Article

Identification of *Bcl2* as a Stably Expressed qPCR Reference Gene for Human Colon Cancer Cells Treated with Cottonseed-Derived Gossypol and Bioactive Extracts and Bacteria-Derived Lipopolysaccharides

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Abstract: Cottonseed contains many bioactive molecules including plant polyphenols. Cottonseed value might be increased by providing high-value bioactive polyphenols for improving nutrition and health. However, there was a lack of molecular evidence for cottonseed bioactivity in mammalian cells. One widely used method for evaluating the bioactivity of natural products is quantitative real-time-PCR (qPCR). The selection of stably expressed internal reference genes is a crucial task of qPCR assay for data analysis. The rationale for reference gene selection is that a lower standard deviation of the cycle of threshold (Cq) among the treatments indicates a more stable expression of the gene. The objective of this study was to select reference genes in human colon cancer cells (COLO 205) treated with cottonseed-derived gossypol and bioactive extracts along with bacterial endotoxin lipopolysaccharides (LPS). SYBR Green qPCR was used to analyze the mRNA levels of a wide range of biomarkers involved in glucose transport, lipid biosynthesis, inflammatory response, and cancer development. qPCR data (10,560 Cq values) were generated from 55 genes analyzed from 64 treatments with triplicate per treatment for each gene. The data showed that B-cell lymphoma 2 (*Bcl2*) mRNA was the most stable among the 55 mRNAs analyzed in the human colon cancer cells. Glyceraldehyde 3 phosphate dehydrogenase (*Gapdh*) and ribosome protein L32 (*Rpl32*) mRNAs were not good qPCR references for the colon cancer cells. These observations were consistent regardless of the treatment comparison between gossypol and LPS, glanded and glandless seed extracts, seed coat and kernel extracts, or treatment for 8 and 24 h. These results suggest that *Bcl2* is a preferable reference gene for qPCR assays in human colon cancer cells treated with cottonseed-derived gossypol and bioactive extracts as well as LPS. The extensive qPCR results firmly support the conclusion that the *Bcl2* gene is stably expressed at the mRNA level in the human colon cancer cells regardless of the treatment, suggesting that *Bcl2* gene expression is not regulated at the mRNA level but at the post-transcriptional level. These results should facilitate studies designated to evaluate bioactivity on gene expression regulation by cottonseed molecules and other natural and synthetic molecules for nutrition and health uses.

Keywords: bioactivity; colon cancer cell; cottonseed extract; gene expression; gossypol; lipopolysaccharides; quantitative real-time PCR; reference gene

1. Introduction

The cotton (*Gossypium hirsutum* L.) plant provides economically important fiber and cottonseed but cottonseed only contributes to approximately 20% of the crop value. It is either glanded or glandless depending on its seed with or without gossypol glands (Figure 1A) [1,2]. Cottonseed contains many bioactive molecules including gossypol (Figure 1B), quercetin, gallic acid, 3,4-dihydroxybenzoic acid, flavonoids, cyclopropenoid

fatty acids, and peptides [3–10]. Most of these value-added products possess health promotion and disease prevention potentials [6,11–15]. Cottonseed value could be potentially increased by providing high-value bioactive products because plant-derived bioactive materials have been used for disease prevention and treatment since ancient history [16–20].
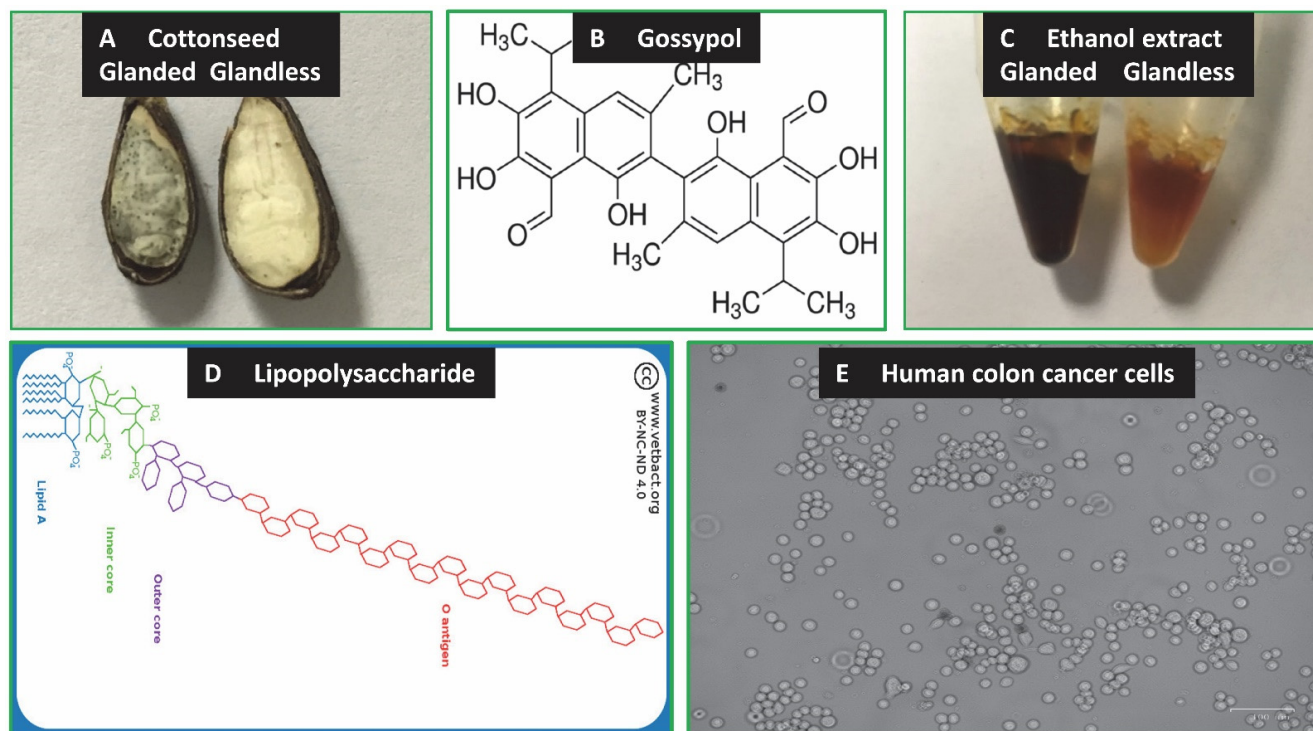
Figure 1. Cottonseed, cottonseed-derived gossypol and polyphenolic extracts, bacteria-derived LPS, and human colon cancer cells. (**A**) Cottonseed (glanded and glandless seed). Glanded seed contains numerous dark-green-colored gossypol glands. (**B**) Cottonseed-derived gossypol (molar mass: 518.56 g/mol). It contains 6 -OH groups and 6 -CH$_3$ groups (image was taken from public domain, Gossypol—Wikipedia.) (**C**) Cottonseed-derived ethanol extracts. Cottonseed extracts were isolated by fractionation, defatting, and ethanol extraction from cottonseed coats and kernels of glanded and glandless seeds [1]. (**D**) Bacteria-derived endotoxin LPS. Intact LPS is made up of three structural components (10–20 kDa) [21]: a hydrophobic lipid section, lipid A, which is responsible for the toxic properties of the molecule; a hydrophilic core polysaccharide chain; and a repeating hydrophilic O-antigenic oligosaccharide side chain that is specific to the bacterial serotype. (http://www.vetbact.org/popup/popup.php?id=73, accessed on 1 October 2022). (**E**) Human colon cancer cells used in the study.

One of the bioactive materials derived from cottonseed is gossypol, a plant polyphenol with a highly colored yellow pigment found in the leaves, stems, roots, and seeds of cotton plants (Figure 1B) [22]. Gossypol and related compounds are reported to have anticancer activities associated with breast cancer [23–25], colon cancer [26,27], pancreatic cancer [28,29], and prostate cancer [30,31]. Gossypol has additional bioactivities such as antiobesity [25], anti-inflammatory [32], and antifungal activities [33]. These new discoveries have generated intensive interest in gossypol and related molecules in the biomedical field.

