

Levels of obesity in Middle Eastern countries are increasing. Phytochemicals have anti-obesogenic properties as evidenced by prevention of adipocyte differentiation and blocking triacylglyceride (TG) accumulation. In Yemen, *Pulicaria jaubertii* E.Gamal-Eldin (PJ) is a food additive and a traditional medicine. We tested the hypothesis that phytochemicals present in PJ inhibit adipocytic responses during differentiation of 3T3-L1 preadipocytes to adipocytes. Methanolic extracts of PJ did not block expression of fatty acid binding protein 4 (FABP4) a marker of differentiation but did inhibit TG accumulation. Treatment of 3T3-L1 preadipocytes increased NADPH:quinone oxidoreductase 1 (NQO1), a suppressor of TG accumulation. Further fractionation of the methanolic PJ extract with hexane and dichloromethane (DCM) demonstrated that bioactivity towards TG reduction and elevated expression of NQO1 and other antioxidant genes (glutamate cysteine ligase catalytic unit, glutathione disulfide reductase, glutathione peroxidase (GPx) 4 resided in the DCM fraction. Activity towards depleting GSH and elevating the expression of catalase and GPx3 were found in the DCM and hexane fractions. Analysis by gas chromatography and liquid chromatography coupled with mass spectrometry demonstrated the presence of catechin-like moieties in the DCM and methanolic fractions and suggest that these components were partially responsible for the bioactivity of these fractions. In summary, our data indicate that fractions derived PJ exhibit anti-adipogenic properties in part through the presence of catechin-like compounds.

Keywords: 3T3-L1; *Pulicaria jaubertii*; NQO1; Glutathione; Catechin, Carvotanacetone, Adipocyte

Abbreviations

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

Dichloromethane (DCM)

Dimethylsulfoxide (DMSO)

Electrospray ionization with high-resolution time of flight mass spectrometry (ESI-TOF-MS)

Fatty acid binding protein (FABP)

Gas chromatography-mass spectrometry (GC-MS)

Glutamate cysteine ligase catalytic unit (GCLc)

Glutathione (GSH)

Glutathione disulfide (GSSG)

Glutathione peroxidase (GPx)

Glutathione reductase (GR)

Liquid chromatography-mass spectrometry (LC-MS)

NADPH:quinone oxidoreductase 1 (NQO1)

N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)

Pulicaria jaubertii Gamal-Eldin (PJ)

Pulicaria jaubertii methanol extract (PJ_M)

Pulicaria jaubertii hexane fraction (PJ_H)

Pulicaria jaubertii dichloromethane fraction (PJ_D)

Pulicaria jaubertii aqueous fraction (PJ_A)

Superoxide dismutase (SOD)

Triacylglyceride (TG)

Trimethylsilyl (TMS)

1. Introduction

In the Middle East, levels of obesity are growing along with the predicted sequelae like insulin resistance [1, 2]. These events have resulted in an increasing attention towards the identification of the anti-obesogenic properties of traditional Middle Eastern herbal medicines. Species of the genus *Pulicaria* are widespread throughout the Mediterranean, Middle East, and Asia, and their extracts are used as traditional medicines [3]. *Pulicaria jaubertii* Gamal-Eldin (PJ) is a Yemeni flavorant and is used in traditional medicines [4, 5]. The extracted oils of PJ have cytotoxic effects towards MCF-7 cancer cells and moderate antimicrobial activities [6]. The extent to which PJ contains phytochemicals with anti-obesogenic properties has not been explored.

Multiple reports indicate that phytochemicals modify adipogenic responses [7-9]. For example, curcumin, isolated from turmeric, inhibits adipocyte differentiation [10]. Several phytochemicals have been studied for their anti-adipogenic activity in vivo and in vitro using the murine 3T3-L1 adipocyte model. Our previous work demonstrates that elevated expression of NADPH:quinone oxidoreductase 1 (NQO1) by sulforaphane, a isothiocyanate derived from brassicates, inhibits triacylglyceride (TG) accumulation in 3T3-L1 adipocytes [11].

Mechanistic evidence indicates that adipocyte differentiation and TG accumulation are controlled by the cellular redox status and the expression of antioxidant enzymes. NQO1 is part of the antioxidant defense system and is extensively studied in relation to chemoprotection and cancer susceptibility [12, 13]. Multiple lines of evidence indicate

that NQO1 plays a mechanistic role in adipocyte function and energy homeostasis. The NQO1 gene is highly expressed in human adipose [14, 15]. NQO1 deficiency modifies abdominal adipose tissues mass in mice [16]. Moreover, the expression of NQO1 is down-regulated during the differentiation of 3T3-L1 preadipocytes into adipocytes and enhancing NQO1 inhibits differentiation [11].

Glutathione (GSH) is one of the most important anti-oxidants in the cell and its roles in obesity and energy metabolism have recently been reviewed [17]. GSH has many functions in regulating the development of obesity. In vitro mechanistic studies using 3T3-L1 preadipocytes demonstrate that GSH depletion with buthionine sulfoximine induces adipogenesis and TG deposition, an effect reversed by GSH ethyl ester [18]. Similarly, *N*-acetyl-cysteine, a GSH precursor, inhibits adipogenesis [19]. Conversely, depletion of the glutathione by BSO in mice prevents diet-induced obesity, increases energy expenditure and enhances insulin sensitivity [20]. Data suggest that GSH depletion in vivo may increase energy expenditure and decrease adiposity via Nrf2-ARE-based mechanisms as reviewed recently [17, 21].

With these data in mind, we tested the hypothesis that phytochemicals present in PJ inhibit adipocytic responses during differentiation of 3T3-L1 preadipocytes to adipocytes. Our results showed that methanolic extracts of PJ blocked accumulation of TG but not differentiation. These effects occurred in the presence of GSH depletion and increases in several antioxidant defense enzymes including NQO1, glutathione peroxidase 3 (GPx3), GPx4, GR, glutamate-cysteine ligase (GCL) and catalase.

Analysis of extract fractions by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) identified multiple compounds with potential biological activity.

