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Categorization and scaling of distinct gossypol classes with respect to gossypol content in cotton (*Gossypium hirsutum* L).

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Abstract
Gossypols are the pigment glands conferring resistance to insect pests; distributed on the plant body covering the stem, leaf, bract, calyx and carpel walls. The objective of the present study was to quantify the gossypol concentration on the unopened bolls of cotton and to devise a quantitative scaling for categorizing different gossypol classes in different genetic backgrounds through spectrophotometry. There were substantial differences among the P_1, P_2, F_1, F_2, BC_1 and BC_2 generations of the two crosses (HRVO-1 × Acala 63-74 and HRVO-1 × HG-142) for total gossypol and total gossypol (%). The mean total gossypol content in the glandless parent (Acala 63-74) and its F_1, in a cross with the normal glanding parent (HRVO-1) was 0.04 $mg.g^{-1}$ (2%) and 0.140 $mg.g^{-1}$ (5%) respectively while in the high glanding parent (HG-142) and its F_1 produced in a cross with the normal glanding parent it was 1.14 $mg.g^{-1}$ (46%) and 0.88 $mg.g^{-1}$ (35%) respectively. The mean total gossypol was estimated as 0.60 $mg.g^{-1}$ (24%) in the commonly used normal glanding parent. From the data and the analytical procedure used for the quantification of gossypol clearly highlighted the importance of the application of these chemo-metric tools. This method of quantification is accurate and may be used in breeding programs to screen the progeny of cotton genotypes showing segregation for gossypol content..
Keywords: *Gossypium hirsutum* L; Gossypol glands; Cotton Bolls; Scaling; Quantification; Spectrophotometry.

Introduction

Some plant traits confer resistance/non-preference to the insect pest infestation. Among them, gossypols are the pigment glands distributed on the plant body covering the stem, leaf, bract, calyx and carpel walls. These pigment glands are visible from both leaf surfaces. Gossypol is a phenolic compound, which acts as an insecticide, repellent and growth retardant (Wilson and Smith, 1976). High gossypol contents had deleterious effects on bollworm/spotted bollworms (Duhoon et al., 1981; Ilango and Uthamasamy, 1989. Gossypols on the margin of the sepals are the most useful in conferring resistance to bollworm and tobacco budworm (Parrott et al., 1983). A high level of gossypol, flavanols, silica and low sugar contents in cotton were reported to have some role in insect resistance (Singh and Agarwal, 1988; Hedin and Mc Carty, 1990). There was the lowest incidence of bollworms in the genotypes with highest gossypol gland density on the ovary (Mohan et al., 1995). Density of glands had an influence on *Heliothis* larval growth. Glandless cottons were more susceptible to bollworms than glanded cottons (Jenkins et al., 1966). Most of the cultivated upland cottons are free of gossypol glands on the sepal margins, such phenotypes were referred as normal glanded and those expressing gossypols on the sepal margins were designated as high glanded (Calhoun et al., 1997).

Studies in quantitative inheritance are generally conducted to analyse the effects of groups of genes acting in concert to produce the character under consideration. It is thus of some interest when the number genes involved in the production of a character can be known through the use of a method (Lee et al., 1967). Mohan et al. (1995) recorded data on the number of gossypol glands per mm^2 on the abaxial leaf surface in Stoneville ranged from 9.2, 8.25 to 23.6 in G-67, which also had the highest number of gossypol glands per mm^2 seed (18.1). Gossypol gland number of cotyledonary leaves was significantly and positively associated with leaf free gossypol content and seed gossypol gland number. Gossypol is a triterpenoid aldehyde (Fig.1) having a molecular weight of 518.6 and melting point of 177-182^{0}C. Total gossypol defines gossypol and gossypol derivatives, both free and bound, which are capable of reacting with 3- amino-1-propanol in dimethylformamide solution to form a diaminopropanol complex, which then reacts with aniline to form dianilinogossypol. The analytical procedures used for quantification of gossypol include spectrophotometry and HPLC (Abou-Donia et al., 1981; Stipanovic et al., 1988; Hron et al., 1990; Tchatchueng et al., 1992). The spectrophotometric method of quantification was applied on the decorticated dried seed lot, (Smith, 1958; Lee, 1973) over $CaC1_2$, (ca. 6%). The kernels were ground to fine meal and returned to cold storage. After all the seed lots had been processed, the samples were extracted and assayed for total gossypol according to the standardized spectrophotometric techniques requiring the formation of a gossypol-aniline complex which led to over-estimated results because of some interferences (Marquie and Bourrely, 1991). But in a study for the validation of spectrophotometric methods making use of a

