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Detection of a Toxic Phenolic Compound in Cottonseed Extract and its Products

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Abstract: Cottonseed is a good source of high quality meal and edible oil. However, it contains a toxic phenolic compound called gossypol. Gossypol is an anti-nutritious factor that limits the use of cottonseed and its products due to its toxicity associated to its reactions with amino acids and minerals. This is the first study on gossypol ever conducted in Pakistan. The purpose of this study was to estimate the level of gossypol in oil and ghee (Hydrogenated oil) samples from local markets so that awareness can be created regarding maintenance of minimum gossypol level in edible oils. During this study gossypol was extracted from cottonseeds and cotton seed cake using different organic solvents. The compound was detected in extracts by applying Chromatographic technique as well as chemical tests with $SbCl_3$, $Pb(CH_3COO)_2$ and $SnCl_3$. Spectrophotometric techniques were also employed for quantitative analysis by measuring absorbance of samples at wavelength of 290 nm. It was found that overall the contents of gossypol were higher in cottonseed oils as compared to Ghee (hydrogenated oil).

Key words: Gossypol, cottonseed extract, phenolic compounds, edible oils, ghee, Pakistan

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) seed is majorly used as a source of edible oil and ghee in Pakistan. Cotton and related species all contain gossypol, a polyphenolic compound that is an integral part of the cotton plant's self-defense system against insect pests and possibly some diseases (Jodi and Gabriela, 2008). Some amount of gossypol tends to react with many natural substances in cottonseed and forms the bound gossypol that is non-harmful. However the unreacted gossypol known as "free gossypol" is toxic. Thus free gossypol is an anti-nutritional factor that limits the use of cottonseed and its products (Hron *et al*., 1987).

Gossypol [2, 2′-Bi (8-formyl-1, 6, 7, trihydroxy 5-isopropyl-3-methyl naphthalene)] is a crystalline compound (Fig. 1). The molecular formula of gossypol is $C_{30}H_{30}O_8$. The inclusion compound formation by gossypol has been studied at different thermodynamic conditions. Most of the investigated molecules form more than one inclusion compounds with gossypol. Polymorphism exhibited by gossypol inclusion compounds is dimorphism and trimorphism.

The toxicity of gossypol is associated to the reaction of its phenolic groups to amino acids and minerals. Hydrogen bonding and oxidation of the carbonyl groups result in easily reactive quinones that bind with proteins. Heating of cottonseeds during oil extraction binds gossypol to proteins. Thus reduces protein availability from cottonseed meal.

Gossypol also reacts with phosphatide-phosphatidylethanol amine present in cottonseed. This reaction product is safer. However gossypol is again converted to its harmful free form in stomach. This conversion of the bound gossypol to free gossypol is called "gossypol reversibility".

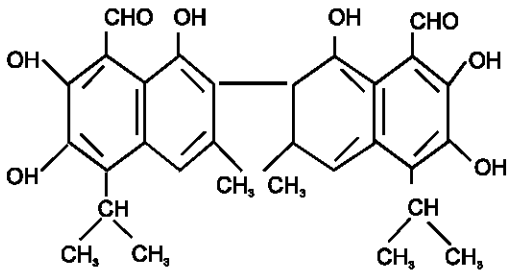

Fig. 1: Structure of gossypol

The main cumulative toxicological effect of gossypol are loss of appetite with consequent weight loss, liver and lung lesions like pulmonary edema preceded by difficult breathing, cardiac irregularity and failure which sometimes become lethal, anemia due to iron complexation and induction of male sterility. At low levels, gossypol blocks spermatogenesis and reduces sperm motility. Reversible antispermatogenic effect of gossypol in langur monkeys (*Presbytis entellus*) has also been studied (Sharma *et al*., 1999).

