The Biologic Potential of Lyophilized Extracts of *Brickellia cavanillesii* (Asteraceae): Apoptosis and Glut 2 Gene Expression

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Abstract

Prior investigations into the therapeutic potential of Lyophilized extracts of *Brickellia cavanillesii* (LBC) are indicative of its possible biologic benefit in the treatment of type 2 diabetes mellitus (T2DB). This manuscript employs in vitro toxicological techniques to explore the effect of LBC on the gene expression of human carcinoma liver cells (HepG2); and attempts to predict a mechanism of action using apoptosis as a therapeutic index. The effect of LBC on the expression of genes associated with human apoptosis pathway and glucose transporter 2 (GLUT 2) were determined using quantitative gene array and real-time PCR (RT2qPCR) respectively. HepG2 cells were exposed to concentrations of LBC (0 mg/mL [control], 0.2 mg/mL for the apoptosis study, 0 mg/mL [control], 0.02 mg/mL, 0.2 mg/mL for the GLUT 2 study), in the absence of PBS 2 h, 4 h, 6 h and 24 h respectively. Results obtained show that several antiapoptotic genes were significantly up-regulated while some apoptotic genes were significantly down-regulated. The most significant up-regulation was by BCL2L1 with a fold change of 46.57; Bcl2l is reputed to be an apoptosis inhibitor. Data acquired from the GLUT 2 gene expression study suggest that LBC may induce GLUT 2 gene expression.

Keywords: Biologic; Lyophilize; *Brickellia cavanillesii*; Apoptosis; GLUT 2; T2DB; Biomarker

Practical Application

*Brickellia cavanillesii* (Asteraceae) is an herbal plant consumed extensively in Neotropical Americas (Central America, Mexico, South-Western USA) for the treatment of Type 2 diabetes mellitus. However, inadequate scientific documentation exists to authenticate its alleged pharmacological properties. This study investigates the biologic potential of lyophilized tea extracts of *Brickellia cavanillesii* (LBC) using apoptosis and GLUT 2 gene expression as biomarkers.

Introduction

Complementary and Alternative Medicine (CAM) has been used as a form of medicinal therapy for as long as history has been recorded. There has been and continues to be significant and growing interest, across the globe, in the introduction and integration of CAM as a complement or substitute to conventional medicinal therapy. While complementary medicine is used together with conventional medicine, alternative medicine is used in place of conventional medicine. Herbalism also known as phytotherapy is a subclass of CAM. Modern pharmacology places emphasis on the active ingredients of herbal plants and research is performed focusing on identifying and isolating these active ingredients. Conversely, herbalism considers the synergistic interaction of all the components of the whole plant for the treatment of acute and chronic conditions [1]. This study is a component part of a research program designed to investigate the efficacy of lyophilized extracts of *Brickellia cavanillesii* (Asteraceae) in the therapy of Type 2 diabetes mellitus (T2DM). Species of the Asteraceae family are used largely for their presumed antidiabetic and anticancer properties among others [2,3]; *Brickellia cavanillesii* is easily one of the more popular herbal plants consumed for the therapy of diabetes in Neotropical Americas. Preliminary studies conducted in our laboratory employed gas chromatographic methods to chemically characterize and identify twenty-one compounds in the methanol extracts of Lyophilized *Brickellia cavanillesii* (LBC) [4]. A succeeding study explored the potential therapeutic benefit and biological activity of LBC *in vitro* [5] using cytotoxicity testing and protein expression.

The focus of this study was to further investigate the biologic potential of LBC utilizing gene expression methodologies. Although *Brickellia cavanillesii* is used widely as an anti-diabetic agent, the precise nature and mechanism of its apoptotic properties has not been fully investigated. The effect of LBC on the expression of genes associated with human apoptosis pathway was determined using quantitative PCR (gene) array. Apoptosis is the physiological process of programmed cell death (PCD) vital for embryonic development and the maintenance of homeostasis in multi-cellular organisms [6,7]. Apoptosis allows cells to undergo characteristic cell changes and death. Typical cell changes include nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum, and membrane blebbing while the mitochondrion remains unchanged morphologically [8]. Apoptosis may be initiated when a cell becomes a threat to an organism’s health, when a cell is no longer needed or for a number of other reasons. The apoptotic process is a complex one. It is centered on a family of proteases caspases which on activation by death receptors (cell-surface receptors transmitting apoptotic signals) cleave key targets in the cell. Death receptors detect the presence of extracellular death signals and respond by rapidly igniting the cells intrinsic apoptosis machinery [9]. Death receptors belong to the tumor necrosis factor (TNF) receptor gene superfamily. TNFR1 (also known as p55 or...
CD120a and the Fas receptor (FasR) (also known as CD95 or Apo1) are the best characterized death receptors [10,11]. The death ligands that activate the death receptors belong to the TNF gene superfamily [9]. Studies indicate that apoptosis is an important therapeutic target and as such may be used as a biomarker in determining the potential of herbal plants in disease therapy [12]. Insulin and beta cell failure are the primary contributors to the development of diabetes mellitus. Reports show that a reduction of islet and insulin containing cell mass or volume are characteristics of T2DM. The regulation of the \( \beta \)-cell mass involves a balance of \( \beta \)-cell replication and apoptosis. However, at the molecular level, pancreatic \( \beta \)-cell loss by apoptosis seems critical in the development of insulin deficiency and disease onset [13,14].