flow injection-mainfold/spectrophotometric technique proved its reproducibility in comparison to the other test methods of conventional types (Vlessidis et al., 2004). Sotelo et al. (2005) reported gossypol content in leaves and seeds in 10 Malvaceae species by HPLC. In *Gossypium hirsutum* L., the gossypol content in leaves (847.00 mg/100g) and seeds (297 mg/100g) was reported. Another sample, fast and cost effective method for isolation, identification and quantification of gossypol, using packed micro-tips columns in combination with HPLC was performed on different parts of the cotton plant comprising of seeds, stems and leaves by Meyer et al. (2004). The minimum detection limit of gossypol was determined to be 10 ng (absolute gossypol). The newly developed competitive direct enzyme-linked immunosorbent assay (cdELISA) technique developed by Wang et al. (2005) could be a valuable and feasible alternative for determination of "free" gossypol, in the condition especially when the available sample is limited with relatively low gossypol concentration. The detection limit for gossypol was 0.005mu $g.mL^{-1}$. A good correlation between the cdELISA method and the AOCS official method for "free" gossypol, analysis of cottonseed meals was also established.

An in-built mechanism of resistance against insects is a safe and secure method according to the bio-safety requirements of the emerging era. The objective of the present study was to quantify the gossypol concentration and to devise a quantitative scaling for categorizing different gossypol classes in different genetic backgrounds in cotton by using spectrophotometry. This technique is rapid and accurate in breeding programs to screen the progeny of cotton genotypes showing segregation in gossypol content.

Results and Discussion

The procedure of quantification of gossypols according to A.O.C.S., Official method (1989) was applied with some modifications. For linear calibration of the standard curve, acetone was used which is a modification to the original protocol, with a view that the decomposition rate of the compound was the lowest in organic solvents like acetone which produced linear array of the concentrations of the standard. The reason for running the standard was to optimise the instrument at the UV- wavelength of 440 nm. There existed precision in the values of the standard curve (Fig. 2) there after the calibration of the spectrophotometer the calibration factors (Table 2) were remained valid for long time. The second modification was the use of glacial acetic acid, which helped in the release of gossypol by rupturing the cell walls protecting the gossypol glands. Solutions of different concentrations of the standard were prepared and absorbance was measured at the wavelength of 440 nm. After the establishment of the standard curves separately for of two crosses, gossypol content was analysed in the samples. On the basis of the quantification of gossypol content and total gossypol percentage in two crosses i.e. HRVO-1 × Acala 63-74 and HRVO-1 × HG-142, significant differences ($P \leq 0.05$) among the P_1, P_2, F_1, F_2, BC_1 and BC_2 generations were found (Tables 3 & 4). Similarly significant differences between the parental means of HRVO-1, Acala 63-74 and HG-142 of the two crosses were also revealed from the Table 4. In the mean comparison of F_1 in these two crosses, the mean of F_1 of the cross HRVO-1 × HG- 142 was closer to the mid parent value.