Randel and co-workers in 1992 studied the effects of gossypol and cottonseed products on reproduction of mammals and came to the conclusion that non-ruminant animals are particularly sensitive to the toxic effects of gossypol, whereas ruminants are somewhat more resistant. Gossypol seems to disturb estrous cycle, pregnancy and early embryo development in females of all non-ruminant species (Randel *et al*., 1992).

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Stephens and fellows in 1983 studied the mechanism of glycolysis inhibition by gossypol. It was found that the only site of glycolytic inhibition was isozyme X of Lactate dehydrogenase enzyme. Gossypol also decreases the concentration of adenine nucleotides, ATP, ADP and AMP and this is most probably the basis for its toxic effect on spermatozoa.

Gossypol usually renders harmless on crushing or heating but may retain minute amounts to which pigs and chickens are sensitive. Gossypol iron-binding properties cause olive green yolks in eggs. It also decreases the hatchability of eggs.

The toxic effect of gossypol can be used against the cancerous cells. It has been found to have antiproliferative activity on tumor cells and is thought to be a potential anticancer drug. Gossypol may provide a potential therapeutic benefit for the treatment of colon carcinoma. Understanding the mechanism of gossypol-induced cytotoxicity on tumor cells can be helpful for including this drug in clinical use (Wang *et al*., 2000).

Racemic gossypol, composed of both (-)-gossypol and (+)-gossypol, is used in herbal medicines in China. Studies on melanoma, breast cancer and colon cancers have shown that racemic gossypol is well tolerated and is moderately effective in reducing tumor volume (Poznak *et al*., 2001; Blackstaffe *et al*., 1997). Keith and his coworkers has also demonstrated that (-)-gossypol can inhibit tumor growth (Keith *et al*., 2006). Since gossypol confers antibiosis type of resistance to the cotton plant against different insects, it is also used as pesticide (Anonymous, 1982).

Gossypol has been the source of scientific interest for over a century. Recent changes in both cotton fiber and cottonseed products markets have focused renewed interest on potential alternate uses for gossypol contained in the seed.

MATERIALS AND METHODS

Sample collection: Samples of different brands of edible oils and ghee prepared from cottonseed oil were purchased from the local market of Multan city of Pakistan. A total of sixteen samples were collected. Out of which 8 samples were of oils and 8 of ghee. The trade names and manufacturers name will not be mentioned so that their business may not be affected.

Extraction of gossypol: For extraction of gossypol three grams of seed kernels obtained manually were crushed and extracted with diethyl ether (5 x 20 ml) (Nazarova and Glushenkova, 1983). The solvent was evaporated at low temperature till an oily material containing gossypol was obtained. This was stored for further use.

Gossypol was extracted with aqueous acetone (Botsoglou, 1991) with the same method. The residual left after the extraction of free gossypol with aqueous acetone was soaked in 2M HCl solution (75 ml) for 10 min and then refluxed for 30 min. After cooling, the solution was filtered. The residue was washed with absolute ethanol (15 ml). The filtrate was extracted with chloroform (4 x 30 ml). Then chloroform was evaporated from the extract at low temperature till an oily material containing gossypol was obtained.

Detection of gossypol by chemical tests: Specific chemical tests were performed for detection of gossypol in samples of edible oils and ghee. For this purpose 5 gram of each sample of oil and ghee was dissolved in small volume of ethanol in 25 ml conical flask and final volume was made up to the mark by adding more ethanol. Two ml of each sample solution was taken in the test tube separately and equal amount of solid antimony chloride was added in each test tube and mixed thoroughly.

Similarly, tests were performed with stannic chloride and lead acetate. The same tests were also performed with the extract of cottonseed as standard.

Thin layer chromatographic studies: Thin layer chromatography was also employed for qualitative analysis of gossypol in the samples of oils and ghee following Ventalchalam's method (Ventalchalam *et al*., 1980). TLC plates were prepared using silica gel F_{254} as adsorbent. The thickness of the plates was 0.02 mm. 1 g of each sample was dissolved in 5ml of diethyl ether. Then equal volume of the diluted samples and ether extract of cottonseeds (standard) were spotted on the plates with the fine capillary jet. The solvent system composed of benzene, dioxane and acetic acid (91:10:4) was used for the development of chromatoplates. The plates were taken out from the tank after two hours and dried in air thoroughly.