2. Materials and methods

2.1 Plant collection. *Pulicaria jaubertii* E.Gamal-Eldin was collected from Alkaa Farm, located near Sana`a, Yemen, in September 2013. The plant was identified and authenticated by Dr. Abdul Wali Al Khulaidi, a plant taxonomist at the Department of Botany, Faculty of Agriculture, Sana`a University, Yemen. Leaves and flowers were separated from the stems. Leaves and flowers were naturally dried, packed in plastic bags, and stored at 4°C with protection from light. The dried material was sent to the USDA-Grand forks Human Nutrition Research Center, in Grand Forks, North Dakota, USA, in December 2013.

2.2 Chemicals. Methanol (LC-MS Optima grade), and dichloromethane (DCM, GC quality) were purchased from Fisher Scientific. *n*-Hexane (LC-MS grade) was obtained from JT Baker, USA. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 99%) with 1% trimethylchlorosilane (TMCS) was purchased from Sigma-Aldrich (St. Louis, MO, USA) . and was used to form trimethylsilyl (TMS) derivatives.

2.3 Preparation of the PJ extract and its fractionation. Dried PJ was finely ground using an electric grinder (Ika Labortechnik M 20 Brand). The resulting green powder was extracted with methanol at 1:5 powder:methanol (w/v) ratio for 48 h with stirring under dark conditions. The resulting extract was filtered, and the resulting filtrate concentrated

by rotary evaporation at 40 °C. The final, dark green viscous extract was transferred into glass amber bottles and stored at 4 °C for subsequent analyses. A 5% yield of final methanolic PJ extract, denoted PJ_M, relative to starting powder was obtained.

Twelve (12) g of PJ_M was mixed with *n*-hexane at a 1:5 w/v ratio and 100 mL of methanol: water (80:20 v/v) was added. The mixture was shaken in a separatory funnel and the hexane layer was removed. This step was repeated once more. Dichloromethane (DCM) was added into the remaining aqueous residue and the process repeated. The resulting DCM and hexane fractions were concentrated to dryness using a rotary evaporator. Both fractions were highly viscous and dark green in color. The aqueous phase was subjected to freeze drying yielding a viscous, dark amber residue. The yields obtained for each PJ fraction with respect to the initial methanolic extract were: *n*-hexane (denoted PJ_H) 5 g (41%), DCM (denoted PJ_D) 2 g (16%) and residual methanol:aqueous fraction (denoted PJ_A) 1 g (8%).

2.4 Chemical characterization of PJ fractions. For GC-MS analysis, the methanolic extract, PJ_M, and each fraction (PJ_H, PJ_D, PJ_A) were analyzed in amounts proportional to the weight of fraction obtained from the extract (i.e., 5 mg of PJ_H, 1.8 mg of PJ_D). Samples were dissolved in 1 mL DCM with and without TMS derivatization with BSTFA. Prior to GC-MS analyses, 25 µL of internal standard (*o*-terphenyl, 1.4 mg/mL) was added to control for volume changes in each sample. The tentative identification of observed compounds was based on comparison with the NIST 05 Mass Spectra library.

For TMS derivatization, appropriate amounts of the methanol extract and its fractions were weighed, dried, and reconstituted in 100 μ L of BSTFA and 50 μ L pyridine. The solution was heated at 70 $^{\circ}$ C for 18 h. After derivatization 25 μ L of internal standard (o-terphenyl) was added together with 0.85 mL of DCM.

GC-MS analyses were performed using 6890N GC with 5975C MS (Agilent Technologies, Santa Clara, CA, USA) equipped with Gerstel MPS2 autosampler (Gerstel, Baltimore, MD, USA). Injections (1 μ L) were performed in a splitless mode for 0.40 min at 250 $^{\circ}$ C. Separation was performed using a DB-5MS capillary column (30 m), with 0.25 mm internal diameter (I.D.) and 0.25 μ L film thickness (J&W Scientific, Folsom, CA, USA). A constant carrier gas (helium) flow rate of 1 mL/min was maintained during the analysis. The temperature program started at 35 $^{\circ}$ C held for 5 min, followed by a gradient of 10 $^{\circ}$ C/min to 300 $^{\circ}$ C and held for 1 min. The MS data in total ion chromatogram (TIC) mode were acquired in the mass range of m/z of 35–1000 at a scan rate 2.66 scan/s using the EI of 70 eV. The solvent delay was based elution of the derivatization agent, their by-products, and solvent retention times, measured by observing the vacuum change. Namely, for BSTFA with pyridine, the MS was off for the first 2.5 min, 2.90–3.60 min, 4.40–7.00 min, 8.00–8.70 min; for samples without derivatization, solvent delay was 2.5 min.

For LC-MS analyses, a stock solution of each sample (P_{JM}, P_{JH}, P_{JD}, P_{JA}) was prepared in methanol in concentration of 2–3 mg/mL. Stocks were further diluted to final concentration of 10 – 16 ppm in acetonitrile and water (50:50 v/v) with ammonium

acetate (final concentration of 5 mM). LC-MS analyses were carried out using electrospray ionization with high-resolution time of flight mass spectrometry (ESI-TOF-MS, G1969A, Agilent, Santa Clara, CA, USA) coupled to an Agilent LC 1100 high performance liquid chromatograph. The column was a C₁₈ Zorbax Eclipse plus (2.1 x 150 mm with 3.5µm particle size) with a 1.2 cm guard column (Agilent). For the chromatographic separations, the mobile phase solutions consisted of 1.0 mM ammonium acetate in water:acetonitrile (95:5, v/v) as solvent A and 1.0 mM ammonium acetate in acetonitrile as solvent B. The flow rate was 0.2 mL/min, and the injection volume was 50 µL with an injector needle wash program to avoid cross-contamination. The gradient elution program started isocratically at 0% B for 2 min, followed by a linear gradient to 80% B from 2 min to 30 min with maintenance of 80% B for 5 min. Following a sharp gradient to 100% A from 35 to 36 min, the column was re-equilibrated with 100% solvent A from 36 to 56 min. The column temperature was maintained at 30 °C. ESI was performed in negative mode with an electrospray voltage of 4500 V, a fragmentor voltage of 175 V, a nebulization pressure of 25 psig, a drying gas flow rate of 12 L/min, and a drying gas temperature of 350 °C.