In the glandless parent (Acala 63-74) and F_1, gossypol content of 0.04 mg/1g and 0.140 mg/1g (Table 4) was recorded respectively. Similarly, the total gossypol (%) in the parent (Acala 63-74) and F_1 was recorded as 2% and 5%, respectively as explained from the Table 3. The mean gossypol yields from the studies of (Lee, 1973), who while crossing a direct normal glanding parent ($Gl_2Gl_2gl_3gl_3$) to four glandless parents ($gl_2gl_2gl_3gl_3$), yielded the gossypol level ranging from 0.068 mg to 0.320 mg in F_1 and in the reciprocal arrangement with four normal glanding parents, the gossypol level ranged from 0.064 mg to 0.253 mg in F_1. In the cross of glandless with four glandless parents, the gossypol level ranged from 0.004 mg to 0.014 mg in F_1. He termed the gossypol yields ranging from 0.004 mg to 0.320 mg as glandless. Mansour et al. (2004) examined the relationship of gossypol content with the bollworm infestation. The range of gossypol content determined was 20-25 mg/100g (0.20-0.25 mg/1g), which was considered low in relation to the non-significant association with bollworm incidence. The statistically significant differences between the parents and their F_1 in the two crosses justified the distinctness of three classes (Table 4). But the studies of (Calhoun, 1997) categorized two main classes (glandless and normal glanding) in F_2 of the cross of normal glanding and glandless and two main classes (normal glanding and high glanding) in the F_2 of the cross between the parents of normal glanding and high glanding. His studies were based on visual observations which were misleading as he was failed to distinguish between the intermediate and glandless classes as obtained in F_2 of the cross HRVO-1 × Acala 63-74 and intermediate and high glanded classes in F_2 of HRVO-1 × HG-142. In the present study a large number of plants with different genetic backgrounds were analysed for gossypol contents and it was found that a similar category/class of gossypol glanding showed similar concentration of gossypol over repeated runs of time. On the basis of such reproducible results a discrete scale was devised ranging from 0 to 4 in ascending order of gossypol concentration ($mg.g^{-1}$) as shown in Table 5 and Fig. 3.

The findings of Calhoun (1997) which were based on visual observation of gossypol glands present on the sepal margins, but the results of the present studies quantified the gossypols which clearly distinguished between the three classes obtained in F_2 of the two crosses. From the data and the analytical procedure used for the quantification of gossypol in this manuscript clearly highlighted the importance of the application of this chemo-

metric tool. This method of quantification is accurate and can be used in breeding programs to screen the progeny of cotton genotypes showing segregation for gossypol content.

Materials and methods

Among thirty-one cotton genotypes/accessions, three cotton genotypes namely HRVO-1 (normal glanding), Acala 63-74 (glandless) and HG-142 (high glanding) were visually selected on the basis of presence of gossypol glands on the surface of unopened cotton bolls. The scheme of crossing is described in Table 1. These genotypes were selfed for four generations to avoid the effect of out crossing at the Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan. The parent HRVO-1 was used as a common parent in the hybridisation scheme with a high glanding parent (HG-142) and a glandless parent Acala 63-74 to produce F_0 seed of two crosses, (HRVO-1 × HG-142 and HRVO-1 × Acala 63-74) during February through March, 2005. The F_1 and their parents were planted during the normal crop season of 2005-06 to produce F_2, backcross (BC_1 and BC_2) generations. Fresh F_1 crosses for each of the two combinations were made through manual crossing. The experimental field was fertilized with N-P-K at the rate of 100-75-00 $kg.ha^{-1}$. Irrigation both by canal and turbine water was applied to the experimental material with the interval of 7-10 days. The six generations of the two crosses were planted in a Randomised Complete Block Design arrangement with three replications. A single plot (4.5 × 0.75 m) per replication was assigned to each of the parents and their respective F_1 while, four plots per replication were assigned to each of the backcrosses and eight plots per replication were assigned to raise the F_2 population of each cross. Five plants were tagged randomly for the parents and their F_1, while 50 and 30 plants in each replication were selected in F_2 and backcross generations respectively to quantify the gossypol glands on the surface of the unopened bolls with a spectrophotometer (Cecil CE-2021) at 440 nm wavelength, according to the protocol of A.O.C.S (1989) during 2006-07.

a) Chemicals

The laboratory grade Isopropyl alcohol (2-propanol), n-hexane (boiling range 68-69^{0}C), gossypol acetic acid (standard), dimethylformamide, 3-amino-1-propanol, glacial acetic acid, 70% aqueous acetone and aniline were purchased from SIGMA suppliers. Complexing reagent was prepared with 2mL of 3-amino-1-propanol and 10 mL glacial acetic acid made to 100 mL volume with dimethylformamide. The standard gossypol acetic acid solution was prepared by dissolving 24 mg of gossypol acetic acid (powder) in the complexing reagent and volume was made to 50 mL with the complexing reagent. Thus the solution contained 0.48 mg gossypol acetic acid per mL. The mg gossypol acetic acid used was multiplied with 0.8962 to obtain mg of gossypol (A.O.C.S, 1989).