Spectrophotometric measurements for quantitative analysis of gossypol: Absorption spectra of cottonseed extract and samples in chloroform were recorded in 250-350 nm regions in quartz cell using UV-visible spectrometer. In this method reaction of the analyte with chromogenic reagent is not required, since the second derivative transformation and measurement of the conventional analytical band around 300 nm permits direct quantification of gossypol in sample extracts (Botsoglou, 1991). For this purpose 1 g of each sample was dissolved in 100 ml chloroform. The absorbance of each sample was noted directly at 290 nm.

RESULTS

Qualitative analysis of gossypol: For detection of gossypol in the samples of edible oils and ghee as well as in pure extract of cottonseeds, three specific chemical tests with $SbCl_3$, Pb $(CH_3COO)_2$ and $SnCl_3$ were performed. Turbid reddish complex appeared in case of

Table 1: Results of detection of gossypol through biochemical tests, TLC and Spectrophotometric studies

Samples	$SnCl_3$ Test	$SbCl_3$ Test	$Pb(CH_3COO)_2$ Test	TLC analysis of samples	Absorbance at 290 nm
Ghee 1	+	+	+	Light violet	1.212
Ghee 2	+	+	+	Light violet	1.338
Ghee 3	+	+	+	Light violet	0.788
Ghee 4	+	+	+	Lightest violet	0.529
Ghee 5	++	++	++	Dark violet	1.126
Ghee 6	+	+	+	Light violet	0.528
Ghee 7	+	+	+	Light violet	0.732
Ghee 8	+	+	+	Lightest violet	0.686
Oil 1	++	++	++	Dark violet	1.034
Oil 2	+	+	+	Lightest violet	0.791
Oil 3	+	+	+	Light violet	0.894
Oil 4	++	++	++	Dark violet	1.460
Oil 5	++	++	++	Dark violet	1.714
Oil 6	+	+	+	Light violet	1.498
Oil 7	+++	+++	+++	Dark violet	1.980
Oil 8	+	+	+	Light violet	1.466

