COTTONSEED BIOACTIVE COMPOUNDS AND PEPTIDES

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Abstract

Cotton seeds have gossypol, a green-yellow polyphenolic pigment in their glands toxic to man and monogastric animals. Gossypol constitutes about 2.5-5.0% of the weight of dehulled cottonseed kernels. Hence, the feeding of the seeds or their products has been limited to cattle and other ruminants. Recently, genetically modified glandless cotton seeds were produced with negligible levels of gossypol. Cottonseed kernels contain about 35% of oil and 40% of protein and if gossypol free, they can be found wide application in food industry. The value of cotton seeds could be increased by finding other bioactive compounds. We have developed methods to prepare polyphenol compounds and peptides, and evaluate their bioactivity in mouse and human cells. The mammalian cells were tested with ethanol extracts and tryptic digests for regulating cell viability and gene expression by microscopic observation, toxic assay, protein content determination, gene expression and immunoblotting methods. Here we summarize the current research progress in cottonseed bioactive compounds and peptides. This information may help to increase cottonseed values by developing bioactive products for improving nutrition and preventing diseases.

Cottonseed

Cottonseed is a secondary product from cotton plant (*Gossypium hirsutum* L.) because it accounts for only 20% of the crop value. Cottonseed can be glanded or glandless depending on the presence of the dark glands containing gossypol (Figure 1A&B) (Luo et al., 2001; Dowd and Pelitire, 2006; Wang et al., 2009; Ma et al., 2016; Cao et al., 2018).

Glanded cottonseed contains about 10% linter, 40% hull and 50% kernel (Figure 1A) (Tharp, 1948). The kernel contains about 35% of oil, 40% of protein and numerous dark-colored gossypol glands (Figure 1B) (Cherry and Leffler, 1984; Cao et al., 2018). The commercial cottonseed meal contains approximately 1% of gossypol (He et al., 2015), which limits its use of cottonseed meal primarily to feed ruminants because they have a high tolerance for the toxic gossypol (Randel et al., 1992; Coutinho, 2002; Gadelha et al., 2014; Zeng et al., 2014; Camara et al., 2015). Gossypol binds to protein and causes difficulties to produce proteins free of gossypol (Alford et al., 1996).

Glandless cottonseed lacks pigment glands (Figure 1B) (Cao et al., 2018) and has only trace levels of gossypol (Cornu et al., 1977; Sneed et al., 1980; Alford et al., 1996). Their proteins are potentially more useful as a food ingredient or as a feed for non-ruminant animals (Cornu et al., 1977; Thomas et al., 1979; Sneed et al., 1980; Lusas and Jividen, 1987). Therefore, glandless cotton has generated considerable interest (Sunilkumar et al., 2006; Rathore et al., 2012; Palle et al., 2013; Zhang et al., 2014; Zhang et al., 2016). Glandless cottonseed and their modified products have been approved for human consumption by the Food and Drug Administration (https://www.gpo.gov/fdsys/pkg/CFR-2012-title21-vol3/pdf/CFR-2012). Glandless seeds may be available for consumption by human and non-ruminant animals in the near future (Lusas and Jividen, 1987; Sunilkumar et al., 2006). However, glandless cottonseed contains growth inhibitors (JOHNSTON and Watts, 1965) such as cyclopropenoid fatty acids, which was reported to cause liver cancer in rainbow trout (Hendricks et al., 1980).