b) Preparation of standard curve of gossypol acetic acid

From the standard gossypol acetic acid solution prepared, the aliquots of 1, 2, 4, 6, 8 and 10 mL were taken and a final volume of 10 mL was made with the complexing reagent. Pure complexing reagent (10 mL) was used as blank. Separate flasks containing a total volume of 10 mL made for each of the aliquots and blank solution were heated in a water bath (95-100^{0}C) for 30 minutes, cooled to room temperature, and finally diluted to a total volume of 50 mL with isopropyl alcohol-hexane solution and mixed well. These aliquots of standard gossypol acetic acid and blank were stored as stock solutions in the refrigerator. 2 mL volume of each of these aliquots of the standard and blank were taken in duplicate into separate volumetric flasks of volume 25 mL. One set of the standard aliquots and the reagent blank were diluted to make the final volume of 25 mL with the isopropyl alcohol-hexane solution and reserved as reference solutions for absorbance measurements. 2 mL aniline was added to the other set of standard aliquots and the blank, heated in a water bath (95-100^{0}C) for 30 minutes, cooled to room temperature, finally diluted up to the volume of 25 mL with the isopropyl alcohol-hexane solution and mixed well. Allowed to cool down for 1 hour at room temperature before determining absorbance. The optical density (OD) of reagent blank and the standard aliquots was determined on a spectrophotometer at 440 nm wavelength absorption. The OD value of reagent blank was subtracted from the OD value of each standard to obtain the corrected value.

Corrected absorbance = OD of each standard – OD of reagent blank

Calibration factor was determined by dividing mg gossypol in standards by corrected OD of the each standard to obtain calibration factors. Average of the factors was determined for each of the standards and used to calculate mg gossypol in sample aliquots (Table 2).

$$\text{Factor} = \frac{\text{mg gossypol in standard}}{\text{Corrected OD}}$$

c) Sample gossypol extraction method

Sample weight and aliquot used for aniline reaction depends on expected total gossypol content. Ideally, the analytical sample should contain 0.5-5.0 mg of gossypol, and the aliquot for the aniline reaction about 0.1 mg gossypol. Before the sample preparation the unopened cotton boll was washed with water. The outer surface of the bolls containing the gossypol glands was peeled off and weighed on a digital balance. About 1 g sample

obtained was crushed in a mortal and pestle using one drop of glacial acetic acid and one drop of 70 % aqueous acetone. The crushed sample was transferred into a test tube and 1 mL of the complexing reagent was added whereas, reagent blank consisted of 1 mL of complexing reagent. Sample and reagent blank were heated in a water bath (95-100^{0}C) for 30 minutes, cooled to room temperature and diluted to 4 mL volume with isopropyl alcohol-hexane mixture and shook well. Sample extract was filtered through 11 cm medium retention paper into a test tube, discarding first 1 mL of filtrate. Two mL duplicate aliquots of sample and blank were taken into test tubes. One set of the sample and blank aliquots was diluted to 10.5 mL volume with the isopropyl alcohol-hexane mixture and reserved as reference solutions for absorbance measurement. One mL aniline was added to the other set of sample and blank aliquots, heated in water bath (95-100^{0}C) for 30 minutes, cooled to room temperature, diluted with volume of 9.5 mL of isopropyl alcohol-hexane solution and mixed well, and allowed to stand for 1 hour at room temperature before determining absorbance. Optical density (OD) of the reagent blank reacted with aniline was determined using blank aliquot without aniline as reference solution. The OD of reagent blank was taken off from the OD of each standard to obtain the corrected absorbance. The OD of the sample aliquots reacted with aniline was determined using diluted sample aliquot without aniline as reference solution. The OD of reagent blank was subtracted from the OD of the sample aliquot reacted with aniline to obtain corrected absorbance.

