BIOACTIVE COMPOUNDS FROM GLANDED AND GLANDLESS COTTONSEED

Heping Cao
USDA-ARS, Southern Regional Research Center
New Orleans, LA

Abstract

Cotton (*Gossypium hirsutum* L.) is an industrially important crop because it provides fiber and cottonseed. Cottonseed accounts for only 20% of the crop value despite of being weighted much more than fiber. Cottonseed are classified as glanded or glandless seeds. Due to presence of toxic gossypol in the glanded cotton, the use of cottonseed is limited to feeding cows. One way to increase cottonseed value is to isolate bioactive extracts and compounds from cottonseed. Cottonseed contains a number of bioactive compounds including gossypol, gallic acid, 3,4-dihydroxybenzoic acid, quercetin, flavonoids, cyclopropenoid fatty acids and protein-derived peptides. Most of these value-added products possess health promotion and disease prevention potentials. However, gossypol and cyclopropenoid fatty acids also have negative effects on nutrition and health. This paper summarized the biologically active compounds and extracts from cottonseed and their effects on nutrition and health related aspects. A working model for future mechanistic study is proposed that links plant extracts and TTP-mediated cytokine instability in mammalian cells.

Introduction

Cotton (*Gossypium hirsutum* L.) is an important economic crop because it provides fiber. Cottonseed is secondary products because they account for only 20% of the crop value despite of being weighted much more than fiber in mass. Cottonseed is divided into glanded or glandless seeds depending on the presence or absence of the dark pigment glands containing polyphenolic gossypol (Figure 1) (Cao et al., 2018;Dowd and Pelitire, 2006;Luo et al., 2001;Ma et al., 2016;Wang et al., 2009).

Glanded cottonseed contains approximately 10% linters, 40% hulls and 50% cotyledons (Figure 1A) (Tharp, 1948). These cotyledons contain about 35% of oil and 40% of protein and numerous dark-colored gossypol glands (Figure 1B) (Cao et al., 2018;Cherry and Leffler, 1984). The commercial cottonseed meal after oil extraction from the seeds contains approximately 1% of gossypol (He et al., 2015). The residual gossypol limits its use of cottonseed meal primarily to feed ruminants because they have a relative high tolerance for the toxic gossypol (Camara et al., 2015;Coutinho, 2002;Gadelha et al., 2014;Randel et al., 1992;Zeng et al., 2014). Gossypol binding to protein also makes it difficult to produce concentrated protein fractions 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 and generally considered safe (Alford et al., 1996;Cornu et al., 1977;Sneed et al., 1980). Their proteins are potentially more useful as a food ingredient or as a feed for non-ruminant animals (Cornu et al., 1977;Lusas and Jividen, 1987;Sneed et al., 1980;Thomas et al., 1979). Therefore, development of glandless cotton has generated considerable interest within the cotton industry (Palle et al., 2013;Rathore et al., 2012;Sunilkumar et al., 2006;Zhang et al., 2016;Zhang et al., 2014). Glandless cottonseed and their modified products have been approved for human consumption as a nut substitute and snack item by the Food and Drug Administration (https://www.gpo.gov/fdsys/pkg/CFR-2012-title21-vol3/pdf/CFR-2012). These seeds may be available for consumption by human and non-ruminant animals in the future (Lusas and Jividen, 1987;Sunilkumar et al., 2006). However, glandless cottonseed contains other growth inhibitors (JOHNSTON and Watts, 1965) such as cyclopropenoid fatty acids, which caused liver cancer in rainbow trout in one study (Hendricks et al., 1980).