2.5 Cell culture.

Mouse 3T3-L1 preadipocytic fibroblasts were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured as previously described [11]. For cell viability determination, the 3T3-L1 preadipocytes were seeded at a density of 6 X 10³ cells/well in 24 well plates. The cells were then treated with increasing doses of extracts for 24 h. Dried extracts and fractions were dissolved in dimethylsulfoxide

(DMSO). The DMSO percentage was <0.1% at final concentration. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) activity assay as described previously [22].

Differentiation of 3T3-L1 pre-adipocytes to adipocytes occurred through a seven-day treatment regimen using insulin, dexamethasone, and isobutylmethylxanthine as previously described [11]. For experiments testing the inhibition of adipocyte differentiation, confluent 3T3-L1 pre-adipocytes were treated with selected doses of the extracts at days 0, 2, 5, and 7 that corresponded to changes in the cell culture media. PJ fractions were diluted in DMSO with final concentrations of DMSO < 0.1%.

2.5.1 Oil red O staining. Lipid accumulation in differentiated adipocytes was assessed by Oil Red O staining [23]. Cells (plated in 6 well plates) were washed with phosphate-buffered saline and fixed with 10% formalin for 30 min. After two washes with distilled water, cells were washed with 60% isopropanol and allowed to completely dry at room temperature. Cells were treated with an Oil Red O working solution for 10 min with rocking. Following incubation of the cells, the Oil Red O solution was removed, and the cells immediately washed four times with distilled water. After the final wash, distilled water was added to each well prior to microscopy. The Oil Red O working solution was made by (1) dissolving 0.35 g of Oil Red O (Sigma-Aldrich, St. Louis, MO) in 100 mL of isopropanol with stirring overnight followed by filtration to make an initial stock solution and (2) diluting 6 mL of this stock solution with 4 mL of distilled water.

2.5.2 Triacylglyceride (TG) analysis. Determination of cellular TG content was performed as described previously [11]. Cells were washed with PBS and lipids were extracted with hexane: isopropanol at 3:2 v/v. Extracted lipids were dissolved in methanol and the TG content was determined on the Roche COBAS Integra 400+ (Roche Diagnostics, Indianapolis, IN). The resulting protein cell pellet was dried at 65 °C for 24 h, re-suspended in 1 M sodium hydroxide and analyzed for protein content. Protein was quantified using the Bio-Rad Protein Dye Kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. TG content was normalized to the cellular protein content. Results are expressed as total mg TG / mg protein.

2.5.3 Leptin determination. Leptin concentration in cell culture media was determined by a mouse-specific enzyme-linked immunosorbent assay (Abcam Cambridge, MA). Briefly, this assay employed an antibody specific for mouse leptin coated on a 96- well plate. Recombinant mouse leptin was used as a standard. All samples were measured in duplicate. Leptin content was normalized to the cellular protein content of the harvested cells.

2.5.4 Gene expression analysis. For determination of gene expression, RNA was isolated using the QIAcube system (Qiagen, Valencia, CA), consisting of a QIA shredder spin column, an RNeasy MiniKit, and on-column DNase I treatment, following the manufacturer's protocols. The quantity and quality of RNA were determined on a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA (1µg) was converted to cDNA using the GoScript reverse transcription system kit

(Promega, Madison, WI). The sequences of the primers used in the study are presenting in Table 1. Real time PCR reactions contained the following: 1X SYBR Green Master Mix (Applied Biosystem, Inc., Carlsbad, CA), 250 nM of forward and reverse primers 1 µg cDNA. The final volume of the reactions was 50 µL and dissociation curves confirmed a single amplification species. Real Time PCR was performed on a 7500 Fast Real Time PCR System (Applied Biosystem, Inc., Carlsbad, CA). Real-time qPCR primers were designed and purchased from IDT (Coralville, IA). Expression of candidate genes was normalized to β -actin expression levels.

2.5.5 Immunoblotting of NQO1 and fatty acid binding protein (FABP) 4. Cells were washed with PBS and homogenized with cold homogenizing buffer (20 mM sodium phosphate, 1mM diethylene triamine pentaacetic acid (DTPA), 0.05 mM butylated hydroxytoluene (BHT), and 0.5% Triton X-100, pH7.4, and 1% Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO)) and sonicated at 3 bursts for 10 s each at 50% amplitude with a sonic dismembrator (Fisher Scientific, Waltham, MA). Total protein was determined using the Bio-Rad protein dye kit (Bio-Rad, Hercules, and CA) using bovine serum albumin as the standard. Immunoblot analysis for NQO1 and FABP4 was performed according to Vomhof-DeKrey and Picklo [11].

2.5.6 GSH and glutathione disulfide (GSSG) analyses. Experiments were performed in 25 cm² flasks. After completion of the treatment period, the cell medium was aspirated and the cells were washed with ice-cold PBS. Cells were scraped into 250 µL of 5% sulfosalicylic acid (Sigma-Aldrich, St. Louis, MO; dissolved in water) and sonicated. The

resulting homogenate was centrifuged at 14000 × g for 10 min at 4 °C. The supernatant was removed and assayed for GSH and GSSG content and the pellet was assayed for total protein. GSH and GSSG content were determined using a modification of the method reported by Dringen and colleagues [22, 24].

2.6 Statistical analysis. Statistical comparisons were made using Prism 5.0 software (GraphPad Software, San Diego California USA, www.graphpad.com). Data were analyzed using one-way ANOVA and statistical significance was taken as $p \leq 0.05$. All data are expressed as the mean $\pm$ the standard deviation (SD) unless otherwise stated.

3. Results

3.1 Inhibition of TG accumulation

In an initial set of experiments, the effect of PJ_M upon cell viability, as determined by the MTT assay, was assessed in order to select PJ_M concentrations for subsequent analyses. Exposure of 3T3-L1 pre-adipocytes to PJ_M for 24 hrs resulted in a dose-dependent decrease in MTT activity (Figure 1). We arbitrarily set the highest concentration of PJ_M (55 µg/mL) used for subsequent experiments to be the concentration which retained 80% of control MTT activity.