The other bioactive materials derived from cottonseed are polyphenolic extracts (Figure 1C). Beneficial plant polyphenolic extracts are present in most diets [34]. They regulate gene expression in numerous studies [35–41]. We recently isolated bioactive ethanol extracts from glanded and glandless cottonseed which were shown to be essentially free of gossypol by HPLC-MS analysis [1]. These bioactive cottonseed extracts also regulate gene expression in mammalian cells [42,43].

The long-term objective of our current research was to explore the potential anti-colon cancer materials from natural sources, especially cottonseed. Colon cancer is one

of the deadliest diseases in the world. The lifetime risk of developing colorectal cancer is approximately 4.0% for men and women in 2022 (https://www.cancer.org/cancer/colon-rectal-cancer/about/key-statistics.html, accessed on 1 October 2022). It is urgently needed. access to fully understand the mechanism of developing colon cancer and explore ways to ease the burden of the healthcare crisis. However, there was a lack of molecular evidence for the bioactivity of cottonseed-derived materials in colon cancer cells.

Quantitative real-time-PCR (qPCR) for gene expression analysis is a widely used method for evaluating the bioactivity of natural products. Some questions can be easily answered by qPCR, e.g., the number of isoforms, the levels of gene expression, and the expression patterns of genes regulated by various stimuli. However, the reliability and reproducibility of qPCR results can be affected by many genetic, environmental, and experimental factors because of the high sensitivity [44]. Therefore, one of the critical tasks of qPCR assay design is to select stably expressed internal reference genes for data analysis due to the inherited variations of gene expression among individual organisms, various tissues, different experimental stages and RNA stability, experimental variations such as RNA extraction methods and cDNA preparations, and human errors [45,46]. Carefully selected internal reference mRNAs are used to normalize transcript levels of test genes to more accurately detect the variations [47–50]. The rationale for qPCR reference gene selection is that the reference gene should be stably expressed without much variation by the experimental treatments. A lower standard deviation of the cycle of threshold (Cq) among the treatments might be an indication of a more stable expression of the gene which could serve as an internal reference.

The objective of this study was to characterize potential reference genes in human colon cancer cells treated with cottonseed-derived gossypol and bioactive extracts. The well-known bacterial endotoxin lipopolysaccharide (LPS) was selected for comparison with cottonseed materials during the qPCR analysis of gene expression. LPS is a major cell wall component of Gram-negative bacteria (Figure 1D). LPS is widely present in the gut and may be derived from colonial bacteria and/or food contamination. LPS was proposed to have an antitumor effect in several experimental models [51]. One study found that LPS induced TGFβ and HGF production mediated by CD14/TLR-2 in cultured human colon cancer cell lines [52]. Another study showed that LPS promoted NFkB activation in colon cancer cells [53]. A third study demonstrated that LPS promoted the migratory capacity of colon cancer cells with the activation of the SDF-1α/CXCR4 axis and epithelial–mesenchymal transition occurrence [54].

In this study, human colon cancer cells (COLO 205) (Figure 1E) were treated with cottonseed-derived gossypol and ethanol extracts, along with LPS. SYBR Green qPCR was used to analyze the mRNA levels of a wide range of biomarkers involved in glucose transport, lipid biosynthesis, inflammatory response, and cancer development. The data showed that B-cell lymphoma 2 (*Bcl2*) mRNA was the most stable among the 55 mRNAs analyzed in human colon cancer cells. Glyceraldehyde 3 phosphate dehydrogenase (*Gapdh*) and ribosome protein L32 (*Rpl32*) mRNAs, two widely used references in mammalian cells, were not good qPCR references for colon cancer cells. These results suggest that *Bcl2* is a preferable reference gene for qPCR assay of gene regulation by cottonseed and bacterial products in human colon cancer cells. The extensive qPCR results firmly support the conclusion that the *Bcl2* gene is stably expressed at the mRNA level in the human colon cancer cells regardless of the treatment, suggesting that *Bcl2* gene expression is not regulated at the mRNA level but at the post-transcriptional level.

2. Results

2.1. Cq mean Distribution in Colon Cancer Cells Treated with Gossypol, LPS, and Cottonseed Extracts

The ideal reference gene should not be expressed at extreme levels. In qPCR technology, cDNA is doubled per cycle of PCR amplification, i.e., one Cq difference equates to a 2-fold difference at the mRNA levels [48]. A lower Cq means a higher mRNA level and vice versa. SYBR Green qPCR assay with the specific primers (Table 1) was used to measure the relative

mRNA levels of 55 genes in the cells treated with plant toxin gossypol, bacterial toxin LPS, and cottonseed extracts with 1% DMSO as the control. The qPCR assay showed that *Bcl2* Cq was 28.82 and *Inos* mRNA was undetectable (mean of 192 independent samples) (Figure 2).

Table 1. Human mRNA targets analyzed by qPCR; these genes are regulated by TTP, plant toxin gossypol, and plant nutrient cinnamon extract as indicated in the references.

ID	mRNA	Name	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Regulator [Reference]
H1	Ahrr1	Aryl hydrocarbon receptor repressor	AGGCTGCTGTT GGAGTCTCTTAA	CGATCGTTGC TGATGCATAAA	TTP [55]
H2	Bcl2	B-cell lymphoma 2	CAGCATGCGG CCTCTGTT	GGGCCAAAC TGAGCAGAGTCT	Gossypol [56]
H3	Bcl2l1	B-cell lymphoma 2 like 1	GTGCGTGGAA AGCGTAGACA	ATTCAGGTAAG TGGCCATCCAA	TTP [57]
H4	Bnip3	BCL2 protein-interacting protein 3	GTCAAGTCGG CCGGAAAATA	TGCGCTTCG GGTGTTTAAAG	Gossypol [27]
H5	Cd36	Cluster of differentiation 36/fatty acid translocase	CTCTTTCCTG CAGCCCAATG	TTGTCAGCCTCTG TTCCAACTG	TTP [58]
H6	Claudin1	Maintain tissue integrity and water retention	GACAAAGTGA AGAAGGCCCGTAT	CAAGACCTGCC ACGATGAAA	TTP [59]
H7	Cox1	Cyclooxygenase 1	CGCCCACG CCAGTGA	AGGCCGAAG CGGACACA	TTP [60]
H8	Cox2	Cyclooxygenase 2	CGATTGTACC CGGACAGGAT	TTGGAGTGGGTT TCAGAAATAATTT	TTP [61]
H9	Csnk2a1	Casein kinase 2 alpha 1	AGCGATGGGA ACGCTTTG	AAGGCCTCAGG GCTGACAA	TTP [62]
H10	Ctsb	Cathepsin B	GACTTGTAGCTGCTGT CTCTCTTTGT	CAAGAGTCGC AAGAACATGCA	TTP [63]
H11	Cxcl1	Chemokine (C-X-C motif) ligand 1	GCCCAAACCG AAGTCATAGC	TGCAGGATTG AGGCAAGCT	TTP [64]
H12	Cyclind1	Cyclin D1	ACACGCGCAG ACCTTCGT	CCATGGAG GGCGGATTG	Gossypol [65]
H13	Cyp19a1	Cytochrome P450 family 19 subfamily A member 1	GACATTGCAA GGACAGTGTGTTG	AGTCTCATCTG GGTGCAAGGA	Gossypol [66]
H14	Dgat1	Diacylglycerol O-acyltransferase 1	ACCTCATCTGGCTCA TCTTCTTCTA	CCCGGTCTC CAAACTGCAT	Cinnamon [40,67]
H15	Dgat2a	Diacylglycerol O-acyltransferase 2a	CCCAGGCAT ACGGCCTTA	CAACACAGG CATTCGGAAGTT	Cinnamon [40,68]
H16	Dgat2b	Diacylglycerol O-acyltransferase 2b	ACTCTGGCCCTTC TCTGTTTTTTA	TCCACCTTGG TTGGGTGTGT	Cinnamon [40,68]
H17	E2f1	E2F transcription factor 1	CGGCGCATCT ATGACATCAC	CAGCCACTGG ATGTGGTTCTT	TTP [69]
H18	Elk1	ETS transcription factor	CTCCTCCGCA TCCCTCTTTAA	AGCGTCACAG ATGGGTCCAT	TTP [70]
H19	Fas	Fas cell surface death receptor	GAACTCCTTGGC GGAAGAGA	AGGACCCGT GGAATGTCA	Gossypol [71]
H20	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	GGGTGTGAAC CATGAGAAGTATGA	GGTGCAGGA GGCATTGCT	[72]
H21	Glut1	Glucose transporter 1	TGCTCATGGGCT TCTCGAA	AAGCGGCC CAGGATCAG	Cinnamon [39]
H22	Glut2	Glucose transporter 2	GCATTTTTCAG ACGGCTGGTA	GCGCCAACT CCAATGGTT	Cinnamon [39]
H23	Glut3	Glucose transporter 3	GAGGATATCACA CGGGCCTTT	CCATGACGCC GTCCTTTC	Cinnamon [39]