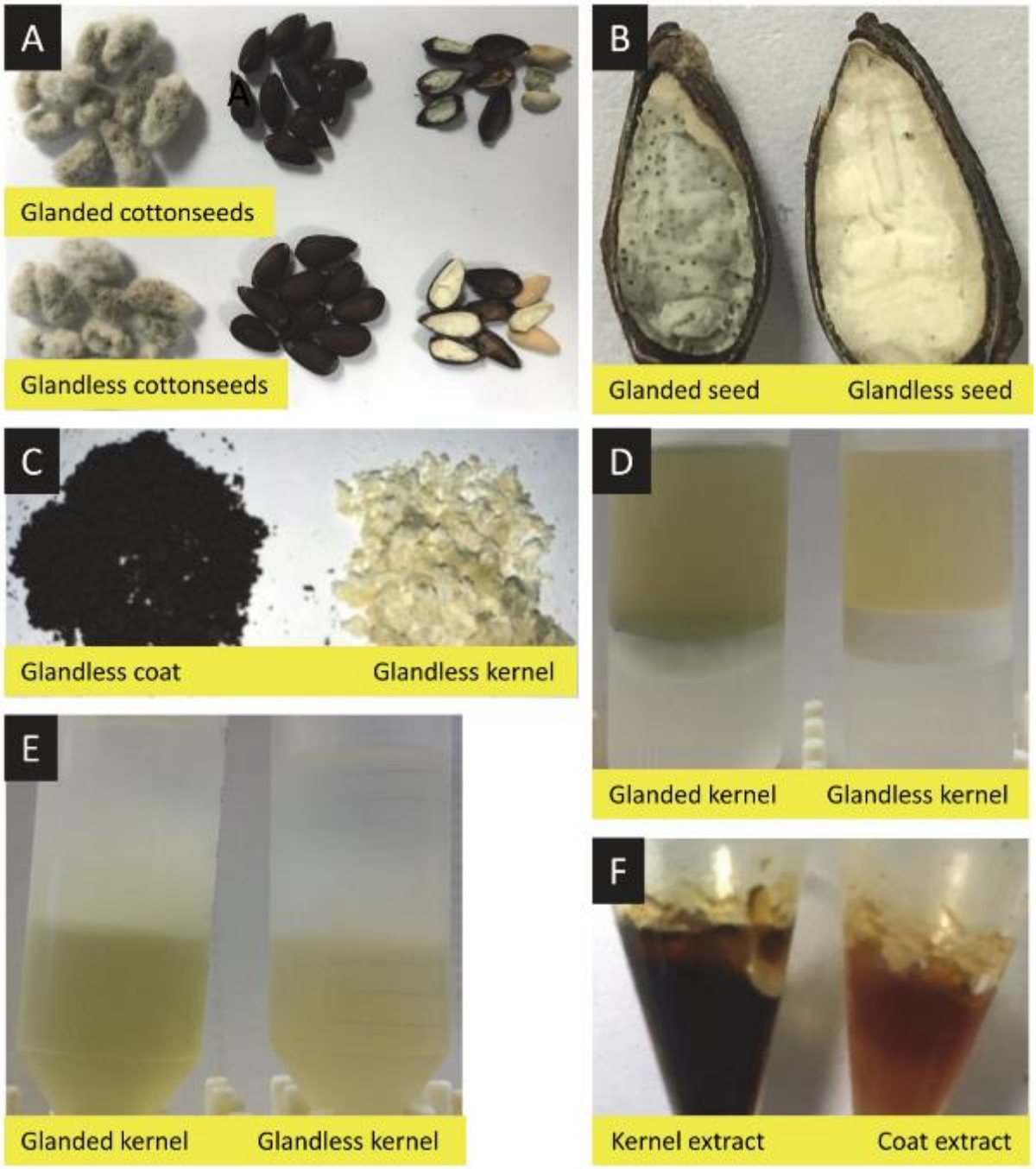

Figure 1. Glanded and glandless cottonseed and isolation of ethanol extracts from the cottonseed. (A) Glanded and glandless cottonseed with short fibers, after sulfuric acid removal of the short fibers, and the seed coat and kernel. (B) Glanded and glandless cottonseed section. Glanded seeds are smaller than glandless seeds and contain numerous dark green-colored glands. (C) Glandless cottonseed coat and kernel. (D) Chloroform extraction. The glanded and glandless kernel homogenates were treated with chloroform followed by centrifugation to separate aqueous (upper) and organic (lower) layers. (E) Hexane extraction: The upper aqueous layer was mixed with hexane followed by centrifugation to separate neutral lipids (upper) and aqueous layers. (F) Ethanol extraction. Seed coat fraction after hexane extraction was suspended in acetic acid, blended, autoclaved and centrifuged. The supernatant was mixed with ethanol followed by centrifugation. The defatted kernel material was directly mixed with ethanol, vortexed and centrifuged. This supernatant was dried under rotoevaporation until all ethanol evaporated (Cao et al., 2018).

Bioactive Compounds from Cottonseed

Obesity and diabetes are epidemic in the US and around the world. It was estimated in 2015 that approximately 40% of the population were obese and 10% of the population have diabetes in the US. Finding ways to slowdown and prevent the occurrence would have tremendous benefits for reducing healthcare cost and improving life quality. Plant bioactive materials can potentially be used to serve these purposes because these value-added products possess health promotion and disease prevention potentials.

Cottonseed contains approximately 70% unsaturated fatty acids (18% monounsaturated and 52% polyunsaturated), 26% saturated fatty acids and a few percentage of bioactive lipids. Oleic acid (w-9=18:1) and linoleic acid (w-6=C18:2) account for almost all of the mono- and polyunsaturated fatty acids, respectively (US National Nutrient Database, Release 28, 2016). The minor bioactive lipids include gossypol, quercetin, gallic acid, 3,4-dihydroxybenzoic acid, flavonoids, and cyclopropenoid fatty acids (Zhang et al., 2001; Dowd and Pelitire, 2006; Piccinelli et al., 2007; Cao, 2019). Most of these value-added bioactive lipids possess health promotion and disease prevention potentials (Zhang et al., 2001; Zhao et al., 2006; Mellon et al., 2012; Cao and Sethumadhavan, 2018; Cao et al., 2018; Cao and Sethumadhavan, 2019; Cao, 2019). However, gossypol and cyclopropenoid fatty acids also have negative effects on nutrition and health (Hendricks et al., 1980; Coutinho, 2002).