The ability of PJ_M to inhibit lipid accumulation was evaluated histochemically through Oil Red O staining and through analysis of cellular TG content. Confluent monolayers of 3T3-L1 pre-adipocytes were treated with increasing concentrations of freshly-diluted

PJ_M on days 0, 2, 5, and 7, days that corresponded to changes in the cell culture media. The resulting differentiated adipocyte cultures were fixed or harvested on day 8. At day 8, untreated differentiated cells had accumulated lipid droplets as demonstrated by Oil Red O staining (Figure 2A), an effect inhibited by PJ_M treatment. In agreement with the staining results, PJ_M (10 µg/mL) lowered TG content by approximately 30% in the differentiated adipocyte cultures (Figure 2B).

Increased expression of FABP4 is recognized as a marker of adipocytic differentiation [25]. We tested whether PJ_M treatment blocked differentiation of preadipocytes to adipocytes vs blocking TG accumulation. As expected FABP4 content increases with differentiation of preadipocytes to adipocytes (Figure 2C). In contrast to its effects on TG, PJ_M did not block expression of FABP4, indicating that PJ_M does not block adipocyte differentiation.

That adipocyte differentiation is not blocked by PJ_M is further supported by analysis of leptin secretion. Following differentiation the leptin concentration in the cellular medium more than doubled for differentiated 3T3-L1 adipocytes undifferentiated 3T3-L1 pre-adipocytes (Figure 2D). While there was a reduction in leptin secretion as a result of PJ_M exposure, leptin levels were nearly 60% greater in adipocytes treated with PJ_M vs pre-adipocytes.

3.2 Changes in redox markers

Previous data shows that suppression of TG content in 3T3 adipocytes by the phytochemical sulforaphane occurs in part through elevating the expression of the antioxidant enzyme NQO1 [11]. In this experiment, we tested the extent to which PJ_M increases the expression of NQO1 (Figure 3). NQO1 gene expression was elevated 5-fold in preadipocytes treated for 48 h with PJ_M (Figure 3A). Moreover, in preadipocytes treated with PJ_M during the seven-day differentiation protocol, NQO1 protein content was elevated > 10-fold by PJ_M treatment vs untreated adipocytes or preadipocytes (Figure 3B).

Elevated expression of NQO1 can occur through stimulation of the Nrf2-ARE pathway that can be activated by depletion of GSH [13, 26]. Following a six-hour treatment with PJ_M, GSH content in preadipocytes was depleted in a dose-dependent manner (Figure 4A). Conversely, the GSSG level (Figure 4B) was increased by PJ_M when compared to control cells. When the GSH: GSSG ratio was calculated, we observed decrease in this ratio starting at 10 µg/mL of PJ_M (Figure 4C) compared to control cells.

3.3 Determination of bioactive fractions

In order to discern the bioactive components responsible for changes noted above, PJ_M was fractioned into *n*-hexane (PJ_H), dichloromethane (PJ_D) and aqueous (PJ_A) fractions. The bioactivity of the resulting fractions was determined using a concentration of fraction comparable to that present in the starting PJ_M. Thus, PJ_M at 55 µg/mL, PJ_H at 25 µg/mL and PJ_D at 10 µg/mL were used in the subsequent assays. PJ_A was used 10 µg/mL, a concentration twice that found in PJ_M.

We examined the extent to which inhibition of TG accumulation was present in the resulting PJ fractions by treatment of preadipocytes during the seven-day differentiation protocol used above. Our results (Table 2) show that the PJ_D but not the PJ_H or PJ_A fractions contained significant activity towards inhibiting TG accumulation. On the other hand, PJ_D and PJ_H fractions both contained compounds that resulted in depletion of GSH.

We further examined the extent to which the PJ_H, PJ_D, and PJ_A fractions modified the expression of NQO1 and other genes that contribute to changes in cellular redox status and anti-oxidant defense responses (Table 2). In these experiments, 3T3-L1 preadipocytes were treated with the fraction and harvested 48 hrs later, similar to experiments described above. PJ_M elevated the expression of NQO1, glutathione disulfide reductase (GR), glutamate cysteine ligase catalytic subunit (GCL_C), glutathione peroxidase 3 (GPx3), GPx4, and catalase. This pattern was replicated by the dichloromethane fraction, PJ_D. The PJ_H fraction, on other hand, only induced expression of GPx3 and catalase. The final, aqueous fraction PJ_A did not affect the gene expression endpoints examined. Expression of GPx1, SOD1 and SOD2 was not modified by any fraction.

3.4 Chemical characterization of PJ fractions

The PJ_M, PJ_H, PJ_D, and PJ_A fractions were analyzed by GC-MS and LC-TOF-MS in order to characterize the bioactive compounds present. GC-MS analyses were

performed directly on the fractions or following TMS derivatization. The formation of TMS derivatives allowed for analysis of hydroxy- and carboxy-containing compounds with limited volatility. Direct GC-MS analysis (without TMS derivatization) (Figure 5) of PJ fractions showed that most fractions contained carvotanacetone, with the highest abundance in PJ_H. Other compounds were observed in limited abundance and are further detailed in supplementary data (Table S1). These results are in agreement with prior reports of the chemical composition of other *Pulicaria* species [4, 6, 27, 28].

In order to further characterize less volatile, polar components by GC-MS, TMS derivatization was performed (Figure 6). Based upon the match with the NIST mass spectra library, catechin-like compounds were found in high-abundance in the PJ_M extract and the PJ_D fractions (Figures 6A,C). These results parallel the bioactivity of these two fractions. Several other compounds, including carvotanacetone, fatty acids and sugars were observed in the fractions analyzed and are further detailed in supplementary data (Table S2).