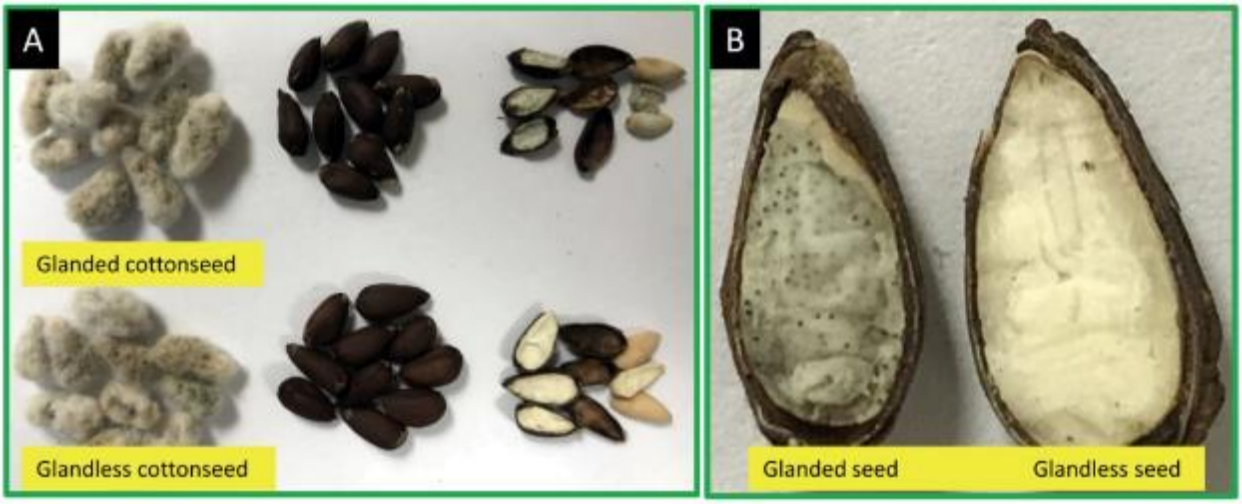

Figure 1. Cottonseed. (A) Glanded and glandless cottonseed with short fibers, after sulfuric acid removal of the short fibers, and the seed coat and cotyledon. Both types of seeds are indistinguishable outside. (B) Glanded and glandless cottonseed sections. Glanded cottonseed is smaller than glandless cottonseed and contains numerous dark green-colored glands in the cotyledon (Cao et al., 2018).

Cottonseed Bioactive Compounds

Cottonseed contains a number of bioactive components including gossypol, quercetin, gallic acid, 3,4-dihydroxybenzoic acid, flavonoids, cyclopropenoid fatty acids, and peptides (Table 1). Most of these value-added products possess health promotion and disease prevention potentials. However, gossypol and cyclopropenoid fatty acids also have negative effects on nutrition and health.

Table 1 Bioactive compounds from cottonseed

Cottonseed compound	Biological effect	Reference
Gossypol	**pro-male infertility anti-breast cancer anti-colon cancer anti-pancreatic cancer anti-prostate cancer anti-obesity anti-inflammation anti-fungi**	**(Coutinho, 2002) (Liu et al., 2002;Zhong et al., 2013) (Chien et al., 2012) (Thakur et al., 2012;Yuan et al., 2013) (Huang et al., 2009;Pang et al., 2011) (Zhong et al., 2010;Zhong et al., 2013) (Chen et al., 2018;Huo et al., 2013;Oskoueian et al., 2011) (Mellon et al., 2012;Puckhaber et al., 2002)**
Gallic acid	**anti-oxidant**	**(Piccinelli et al., 2007)**
3,4-dihydroxybenzoic acid	**anti-oxidant**	**(Piccinelli et al., 2007)**
Quercetin	**anti-depressant/ anxiety anti-Alzeheimer's disease**	**(Zhang et al., 2001) (Zhao et al., 2006)**
Flavonoids	**identification anti-diabetes other conditions**	**(Yuan et al., 2012;Zhang et al., 2001) (Anjaneyulu et al., 2003) (Gong et al., 2014)**
Cyclopropenoid fatty acids	**caused liver cancer in rainbow trout**	**(Hendricks et al., 1980)**
Peptides	**anti-oxidant**	**(Gao et al., 2010)**

Gossypol

Gossypol is the best studied minor component from glanded cottonseed, Gossypol is a complex polyphenol with a highly colored yellow pigment found in the small intercellular pigment glands in cotton leaves, stems, roots, and seeds (Figure 2A) (Kenar, 2006). Gossypol has anti-nutritional property. Long-term consumption of gossypol-containing cottonseed oil contributes to its toxicity resulting in male infertility (Coutinho, 2002). Therefore, gossypol is regarded as unsafe for most animal and human consumption. Significant research efforts have been directed to reduce gossypol content in cottonseed by selecting glandless cotton varieties (Alford et al., 1996;Cornu et al., 1977;Hendricks et al., 1980;Lusas and Jividen, 1987;Sneed et al., 1980;Thomas et al., 1979) and genetic engineering of gossypol-free seeds of cotton plants (Palle et al., 2013;Rathore et al., 2012;Sunilkumar et al., 2006).