Corrected absorbance = OD of sample aliquot – OD of reagent blank

From the corrected absorbance gossypol (mg) in sample aliquot were determined by multiplying OD with either the mean calibration factor, or reference to calibration graph.
Total gossypol % was calculated by the formula (A.O.C.S, 1989).

$$\text{Total gossypol \%} = \frac{5 \times G}{W \times V}$$

Where,

G = mg gossypol in sample aliquot.
W = weight of sample in grams.
V = volume of sample aliquot used for analysis.

Statistical Analyses

The data were analysed using analysis of variance technique (Steel et al., 1996) using MSTATC (1989) version 1.5. A generation means analysis was performed following the method described by (Mather and Jinks, 1982) using a computer program. Means and variances of each population (parents, backcrosses, F_1 & F_2) used in the analysis were calculated from individual plants pooled over replications.

Safety

Isopropyl alcohol and n-hexane are flammable solvents. They should not be used near an open flame. The use of a properly operating fume hood is recommended when using these solvents. Hexane vapour causes lung irritation and produces neurotoxic effects. Aniline is an allergin and is toxic if absorbed through the skin. Protective clothing and a properly operating fume hood should be used when using aniline. Dimethylformamide is a strong irritant to skin and tissue. It is toxic by skin absorption. It is a moderate fire risk. 3-amino-1-propanol (propanolamine) is a tissue irritant. Avoid breathing vapours and contact with the skin. Glacial acetic acid is moderately toxic by ingestion and inhalation. It is strong irritant to skin and tissue.

Conclusions

The work presented here has an impact, as the earlier studies defined the genetics of gossypols on visual observation. But the results of the present study clearly elaborated the segregating classes in F_2; by making use of the application of the analytical procedure for the quantification of gossypol, which is accurate and can be used in breeding programmes to screen the genotypes showing segregation and further scaling of the genotypes for gossypol content which will in turn help in classifying genotypes on the basis of gossypol glands. The genetic studies pertaining to the inheritance of gossypols will be further helpful in regulating gossypol gland density on the plant body as well as on the seeds.

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Table 1: Scheme of crossing

S. No.	CROSS	TRAIT CONSIDERED
1	HRVO-1 × Acala 63-74	Normal glanding × Glandless
2	HRVO-1 × HG-142	Normal glanding × High glanding

Table 2: Computation of the standard aliquots for the development of standard curve in HRVO-1 × Acala 63-74 (Normal × glandless) & HRVO-1 × HG-142 (Normal × High glanding)

Conc. of stock Solution	Gossypol in gossypol acetic acid in standard solutions (mg)	(mg) of gossypol = gossypol acetic acid (mg) × 0.8962	OD Reading (A)		Corrected Absorbance = (A-B)		Calibration factor	
			HRVO-1 × Acala 63-74	HRVO-1 × HG-142	HRVO-1 × Acala 63-74	HRVO-1 × HG-142	HRVO-1 × Acala 6374	HRVO-1 × HG-142
1mL	0.048	0.043	0.132	0.107	0.13	0.087	0.3308	0.4943
2mL	0.096	0.086	0.188	0.17	0.19	0.15	0.4600	0.5733
4mL	0.192	0.172	0.239	0.24	0.237	0.22	0.7257	0.7818
6mL	0.280	0.251	0.292	0.31	0.29	0.29	0.8655	0.8655
8mL	0.380	0.341	0.363	0.4	0.361	0.38	0.9446	0.8974
10mL	0.480	0.430	0.418	0.49	0.42	0.47	1.0300	0.9149
Blank OD reading = Zero						**Mean**	**0.7261**	**0.7545**
(HRVO-1 × Acala 63-74) Blank OD reading (A) – (Blank Aniline treated "B")					**=**	**0.002**		
(HRVO-1 × HG-142) Blank OD reading (A) – (Blank Aniline treated "B")					**=**	**0.02**		

Table 3: Mean squares from analysis of variance for gossypol content and gossypol percentage for six generations