The investigation of the apoptotic effect, and the gene expression, of HepG2 cells exposed to LBC is predicated on the outcome of previous studies [4,5] which suggest that LBC may possess potential therapeutic benefit and biological activity in the therapy of T2DM. HepG2 cells have been employed severally to understudy therapeutic activity [15,16] in herbal decoctions; and is demonstrated to be an excellent model used in examining therapeutic activity [17,18]. Apoptosis has been used to determine anti-carcinogenic property of decoctions in vitro [19,20]; Glut 2 gene, and its expression, is traditionally a candidate gene explored for in vitro T2DM studies [21,22]. Therefore, this study examines; 1) the effect of LBC on the expression of genes associated with human apoptosis pathway; and 2) determines the effect of LBC on the expression of glucose transporter 2 (GLUT2) genes at different concentrations and time points. GLUT 2 genes code proteins that play a fundamental role in glucose homeostasis of the body, and are not included in the apoptosis pathway. It is critical that a continuum of research procedure and evaluation is performed to assure the efficacy of scientific determination.

### Materials and Methods

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#### Materials
Human liver carcinoma cell line (HepG2) (ATCC HB-8065) and fetal bovine serum (FBS) was obtained from ATCC (Manassas, VA, USA). Dulbecco’s Modified Eagle Medium- high glucose (DMEM) was obtained from Hyclone (Logan, UT, USA). Trypsin, 0.25% (1X) with 0.03% EDTA solution was purchased from Gibco (Newport Beach, CA, USA). Other supplies and reagents were purchased from Fisher Scientific (Hampton, NH, USA). Lyophilized extracts of *Brickellia cavanillesii* were prepared [4], filter-sterilized using 0.2 µm filter (Corning, NY, USA) and diluted with plain media to produce serial concentrations of LBC solution without FBS. Fresh preparations were made for each experiment.

#### Cell Culture and treatment

The method used to culture and treat cells for this study is outlined in Eshiet, et al. [5]. Human carcinoma liver cells (HepG2) were cultured in 25 cm² flasks to 80-100% confluence state: a) to study the effect of LBC on the expression of genes associated with human apoptosis pathway, cells were exposed to Dulbecco’s minimum essential media—High Glucose (DMEM) at concentrations of 0 mg/mL (control), 0.02 mg/mL and 0.2 mg/mL LBC extract in the absence of FBS 2h, 4h, 6h and 24h. This was done in three replicates; b) to study the effect of LBC on the expression of glucose transporter 2 (GLUT2) genes, cells were exposed to Dulbecco’s minimum essential media—High Glucose (DMEM) at concentrations of 0 mg/mL (control), 0.02 mg/mL and 0.2 mg/mL LBC extract in the absence of FBS 2 h, 4 h, 6 h and 24 h. This was done in three replicates.

#### Experimental procedure

**Ribonucleic acid (RNA) isolation purity and integrity:** Total cellular RNA was isolated from control and treated HepG2 samples after respective times of exposure using Trizol reagent (Life Technologies, Carlsbad, CA, USA); 24h for study (a) and 2h, 4h, 6h and 24h for study (b). Trizol procedure was performed according to manufacturer’s instructions. RNA purity and concentrations were measured using Thermo Scientific Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Highly purified RNA was indicated by A260/280 values between 1.8 and 2.1. RNA integrity was determined by running the RNA samples on a 1% formaldehyde agarose gel. Sharp clear 28S and 18S bands on the denaturing gel are indicative of high RNA integrity. For our RNA integrity analysis, a sample solution containing 1 μg RNA, 8-10 μL of formaldehyde loading buffer, and 0.3 μL of ethidium bromide was prepared for each sample and incubated at 75°C for 15 min in a thermocycler (Whatman Biometra TGradient, Goettingen, Germany). Electrophoresis was performed using electrophoresis equipment IBI QS-710 (Shelton Scientific, CT, USA) at 80 v for 2h in 1X MOPS-EDTA-Sodium Acetate (MES) buffer. These gels were photographed under ultra violet (UV) light with Chemilinager 4400 (Alpha Innotech Corporation, San Leandro, CA, USA). Only samples for which two clear bands were resolved were used for downstream applications.

