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Isolation of Gossypol and Analysis of Phytochemicals in Seed Extract of Bt and Non-Bt Varieties of Cotton

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The purpose of this study was to isolate the gossypol (Phenolic compound) and screening of phytochemical constituents from seed extract. During this study gossypol was extracted from cotton seeds and cotton seed cake using different organic solvents like acetone, ethanol, methanol, pet ether, chloroform and hot water and screened for phytochemical constituents. Analysis revealed the presence of phenols, glycosides, flavonoids, and steroids. Specific tests were conducted for each group of the phytochemicals. Among the extracts tested polar solvents like acetone, ethanol, methanol extracts showed more phytochemicals than others followed by pet ether, hot water, chloroform. The phytochemicals like saponins, flavonoids, tri-terpenoids, and tannins were not found in seed extract, specifically showed phenols with more quantity in polar solvent extract like acetone, ethanol and methanol cardiac glycosides and steroids are observed in both polar and non-polar solvent of seed extracts. Similar kind of compounds are present in Bt and non-Bt but the appearance of test coloration of seed extracts predicted as is slightly darker for Bt variety. This could be due to more amount of the component may be present in Bt cotton seed extract than non-Bt cotton seed extract. The compound Gossypol was detected in extracts by applying Chromatographic technique as well as chemical tests with antimony chloride (SbCl3), and stannic chloride (SnCl3) and leadacetate (Pb(CH3COO)2) . Spectrophotometric techniques were also employed for quantitative analysis by measuring absorbance of samples at wavelength of 290nm.

Keyword: Gossypol, Cotton Seed, Phytochemical Analysis

1. Introduction

Cotton seed is used as a source of edible oil. 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)[5]. Gossypol [2, 2N-Bi (8-formyl-1, 6, 7, trihydroxy5-isopropyl-3-methyl naphthalene)] is a crystalline compound (fig1). 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 from more than one inclusion compounds with gossypol. Polymorphism exhibited by gossypol inclusion compounds is dimorphism and trimorphism.

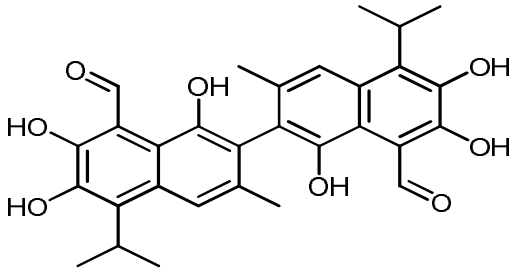


Fig 1: Structure of Gossypol

2. Materials and Methods:

Preliminary Phytochemical Analysis:

The healthy and disease free Cotton seed material was collected from the Regional Agricultural Research Station, NANDYAL, Kurnool dist, Andhra Pradesh. The seed material was washed thoroughly in tap water, shade dried in open air. Powder of the seeds obtained by grinding them mechanically. About 100 gm of each dried powder of the seed powder were soaked separately in 100 ml of different solvents like methanol, ethanol, chloroform, pet ether, acetone and hot water in conical flasks and then subjected to agitation on a rotary magnetic shaker for about 72 hours. After three days the seed extracts were subjected to filtration, filtered with No 42 what man filter paper separately. Concentrated extracts was preserved in sterilized air tight labeled bottles and preserved in refrigerator at 4˚c until required for further use. The extract was filtered under reduced pressure using rotary flash evaporator and subjected for further preliminary phytochemical tests.

Different tests conducted for the identification of phytochemicals is adopted by using the methods described by Edeogal et al (2005)[19] and B.Thamilmrlai selvi et al (2011)[18].

To the 5ml of extract 5ml of 2N HCL is added and boiled and then the mixture is filtered. To the filtrate a few drops of Mayer's reagent is added. A cream colour precipitate was produced immediately indicating the presence of alkaloids.

Saponins are tested by boiling 5ml of extract in 10ml of distilled water in a test tube and are shaken vigorously for about 30 seconds. The test tube is allowed to settle for half an hour. Formation of froth indicates the presence of saponins.

Tannins are tested by adding a few drops of 1% lead acetate to 5 ml of plant extract. Appearance of yellow precipitate indicates the presence of tannins.

Phenols are tested by adding 2ml of ferric chloride solution to 2ml of plant extract. Appearance of bluish green colour solution indicates the presence of phenols.

For testing the presence of steroids 1ml extract was dissolved in 10ml of chloroform and equal volume of concentrated sulphuric acid was added from the walls of the test tube. Appearance of red colour in the upper layer and yellow with green fluorescence indicates the presence of steroids.

To 1ml of extract glacial acetic acid, few drops of ferric chloride and then finally concentrated sulphuric acid were added from the walls of the test tube. Appearance of the reddish brown at the junction of two layers and the bluish green colour in the upper layer indicates the presence of cardiac glycosides.