Source	DF	Gossypol content		Gossypol percentage	
		HRVO-1 × Acala 63-74	HRVO-1 × HG-142	HRVO-1 × Acala 63-74	HRVO-1 × HG-142
Replication	2	0.000	0.000	0.000	0.000
Genotypes	5	0.126**	0.109**	0.020**	0.018**

****** Highly significant

Table 4: Generation means for gossypol content (mg/g) and gossypol percentage in two single crosses

Generation	HRVO-1 × HG-142		Generation	HRVO-1 × Acala 63-74	
	Gossypol content (Mean)	Gossypol percentage (Mean)		Gossypol content (Mean)	Gossypol percentage (Mean)
P_1 (HRVO-1)	0.60	0.240	**P_1 (HRVO-1)**	0.590	0.233
P_2 (HG-142)	1.14	0.455	**P_2 (Acala 63-74)**	0.040	0.020
F_1	0.88	0.351	**F_1**	0.140	0.050
F_2	0.88	0.351	**F_2**	0.200	0.081
BC_1	0.74	0.295	**BC_1**	0.373	0.149
BC_2	1.03	0.411	**BC_2**	0.11	0.041
LSD (0.05)	**0.018**	**0.018**	**LSD (0.05)**	**0.057**	**0.018**

Table 5: Categorization and scaling of distinct gossypol classes with respect to gossypol content.

S.No.	Class/Category	Gossypol range (mg/g)	Scale
1	Glandless	0.00-0.09	0
2	Intermediate glandless	0.10-0.29	1
3	Normal glanding	0.30-0.69	2
4	Intermediate glanding	0.70-0.99	3
5	High glanding	1.00 & above	4

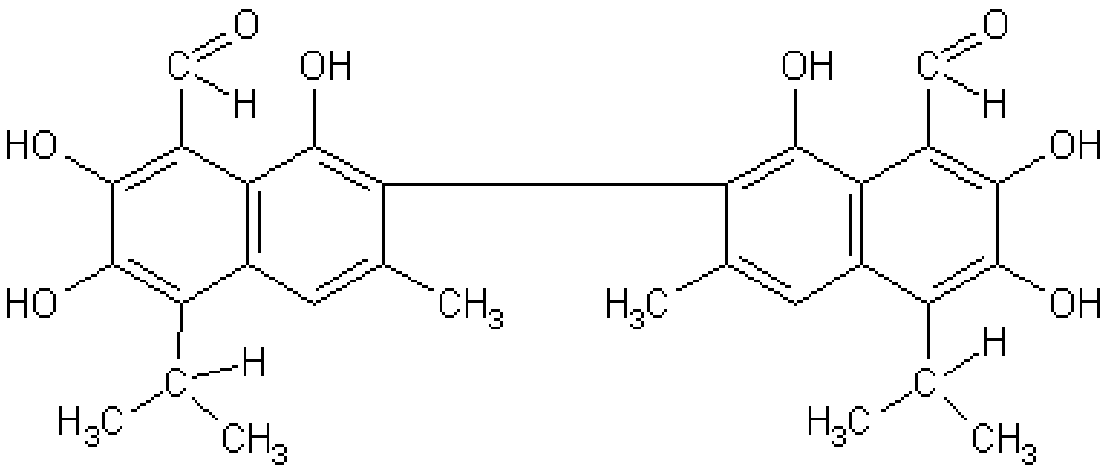
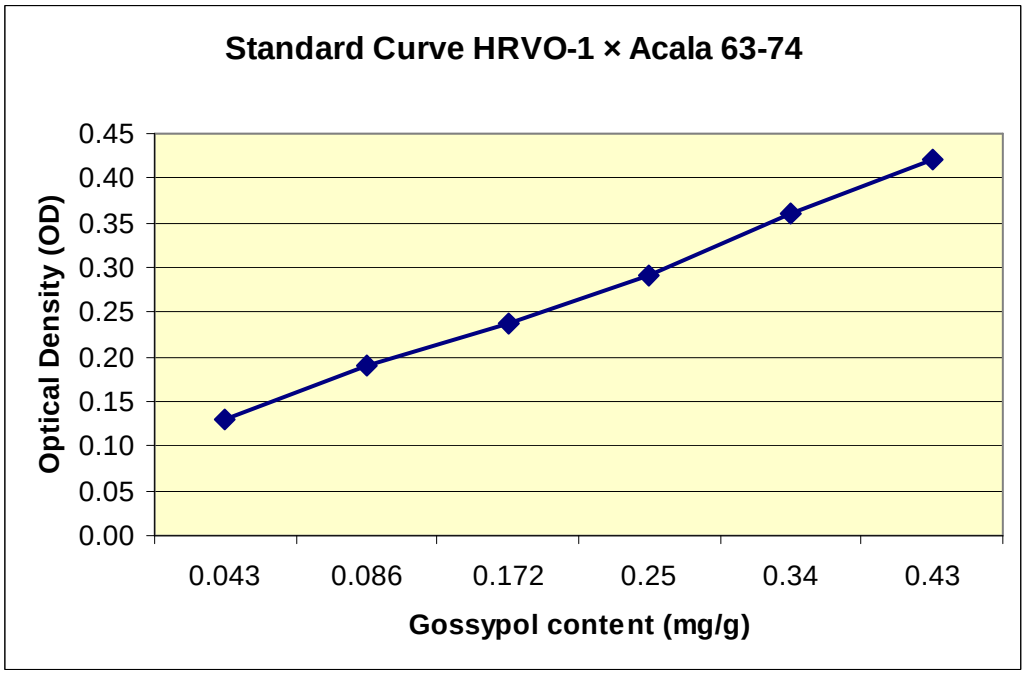