Recent research has demonstrated that gossypol have potential biomedical applications. Gossypol and related compounds are shown to have anticancer activities, including breast cancer (Liu et al., 2002;Zhong et al., 2013), colon cancer (Chien et al., 2012), pancreatic cancer (Thakur et al., 2012;Yuan et al., 2013), and prostate cancer (Huang et al., 2009;Pang et al., 2011). Gossypol has additional bioactivities such as anti-obesity activities (Zhong et al., 2010;Zhong et al., 2013), anti-inflammatory activities (Huo et al., 2013;Oskoueian et al., 2011), and anti-fungal activities (Mellon et al., 2012;Puckhaber et al., 2002). The molecular mechanism(s) of anti-cancer effects of gossypol has been studied extensively. It has been shown that gossypol inhibits breast cancer cells via DNA synthesis (Hu et al., 1993), suppressing Bcl-2 and Bcl-xL expression (Li et al., 2011). Gossypol also inhibits Bcl-2 and Mcl-1 gene expression in pancreatic cancer cells (Banerjee et al., 2010). These new discoveries have generated intensive interest in biomedical field and extensive research has been directed at understanding the medical utilization of gossypol and related compounds.

We recently showed that gossypol significantly decreased breast cancer cell growth by approximately 30% after 24 h treatment (Cao et al., 2018). We also showed that gossypol treatment for 2 h significantly decreased cell viability of pancreatic cancer cell by up to 50% (Cao et al., 2018). These results are in agreement with previous findings that gossypol has anticancer property.

However, gossypol significant inhibited mouse RAW macrophage growth under higher concentration or longer time of treatments; the higher the concentration or the longer treatment resulted in more severe reduction of mitochondrial activity (data not shown). Mitochondrial activity in RAW macrophages was almost completely inhibited by gossypol after treatment for 24-72 h at 5-50 µg/mL or 2-72 h at 100 µg/mL (data not shown). However, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assays showed that gossypol did not appear to have significant inhibitory effect on adipocyte survival after 2-24 h treatments (data not shown). These results suggest that long time consumption of high dose of gossypol could decrease host's immunity by destroying macrophages.

We also showed that gossypol strongly induced diacylglycerol acyltransferase (DGAT) gene expression in mouse macrophages (Cao and Sethumadhavan, 2018). DGAT is the rate-limiting enzyme for triacylglycerols (TAGs) biosynthesis in eukaryotic organisms (Cao, 2011;Cao et al., 2013;Cao, 2018;Liu et al., 2012). Plants and animals deficient in DGAT accumulate less TAG (Smith et al., 2000;Stone et al., 2004;Zou et al., 1999). Animals with reduced DGAT activity are resistant to diet-induced obesity (Chen et al., 2004;Smith et al., 2000), lack milk production (Smith et al., 2000) and maybe subject to a congenital diarrheal disorder (Haas et al., 2012). Over-expression of DGAT enzymes increases TAG content in plants (Burgal et al., 2008;Durrett et al., 2010;Lardizabal et al., 2008), animals (Liu et al., 2007;Liu et al., 2009;Roorda et al., 2005), and yeast (Kamisaka et al., 2007). These results suggest that gossypol may help to stimulate oil/milk production by up-regulating DGAT gene expression.