We developed protocols for isolating ethanol extracts from cottonseed (Cao et al., 2018) (Figure 1C-F). Cottonseed extract from seed kernel was isolated with a protocol of three steps: fractionation, defatting, and ethanol extraction (Figure 2). Cottonseed extract from seed coat was isolated with a protocol of four steps: fractionation, defatting, acetic acid extraction, and ethanol extraction (Figure 2). The ethanol extracts were dried under rotoevaporation until all acetic acid and ethanol evaporated. This procedure yielded 0.39 g of ethanol extract from seed coat and 3.66 g of ethanol extract from seed cotyledon per 100 g of glanded cottonseed, and 0.98 g of ethanol extract from seed coat and 1.12 g of ethanol extract from seed cotyledon per 100 g of glandless cottonseed (Cao, 2019). HPLC-MS identified more than 20 compounds in cottonseed extracts. Gossypol (a polyphenolic metabolite present in regular cottonseed) was shown to be extremely low with less than 1 ppm in cottonseed extracts, which was much less than the US federal government limit of 450 ppm. Bioactive quercetin was only detected in glandless seed coat extract (Cao et al., 2018; Cao, 2019).

No cytotoxicity effect was observed in macrophages treated with extracts from the coat or kernel of glanded and glandless cottonseed (Cao and Sethumadhavan, 2020). Similarly, the viability of mouse adipocytes was not affected by cottonseed extracts (Cao and Sethumadhavan, 2020). qPCR assay and immunoblotting assays showed that gossypol but not cottonseed extracts increase the expression of pro-inflammatory human antigen R (HuR) which stabilizes cytokine mRNAs and plays an important role in tumorigenesis and inflammation (Cao and Sethumadhavan, 2019) (Figure 3). These results suggest that the gossypol-free ethanol extracts are safe for macrophages and adipocytes.

Ethanol extracts from glanded and glandless cottonseed kernels significantly decreased the mitochondrial activity (energy production) of breast cancer cells and pancreas cancer cells (Cao et al., 2018). Ethanol extracts from glanded cottonseed kernels are stimulators of DGAT2 gene expression and that they may be novel agents for intervention of lipid-related diseases (Cao and Sethumadhavan, 2018) (Figure 4). Cottonseed extracts exhibited modest effects on anti-inflammatory TTP family gene expression in macrophages but glandless cottonseed coat extract significantly increased TTP gene expression with a magnitude similar to cinnamon and green tea polyphenol extract and insulin (Cao and Sethumadhavan, 2020) (Figure 5).

These results suggest that ethanol extracts from cottonseed have anti-inflammatory effects (Cao and Sethumadhavan, 2020). In particular, ethanol extract from glandless cottonseed do not have gossypol but has quercetin (Cao et al., 2018). It was reported that aqueous extract from glandless cottonseed meal had antidepressant effect and subsequently the authors identified the major bioactive compound being quercetin (CTN-986) (Zhang et al., 2001). This compound has antidepressant effects in pharmacological tests (Li et al., 2000; Zhang et al., 2009). It has potential applications in treating anxiety, depression and Alzeheimer's disease (Zhao et al., 2006).