Table 1. *Cont.*

ID	mRNA	Name	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Regulator [Reference]
H24	Glut4	Glucose transporter 4	CGTGGGCG GCATGATT	CCAGCATGG CCCTTTTCC	Cinnamon [39]
H25	Hif1a	Hypoxia inducible factor 1 subunit alpha	GGTGGATATGT CTGGGTTGAAAC	ATGCACTGTGGT TGAGAATTCTTG	TTP [73]
H26	Hmgr	3-Hydroxy-3-methylglutaryl-CoA reductase	AAGTGAAAG CCTGGCTCGAA	CTAGTGCTGTCAA ATGCCTCCTT	[74]
H27	Hmox1	Heme oxygenase 1	CTTCTCCGATGG GTCCTTACACT	TCACATGGCATA AAGCCCTACA	TTP [75]
H28	Hua	Human antigen a	GATCCTCTGG CAGATGTTTGG	CGCGGATCAC TTTCACATTG	Gossypol [41]
H29	Icam1	Intercellular adhesion molecule 1/CD54	GGAGCTTCGTG TCCTGTATGG	TTTCTGGC CACGTCCAGTTT	[76]
H30	Inos	Inducible nitric oxide synthase	AGATCCGGTTCA CAGTCTTGGT	GCCATGACCT TCCGCATTAG	[77]
H31	Insr	Insulin receptor	CAACGGGCA GTTTGTCGAA	TGGTCGGGCA AACTTTCTG	[38]
H32	Il2	Interleukin 2	TATGCAGATGAG ACAGCAACCAT	TTGAGATGATGCT TTGACAAAAGG	TTP [78]
H33	IL6	Interleukin 6	CCCACACAGA CAGCCACTCA	CCGTCGAGGA TGTACCGAAT	TTP [79]
H34	IL8	Interleukin 8	CCATCTCACTGTGTGTAA ACATGACTT	ATCAGGAAGGC TGCCAAGAG	TTP [80]
H35	Il10	Interleukin 10	GCCGTGGAG CAGGTGAAG	TGGCTTTGTAGA TGCCTTTCTCT	TTP [81]
H36	Il12	Interleukin 12	TGCCTTCAC CACTCCCAAA	TGTCTGGCCT TCTGGAGCAT	TTP [82]
H37	Il16	Interleukin 16	CAGGGCC TCACACGGTTT	GACAATCGTGAC AGGTCCATCA	TTP [83]
H38	Il17	Interleukin 17	CCCAAAAGGTCCT CAGATTACTACA	TCATTGCGGTG GAGATTCC	TTP [84]
H39	Leptin	Body fat and obesity hormone	AGGGAGAC CGAGCGCTTT	CACATCCCTCA CCTCCTTCAAA	[85]
H40	Map1lc3a	Microtubule-associated proteins 1 light chain 3A	GTGAACCAG CACAGCATGGT	CCTCGTCTTTCT CCTGCTCGTA	[86]
H41	Map1lc3b	Microtubule-associated proteins 1 light chain 3B	AGGCGCTTA CAGCTCAATGC	ACCATGCTGTG TCCGTTCAC	[86]
H42	Nfkb	Nuclear factor kappa B	GGTGCCTCTAGT GAAAAGAACAAGA	GCTGGTCCCA CATAGTTGCA	[87]
H43	P53	Tumor suppressor	CTTGCAATAGGT GTGCGTCAGA	GGAGCCC CGGGACAAA	Gossypol [88]
H44	Pim1	Proto-oncogene serine/threonine-protein kinase	TGCTCCACC GCGACATC	TGAGCTCGC CGCGATT	TTP [89]
H45	Pparr	Peroxisome proliferator-activated receptor γ	GAACGACCAAGT AACTCTCCTCAAA	CAAGGAGGCC AGCATTGTGT	Gossypol [90]
H46	Rab24	Ras-related oncogene 24	TCGGTCGGA GACGCACTT	TGGCCTCAT AGCGCTCAGA	[91]
H47	Rpl32	Ribosomal protein L32 (60S ribosomal unit)	CCTCCAAGAAC CGCAAAGC	GGTGACTCTGA TGGCCAGTTG	[92]
H48	Tnf	Tumor necrosis factor	GGAGAAGGGT GACCGACTCA	CAGACTCGGC AAAGTCGAGAT	TTP [79]
H49	Tnfsf10	Tumor necrosis factor superfamily, member 10	GCTCTGGGCC GCAAAAT	AGGAATGAATG CCCACTCCTT	Gossypol [93]

Table 1. *Cont.*

| ID	mRNA	Name	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Regulator [Reference]
H50	Ulk2	Unc-51 like autophagy activating kinase 2	ACAGCTCCTT TCAAAATCCCTAAA	AGGCCCATGAC GAGTAACCA	[94]
H51	Vegf	Vascular endothelial growth factor	CCCACTGAG GAGTCCAACATC	GGCCTTGGT GAGGTTTGATC	TTP [95]
H52	Zfand5	Zinc finger AN1-type containing 5	AGGGTTTGACT GCCGATGTG	ACTGGATTCTCT TTTCTGATTTTTGC	TTP [96]
H53	Zfp36/Ttp	Zinc finger protein 36/Tristetraprolin	GGCGACTCCCC ATCTTCAA	GACCGGGCAGT CACTTTGTC	TTP [38]
H54	Zfp36l1	Zinc finger protein 36 like 1	TCTGCCACCATC TTCGACTTG	TGGGAGCACTATA GTTGAGCATCT	TTP [38]
H55	Zfp36l2	Zinc finger protein 36 like 2	CCTTTCATACCAT CGGCTTCTG	TCGTCCGC GTTGTGGAT	TTP [38]
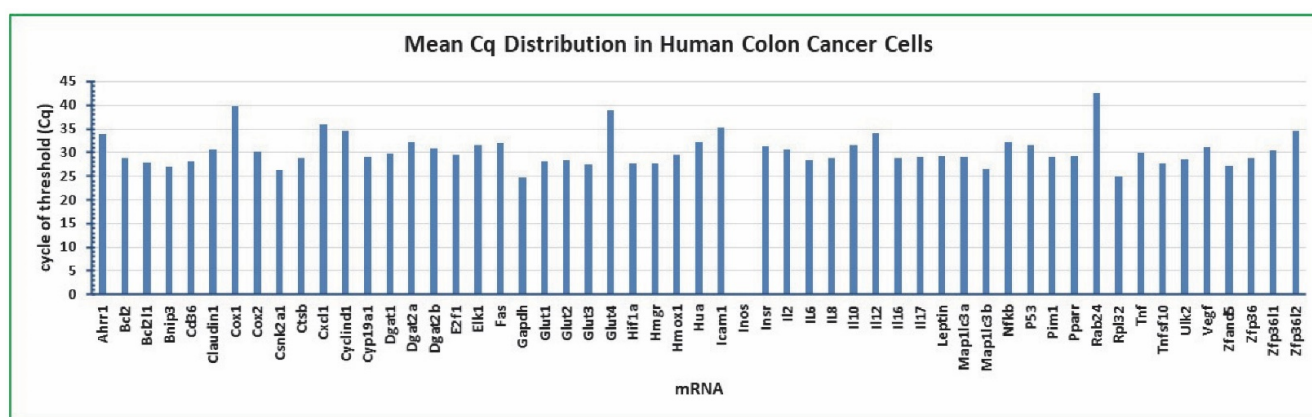
Figure 2. Mean Cq distribution of 55 mRNAs in human colon cancer cells. The cancer cells were treated with multiple concentrations of gossypol (0, 0.1, 0.5, 1, 5, 10, 50, and 100 µg/mL), LPS (0, 5, 10, 20, 50, 100, 500, and 1000 ng/mL) and cottonseed extracts (0, 5, 10, 20, 30, 40, 50, and 100 µg/mL) for 8 h and 24 h. Total mRNAs were extracted from the cells, converted into cDNAs, and the relative abundance was analyzed by SYBR Green qPCR. The Cq values represent the mean of 192 independent samples.