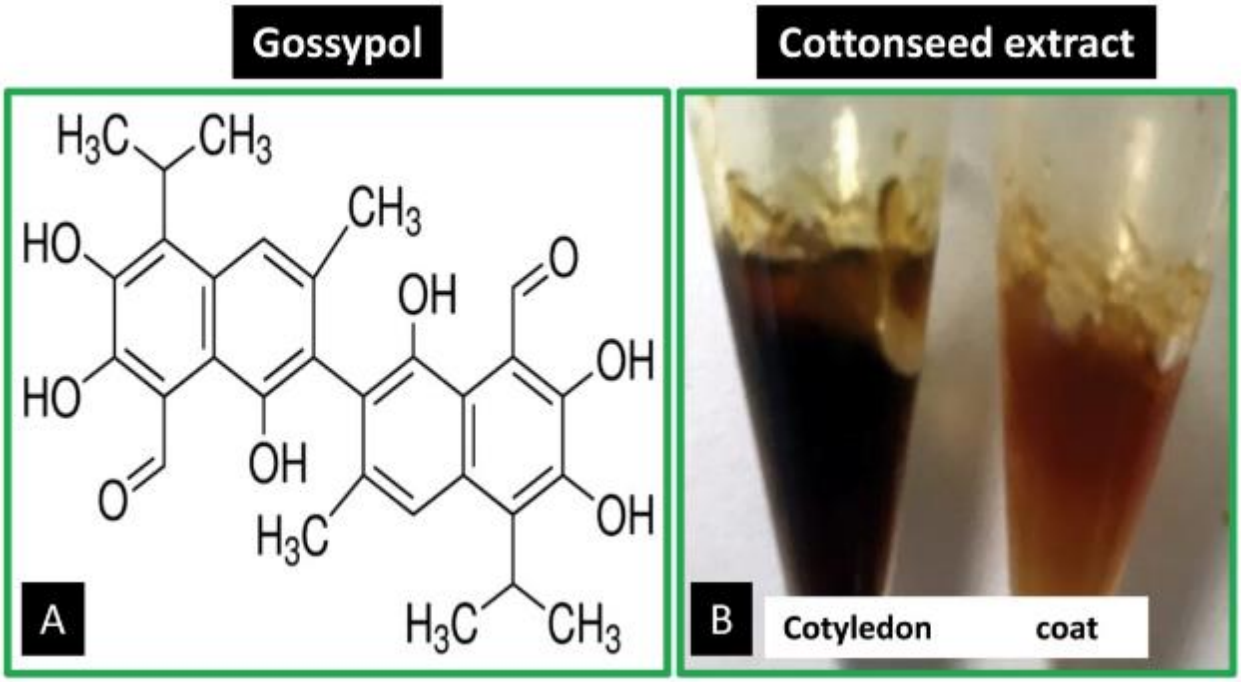

Figure 2. Gossypol and cottonseed extracts. (A) Gossypol is derived from cottonseed. (B) Cottonseed extracts are isolated from defatted cottonseed cotyledon and coat of glanded and glandless cottonseed which are shown to be essentially free of gossypol (Cao et al., 2018).

Antioxidants

Besides the well-studied gossypol, anti-oxidant compounds from glanded cottonseed exhibit significant bioactivities. For example, gallic acid and 3,4-dihydroxybenzoic acid have been identified from cottonseed that have positive effects on human health (Piccinelli et al., 2007).

Quercetin

Flavonol glycosides (flavonoids) are the best studied compounds from glandless cottonseed. We recently identified quercetin in the coat extract from glandless cottonseed (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).

Flavonoids

Seven flavonol glycosides have been identified from whole cottonseed (Piccinelli et al., 2007). Five flavonoids have been identified from glandless cottonseed (Yuan et al., 2012;Zhang et al., 2001). Independent studies have confirmed that flavonoids could be used as a therapy for depression associated with diabetes (Anjaneyulu et al., 2003) and under other conditions (Gong et al., 2014).

Cyclopropenoid Fatty Acids

Cyclopropenoid fatty acids are active as synergists with aflatoxins and primary liver carcinogens in rainbow trout in one study (Hendricks et al., 1980). It is a known toxic compound in cottonseed oil (Author not listed, 2001).

Peptides

Cottonseed protein is a potential source of nutrients for humans and animals (He et al., 2018;He et al., 2014). Cottonseed protein is being tested to make adhesive materials for industrial uses (Cheng et al., 2013;He et al., 2013;He

and Chapital, 2015). It was shown that the hydrolysate derived from cottonseed protein has antioxidant activity (Gao et al., 2010). Therefore, it is possible to increase cottonseed value by producing antioxidant peptides.

Cottonseed Bioactive Extracts

Bioactive plant extracts have been used for the prevention and treatment of various diseases. One of the major classes of bioactive compounds is plant polyphenols, which are found in plant seeds, fruits, leaves and bark. Plant polyphenols with nutritional properties are mostly water-soluble and can be extracted by ethanol from plant materials such as cinnamon bark and green tea leaves, and that some toxic compounds such as cinnamaldehyde (essential oil) are extracted from cinnamon bark by organic solvent (Anderson et al., 2004;Anderson and Polansky, 2002;Cao et al., 2007;Cao et al., 2010).