5ml extract was boiled with 10ml of sulphuric acid and filtered while hot. The filterate was shaken with 5ml of chloroform the chloroform layer was pipetted out into another test tube then 1ml of dilute ammonia is added. The resulting solution was observed for colour changes. The change in colour indicates the presence of anthraquinones.

To one ml of the extract, a few drops of dilute sodium hydroxide are added. An intense yellow colour was produced in the plant extract, which became colorless on addition of few drops of dilute acid. This indicates the presence of flavonoids.

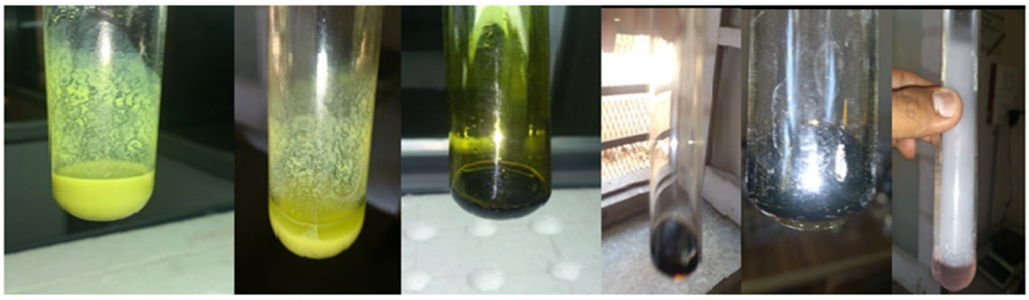
1ml of the extract was dissolved in 1ml of chloroform; 1ml of acetic anhydride was added following the addition of 2ml of concentrated sulphuric acid. Formation of reddish colour indicates the presence of terpenoids.

1ml of the extract was treated with few drops of Ninhydrin reagent. Appearance of purple colour indicates the presence of amino acids.

1ml of extract was added 5 to 10 drops of Fehling's solution. Mixture was then subjected to boiling for 15 minutes. Appearance of brick red precipitate indicates the presence of reducing sugars.

To the 1ml of extract, 1ml of Barfoed´s reagent was added and heated on water bath. Formation of brown precipitate indicates the presence of monosaccharaides.

2) Extracts of Cotton Seed (Bt and Non-Bt)



A B C D E F

3) Preliminary Phytochemical Tests

A) & B) - Yellow color Steroids confirm color test
C) - Bluish green Phenols confirm color test
D) & E) - Bluish red Cardio glycosides confirm color test
F) - Color less solution Flavonoids confirm color test

Table 2: Phytochemical screening test of Bt Cotton Seed extracts

S.NO	phytochemicals	Methanol	Ethanol	Chloroform	Pet ether	water	Acetone
1	Tannins	--	--	--	--	--	--
2	Phenols	+++	+++	--	--	--	+++
3	Saponins	--	--	--	--	--	--
4	Alkaloids	--	--	--	--	--	--
5	Flavonoids	+++	++	--	--	--	++
6	Anthraquinones	--	--	--	--	--	--
7	Amino acids	--	--	--	--	--	--
8	Carbohydrates	--	--	--	--	--	--
9	Terpenoids	--	--	--	--	--	--
10	Cardiac glycosides	+++	+++	+++	+++	+++	+++
11	Steroids	++	++	--	+	+	+

(+++)= strongly present, (+) = poorly present
(++)= moderately present (-) = absent

3. Isolation of a Phenolic Compound:

3.1 Extraction of Gossypol: For extraction of gossypol three grams of Bt (Vibha, Kaveri, JK, Rudra, Tulasi and Bhasker) and non-Bt (Gossypium hirusutum) cotton seed kernels obtained manually were crushed and extracted with diethyl ether (5 x 20 ml) (Nazarova and Glushenkova, 1983)[9]. The solvent was

evaporated at low temperature till an oily material containing gossypol was obtained. This was preserved for further use. Gossypol was extracted with aqueous acetone (Botsoglou, 1991)[3] with the same above method. The residual left after the extraction of free gossypol with aqueous acetone was soaked in 2M HCl solution (75 ml) for 10 min then refluxed for 30 min. After cooling, the solution was filtered. The residue was washed with absolute ethanol (15ml). Then chloroform was evaporated from the extract at low temperature till an oily material containing gossypol was obtained.

3.2 Detection of Gossypol: Specific chemical tests were performed for detection of gossypol in samples of seed extracts. For this purpose 5 ml of seed extract crude 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 other types of tests were also performed with stannic chloride and lead acetate.

3.3 Thin Layer Chromatographic Studies:
Thin layer chromatography was also employed for qualitative analysis of gossypol in the samples of seed extracts following Ventalchalam's method (Ventalchalam *et al*., 1980)[15]. TLC plates were prepared using silica gel F254 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 were spotted on the plates with the fine capillary jet. The solvent system composed of Ethyl acetate, pet ether and acetic acid (91:10:4) was used for the development of chromatoplates. The plates were taken out from the chamber and dried in air thoroughly.