Further analysis of these catechin-like compounds was performed (Figure 7). Focusing in on the relevant section of the chromatogram derived from GC-EI/MS of TMS-derivatized PJ_D fraction, six catechin-like compounds were observed (Figure 7A). Three of these compounds, peaks D2, D3, D4 (Figure 7B-D), had a characteristic common ion 368 *m/z* that matched the EI mass spectra of catechin and epi-catechin analytical standards (Figure 7E) [29]. However, the retention time of these catechin-like species

did not match the retention time of the catechin and epicatechin standards (Figure 8). The low resolution of the EI-MS data prevented further identification of these compounds.

The PJ_D fraction was analyzed using LC with high resolution TOF-MS. Similar to GC-MS, two peaks with ions of 289.07 *m/z* (corresponding to [M-H]⁻ ion for catechin) were observed (Figure 9A), and similar to GC, they eluted at later retention times. The low mass accuracy error of 14 ppm supported the molecular structure of this ion as the same as that of catechin (Figure 9A). The mass spectra of these peaks (Figure 9B,C) showed the most abundant fragments as 317.05 *m/z* and 359.19 *m/z*. The first peak (L1, *t_R* 19.15) with the ions of 289 *m/z* and 317 *m/z* may be related to GC-MS peaks D2 and D4 (Figure 7), respectively. The 317 *m/z* corresponds to catechin with a CO group, with 40 ppm error. The second peak (L2) had an abundant ion of 359 *m/z*, suggesting that the parent molecule possessed a mass of 360 with a proposed structure C₂₀H₂₄O₆ with 111 ppm error.

4. Discussion

Obesity is a global health concern, including in the Middle East. Therapeutic strategies include nutritional, pharmacological, and surgical interventions. Phytochemicals from traditional medicinal herbs offer an alternative approach. Pharmacological characterization of *Pulicaria jaubertii* E.Gamal-Eldin (PJ) is lacking even though it has been used by the Yemeni people as a traditional medicine. In this study, we examined the anti-obesogenic properties of PJ. Our data demonstrate that organic extracts of PJ attenuated TG accumulation and increased the expression of multiple antioxidant

genes. Chemical fractionation and MS-based characterization indicate the catechin-like compounds present in the DCM fraction of PJ are responsible in part for this activity.

PJ_M blocks accumulation of TG but not expression of the adipocyte differentiation marker FABP4. FABP4 is a key factor for intracellular fatty acid transport and lipid metabolism [30]. These data indicate that decreases in TG accumulation were not the result of inhibiting differentiation of the pre-adipocytes into adipocytes per se but by inhibition of TG accumulation itself. Treatment of 3T3-L1 preadipocytes with epigallocatechin yields a similar response [31]. These data are in contrast to other phytochemicals like sulforaphane and curcumin that inhibit TG synthesis in addition to inhibiting expression of FABP4 [11, 23].

PJ_M increased the expression of several genes NQO1, GR, GCL_C, and GPx4 associated with antioxidant defense and activation of the Nrf2-ARE pathway [32]. Previous data from this laboratory demonstrate the isothiocyanate sulforaphane, a known activator of the Nrf2-ARE, inhibits TG accumulation in part through elevation of NQO1 activity [11]. Given these previous data, our findings suggest that activation of the Nrf2-ARE pathway contributed to the decrease in TG accumulation. While our experiments do not specifically test for activation of an Nrf2-ARE reporter or movement or accumulation of Nrf2 to the nucleus, the activation of a number of Nrf2-ARE inducible gene tested strongly support the activation of this pathway

The role of GSH in adipogenesis and obesity development is complex [17]. Previous in vitro experiments demonstrate that GSH depletion increases adipogenesis and TG accumulation and that supplementation with GSH precursors can block adipogenesis [18, 19]. Our data do not support these findings since PJ_H while reducing cellular GSH did not significantly modify (increase or decrease) TG accumulation. We recognize however that our experiments utilized a complex mixture of bioactive compounds with potential pro-adipogenic and anti-adipogenic properties. In this context, GSH depletion itself may not be sufficient to modulate TG accumulation.

Consistent with activity in PJ_M , the DCM fraction PJ_D contained catechin-like compounds to a similar degree as found in PJ_M . The effect of catechin species in preventing the differentiation of adipocytes is well-documented [31, 33-35]. Catechins and related flavonoid phytochemicals have not been reported in previous analyses of PJ, likely as the result of using GC-MS without TMS derivatization. However, a number of flavonoids and sterols have been detected in other *Pulicaria* species [3].

GC-MS analysis of PJ_M and the PJ_H and PJ_D fractions resulted in the identification of carvotanacetone as being a major component. Carvotanacetone is an α,β -unsaturated ketone. Given its structure, carvotanacetone has an electrophilic center and thus likely can form a Michael adduct with GSH. While carvotanacetone is not commercially available, treatment of 3T3-L1 cells with the structural analog carvone confirms that carvone-type compounds deplete cellular GSH (data not shown).

In conclusion, this study provides in vitro evidence of anti-adipogenic properties of *Pulicaria jaubertii* E.Gamal-Eldin. Our results show that the methanol extract of PJ and its subsequent DCM fraction, likely through the presence of catechin-like compounds, possessed bioactivity towards inhibiting TG accumulation in adipocytes. Our data provide new knowledge regarding the abundance of compounds in this Yemeni herb and their ability to modify cellular anti-oxidant properties.

5. Acknowledgments

The authors are grateful to Mary Briske-Anderson, Kim Michelson, Gregoire Brian and Dale Christopherson for their technical assistance with this work. Funding was provided through USDA-ARS Projects 5450-51000-048-00D. The U.S. Department of Agriculture, Agricultural Research Service, Plains Area is an equal opportunity/affirmative action employers and all agency services are available without discrimination. Mention of trade names or commercial products in this article is solely for providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

6. References

- [1] M. Ng, T. Fleming, M. Robinson, B. Thomson, N. Graetz, C. Margono, E.C. Mullany, *et al.*, Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013, *Lancet* 384 (2014) 766-781.
- [2] B.M. Popkin, M.M. Slining, New dynamics in global obesity facing low- and middle-income countries, *Obes Rev* 14 Suppl 2 (2013) 11-20.
- [3] L.L. Liu, J.L. Yang, Y.P. Shi, Phytochemicals and biological activities of *Pulicaria* species, *Chem Biodivers* 7 (2010) 327-349.
- [4] M.N. Algabr, A. S., A. Menad, R. Mekkiou, J.C. Chalchat, B. S., F. Benayache, Essential oil composition of *Pulicaria jaubertii* from Yemen, *Int J Med Arom Plants* 2 (2012) 688-690.
- [5] A.S. Dubaie, A.A. Al-Khulaida, Studies on the Flora of Yemen On the flora of Tihama plain *J Botan Taxon Geobot* 104 (1993) 259-265.
- [6] G.A. Fawzy, H.Y. Al Ati, A.A. El Gamal, Chemical composition and biological evaluation of essential oils of *Pulicaria jaubertii*, *Pharmacogn Mag* 9 (2013) 28-32.
- [7] M. Gonzalez-Castejon, A. Rodriguez-Casado, Dietary phytochemicals and their potential effects on obesity: a review, *Pharmacol Res* 64 (2011) 438-455.