Cottonseed Extract Isolation

We developed protocols for isolating ethanol extracts from cottonseed (Figure 2B). Cottonseed extract from seed cotyledon was isolated with a protocol of three steps: fractionation, defatting, and ethanol extraction. Cottonseed extract from seed coat was isolated with a protocol of four steps: fractionation, defatting, acetic acid extraction, and ethanol extraction. The seed coat and cotyledon of glanded and glandless cottonseed were fractionated by grinding and homogenization. The seed cotyledon fractions were defatted with equal volume of chloroform and hexane followed by centrifugation. The aqueous layer from glanded cotyledon appeared much darker/greener color than that from glandless cotyledon. The seed coat fraction was suspended in acetic acid, autoclaved and centrifuged before ethanol extraction. The ethanol extracts were dried under rotoevaporation until all acetic acid and ethanol evaporated. The seed cotyledon extract was much darker in color than the seed coat extract (Figure 2B). 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 (Table 2).

Table 2 Isolation of ethanol extracts from cottonseed

Cottonseed	Cottonseed extract (g/100g seed)	Gossypol concentration (ng/mg extract)	Quercetin detection
Glanded seed coat	**0.39**	**0.82**	**no**
Glanded seed cotyledon	**3.66**	**0.03**	**no**
Glandless seed coat	**0.98**	**0.37**	**yes**
Glandless seed cotyledon	**1.12**	**0**	**no**

Cottonseed Extract Composition

HPLC-UV-MS was used to analyze the four ethanol extracts from glanded and glandless cottonseed. Many peaks were observed with 27 peaks having mass values equivalent to flavonols or derivatives (Cao et al., 2018). Because only 11 flavonol standards were available for comparison, only quercetin was positively identified by retention time, ESI-neg, and ESI-pos. The other 10 flavonol standards were not detected in these extracts based on retention times. The other 16 possible matches to apiosyl, rhamnosyl, and glucosyl-derivative masses could not be verified. Quercetin was only identified in the glandless seed coat extract (Table 2) (Cao et al., 2018). Gossypol was quantified in extracts from cottonseed coat and cotyledons. Seed coats from glanded and glandless seeds contained 8.2 and 3.7 pg/µL of gossypol, respectively. Seed cotyledons from glanded and glandless seeds contained 0.3 and 0 pg/µL, respectively (Cao et al., 2018). These cottonseed extracts were essentially free of gossypol with only 0.82, 0.03, 0.37 and 0 ng of gossypol per mg of the extracts from glanded cottonseed coat, glanded cottonseed cotyledon, glandless cottonseed coat and glandless cottonseed cotyledon, respectively (Table 1).

Cottonseed Extract on Breast Cancer Cell Growth

Human breast cancer cell viability was determined with MTT method after the cells (MCF7) were treated for 2-24 h with 5-100 µg/mL of cottonseed extracts from the coat and cotyledon of glanded and glandless cottonseed. The cell viability was increased up to 60% by extract from glanded cottonseed coat with 100 µg/mL treatment for 2 h but declined by 15% after treatment for 24 h. However, glanded cottonseed cotyledon extract significantly reduced the cell viability by 25% after 24 h treatment with 10-100 µg/mL concentrations (Cao et al., 2018). Glandless cottonseed coat extract did not have significant effect on breast cancer cell viability after treatment for 2 h or 24 h with 5-100

µg/mL of the extract concentrations but glandless cotyledon extract significantly reduced cell viability by up to 50% after 5-100 µg/mL treatment for 2 h although it did not affect breast cell viability after 24 h treatment (Cao et al., 2018). These analyses suggest that cottonseed cotyledon extracts prohibit human breast cancer cell growth.

Cottonseed Extract on Pancreatic Cancer Cell Growth

Cottonseed extracts also tested the viability of pancreatic cancer cell. The cells (MIA PaCA-2) were treated with the extracts from coat and cotyledon of glanded and glandless cottonseed (5-100 µg/mL) for 2 h and 24 h. The cell viability was not significantly affected by extract from glanded cottonseed coat. Similarly, extract from glanded cottonseed cotyledon did not affect cancer cell growth by 2 h treatment. However, glanded cotyledon extract significantly decreased pancreatic cancer cell viability by approximately 50% after 24 h treatment (Cao et al., 2018). Extracts from glandless cottonseed coat or cotyledon did not have significant effects on pancreatic cancer cell viability. These analyses suggest that only glanded cottonseed cotyledon extract prohibits human pancreatic cancer cell growth.