3.4 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 analyses 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)[3]. For this purpose 1 g of each sample was dissolved in 100 ml chloroform. The absorbance of each sample was noted directly at 254 nm.

Fig 4: TLC Chamber

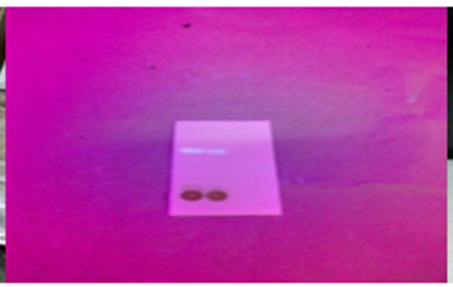


Fig 5: TLC Plate

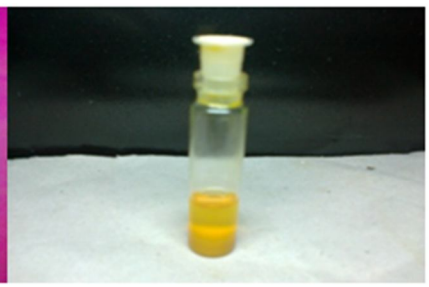


Fig 6: Yellow PPT formed Gossypol confirm test

4. Results and Discussion:
For Phytochemical results Table 1 shows the phytochemicals that are present in the non-Bt Cotton seed extracts screened by different screening tests. The seed extracts revealed the presence of phenols, cardio glycosides, flavonoids, steroids commonly from methanol, ethanol, acetone and water that were not observed in chloroform and pet ether. From the table 2 the screening tests of Bt cotton seed extracts revealed

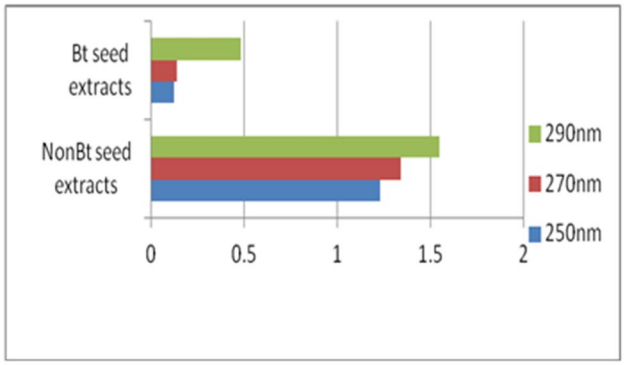
the presence of phenols, cardio glycosides, flavonoids, steroids in more quantity when compared with non-Bt cotton seed extracts. Analysis revealed the presence of phenols, glycosides, flavonoids, and steroids. Specific tests were conducted for each group of the phytochemicals. Among the extracts tested polar solvents like acetone, ethanol, methanol extracts showed more phytochemicals than others followed by pet ether, hot water and chloroform. The phytochemicals like saponins, flavonoids, triterpenoids, and tannins were not found in seed extract, specifically showed phenols with more quantity in polar solvent extract, cardiac glycosides and steroids are observed in both polar and non-polar solvent of seed extracts. Similar kind of compounds are present in Bt and non-Bt but the appearance of test coloration is slightly darker for Bt variety. This could be due to more amount of the component that is present in Bt cotton seed extract than non-Bt cotton seed extracts. Due to presence of phytochemicals seed extracts has an antimicrobial, antifungal, antidiabetic and anticancer activity. Further phytochemicals found in seed will be tested for their antimicrobial activity.

4.1 Qualitative Analysis of Gossypol: For detection of gossypol in the samples of Bt and non-Bt cottonseed extracts three specific chemical tests with SbCl3, Pb (CH3COO)2 and SnCl3 were performed. Turbid reddish complex appeared in case ofSbCl3 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.

4.2 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; it was observed that cottonseed extract had spectral maxima at 270 nm having highest extinction coefficient at 285nm(Harborne, 1985)[5]. All the samples also have two spectral maxima at 273-274 nm and 285nm 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 290nm to avoid the interference of other aromatic compounds that usually show absorbance at about 270nm.

Table 3: Results of detection of gossypol through biochemical tests, TLC and Spectrophotometric studies

Samples	SnCl3 Test	SbCl3 Test	Pb(CH3COO)2 Test	TLC analysis of samples	Absorbance at 290nm
Non Bt	++	++	++ ++	Light violet	1.547
Bt	++	++	++ ++ ++	Light violet	0.483



X-axix : OD values
Y-axix : Wavelength

5. Conclusion:

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). During these studies it was observed that the amount of gossypol was less Non Bt cotton seed extracts due to the OD values of non-Bt seed extracts is high when compare

with Bt seed extracts. The low level of OD values may represents low amount of component present in extracts which is shown in table3. The appearance of test coloration of seed extracts predicted as is slightly darker for Bt variety. This could be due to more amount of the component may be present in Bt cotton seed extract than non-Bt cotton seed extract. 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)[14].

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