The mean Cq values of 11 mRNAs (mean of 192 independent samples) were at least one Cq less than *Bcl2* Cq including *Bcl2l1* (−1.00), *Bnip3* (−1.75), *Csnk2a1* (−2.43), *Gapdh* (−4.17), *Glut3* (−1.42), *Hif1a* (−1.13), *Hmgr* (−1.21), *Map1lc3b* (−2.17), *Rpl32* (−3.92), *Tnfsf10* (−1.19), and *Zfand5* (−1.67) (Figure 2). The mean Cq values of 24 mRNAs (mean of 192 independent samples) were at least one Cq larger than *Bcl2* Cq including *Ahrr1* (5.15), *Claudin1* (1.92), *Cox1* (10.99), *Cox2* (1.29), *Cxcl1* (7.07), *Cyclind1* (5.71), *Dgat2a* (3.52), *Dgat2b* (2.15), *Elk1* (2.87), *Fas* (3.17), *Glut4* (10.21), *Hua* (3.38), *Icam1* (6.40), *Inos* (undetected), *Insr* (2.59), *Il2* (1.88), *Il10* (2.79), Il12 (5.17), *Nfkb* (3.50), *P53* (2.79), *Rab24* (13.82), *Tnf* (1.23), *Vegf* (2.35), *Zfp36l1* (1.69), and *Zfp36l2* (5.66) (Figure 2).

The above Cq data suggest that the *Bcl2* mRNA level was within the middle range of the tested 55 genes in the human colon cancer cells treated with gossypol, LPS, and cottonseed extracts. The mRNA levels of *Gapdh* and *Rpl32* were the most abundant in the cells with approximately 18- and 15-fold of *Bcl2* mRNA, respectively, whereas *Inos* mRNA was undetectable and those of *Ahrr1*, *Cox1*, *Cxcl1*, *Cyclind1*, *Dgat2a*, *Glut4*, *Hua*, *Icam1*, *Il12*, *Nfkb*, *Rab24*, and *Zfp36l2* mRNAs were minimally detected with less than 10% of *Bcl2* mRNA in the colon cancer cells. These results suggest that the *Bcl2* gene is modestly expressed (i.e., not at an extreme level among the selected biomarkers analyzed) in the human colon cancer cells treated with gossypol, LPS, and cottonseed extracts and that the *Bcl2* gene could serve as a good reference gene for qPCR analysis of gene expression in the human colon cancer cells.

2.2. Cq standard Deviation Distribution in Colon Cancer Cells Treated with Gossypol, LPS, and Cottonseed Extracts

The qPCR assay showed that *Bcl2* Cq was the least varied mRNA among the 55 genes analyzed. The standard deviation of Cq for *Bcl2* was 1.09 (mean of 192 independent samples) (Figure 3). The standard deviations of Cq for *Gapdh* and *Rpl32* were 3.12 and 3.16 (mean of 192 independent samples), respectively (Figure 3). All of the other mRNAs had larger standard deviations of Cq than *Bcl2* (Figure 3). The mRNAs with closest standard deviations of *Bcl2* Cq (less than 1.50) included *Bnip3* (1.21), *Glut3* (1.30), *Hmgr* (1.49), *Il2* (1.12), *Il6* (1.37), *Il8* (1.32), *Il17* (1.48), *Pim1* (1.26), *Pparr* (1.32), *Tnf* (1.39), *Tnfsf10* (1.40), *Ulk2* (1.16), and *Zfand5* (1.34) (Figure 3). These results suggest that *Bcl2* is the most stably expressed gene and therefore, could serve as a reliable reference for qPCR analysis of gene expression in human colon cancer cells treated with gossypol, LPS, and cottonseed extracts, regardless of the treatments.
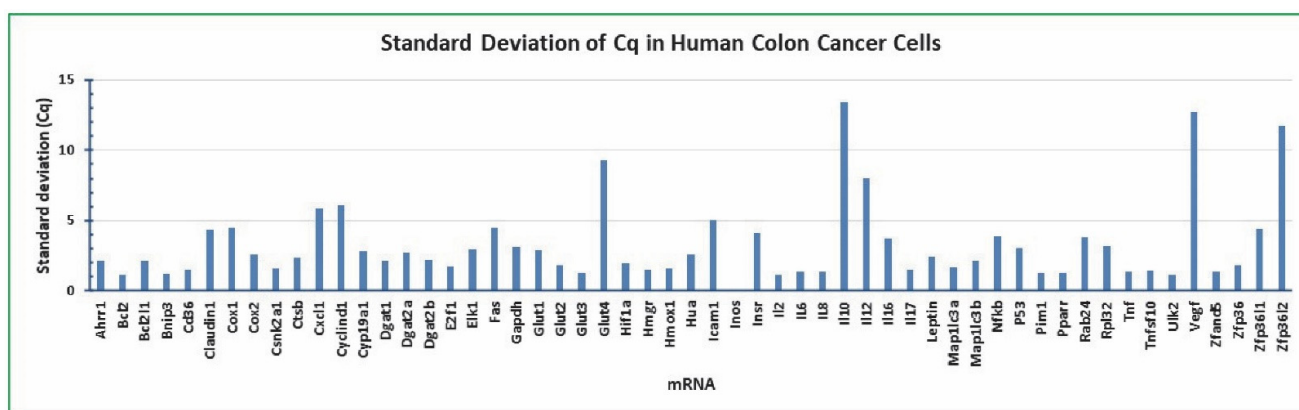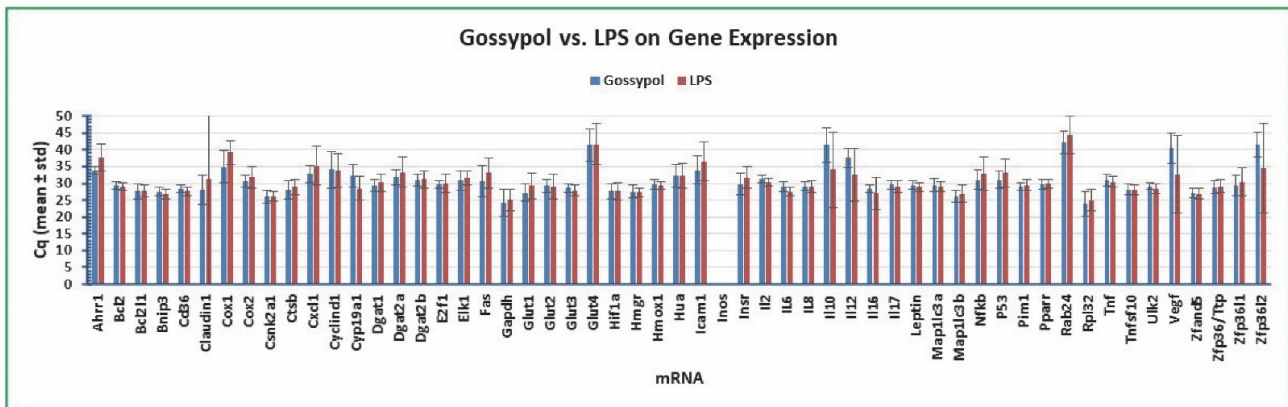

Figure 3. Mean standard deviation distribution of 55 mRNAs in human colon cancer cells. The cancer cells were treated with multiple concentrations of gossypol (0, 0.1, 0.5, 1, 5, 10, 50, and 100 µg/mL), LPS (0, 5, 10, 20, 50, 100, 500, and 1000 ng/mL) and cottonseed extracts (0, 5, 10, 20, 30, 40, 50, and 100 µg/mL) for 8 h and 24 h. Total mRNAs were extracted from the cells, converted into cDNAs, and the relative abundance was analyzed by SYBR Green qPCR. The standard deviations of Cq values represent the mean of 192 independent samples.