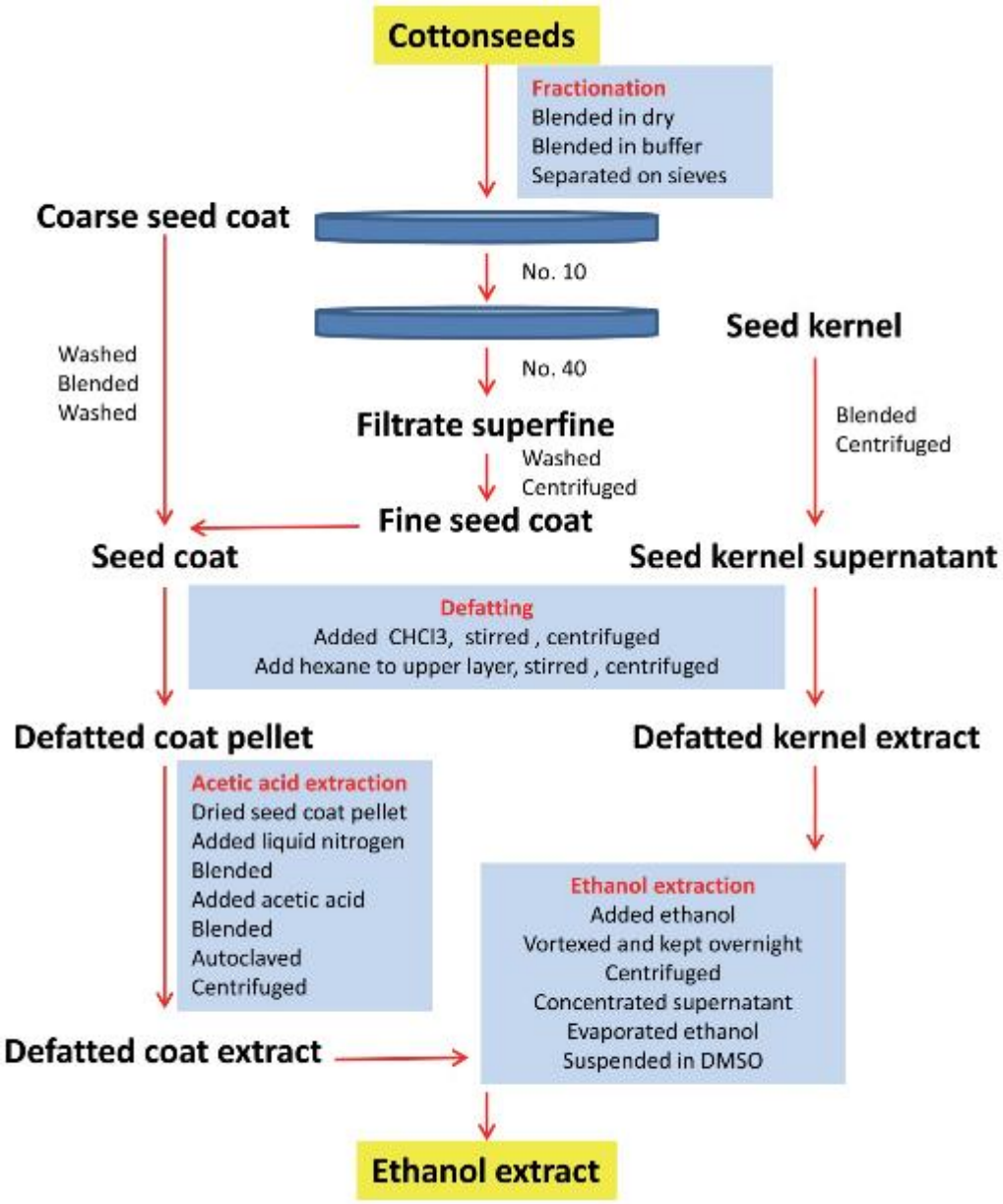

Figure 2. The protocol for isolating ethanol extracts from cottonseeds. This method was consistent of three steps for seed kernel extraction (fractionation, defatting, and ethanol extraction) and four steps for seed coat extraction (fractionation, defatting, acetic acid extraction, and ethanol extraction). Briefly, cottonseeds were ground in dry and in a buffer. The homogenate was separated successively through No. 10 sieve to retain coarse seed coat and No. 40 sieve to retain fine seed kernel and colored superfine filtrate. The coarse seed coat was washed with water and blended several times until clear seed coat pellet was obtained. The colored superfine filtrate was mixed with water and allowed to stand and centrifuged. The red pellet of fine seed coat was pooled together with the coarse seed coat and suspend in the buffer. The seed kernel was suspended in the same buffer followed by grinding and centrifugation. The seed coat suspension and kernel supernatant were defatted with chloroform and hexane followed by centrifugation. Defatted seed coat pellet was air-died after defatting and grinded into fine powder under liquid nitrogen. The fine powder was suspended in acetic acid, ground again, autoclaved, and centrifuged. The supernatant was filtrated through glass wool, mixed with ethanol, stirred well, refrigerated overnight and centrifuged. The supernatant was concentrated in a rotovap and residue ethanol was removed by rotoevaporation. The preparation of ethanol extract from the defatted kernel extract was similar to those of defatted coat extract without acetic acid treatment. The dried ethanol extract pellet was reconstituted in DMSO (Cao et al., 2018).