- [8] J. Lin, M.A. Della-Fera, C.A. Baile, Green tea polyphenol epigallocatechin gallate inhibits adipogenesis and induces apoptosis in 3T3-L1 adipocytes, *Obes Res* 13 (2005) 982-990.
- [9] N. Siriwardhana, N.S. Kalupahana, M. Cekanova, M. LeMieux, B. Greer, N. Moustaid-Moussa, Modulation of adipose tissue inflammation by bioactive food compounds, *J Nutr Biochem* 24 (2013) 613-623.
- [10] A. Ejaz, D. Wu, P. Kwan, M. Meydani, Curcumin inhibits adipogenesis in 3T3-L1 adipocytes and angiogenesis and obesity in C57/BL mice, *J Nutr* 139 (2009) 919-925.
- [11] E.E. Vomhof-DeKrey, M.J. Picklo, NAD(P)H:quinone oxidoreductase 1 activity reduces hypertrophy in 3T3-L1 adipocytes, *Free Radic Biol Med* 53 (2012) 690-700.
- [12] A.K. Jaiswal, Regulation of genes encoding NAD(P)H:quinone oxidoreductases, *Free Radic Biol Med* 29 (2000) 254-262.
- [13] D. Ross, J.K. Kepa, S.L. Winski, H.D. Beall, A. Anwar, D. Siegel, NAD(P)H:quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms, *Chem Biol Interact* 129 (2000) 77-97.
- [14] J. Palming, K. Sjoholm, M. Jernas, T.C. Lystig, A. Gummesson, S. Romeo, L. Lonn, *et al.*, The expression of NAD(P)H:quinone oxidoreductase 1 is high in human adipose tissue, reduced by weight loss, and correlates with adiposity,

- insulin sensitivity, and markers of liver dysfunction, *J Clin Endocrinol Metab* 92 (2007) 2346-2352.
- [15] D. Siegel, W.A. Franklin, D. Ross, Immunohistochemical detection of NAD(P)H:quinone oxidoreductase in human lung and lung tumors, *Clin Cancer Res.* 4 (1998) 2065-2070.
- [16] A. Gaikwad, D.J. Long, 2nd, J.L. Stringer, A.K. Jaiswal, In vivo role of NAD(P)H:quinone oxidoreductase 1 (NQO1) in the regulation of intracellular redox state and accumulation of abdominal adipose tissue, *J Biol Chem.* 276 (2001) 22559-22564. Epub 22001 Apr 22517.
- [17] M.J. Picklo, E.K. Long, E.E. Vomhof-DeKrey, Glutathionyl systems and metabolic dysfunction in obesity, *Nutr Rev.* 73 (2015) 858-868. doi: 810.1093/nutrit/nuv1042. Epub 2015 Oct 1022.
- [18] P. Vigilanza, K. Aquilano, S. Baldelli, G. Rotilio, M.R. Ciriolo, Modulation of intracellular glutathione affects adipogenesis in 3T3-L1 cells, *J Cell Physiol* 226 (2011) 2016-2024.
- [19] P. Calzadilla, M. Gomez-Serrano, E. Garcia-Santos, A. Schiappacasse, Y. Abalde, J.C. Calvo, B. Peral, *et al.*, N-Acetylcysteine affects obesity-related protein expression in 3T3-L1 adipocytes, *Redox Rep* 18 (2013) 210-218.
- [20] H.M. Findeisen, F. Gizard, Y. Zhao, H. Qing, K.L. Jones, D. Cohn, E.B. Heywood, *et al.*, Glutathione depletion prevents diet-induced obesity and enhances insulin sensitivity, *Obesity (Silver Spring)* 19 (2011) 2429-2432.

- [21] E.E. Vomhof-Dekrey, M.J. Picklo, Sr., The Nrf2-antioxidant response element pathway: a target for regulating energy metabolism, *J Nutr Biochem* 23 (2012) 1201-1206.
- [22] E.K. Long, T.C. Murphy, L.J. Leiphon, J. Watt, J.D. Morrow, G.L. Milne, J.R. Howard, *et al.*, Trans-4-hydroxy-2-hexenal is a neurotoxic product of docosahexaenoic (22:6; n-3) acid oxidation, *J Neurochem* 105 (2008) 714-724.
- [23] J. Ahn, H. Lee, S. Kim, T. Ha, Curcumin-induced suppression of adipogenic differentiation is accompanied by activation of Wnt/beta-catenin signaling, *Am J Physiol Cell Physiol* 298 (2010) C1510-1516.
- [24] R. Dringen, B. Pfeiffer, B. Hamprecht, Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of CysGly as precursor for neuronal glutathione, *J Neurosci* 19 (1999) 562-569.
- [25] K. Schroder, K. Wandzioch, I. Helmcke, R.P. Brandes, Nox4 acts as a switch between differentiation and proliferation in preadipocytes, *Arterioscler Thromb Vasc Biol.* 29 (2009) 239-245. doi: 210.1161/ATVBAHA.1108.174219. Epub 172008 Dec 174214.
- [26] A.H. Lin, H.W. Chen, C.T. Liu, C.W. Tsai, C.K. Lii, Activation of Nrf2 is required for up-regulation of the pi class of glutathione S-transferase in rat primary hepatocytes with L-methionine starvation, *J Agric Food Chem* 60 (2012) 6537-6545.