2.3. Variation of Gene Expression between Plant Toxin Gossypol and Bacterial Toxin LPS Treatment

To confirm the above conclusion that *Bcl2* was a preferable reference gene for qPCR analysis of gene expression in the human colon cancer cells treated with gossypol, LPS, and cottonseed extracts, we selected subsets of data for comparison. We first compared the variation of gene expression in the human colon cancer cells under the treatment with plant toxin gossypol and bacterial toxin LPS. As shown in Figure 4, *Bcl2* Cq was the least varied among the 55 targets with a standard deviation of 1.08 and 1.16 for gossypol and LPS treatment (mean of 24 independent samples), respectively. The standard deviations of *Gapdh* Cq were 4.01 (gossypol treatment) and 3.15 (LPS treatment) and those of *Rpl32* Cq were 3.68 (gossypol treatment) and 3.10 (LPS treatment) (mean of 24 independent samples) (Figure 4). All of the other mRNAs had larger standard deviations of Cq than *Bcl2* Cq (Figure 4). The mRNA levels of genes regulated by gossypol that were at least 2-fold of those regulated by LPS could be interpreted as gossypol regulation of gene expression being significantly higher than LPS. There were 14 genes with more abundantly expressed mRNA levels under gossypol treatment than LPS treatment including *Ahrr1*, *Claudin1*, *Cox1*, *Cox2*, *Cxcl1*, *Dgat2a*, *Fas*, *Glut1*, *Icam1*, *Insr*, *Nfkb*, *P53*, *Rab24*, and *Zfp36l1* (Figure 4). Similarly, the mRNA levels of genes regulated by gossypol less than 0.5-fold of those regulated by LPS could be interpreted as their expression being regulated by gossypol significantly less than the LPS effect. There were 7 genes with less abundantly expressed mRNA levels under gossypol treatment than under LPS treatment including *Cyp19a1*, *Il6*, *Il10*, *Il12*, *Il16*, *Vegf*, and *Zfp36l2* (Figure 4). The above results of modest expression of the *Bcl2* gene with minimal variation

agree with the conclusion that *Bcl2* is a suitable reference gene for qPCR analysis of gene expression in human colon cancer cells regardless of treatments with gossypol or LPS.

Figure 4. Variation of gene expression between gossypol and LPS treatment. The cancer cells were treated with multiple concentrations of gossypol (0, 0.1, 0.5, 1, 5, 10, 50, and 100 μg/mL) and LPS (0, 5, 10, 20, 50, 100, 500, and 1000 ng/mL) for 8 h. Total mRNAs were extracted from the cells, converted into cDNAs, and the relative abundance was analyzed by SYBR Green qPCR. The Cq values represented the mean and standard deviation of 24 independent samples.

2.4. Variation of Gene Expression between Glanded and Glandless Cottonseed Extract Treatment

We further compared the variation of gene expression in the human colon cancer cells under the treatment with glanded and glandless cottonseed extracts. Again, *Bcl2* Cq was the least varied among the 55 targets with a standard deviation of 1.07 and 0.98 for glanded seed extract and glandless seed extract (mean of 48 independent samples), respectively (Figure 5). The standard deviations of *Gapdh* Cq were 3.34 (glanded seed extract) and 3.51 (glandless seed extract) and those of *Rpl32* Cq were 3.22 (glanded seed extract) and 3.43 (glandless seed extract) (mean of 48 independent samples) (Figure 5). All of the other mRNAs had larger standard deviations of Cq than *Bcl2* Cq (Figure 5). There were only two genes more abundantly expressed under glanded seed extract treatment than glandless seed extract treatment including *Vegf* and *Zfp36l1* (Figure 5). There were 15 genes with lower mRNA levels under glanded seed extract treatment than under glandless seed extract treatment including *Claudin1*, *Cox1*, *Cyclind1*, *Elk1*, *Fas*, *Gapdh*, *Glut1*, *Insr*, *Il10*, *Il12*, *Nfkb*, *P53*, *Rab24*, *Zfp36l1*, and *Zfp36l2* (Figure 5). These results support the conclusion that *Bcl2* is a suitable reference gene for qPCR analysis of gene expression in human colon cancer cells regardless of treatment with glanded or glandless cottonseed extracts.
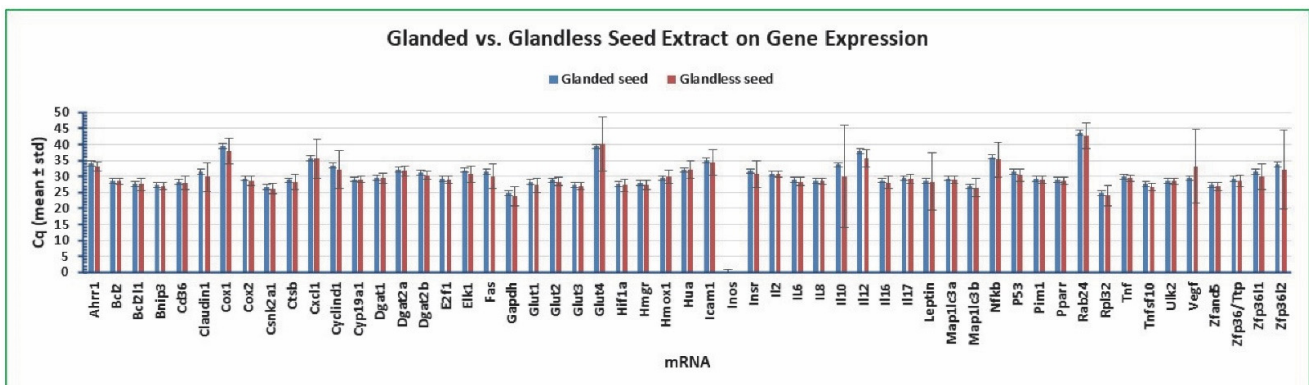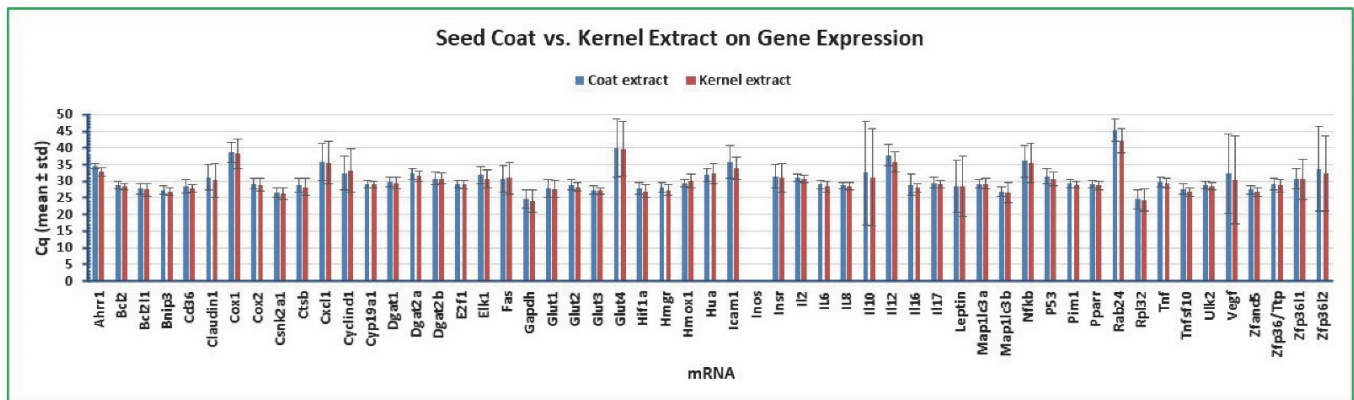

Figure 5. Variation of gene expression between glanded and glandless cottonseed extract treatment. The cancer cells were treated with multiple concentrations of cottonseed extracts (0, 5, 10, 20, 30, 40, 50, and 100 μg/mL) for 8 h. Total mRNAs were extracted from the cells, converted into cDNAs, and the relative abundance was analyzed by SYBR Green qPCR. The Cq values represented the mean and standard deviation of 48 independent samples.