Cottonseed Extract on Macrophage Growth

Mouse macrophage viability was determined with MTT method after being treated with cottonseed extracts (5-100 µg/mL) for 2, 5, 24 and 72 h. The cell viability of macrophages was not statistically affected by extracts from glanded or glandless cottonseed coat or cotyledon (data not shown).

Cottonseed Extract on Adipocyte Growth

Adipocyte viability was also determined with MTT method after being treated with cottonseed extracts (5-100 µg/mL) for 2 h and 24 h. The cell viability was not statistically affected by extracts from glanded or glandless cottonseed coat or cotyledon (data not shown).

Cottonseed Extract on Mammalian Gene Expression

Plant polyphenols can regulate gene expression in mammalian cells. For example, green tea polyphenols have affected multiple gene expression in rats fed with a high fructose diet (Cao et al., 2007;Cao et al., 2007). Cinnamon polyphenols can regulate the expression of insulin signaling pathway and inflammatory response genes (Cao et al., 2007;Cao et al., 2008;Cao et al., 2010;Cao and Anderson, 2011). We tested the effects of the isolated bioactive extracts from glanded and glandless cottonseed on gene regulation. These bioactive cottonseed extracts, essentially free of gossypol (Table 1), regulate DGAT gene expression in mouse macrophages (Cao and Sethumadhavan, 2018).

We also demonstrated that cottonseed extracts exhibited modest effects on anti-inflammatory tristetraprolin (TTP) gene expression in macrophages but glandless cottonseed coat extract significantly increased TTP mRNA and protein levels with a magnitude similar to cinnamon and green tea polyphenol extract and insulin (data not shown).

A Working Model for Plant Extracts on TTP-Mediated Cytokine Instability

Plant extracts have played major roles in disease prevention. They are important means for maintaining long-term health and decreasing risks of chronic diseases. However, there is insufficient evidence at the molecular level to support the practice of using bioactive food components to alleviate or prevent diseases. It is known that tristetraprolin (TTP)/zinc finger protein 36 (ZFP36) protein is an anti-inflammatory, low abundance, cytosolis and highly phosphorylated protein (Cao et al., 2004;Cao et al., 2006;Fu and Blackshear, 2017;Patial and Blackshear, 2016). TTP binds to AU-rich elements in some cytokine mRNAs and destabilizes those transcripts (Cao, 2004;Cao et al., 2007;Cao et al., 2014). TTP knockout mice accumulate excessive levels of the proinflammatory cytokines and develop a severe systemic inflammatory syndrome including arthritis, autoimmunity, and myeloid hyperplasia (Phillips et al., 2004;Taylor et al., 1996). Upregulation of TTP decreases inflammatory responses in macrophages (Sauer et al., 2006). Agents that induce TTP gene expression may have potential therapeutic value for the prevention and/or treatment of inflammation-related diseases.

We have shown that cinnamon polyphenol extract (CPE), like insulin (Cao et al., 2008), increases TTP expression in mouse adipocytes (Cao et al., 2007;Cao et al., 2008). However, unlike insulin, CPE also increases TTP expression in mouse macrophages (Cao et al., 2008). We showed that green tea extract (GTE) also increased TTP mRNA levels and decreased pro-inflammatory tumor necrosis factor (TNF) mRNA levels in the liver and skeletal muscle of rats fed a high-fructose diet and given 1 g GTE/kg in the diet (Cao et al., 2007). GTE consumption regulates gene expression in glucose uptake and insulin signaling pathways in the rats (Cao et al., 2007). Finally, we demonstrated that cottonseed

extracts, especially glandless cottonseed coat extract, increased TTP gene expression in mouse macrophages (data not shown).

Based on these studies using extracts from cinnamon bark, green tea leaf, and cottonseed, a working model for future mechanistic study is proposed that links plant extracts and TTP-mediated cytokine instability in mammalian cells (Figure 3).