- [27] N.A. Ali, F.S. Sharopov, M. Alhaj, G.M. Hill, A. Porzel, N. Arnold, W.N. Setzer, *et al.*, Chemical composition and biological activity of essential oil from *Pulicaria undulata* from Yemen, *Nat Prod Commun* 7 (2012) 257-260.
- [28] J. Sharifi-Rad, A. Miri, S.M. Hoseini-Alfatemi, M. Sharifi-Rad, W.N. Setzer, A. Hadjiakhoondi, Chemical composition and biological activity of *Pulicaria vulgaris* essential oil from Iran, *Nat Prod Commun* 9 (2014) 1633-1636.
- [29] C. Proestos, M. Komaitis, Analysis of Naturally Occurring Phenolic Compounds in Aromatic Plants by RP-HPLC Coupled to Diode Array Detector (DAD) and GC-MS after Silylation, *Foods* 2 (2013) 90-99.
- [30] E.D. Rosen, O.A. MacDougald, Adipocyte differentiation from the inside out, *Nat Rev Mol Cell Biol* 7 (2006) 885-896.
- [31] N. Sakurai, K. Mochizuki, H. Kameji, M. Shimada, T. Goda, (-)-Epigallocatechin gallate enhances the expression of genes related to insulin sensitivity and adipocyte differentiation in 3T3-L1 adipocytes at an early stage of differentiation, *Nutrition* 25 (2009) 1047-1056.
- [32] J.D. Hayes, A.T. Dinkova-Kostova, The Nrf2 regulatory network provides an interface between redox and intermediary metabolism, *Trends Biochem Sci* 39 (2014) 199-218.
- [33] H.C. Ku, H.H. Chang, H.C. Liu, C.H. Hsiao, M.J. Lee, Y.J. Hu, P.F. Hung, *et al.*, Green tea (-)-epigallocatechin gallate inhibits insulin stimulation of 3T3-L1

preadipocyte mitogenesis via the 67-kDa laminin receptor pathway, *Am J Physiol Cell Physiol* 297 (2009) C121-132.

- [34] H. Lee, S. Bae, Y. Yoon, The anti-adipogenic effects of (-)epigallocatechin gallate are dependent on the WNT/beta-catenin pathway, *J Nutr Biochem* 24 (2013) 1232-1240.
- [35] H.S. Liu, Y.H. Chen, P.F. Hung, Y.H. Kao, Inhibitory effect of green tea (-)-epigallocatechin gallate on resistin gene expression in 3T3-L1 adipocytes depends on the ERK pathway, *Am J Physiol Endocrinol Metab* 290 (2006) E273-281.

Table 1. Primer sequences used in the study

Gene name	forward primer Sequence	reverse primer Sequence
β-actin	TGTCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTAGA
NQO1	TATCCTTCCGAGTCATCTCTAGCA	TCTGCAGCTTCCAGCTTCTTG
GLC _C	CATCGACCTGACCATCGATAAG	AGGGTGAGTGGGTCTCTAATAA
GR	CTT TCA GCT GGA GGA CTT GC	CCA GGC CTG ATG ATG TCT TT
GPx1	GGG ACT ACA CCG AGA TGA ACG A	ACC ATT CAC TTC GCA CTT CTC A
GPx3	GCCAGCTACTGAGGTCTGACAGA	CCACCTGGTCGAACATACTTGAG
GPx4	TCT GGC AGG CAC CAT GTG T	CGG GCA TGC AGA TCG ACT A
SOD1	TGG GTT CCA CGT CCA TCA GTA	ACC GTC CTT TCC AGC AGT CA
SOD2	ATT AAC GCG CAG ATC ATG CA	TGT CCC CCA CCA TTG AAC TT
CAT	GCG TCC AGT GCG CTG TAG A	TCA GGG TGG ACG TCA GTG AA

Table 2. Determination of bioactive fractions of *Pulicaria jaubertii*

Endpoint	Fraction¹				
	Control	PJ_M	PJ_H	PJ_D	PJ_A
Triacylglyceride² (mg/mg protein)	1.45 (0.18)	0.61 (0.15)*	1.15 (0.24)	0.90 (0.07)*	1.01 (0.27)
GSH³ (nmol/mg protein)	28.5 (2.3)	10.9 (1.2)*	14.2 (2.5)*	12.2 (3.7)*	24.5 (4.4)
GSSG³ (nmol/mg protein)	0.53 (0.12)	0.95 (0.29)	1.03 (0.24)	1.01 (0.22)	0.65 (0.18)
MTT Activity⁴	100	111 (4)	109 (7)	98 (10)	100 (6)
<u>Gene Expression⁵</u>					
NQO1	1.00	6.2 (0.8)*	2.4 (0.5)	7.6 (0.8)*	1.6 (0.1)
GR	1.00	2.7 (0.1)*	1.7 (0.1)	3.3 (0.5)*	1.3 (0.1)
GCL _C	1.00	5.7 (0.1)*	1.6 (0.5)	7.1 (1.2)*	1.4 (0.4)
GPx1	1.00	1.2 (0.2)	1.1 (0.1)	1.5 (0.6)	1.5 (0.7)
GPx3	1.00	7.0 (0.5)*	5.7 (0.1)*	12.2 (0.9)*	1.4 (0.1)
GPx4	1.00	1.6 (0.2)*	1.3 (0.2)	1.7 (0.2)*	1.6 (0.0)
Catalase	1.00	3.2 (0.3)*	2.8 (0.7)*	2.9 (0.3)*	1.6 (0.0)
SOD1	1.00	1.4 (0.5)	1.5 (0.5)	1.1 (0.4)	1.6 (0.1)
SOD2	1.00	1.5 (0.4)	1.5 (0.2)	1.5 (0.1)	1.1 (0.1)

* significantly ($p \leq 0.05$) different from the Control (vehicle only) treatment using one way ANOVA.