2.5. Variation of Gene Expression between Cottonseed Coat and Kernel Extract Treatment

We also compared the variation of gene expression under the treatment with seed coat and kernel extracts in the human colon cancer cells. As shown in Figure 6, *Bcl2* Cq was the least varied mRNA among the 55 targets with a standard deviation of 1.06 and 0.96 for coat extract and kernel extract (mean of 48 independent samples), respectively. The standard deviations of *Gapdh* Cq were 3.05 (coat extract) and 3.83 (kernel extract) and those of *Rpl32* Cq were 3.18 (coat extract) and 3.49 (kernel extract) (mean of 48 independent samples), respectively (Figure 6). All of the other mRNAs had larger standard deviations of Cq than *Bcl2* Cq (Figure 6). There were none of the genes with more abundantly expressed mRNA levels under the coat extract treatment than the kernel extract treatment (Figure 6). There were 13 genes with less abundantly expressed mRNA levels under coat extract treatment than kernel extract treatment including *Ahrr1*, *Cox1*, *Elk1*, *Gapdh*, *Icam1*, *Insr*, *Il10*, *Il12*, *Nfkb*, *Rab24*, *Vegf*, *Zfp36l1*, and *Zfp36l2* (Figure 6). The conclusion that *Bcl2* is a suitable reference gene for qPCR analysis of gene expression in human colon cancer cells is confirmed with this comparison regardless of the cells treated with seed coat or kernel extract.

Figure 6. Variation of gene expression between cottonseed coat and kernel extract treatment. The cancer cells were treated with multiple concentrations of cottonseed extracts (0, 5, 10, 20, 30, 40, 50, and 100 μg/mL) for 8 h. Total mRNAs were extracted from the cells, converted into cDNAs, and the relative abundance was analyzed by SYBR Green qPCR. The Cq values represented the mean and standard deviation of 48 independent samples.

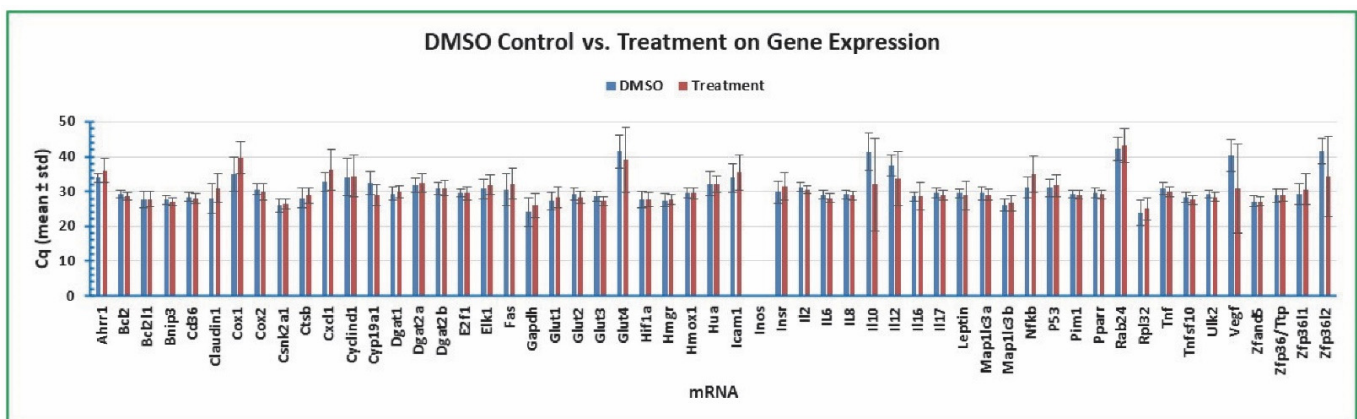*2.6. Variation of Gene Expression between Cottonseed Extract Treatment Time*

In addition, we compared the variation of gene expression in the human colon cancer cells between 8 and 24 h treatment with seed coat extract. As shown in Figure 7, *Bcl2* Cq was the least varied mRNA among the 55 targets with a standard deviation of 1.06 and 0.95 for 8 h and 24 h (mean of 48 independent samples), respectively. The standard deviations of *Gapdh* Cq were 3.05 (8 h) and 2.90 (24 h) and those of *Rpl32* Cq were 3.18 (8 h) and 2.93 (24 h) (mean of 48 independent samples), respectively (Figure 7). All of the other mRNAs had larger standard deviations of Cq than *Bcl2* Cq (Figure 7). There were 21 genes with more abundantly expressed mRNA levels under 8 h treatment than 24 h treatment including *Bcl2l1*, *Claudin1*, *Cox1*, *Cox2*, *Cstb*, *Cxcl1*, *Cylind1*, *Dgat1*, *Elk1*, *Fas*, *Gapdh*, *Glut1*, *Hua*, *Icam1*, *Insr*, *Il8*, *Nfkb*, *P53*, *Rpl32*, *Zfp36l1*, and *Zfp36l2* (Figure 7). There were 5 genes with less abundantly expressed mRNA levels under 8 h treatment than 24 h treatment including *Ahrr1*, *Glut4*, *Il12*, *Rab24*, and *Vegf* (Figure 7). These results of gene expression variation in the human colon cancer cells under 8 or 24 h treatment with seed coat extract also support the conclusion that *Bcl2* but not *Gapdh* or *Rpl32* or any other gene is a preferable reference gene for qPCR analysis
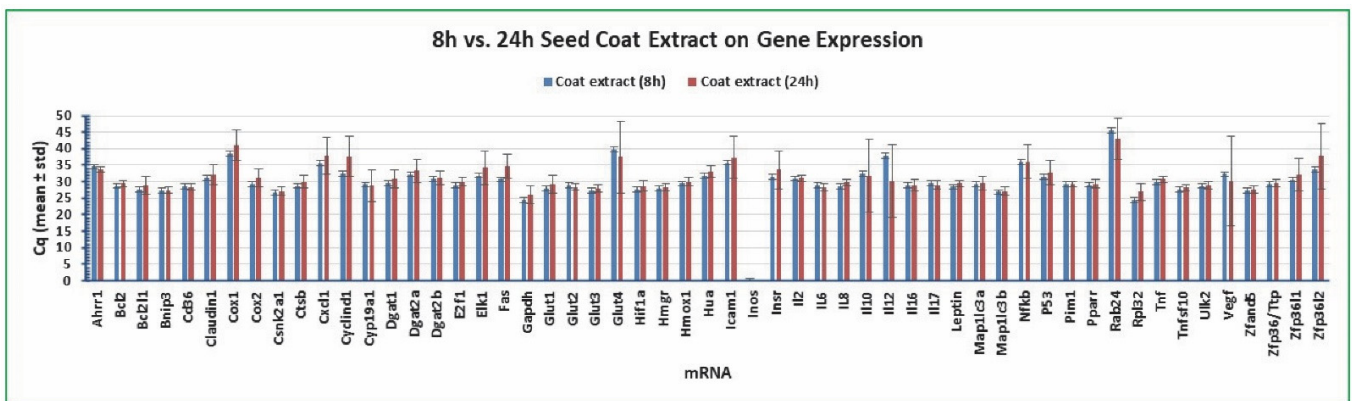
Figure 7. Variation of gene expression between 8 and 24 h treatment of coat extract of cottonseed. The cancer cells were treated with multiple concentrations of cottonseed extracts (0, 5, 10, 20, 30, 40, 50, and 100 µg/mL) for 8 h. Total mRNAs were extracted from the cells, converted into cDNAs, and the relative abundance was analyzed by SYBR Green qPCR. The Cq values represented the mean and standard deviation of 48 independent samples.