Fig. 1. Chemical structure of gossypol



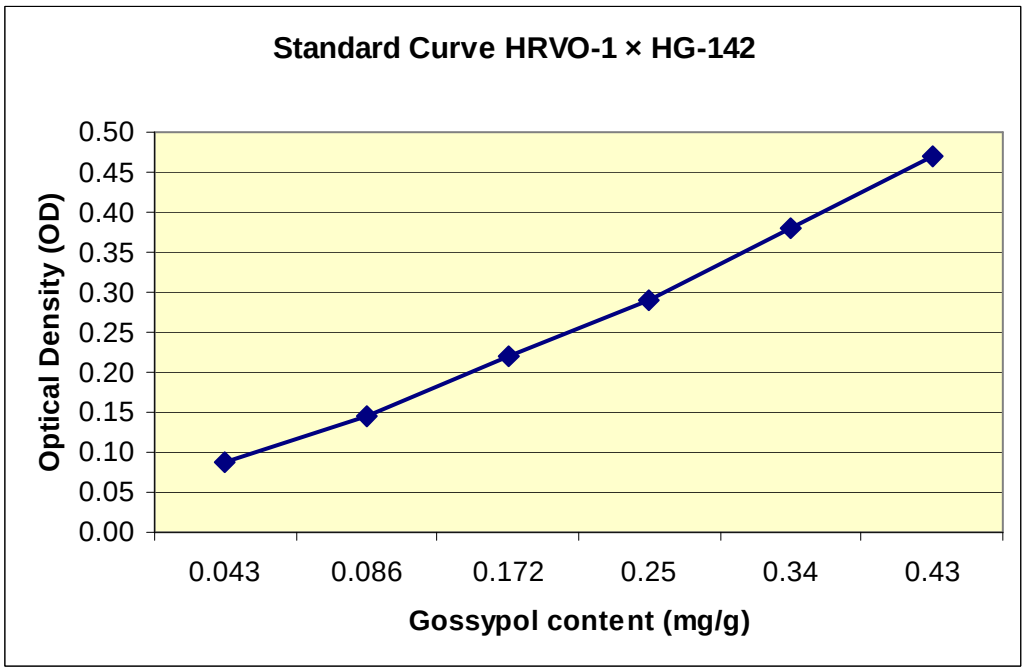


Fig. 2 Optical density versus Gossypol contents (mg/g)

Glandless
(Scale-0)

Intermediate glandless
(Scale-1)

Normal glanding
(Scale-2)

Intermediate glanding
(Scale-3)

High glanding
(Scale-4)

Fig. 3: Pictorial view of gossypol classes and scaling