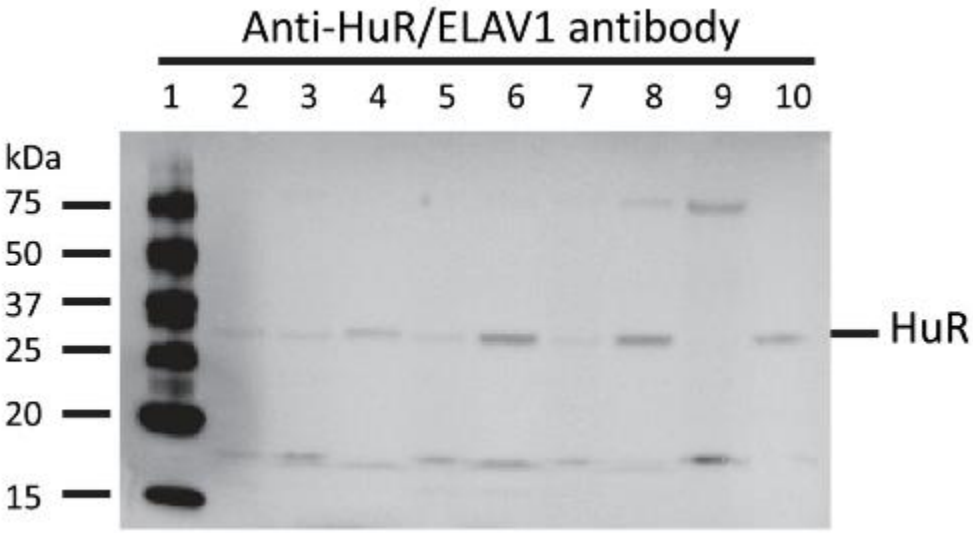

Figure 3. Effect of Gossypol and lipopolysaccharides (LPS) on HuR protein in mouse macrophages. RAW264.7 cells were stimulated with 100 µg/mL gossypol or 100 ng/mL LPS for 2, 4, 8 and 24 h. Cell extract from a 10,000*g* supernatant (100 µg of protein per lane except lane with 50 µg of protein) was used for immunoblotting using the anti-HuR/ELAV1 antibody ab28660. Lane 1: protein standards; lane 2: 1% DMSO; lanes 3, 5, 7 and 9: LPS treatment for 2, 4, 8 and 24 h, respectively; lanes 4, 6, 8 and 10: gossypol treatment for 2, 4, 8 and 24 h, respectively (Cao and Sethumadhavan, 2019).