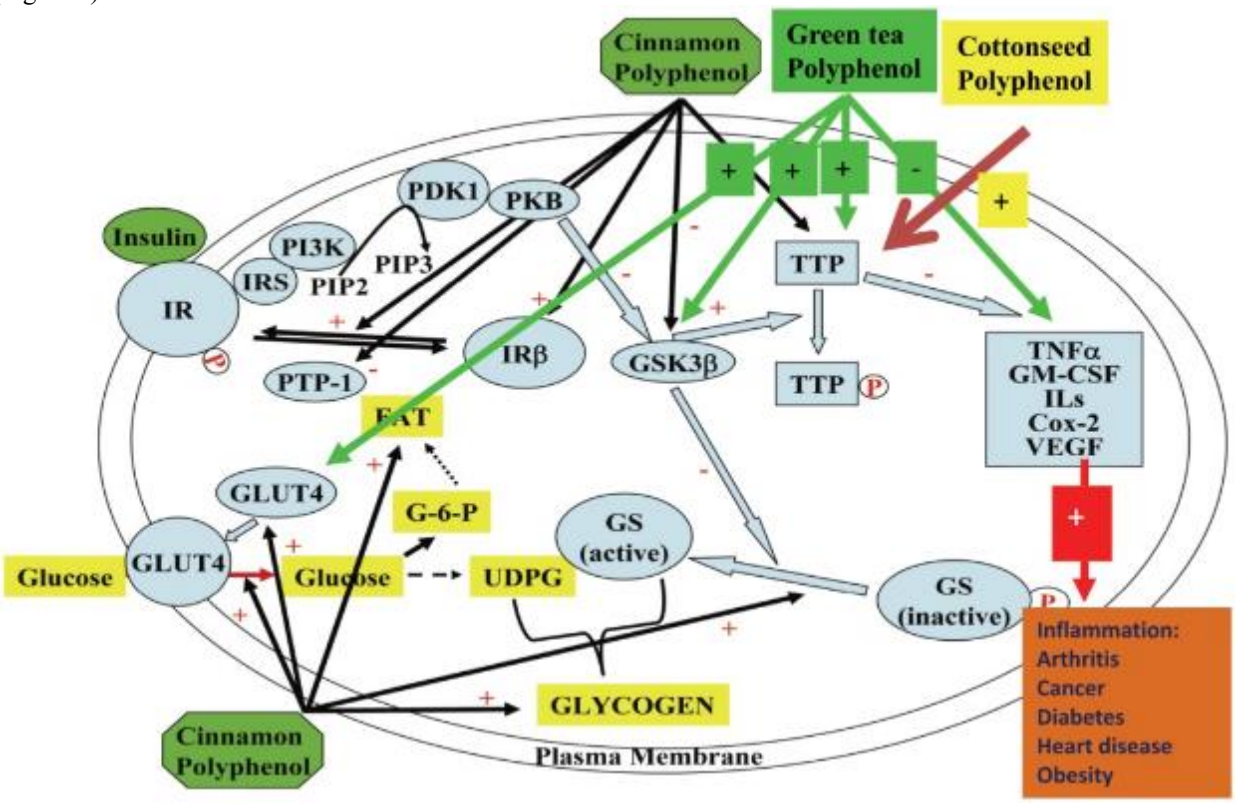

Figure 3. A model that links bioactivity from cinnamon bark, green tea leaf and cottonseed extracts and insulin, TTP, and cytokines to inflammatory diseases ("+" represents positive effect and "-" represents negative effect.). The model is modified from Cao et al. (Cao et al., 2007;Cao et al., 2007).

Conclusions

Cottonseed usage is limited to feeding ruminant animals and not for human or other animal consumption due to toxic gossypol in glanded cottonseed, the commonly cultivated cotton. Cottonseed contain a number of minor bioactive compounds such as gossypol, quercetin, gallic acid, 3,4-dihydroxybenzoic acid, flavonoids, cyclopropenoid fatty acids and protein-derived peptides. Most of these value-added products possess health promotion and disease prevention potentials. Gossypol is a potent compound for regulating gene expression and inhibiting cancer cell growth. Cottonseed extracts are harmless towards mouse cells and glandless cottonseed coat extract stimulates TTP gene expression. These results suggest that cottonseed is a safe source of plant polyphenols with anti-inflammatory property. Therefore, cottonseed value can be increased by extracting polyphenolic extracts and bioactive compounds for potential nutritional and biomedical uses.

List of Abbreviations

CPE: cinnamon polyphenol extract; DGAT: diacylglycerol acyltransferases; GTE: green tea extract; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; qPCR: quantitative real-time PCR; TAG: triacylglycerol; TNF: tumor necrosis factor; TTP: tristetraprolin; ZFP36: zinc finger protein 36.

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