2.7. Variation of Gene Expression between DMSO Control and Various Treatments

Finally, we compared the variation of gene expression in the human colon cancer cells under the 1% DMSO control and various treatments with plant toxin gossypol, bacterial toxin LPS, and cottonseed-derived ethanol extracts. Figure 8 confirms that *Bcl2* was the most stable gene among the 55 targets with standard deviations of 1.08 and 1.08 for DMSO control (mean of 24 independent samples) and treatment (mean of 168 independent samples). The standard deviations of *Gapdh* Cq were 4.01 (DMSO control) and 3.57 (treatment) and those of *Rpl32* Cq were 3.68 (DMSO control) and 3.07 (treatment) (Figure 8). All of the other mRNAs had larger standard deviations of Cq than *Bcl2* Cq (Figure 8). These qPCR data analyses further support the above conclusion that *Bcl2* gene is stably expressed at the mRNA level in human colon cancer cells regardless of the treatment.

Figure 8. Variation of gene expression between DMSO control and treatment. The cancer cells were treated with 1% DMSO and multiple concentrations of gossypol (0.1, 0.5, 1, 5, 10, 50, and 100 µg/mL), LPS (5, 10, 20, 50, 100, 500, and 1000 ng/mL) and cottonseed extracts (5, 10, 20, 30, 40, 50, and 100 µg/mL) for 8 h and 24 h. Total mRNAs were extracted from the cells, converted into cDNAs, and the relative abundance was analyzed by SYBR Green qPCR. The Cq values represented the mean and standard deviation of 24 independent samples.

3. Discussion

Cottonseed is a low-value commodity and contributes to approximately 20% of the cotton value. Cottonseed value could be increased by serving as a cheap source of high-value bioactive materials for improving nutrition and preventing diseases. However, molecular evidence to support cottonseed bioactivity in mammalian cells is not substantial.

qPCR is widely used to evaluate the bioactivity of natural products at the gene expression level. However, many factors affect the calculation of gene expression data due to the inherited variations of gene expression among individual organisms, various tissues, different experimental stages, and RNA stability. It is also affected by experimental variations such as RNA extraction methods and cDNA preparations, and human errors [44,46]. Therefore, the selection of stably expressed internal reference genes is a critical task for qPCR assay design for normalizing transcript levels of test genes during the post-qPCR data analysis [48,49].

The hallmark of qPCR reference gene selection is that a reference gene should be stably expressed without much variation by experimental conditions and ideally its expression level is like those of the target genes [45,97]. A lower standard deviation of Cq among the treatments indicates a more stable expression of the gene which could serve as a better internal reference. In this study, we used the qPCR method to screen reference genes from 55 genes involved in glucose transport, lipid biosynthesis, inflammatory response, and cancer development using human colon cancer cells treated with multiple concentrations of plant toxin gossypol, bacterial toxin LPS, and bioactive cottonseed extracts. Our results consistently showed that *Bcl2* is a very stably expressed gene with minimal variation and expressed at adequate mRNA levels similar to most of the other gene targets. On the other hand, two widely used reference genes (*Gapdh* or *Rpl32*) were the most abundantly expressed genes with much larger variations among the treatments. These expression differences among them make *Bcl2* rather than *Gapdh* or *Rpl32* a preferable qPCR reference for colon cancer cells. These conclusions were confirmed by various comparisons between gossypol and LPS, glanded and glandless seed extracts, seed coat and kernel extracts, or treatment for 8 and 24 h. A previous study analyzed the effect of gossypol on gene expression in human colon cancer cells, using *Bcl2* as the reference gene [26]. The current study is technically oriented rather than looking at the regulation of specific genes with specific agents. This study has analyzed much larger data sets with a broader new view. The comparisons among the treatments are also much more comprehensive. All of the data support the conclusion that *Bcl2* is a preferable reference gene for qPCR assay of gene expression in human colon cancer cells.

Our current results firmly support the conclusion that *Bcl2* is a preferable reference gene for qPCR assay of gene expression in human colon cancer cells treated with cottonseed molecules and bacterial endotoxin LPS. This conclusion is drawn from extensive qPCR data with 55 genes analyzed and 192 individual samples (64 treatments with triplicate for each treatment) for each gene for a total of 10,560 Cq values. Since our study used regulatory molecules ranging from plant toxin gossypol to bacterial endotoxin LPS as well as bioactive cottonseed mixtures, it is expected that the technical advance should be applicable to evaluating a wide range of biomolecules' modulation of gene expression in the human colon cancer cells. However, further research is required if *Bcl2* is a preferable reference gene for qPCR assay of gene expression in other cell lines since responses of stimuli on gene expression are widely different in different cell types.

Our extensive qPCR results support the conclusion that the *Bcl2* gene is stably expressed at the mRNA level in human colon cancer cells regardless of the treatment with plant toxin gossypol, bacterial endotoxin LPS, or cottonseed-derived bioactive extracts. However, a number of previous studies reported the regulation of *Bcl2* gene expression in other testing systems, even though the findings in those reports were mostly generated by less sensitive methods such as immunoblotting, immunostaining, and end-point PCR techniques. For example, Western blotting showed that *Bcl2* protein levels in colorectal cancer HCT-116 cells were reduced to less than half of the control by 5 days of treatment with 25–100 μg/mL of 3,6-anhydro-L-galactose derived from red seaweed agarose [98]. Similarly, fermented Pu-erh tea (Xiaguan bowl tea [X]) decreased *Bcl2* gene expression in HT-29 colon cancer cells [99]. In situ hybridization and immunostaining showed that tea polyphenol treatment significantly reduced the percentage of *Bcl2* expressing cells and reduced the level of *Bcl2* mRNA and protein in the *Bcl2* positive cells in lung preneoplastic

lesions of Sprague Dawley rats [100]. Curcumin (diferuloylmethane), the yellow pigment in turmeric (*Curcuma longa*), at 25 μM for 4 h suppressed NFκB-regulated gene products (*Bcl2*, *BclxL*, *Cox2*, cyclin D1, and inhibitor of apoptosis protein-2) in HCT-116 cells [101]. Western blotting showed that curcumin treatment at 20 μM for 24 h but not 6 h or 12 h inhibited the expression of *Bcl2* by about 50% in COLO 205 cells [102]. Regular end-point PCR showed that methanol extract from the plant *Drimia calcarata* significantly downregulated *Bcl2* gene expression in both Caco-2 and HT-29 cells [103]. Finally, Western blot and qPCR showed that treatment with berberine (40 μM), a natural isoquinoline alkaloid derived from *Berberis* genus plants, decreased the expression of *Bcl2* protein but not mRNA in human colorectal cancer cell lines HT-29 and HCT-116 [104]. Taken together, all of the above results strongly suggest that *Bcl2* gene expression is not regulated at the mRNA level but at the post-transcriptional level.

In conclusion, this study identified *Bcl2* as a preferable reference gene for qPCR assays in human colon cancer cells treated with cottonseed-derived gossypol and bioactive extracts as well as LPS. Our extensive qPCR results firmly support the conclusion that the *Bcl2* gene is stably expressed at the mRNA level in human colon cancer cells regardless of the treatment with plant toxin gossypol, bacterial endotoxin LPS, or cottonseed-derived bioactive extracts, suggesting that *Bcl2* gene expression is not regulated at the mRNA level but at the post-transcriptional level. These results should facilitate studies designated to evaluate the bioactivity of cottonseed molecules and other natural and synthetic molecules for nutrition and health uses.

4. Materials and Methods

4.1. Colon Cancer Cell Line

American Type Culture Collection (Manassas, VA, USA) provided the human colon cancer cell line (COLO 205-ATCC CCL-222). For long-term storage, the cells were kept under liquid nitrogen vapor in a cryogenic storage vessel (Thermo Fisher Scientific, Waltham, MA, USA). During the experiment, the cells were maintained at 37 °C in a humidified incubator with 5% CO_2 in RPMI-1640 medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v:v) fetal bovine serum, 0.1 million units/L penicillin, 100 mg/L streptomycin, and 2 mmol/L L-glutamine.