**Deoxyribonuclease (DNase) treatment:** Removal of genomic deoxyribonucleic acid (gDNA) from RNA samples was done by treating the samples with DNase free kit (Ambion, Austin, TX, USA) prior to reverse transcription. This was performed according to manufacturer’s instructions.

- a) Reverse Transcription on RNA samples to study the effect of LBC on the expression of genes associated with human apoptosis pathway. Reverse transcription was performed using Super Array’s RT² First Strand Synthesis kit (Cat. No. C-03) (SABiosciences, Frederick, MD, USA) according to manufacturer’s instructions.

**Performing array-based SYBR® green real-time PCR:** Constitutive gene expression profiling was completed using RT² Profiler TM PCR Array Human Apoptosis purchased from SuperArray (SABiosciences, Frederick, MD, USA). For this study the effects on the expression of 84 apoptosis regulated genes (4PAHS-012A) were examined according to the manufacturer’s instructions.

- a) Reverse Transcription of RNA samples to study the effect of *Brickellia cavanillesii* on the expression of glucose transporter 2 (GLUT2) genes. Reverse transcription was performed using RETRO First Strand Synthesis kit (Ambion, Austin, TX) according to manufacturer’s instructions.

**SYBR® Green Real-Time PCR:** Quantitative real-time PCR analysis by standard curve quantitation was performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA).

### Data Analysis
Data was imported into an Excel database [23,24] and analyzed using the comparative cycle threshold method with normalization of the raw data to housekeeping genes (β2M, hypoxanthine, phosphoribosyl transferase 1, ribosomal protein L13a,
GAPDH, and ACTB (β-actin)). Results were compared between groups using t-test. A p value of < 0.05 is considered statistically significant.

Results

Determination of the effect of lyophilized *Brickellia cavanillesii* on the expression of genes associated with human apoptosis pathway

Expression levels of genes associated with human apoptosis pathway were calculated as fold change relative to the gene expression of control samples. An array of 84 genes associated with human apoptosis was screened. Nine genes; AKT1, APAF1, BCL2L1, BNIP2, CASP10, FADD, NOL3, TNFRSF10A, and TNFRSF10B were significantly up-regulated by LBC. Conversely, seven genes; BIRC3, BIRC6, CARD8, CASP1, PYCARD, TNF, and TP73 were significantly down-regulated (Figure 1). However the downward-regulation of BIRC3, CARD8, CASP1, and TNF needs further validation using more biological replicates. In addition, we examined the profile of each functional gene group within the human apoptosis pathway. The results are illustrated in Figures 2-13.

![Figure 1: Fold change differences in the expression of genes regulated by human apoptosis pathway.](image)

![Figure 2: Fold change differences in the expression of genes associated with Tumor Necrosis Family (TNF) ligand family.](image)

![Figure 3: Fold change differences in the expression of genes associated with Tumor Necrosis Family Receptor Family (TNFRF).](image)

![Figure 4: Fold change differences in the expression of genes associated with β-cell lymphoma 2 (βcl-2) family.](image)

![Figure 5: Fold change differences in the expression of genes associated with Caspase family.](image)
Effect of lyophilized *Brickellia cavanillesii* on the expression of GLUT 2 genes

The effect of LBC on GLUT 2 gene expression was investigated using Real-time RT-PCR (RT-qPCR). GLUT 2 expressions was down-regulated at 2 h, 4 h, 6 h and 24 h for concentrations of 0.02 mg/mL LBC in the absence of FBS. The expression of GLUT 2 was also down-
regulated at 2 h for concentrations of 0.2 mg/mL LBC in the absence of FBS. However, at exposures of 0.2 mg/mL LBC in the absence of FBS at 4 h, 6 h and 24 h, the expression of GLUT 2 was up-regulated. Significant up regulation of GLUT 2 gene expression was observed at 6 h and 24 h. This is illustrated in Figure 14. All fold change values greater than 1 are reported as an up-regulation. If the fold change value is less than 1, then the negative inverse of the result may be reported as a fold down-regulation [23,24]. For this study, fold change values above 2 are considered biologically significant.