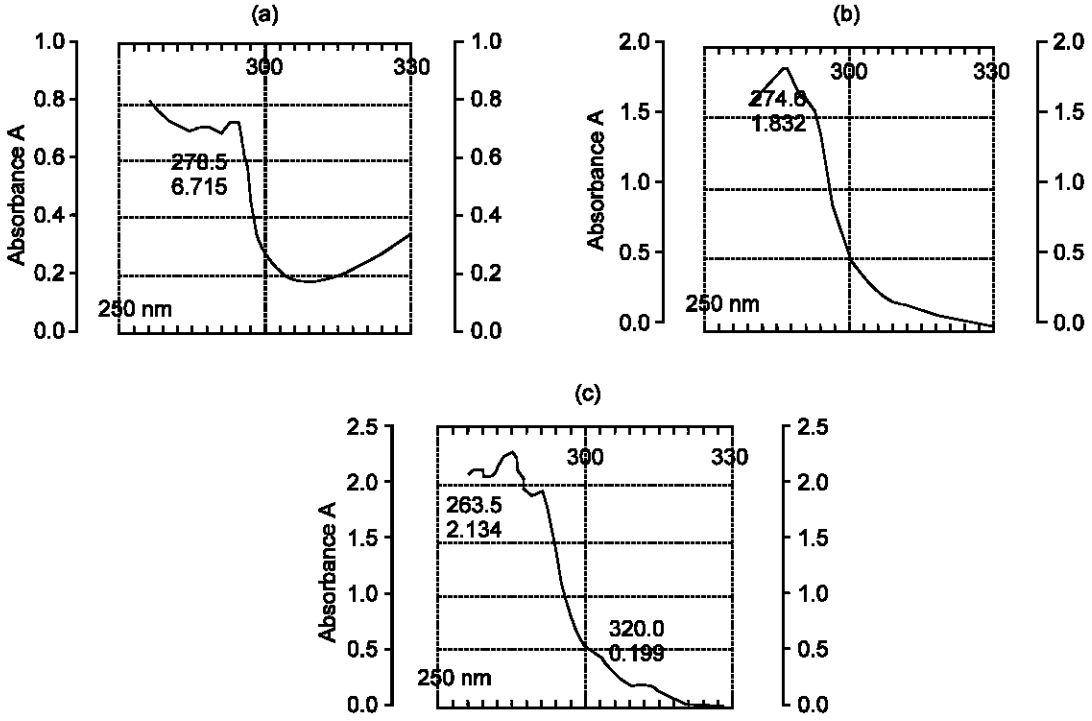

Fig. 2: Absorption spectra of (a) Cottonseed extract (b) Ghee sample 1 (c) Oil sample 8 in chloroform

$SbCl_3$ after 15 min. While reddish precipitate appeared after 10 min in case of test with Stannic chloride. Test with lead acetate gave yellowish precipitate that appeared after 20 min. The intensity of colour increased with the passage of time in case of all tests. Since cottonseed is a richest source of gossypol, therefore its extract was used as standard in these studies. Gossypol was detected in all the samples since these gave positive test with all the reagents with different intensities comparable to that of cottonseed extract. The results are given in Table 1.

For further confirmation of gossypol in the samples TLC was performed. A single light blue spot was detected on chromatoplates under UV-light. The Rf values of the spot in all the samples was approximately 0.97, which was comparable to that of the spot in the standard pure cottonseed extract. It is therefore concluded that gossypol is present in all the brands of oils and ghee having cottonseed oil.

Quantitative analysis of gossypol by spectrophotometry: Because of the phenolic nature of gossypol, spectrophotometric measurements of cottonseed extract and samples were made in UV region (Fig. 2). It was observed that cottonseed extract had spectral maxima at 278.5 and 288 nm having

highest extinction coefficient at 288 nm. All the samples also have two spectral maxima at 273-274 nm and 285 nm having higher extinction coefficient at 273-274 nm, comparable to that of pure gossypol reported in literature, thus permitting the direct quantification of gossypol. The appearance of spectral maxima in this region of UV light is indicative of gossypol in the samples. The spectrophotometric studies were carried directly at 290 nm to avoid the interference of other aromatic compounds that usually show absorbance at about 270 nm. Results are given in Table 1.

DISCUSSION

Gossypol is a phenolic compound that is why spectrophotometric method was applied for its detection. The reason for this is that all phenolic compounds are aromatic and they show intense absorption in the UV region (200-350 nm) of the spectrum. Spectral methods are therefore especially important for identification and quantitative analysis of phenolic compounds (Harborne, 1985).

Overall during these studies it was observed that the amount of gossypol was less in ghee samples than in oils due to conversion of free gossypol to bound gossypol during the process of ghee formation including hydrogenation of oils. The variation in the contents of gossypol in various brands of oils and ghee may be due to different methods of oil extraction and refining.

In food the accepted level of gossypol is 0.045% (Cherry, *et al*., 1981). Different brands of edible oils and ghee are used for cooking and frying of food and also used in sweets, bakery products etc. If gossypol is accumulated in human body, it may cause harmful effects. It is therefore suggested that one should be careful when using the products of cottonseed.

Food and animal feed industries must minimize cotton-derived product levels to prevent toxicity. Scientists at Texas A&M University have genetically modified cotton plants that contain less gossypol in the seed, but the compound remains in the stems and leaves. This protects the plant from pests. This gossypol-free cottonseed can be used as a high-quality protein source for human as well as animals. (USDA/Agricultural Research Service, 2007).

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