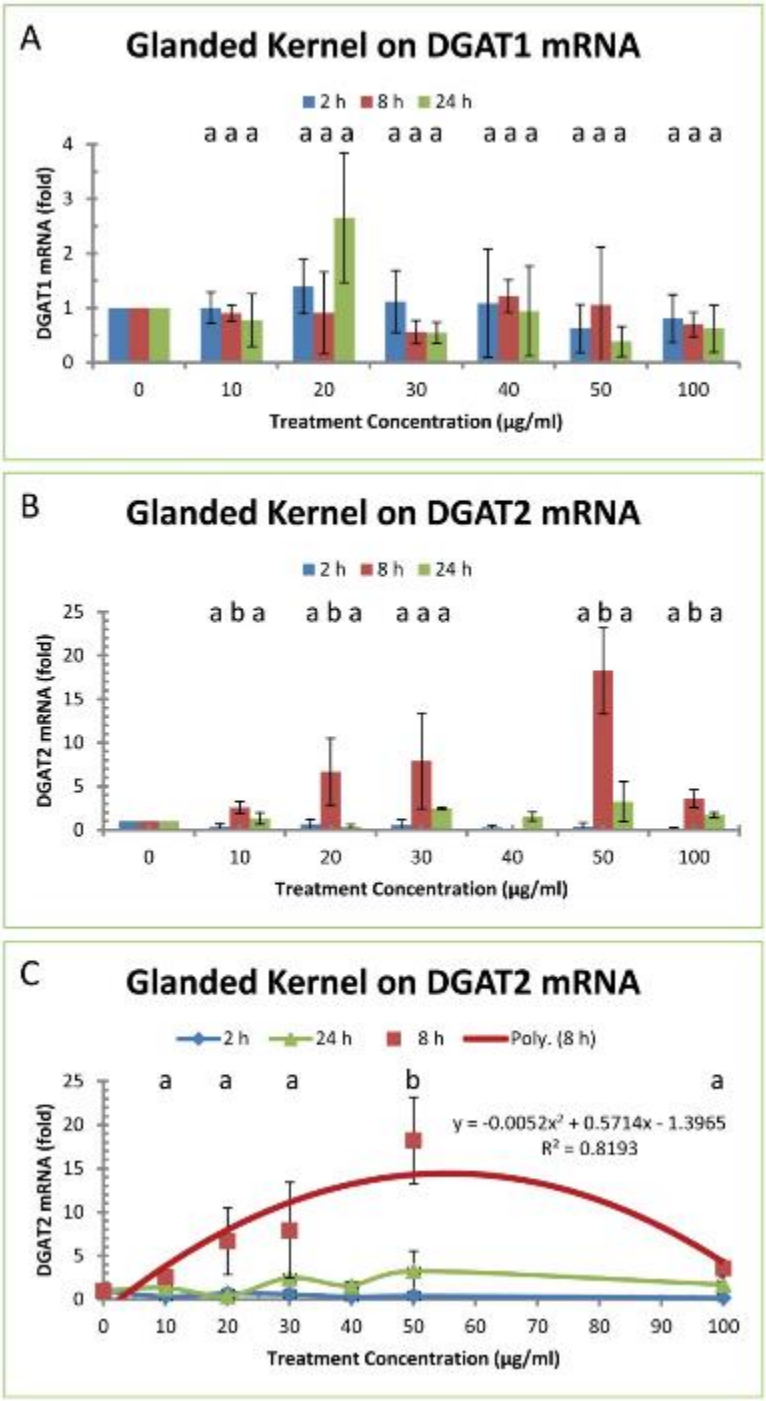

Figure 4. Effect of glanded cottonseed kernel extract on DGAT gene expression. (A) DGAT1 mRNA levels, (B) DGAT2 mRNA levels, (C) Calculation of equation and efficient correlation (R^2) between DGAT2 mRNA levels and extract concentration. Experimental procedures and data analysis were described under Figure 2 legend. The data represent the mean and standard deviation of three independent samples. Different lower case letters displayed above each of the treatment concentration on the figures are significantly different between the treatment times at $p < 0.05$. The calculated equation was $y = -0.0052x^2 + 0.5714x - 1.3965$ ($R^2 = 0.8193$), where y is DGAT2 mRNA levels in fold and x was glanded kernel extract concentration used in the culture medium (Cao and Sethumadhavan, 2018).