### Discussion

In recent times, it has become increasingly important to provide proof of mechanism of herbal plants used in disease therapy. The selective activation of apoptosis is often cited as one of the principal indices of therapeutic drugs. Pancreatic β-cell loss by apoptosis could be a possible mechanism for onset of diabetes. Therefore, we examined the effect of LBC on genes associated with the apoptosis pathway. Our results indicate that several genes were either up or down regulated by LBC while one gene, baculoviral IAP repeat-containing 4 (BIRC4) was not detected. The analysis of each functional gene group within the human apoptosis pathway showed that whereas all the genes belonging to the functional gene group TNF Receptor Associated Factor Family (TRAFF) and Death Effector Domain family were up-regulated, all the genes belonging to the Cell Death Inducing DNA fragmentation factor α-like Effector family was down-regulated. However, for this study we concentrated our efforts on understanding the implications of only the genes that were significantly up or down regulated by treatment of HepG2 cells with LBC. The expressions of the following genes; AKT1, APAF1, BCL2L1, BNIP2, CASP10, FADD, NOL3, TNFRSF10A and TNFRSF10B were significantly up-regulated. AKT1 is vital to cell survival and has been shown to be required for cell migration in different organisms [25,26]. AKT1 has been observed to increase fibroblast motility by phosphorylating gridin, an actin-binding protein that enhances fiber formation and lamellapodia [26]. AKT1 signaling promotes matrix metalloproteinase-2 (MMP2) activity in mouse mammary epithelial cells, thereby augmenting invasion [27]. Also, AKT1 promotes cell motility and MMP9 production via NF-KB (Nuclease-factor KB) in fibro-carcinoma cells [28]. Furthermore, AKT1 and AKT2 have been observed to increase invasion of human pancreatic cancer cells by up-regulating IGF-1 receptor (IGF-IR) expression [29]. Therefore, in a range of cell types, AKT1 signaling is associated with enhanced motility and invasion [25]. AKT1 plays an important role in insulin signaling and glucose metabolism. It is involved in the signal cascade that includes glycogen synthase kinase 3β (GSK3β) and Foxol. Studies have revealed that the expression of constitutively active AKT1 (CA-AKT) in human islets induced a significant increase in β-cell replication and significant decrease in β-cell death [30]. Apoptotic protease activity factor (APAF1) is an important component of the intrinsic apoptotic pathway. A deficiency of APAF1 results in an accumulation of neural progenitor cells (NPCs) in the developing central nervous system and therefore in perinatal lethality [31]. BCL2L1 is a member of the BCL-2 family and is known to have anti-apoptosis properties. Studies have indicated that BCL2L1 may play a very important role in regulating the apoptosis of normal liver and cancer liver cells [32]. The specific function of the BNIP2 gene is unclear. However, BNIP2 is known to interact with the E1B 19 kDa protein which is responsible for the protection of virally-induced cell death. BNIP2 is also known to interact with another apoptotic protector, the E1B 19 kDa-likesequences of Bcl-2 [33]. CASP10 is reported to be important in the execution phase of cell apoptosis. CASP10 activates caspase 3 and 7 and is processed by caspases 8. The activation of caspase 3 is necessary because if unregulated, caspase...
activity will kill cells arbitrarily [34]. Mutations in CASP10 are associated with apoptosis defects and have been observed in type II autoimmune lymphoproliferation. FADD is the main adaptor transmitting apoptotic signals mediated by all known death receptors (DRs). Evidence supports an indirect control of FADD on cellular homeostasis, elimination of auto-reactive cells, infected cells and tumor cells. FADD has also been proven to be effective in embryonic development [35,36]. NOL3 encoded for nucleolar protein 3, selectively interacts with Bax but not caspase 3 or 9. By suppressing the enzyme activity of Caspase 8, NOL3 inhibits Fas-induced apoptosis mediated by FADD and TRADD [36,37]. TNFRSF10A on the other hand is activated by tumor necrosis-factor-related apoptosis inducing ligand (TRFRSF10/TRA1). TNFRSF10A transduces cell death signal and induces cell apoptosis [38]. The TNFRST11A gene encodes the receptor activator of nuclear factor-kappa B (RANK) which has been demonstrated to be essential in bone remodeling and osteolysis [39]. The expressions of BIRC3, BIRC6, CARD8, CASP1, PYCARD, TNF, and TP73 were significantly down-regulated. BIRC3 (or cIAP2) is a member of the inhibitor of apoptosis (IAP) family. BIRC3 inhibits apoptosis induced by serum deprivation. It however does not affect apoptosis