¹ *Pulicaria jaubertii* methanol fraction (PJ_M); PJ hexane fraction (PJ_H); PJ dichloromethane fraction (PJ_D); PJ aqueous fraction (PJ_A); NADPH;Quinone Oxidoreductase 1 (NQO1); Glutathione reductase (GR); glutamate cysteine ligase catalytic unit (GCL_C); Glutathione peroxidase (GPx); Superoxide dismutase (SOD).

²Triacylglyceride content was measured after treating 3T3-L1 preadipocytes with the fractions following the seven-day differentiation regimen. Data are the mean $\pm$ the SD for three independent experiments ($n = 3$).

³ GSH and GSSG were measured following a 6 hr exposure of 3T3-L1 preadipocytes to the fractions. Data are the mean $\pm$ the SD for three independent experiments ($n = 3$).

⁴ MTT activity as a measure of cell viability was measured 24 hrs after exposure to the fractions. Data are the mean $\pm$ the SD for three independent experiments ($n = 3$) performed in quadruplicate.

⁵ Gene expression analysis was performed on 3T3-L1 preadipocytes 48 hrs following a single exposure to the fractions. Data are the mean $\pm$ the SD for three independent experiments ($n = 3$).

Figure Legends

Figure 1. Effect of methanolic extract of *Pulicaria Jaubertii* (PJ_M) on preconfluent 3T3-L1 preadipocytes. Cells were exposed to PJ_M (1-250 µg/ml) for 24 h. Cell viability was determined using the MTT colorimetric assay. All data are reported as the percentage change in comparison with the control cells. The values represent means ± SD for three independent experiments ($n = 3$). * = significantly different ($p \leq 0.05$) from control (0 µg/mL) using one way ANOVA.

Figure 2. *Pulicaria jaubertii* extract reduces TG synthesis but not adipocyte differentiation. Preadipocytes were differentiated into adipocytes with and without treatment with the methanolic PJ extract (PJ_M) at increasing concentrations on day 0, 2, 5, and 7. The following cells were processed for (A) Oil red O staining, (B) TG content, (C) FABP4 content, and (D) leptin content. In (A) note the decrease in Oil red O with PJ_M treatment that is reflected by decreased TG content (B). Expression of the adipocyte marker FABP4 is not blocked by PJ_M exposure (C). Leptin secretion is reduced but not abolished by PJ treatment (D). Sample sets with different letters are significantly different ($p \leq 0.05$) using one way ANOVA. The values represent means ± SD for three independent experiments ($n = 3$).

Figure 3. Expression of NQO1 is elevated by PJ exposure. Preadipocytes were differentiated into adipocytes with and without treatment with the methanolic PJ extract (PJ_M) at increasing concentrations on day 0, 2, 5, and 7. Following harvest, expression of the NQO1 gene (A) and levels of NQO1 protein (B) were determined by PJ extract in differentiated 3T3-L1. The cells were treated with control vehicle or PJ_M 55 µg/mL at day

0,2,5,7. A representative western blot for NQO1 is provided in (B). The data represent the means $\pm$ SD of 2 different experiments performed in triplicate. Sample sets with different letters are significantly different ($p \leq 0.05$) using one way ANOVA.

Figure 4. *Pulicaria jaubertii* extract exposure reduces GSH content. The effects of the PJ_M and its fractions on (A) GSH depletion (A) GSSG level (B) and GSH/GSSG ratio (C) in 3T3 L1 cells. Confluent 3T3-L1 preadipocytes were treated with increasing concentrations of PJ_M for 6 h. The lines show significant differences between different groups ($p < 0.05$). The values represent means $\pm$ SD for three independent experiments ($n = 3$). * = significantly different ($p \leq 0.05$) from control (0 $\mu\text{g/mL}$) using one way ANOVA.

Figure 5. GC-MS total ion current chromatograms of methanol extract and its fractions without TMS derivatization. (A) methanol extract (PJ_M), (B) DCM fraction (PJ_D), (C) *n*-hexane fraction (PJ_H), (D) water/methanol fraction (PJ_A). All chromatograms are normalized to the response of internal standard, thus providing proportional response based on the quantities extracted.

Figure 6 . GC-MS total ion current chromatograms of methanol extract and its fractions with TMS derivatization. (A) methanol extract (PJ_M), (B) DCM fraction (PJ_D), (C) *n*-hexane fraction (PJ_H), (D) water/methanol fraction (PJ_A). Note that analysis of TMS derivatives allowed for enhanced characterization of the fractions and allowed for identification of catechin-like compounds in the methanol extract and the DCM fraction. All chromatograms are normalized to the response of internal standard, thus providing proportional response based on quantities extracted.

Figure 7. GC-MS characterization of DCM fraction (PJ_D) and EI-MS spectrum of most abundant catechin-like compounds. (A) Total ion current chromatogram (B-G) EI-MS spectra of peaks D1-D6. Compounds D2, D3, D4 showed high identity (> 80%) with the EI/MS NIST library for catechin. Compounds were analyzed as TMS derivatives.

Figure 8. GC-MS characterization of catechin and epicatechin standards. (A) TMS-derivatives of catechin and epicatechin standards were analyzed by GC-MS in comparison to the TMS-derivatized DCM fraction, PJ_D. Note that the retention times for the standards do not match that of the compounds observed in the PJ_D. The EI-MS spectra of catechin and epicatechin are provided in (B) and (C). Note that of the 368.2 ions present in the standards is also enriched in the TMS-derivatized compounds observed in the DCM fraction detailed in Figure 7.

Figure 9. Extracted ion chromatograms and mass spectra of LC-HR-MS analysis of the DCM fraction PJ_D. (A) Extracted ion chromatogram (EIC) of ions 289.07, 317.05 and 359.19. (B) Time of flight (TOF) mass spectrum of L1 peak, C) TOF mass spectra of L2 peak and mass accuracy confirming the catechin-like structure. The structure of catechin (m/z 289.0718) is provided for comparison.

Figure 1

Figure 2

A

B

C

D

Figure 3

Figure 4

Figure 5.

Figure 6

Figure 7

Figure 8.

B) EI-MS spectra of catechin standard

C) EI-MS spectra of epicatechin standard

Figure 9