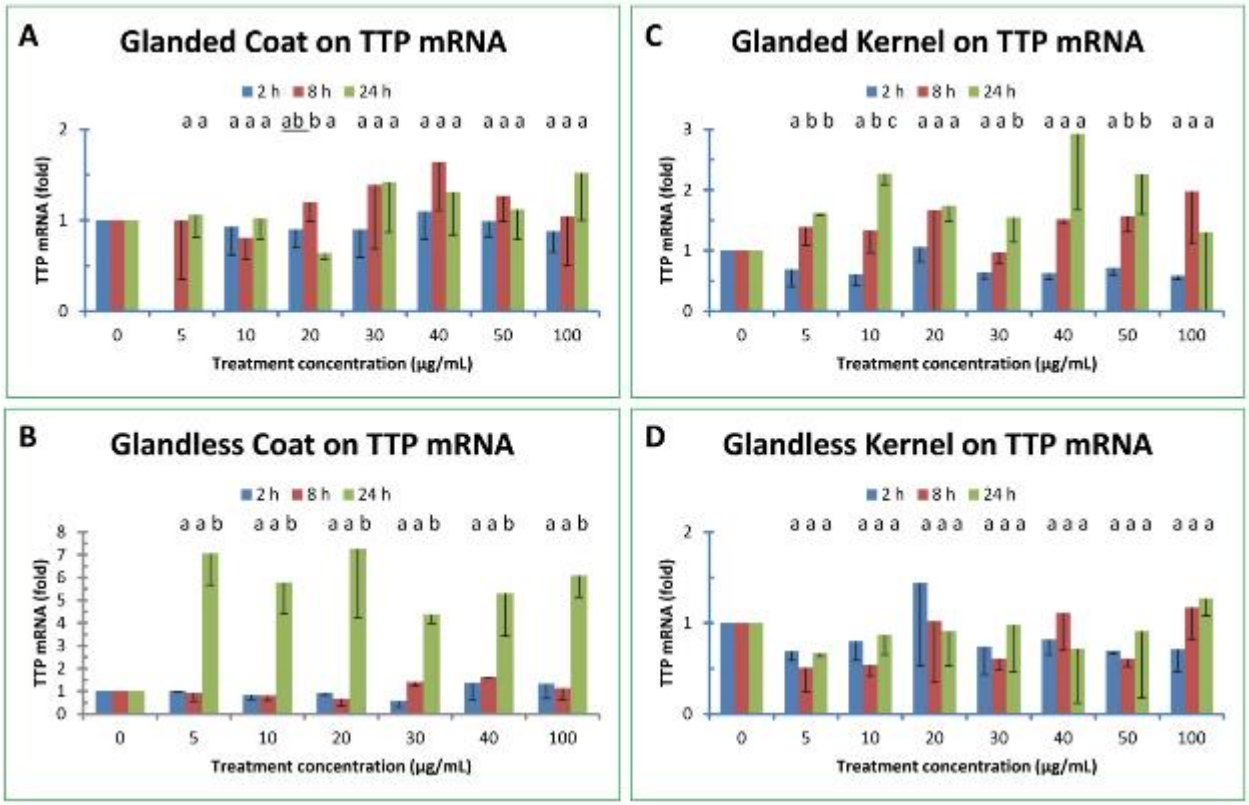

Figure 5. Effect of cottonseed extracts on TTP mRNA expression. Mouse RAW264.7 macrophages were treated with the extract (0-100 µg/mL, "0" treatment corresponding to 1% DMSO in the culture medium) for 2, 8 and 24 h. Total RNAs were isolated from the cells and used for cDNA synthesis. The SYBR Green qPCR reaction mixtures contained 5 ng of RNA-equivalent cDNAs from each sample and 200 nM of each primer. The $2^{-\Delta\Delta CT}$ method of relative quantification was used to determine the fold change in expression using RPL32 mRNA as a reference mRNA. The data represent the mean and standard deviation of three independent samples. Different lower case letters displayed above each of the treatment time on the figures are significantly different between the LPS concentrations at $p < 0.05$. (A) glanded cottonseed coat extract, (B) glanded cottonseed kernel extract, (C) glandless cottonseed coat extract, (D) glandless cottonseed kernel extract (Cao and Sethumadhavan, 2020).

Bioactive Peptides from Cottonseed

Bioactive peptides are short peptides with 2 - 20 amino acid residues (Karami and Akbari-Adergani, 2019). They are detected in many foods such as milk (Aspri et al., 2018), fermented products (Beermann and Hartung, 2013), plant and marine proteins (Orona-Tamayo et al., 2018). Bioactive peptides affect pro-health or functional properties of food products. They are potential high-value products with health and nutritional benefits because of their functional properties such as anti-hypertensive, anti-oxidative, hypocholesterolemic, water-holding capacity, foaming capacity, emulsifying properties and solubility (Karami and Akbari-Adergani, 2019). Bioactive peptides from food protein sources can be used as potential ingredients of health promoting functional foods.

Cottonseed protein is a potential source of nutrients for humans and animals (Alford et al., 1996; He et al., 2018), but its use is limited to feed ruminants due to high concentration of toxic gossypol in cottonseed meals (Randel et al., 1992; Coutinho, 2002; Zeng et al., 2014; He et al., 2015; Camara et al., 2015). The development of glandless and extra-low gossypol cottons provides a unique opportunity of exploring the use as a food source (Lusas and Jividen, 1987; Sunilkumar et al., 2006; Palle et al., 2013; Zhang et al., 2016). It was reported that cottonseed proteins digested with neutrase resulted in the identification of a hydrolysate with antioxidant activity suitable for conversion to high-value products and suggested that the hydrolysate fraction III derived from cottonseed protein could be a natural antioxidant source suitable for use as a food additive (Gao et al., 2010). Optimum enzymatic hydrolysate of cottonseed protein supplementation increased growth and decreased diarrhea incidence, and improved intestinal morphology and

antioxidant capacity of nursery pig in Thailand (Cao and Sethumadhavan, 2018; Tanumtuen et al., 2019). Cottonseed proteins are arginine-rich polypeptides, and arginine accounts for 15% to 34% of total protein, which is higher than soybean meal (He et al., 2015). This information suggests that cottonseed proteins may contain unique bioactive peptides. In fact, a peptide from cottonseed meal protein hydrolysate was identified with antioxidant activity and immunoenhancement for fish (Yuan et al., 2020).

We developed methods to prepare peptides, and evaluate its bioactivity in mouse macrophages. Cotton seeds were grounded in alkaline media and defatted using organic solvents. The protein pellet was sonicated in presence of Tris buffer, pH 7.4 and the proteins were digested to peptide fragments using trypsin enzyme. The electrophoresis of the digest showed similar pattern in both glanded and glandless cotton types. The mouse macrophages were tested with tryptic digests for regulating cell viability and gene expression. Cell viability assay showed that higher peptide concentrations caused some inhibition of mitochondrial activity in mouse RAW264.7 macrophages. The effects of these peptides on immunological responses were evaluated by studying mRNA levels related to signaling pathways. This will be a useful tool in identifying the biological nature of biopeptides and hence its usefulness in nutrition.