resulting from exposure to menadione, a potent inducer of free radicals [40,41]. BIRC6 encodes a protein with a BIR (baculoviral inhibition of apoptosis protein repeat) domain and an UBC (ubiquitous conjugating enzyme E2 catalytic domain) and inhibits apoptosis by facilitating the degradation of apoptotic proteins by ubiquitination [42]. CARD8 is involved in pathways leading to the activation of caspases or nuclear factor kappa B (NFKB) in apoptosis by regulating the degradation of caspase-8, but not caspase 3 or 9. By family (caspases). CASP1 is known to interact with a member of the CARD domain, PYCARD and is involved in inflammation and activation of inflammatory processes [44]. The PYCARD gene encodes an adaptor protein composed of two protein-protein interaction domain; the PYD (PYRIN-PADD DAPIN) and CARD (C-terminal caspases recruitment). PYCARD mediates assembly of large signaling complexes in inflammatory and apoptotic signaling pathways via activation of caspases [45]. The fundamental role of TNF is in the regulation of immune cells. TNF can induce apoptotic cell death and inflammation and can also inhibit tumorigenesis and viral replication. The dysregulation of TNF production has been known to lead to a number of human diseases including cancer [46]. Tumor protein 73 (TP73 or p73) is considered to be a tumor suppressor. It is involved in cell cycle regulation, and induction of apoptosis. The analyses of many tumors mainly found in humans such as breast and ovarian cancer have been shown to have high expression of TP73 compared to normal tissues in corresponding areas [47,48]. There are several factors that contribute to apoptosis. The major elements are characterized into two main families of proteins, caspase enzymes and Bcl-2 family [49]. The Bcl-2 (B-cell lymphoma 2) family of apoptosis regulated proteins is encoded by the Bcl-2 gene. The two groups of this family, Bcl-2 and Bax are diametrically opposed; Bcl-2 and Bcl-xl act to inhibit apoptosis while Bax activates apoptosis. Other studies have employed Bax/Bcl-2 and Bax/Bcl-xl ratios as the key index of apoptotic cell death [43]. Studies suggest that over expression of Bax can induce apoptosis in cells both in vivo and in vitro [50,51]. Other studies on PC12 cells indicated that apoptosis was induced when excess concentration of glucose led to an increase in Bax expression [52]. Investigations by Anarkooli et al. revealed the activation of proapoptotic proteins and suppression of antiapoptotic proteins in hippocampus of STZ-induced diabetic rats at both mRNA and protein levels using Bax/Bcl-xl ratios [43]. An analysis of the genes that were significantly regulated by LBC indicates that while several antiapoptotic genes were significantly up-regulated, some apoptotic genes were also significantly down-regulated. However the most significant up-regulation was by BCL2L1 with a fold change value of 46.57. Using the Bax/Bcl-xl ratio as the key index of apoptotic cell death, data shows that there is an increased expression of BCL2L1 over Bax (46.57:1.90) as illustrated in Figure 15. BCL2L1 may be useful as a prognostic marker for understanding the effect of LBC on apoptosis. Furthermore, we hypothesize that LBC may have antiapoptotic properties, and could possibly be a facilitator of β-cell replication. However, the mechanism for this is unclear. An analysis of the data obtained from the effect of LBC on the GLUT 2 gene expression of HepG2 cells suggests that B. cavanillesii may induce GLUT 2 gene expression. Although increase in gene expression did not achieve statistical significance (p=0.05422), it attained biological significance for concentrations of 0.2 mg/mL LBC at 6h and 24 h. We postulate that 0.2 mg/mL LBC may be a better therapeutic dose than 0.02 mg/mL LBC. Two previous studies exploring the potential of B. cavanillesii in diabetes therapy, performed in our laboratory at The Institute of Environmental and Human Health, Texas Tech University (TIEHH), indicate that there may be some validity in the acclaimed therapeutic effect of B. cavanillesii [4,5]. This study reinforces this observation and attempts to propose a mechanism of action. However, further studies are needed to clearly delineate a detailed mechanism of action.

Conclusion

B. cavanillesii is one of the more popular herbal plants consumed for the treatment of Type 2 diabetes mellitus (T2DM). Experimental methods employed in the determination of the expression of genes associated with the human apoptosis pathway and glucose transporter 2 (GLUT 2) suggest that LBC may possess benefit in the therapy of T2DM. Bcl-2 may be useful as a prognostic maker for understanding the effect of B. cavanillesii on apoptosis.

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