4.2. Gossypol, LPS, and Cottonseed Extracts

Gossypol (molar mass: 518.56 g/mol) was purified from cottonseed by HPLC (Sigma, St. Louis, MO, USA). LPS was extracted from *E. coli* serotype K235 and purified by gel filtration (Sigma, St. Louis, MO, USA). Cottonseed extracts were isolated by fractionation, defatting, and ethanol extraction from cottonseed coats and kernels of glanded and glandless seeds [1]. Briefly, the cottonseed coat or kernel was ground into a fine powder and homogenized. The kernel fraction was defatted with chloroform and hexane. The coat fraction was treated with acetic acid followed by autoclave and centrifugation. The defatted materials were extracted with ethanol followed by evaporation to remove acetic acid and ethanol. Ethanol extracts were reconstituted in 100% DMSO (100 mg/mL) (Sigma, St. Louis, MO, USA) and analyzed by HPLC-MS. The ethanol extracts contained trace amounts of gossypol (0.82 ng gossypol/mg extract in glanded seed coat, 0.03 ng gossypol/mg extract in glanded seed kernel, 0.37 ng gossypol/mg extract in glandless seed coat, and 0 ng gossypol/mg extract in glandless seed kernel) [1]. Gossypol, LPS, and cottonseed extract stocks were prepared in 100% DMSO and diluted before use.

4.3. Reagents and Equipment

Tissue culture reagents (RPMI-1640, fetal bovine serum, penicillin, streptomycin, and L-glutamine) were from Gibco BRL. The tissue culture incubator (water jacket CO_2 incubator, Forma Series II, Model 3100 Series) was from Thermo Fisher. The tissue culture workstation (Logic+ A2 hood) was from Labconco (Kansas City, MO, USA). Tissue culture plastic (flasks, plates, cell scraper) was from CytoOne (USA Scientific, Ocala, FL, USA). Cell counting

reagent (trypsin blue dye), slides (dual chamber), counter (TC20 Automatic Cell Counter), and microscope (Zoe Florescent Cell Imager) were from Bio-Rad (Hercules, CA, USA). The microplate spectrophotometer (Epoch) was from BioTek Instruments (Winooski, VT, USA).

4.4. Cell Culture and Chemical Treatment

Cancer cells were dissociated from the T-75 flask with 0.25% (*w/v*) trypsin−0.53 mM EDTA solution, stained with an equal volume of 0.4% trypsin blue dye before counting the number of live cells with a TC20 Automatic Cell Counter. Cancer cells (0.5 mL) from trypsin-dissociated flasks were subcultured at approximately 1×10^5 cells/mL density in 24-well tissue culture plates. The cancer cells were routinely observed under a Zoe Florescent Cell Imager before and during the treatment. Cancer cells in 24-well plates (triplicate for every treatment) were treated with gossypol (0, 0.1, 0.5, 1, 5, 10, 50, and 100 μg/mL), LPS (0, 5, 10, 20, 50, 100, 500, and 1000 ng/mL), or ethanol extracts (0, 5, 10, 20, 30, 40, 50, and 100 μg/mL) for 8 and 24 h. The experimental control "0" treatment corresponded to 1% DMSO present in all of the culture mediums. Gossypol concentrations were in the range of previously published concentrations for gossypol (up to 100 μM) [23,25,105], (-) gossypol (up to 100 μM) [106], apogossypolone (up to 40 μM) [107], and gossypol derivatives (IC_{50} concentrations of 6–28 μM) [108].

4.5. Real-Time qPCR Primers and Probes

The selection of 55 genes for qPCR analysis was based on the literature showing those gene expressions regulated by TTP, gossypol, or cinnamon extract in cancer cells and macrophages (relevant references are listed in the right column in Table 1). RNA sequences were obtained from the National Center for Biotechnology Information (NCBI)'s non-redundant protein sequence databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The qPCR primers were designed with Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA) using the default parameter values including amplicon length (50 to 150 bases for optimum PCR efficiency), optimal primer length (20 bases), Tm (58 °C to 60 °C), % GC (30% to 80%), 3′ end (the last five nucleotides at the 3′ end contain no more than two G + C residues), and repeating oligonucleotides (void runs of identical nucleotides; if repeats are present, there must be fewer than four consecutive G residues). The primers were synthesized by Biosearch Technologies, Inc. (Navato, CA, USA). The names of mRNAs and their nucleotide sequences (5′ to 3′) of the forward primers and reverse primers, and corresponding references are described in Table 1.

4.6. RNA Isolation and cDNA Synthesis

RNA isolation and cDNA synthesis were essentially as described [13]. Human colon cancer cells in 24-well plates treated with various concentrations of gossypol, LPS, or cottonseed extracts for 8 h (triplicate in every concentration). The dishes were washed twice with 1 mL 0.9% NaCl and lysed directly with 1 mL of TRI_{ZOL} reagent (Invitrogen, Carlsbad, CA, USA). RNA was isolated according to the manufacturer's instructions without DNase treatment and stored in a −80 °C freezer. RNA concentrations were quantified with an Implen NanoPhotometer (Munchen, Germany). The mean and standard deviations of A260/A280 for the 192 independent RNA samples was 1.73 ± 0.17, indicating some contaminations of the RNA samples with protein, phenol, or other contaminants that have an absorbance close to 280 nm. RNA quality was evaluated by electrophoretic gel and electropherogram analyses. In our typical analysis, RNA isolated from mammalian cells with the Trizol reagent without DNase treatment resulted in high-quality RNA as evidenced by sharp 28S and 18S rRNA bands on electrophoretic gel and sharp peaks on electropherogram [109]. The total RNA was used to synthesize cDNAs using SuperScript II reverse transcriptase at 42 °C for 50 min. The cDNA synthesis mixture (20 μL) contained 5 μg total RNA, 2.4 μg oligo(dT)$_{12–18}$ primer, 0.1 μg random primers, 500 μM dNTPs, 10 mM DTT, 40 u RNaseOUT and 200 u SuperScript II reverse transcriptase in 1X first-

strand synthesis buffer (Life Technologies, Carlsbad, CA, USA). The cDNA was stored in a −80 °C freezer and diluted with water to 1 ng/μL before qPCR analyses.

4.7. Quantitative Real-Time PCR Analysis

The qPCR assays followed the MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments [46]. The qPCR assays were described in detail previously [26]. The qPCR efficiency was performed with variable amounts of template cDNA concentrations (0, 0.05, 0.5, 2.5, 5, 12.5, and 25 ng) essentially as described [109]. The correlation coefficiencies between Cq and cDNA concentration were over 0.99 for the BCL2 gene and tested genes. SYBR Green qPCR reaction mixture (12.5 μL) contained 5 ng of total RNA-derived cDNA, 200 nM each of the forward primer and reverse primer, and 1× iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The reactions in 96-well clear plates sealed by adhesives were performed with CFX96 real-time system-C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The thermal cycle conditions were as follows: 3 min at 95 °C, followed by 40 cycles at 95 °C for 10 s, 65 °C for 30 s and 72 °C for 30 s. The specificity of qPCR products was evaluated by melt curve analysis and 3% agarose gel electrophoresis as described [109]. Primer pair specificity for cDNA was evident from the analysis of qPCR products with sharp peaks on the melt curve and a single band on the electrophoretic gel.

4.8. Data Analysis and Statistics

qPCR data (10,560 Cq values) were generated from 55 genes analyzed and 64 treatments with triplicate per treatment for each gene. The data in the figures and tables represent the mean and standard deviation of 24–192 independent samples.

Author Contributions: Conceptualization, H.C.; methodology, H.C. and K.S.; validation, H.C. and K.S.; investigation, H.C. and K.S.; data curation, H.C.; writing—original draft preparation, H.C.; writing—review and editing, H.C.; supervision, H.C.; project administration, H.C.; funding acquisition, H.C. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets generated during the current study are available in the NIH Gene Expression Omnibus (GEO) Database, accession number GSE200980, GSE203027, GSE204818, and GSE204820.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are available from the authors.

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