Conclusions

Obesity and diabetes are epidemic in the US and around the world. It was estimated in 2015 that approximately 40% of the population were obese and 10% of the population have diabetes in the US. Finding ways to slowdown and prevent the occurrence would have tremendous benefits for reducing healthcare cost and improving life quality. Plant bioactive materials can potentially be used to serve these purposes. Cottonseed accounts for only 20% of the crop value due to presence of toxic gossypol. Current work has provided useful information to increase cottonseed value by isolating bioactive extracts and compounds from cottonseed because these value-added products possess health promotion and disease prevention potentials. We have developed protocols for isolating bioactive ethanol extracts with a yield of ~ 2% from cottonseed. HPLC-MS identified more than 20 compounds in cottonseed extracts. Gossypol was shown with less than 1 ppm in cottonseed extracts, which was much less than the US federal government limit of 450 ppm. Bioactive quercetin was only detected in glandless seed coat extract. No cytotoxicity effect of cottonseed extracts was observed in mouse macrophages or adipocytes. Molecular and immunoblotting assays showed that cottonseed extracts did not increase the expression of pro-inflammatory HuR expression which stabilizes cytokine mRNAs and plays an important role in tumorigenesis and inflammation. We have further demonstrated that ethanol extracts decreased breast cancer and pancreas cancer cell growth, stimulated key lipid-synthesizing DGAT2 gene expression which may be a novel target for intervention of lipid-related diseases, and increased anti-inflammatory TTP gene expression with a magnitude similar to cinnamon and green tea polyphenol extract and insulin. In particular, ethanol extract from glandless cottonseed did not have toxic gossypol but had beneficial quercetin. These results suggest that the gossypol-free ethanol extracts are safe with anti-inflammatory and anticancer properties. This information should lead a foundation for developing glandless cottonseed as a functional food for promoting human nutrition and preventing human diseases associated with inflammation such as arthritis, diabetes, obesity, cardiovascular diseases and cancers (Figure 6).

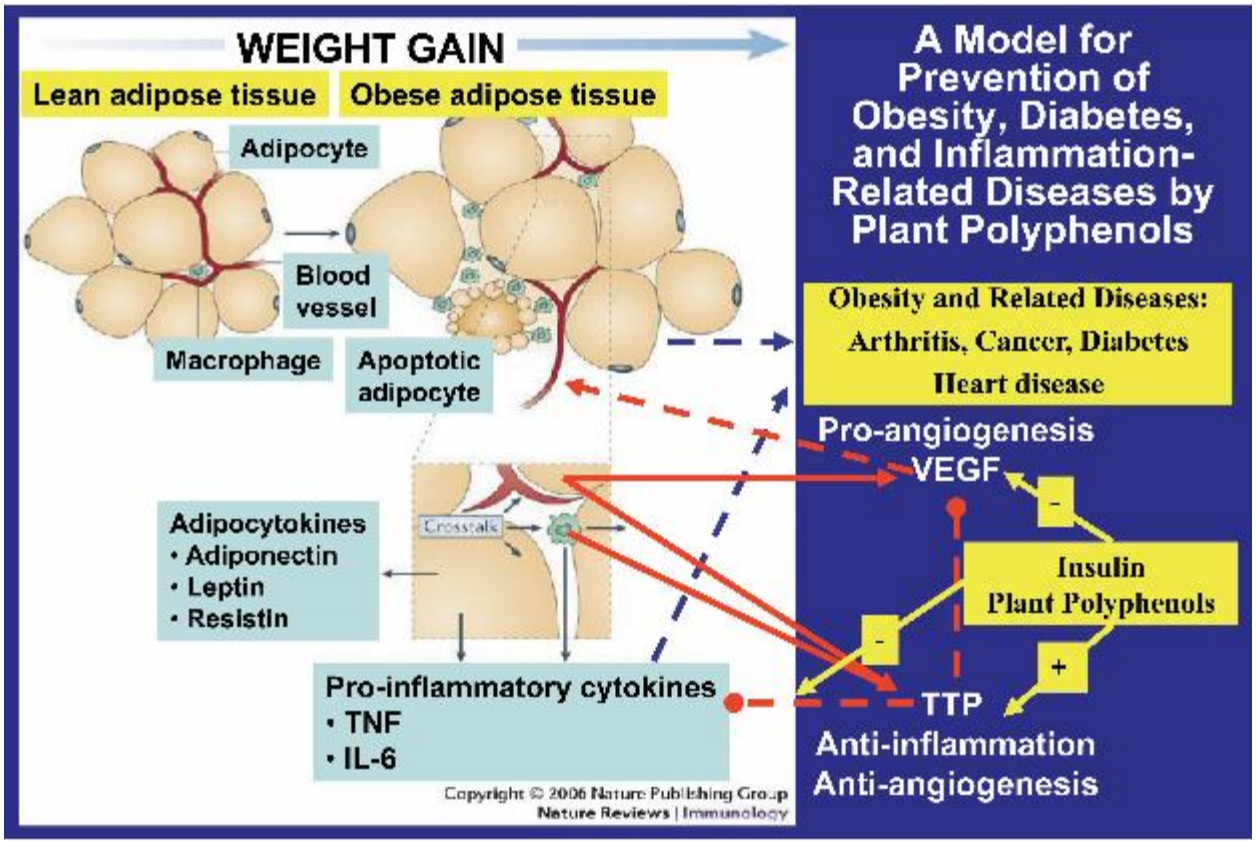

Figure 6. A model for plant polyphenols to prevent obesity, diabetes, and inflammation-related diseases.

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