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Effect of cottonseed milk on growth performance, hematological and semen characteristics in male Wistar albino rats

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Abstract

Various plant based milk extract is appropriate for human nutrition among which cottonseed is one of the potential crops with the advantages of stable milk emulsion, improved nutrient profile with affordable waste utilization. Although cottonseed milk is a popular indigenous beverage, it has not been exploited worldwide for regular consumption owing to the presence of gossypol. The gossypol toxicity and associated gossypol-iron complex formation in the intestine lead to changes in hematological characteristics and alternation of sperm motility in monogastric animals. Male Wistar albino rats weighing 60 to 70 g were divided into four groups of five animals each. The group fed the standard diet (STD) served as control, and the experimental groups included the group (i) rats fed cottonseed diet (CSD) supplemented at 10% of cottonseed level (ii) rats fed conventional aqueous extracted cottonseed milk (CCM) diet (CAD) and (iii) rats fed enzymatic assisted aqueous extracted cottonseed milk (ECM) diet (EAD). The CAD and EAD feed was administrated @ 1 ml/100 g of animal body weight /day for the study period of 45 days among the experimental groups and control group. A significant difference in weight gain of the experimental rats was noticed between the CCM and ECM cottonseed milk extracts fed experimental groups compared with the standard and cottonseed fed groups. The rats fed with CSD, CCM and ECM diet exhibited higher white blood cell counts, also reducing the red blood cells count, hemoglobin hematocrit and platelet in the group compared with STD. No significant difference in semen motility characteristics was noticed among the CSD, CCM and ECM fed groups. In conclusion, the intake of less than the permissible level of gossypol from selected cottonseed and its aqueous extracted milk samples has influenced the hematological parameters and whereas an improved effect was shown in semen characteristics.

Keywords: Cottonseed, Gossypol, Growth performance, Hematological parameters, Semen characteristics, Wistar albino rats

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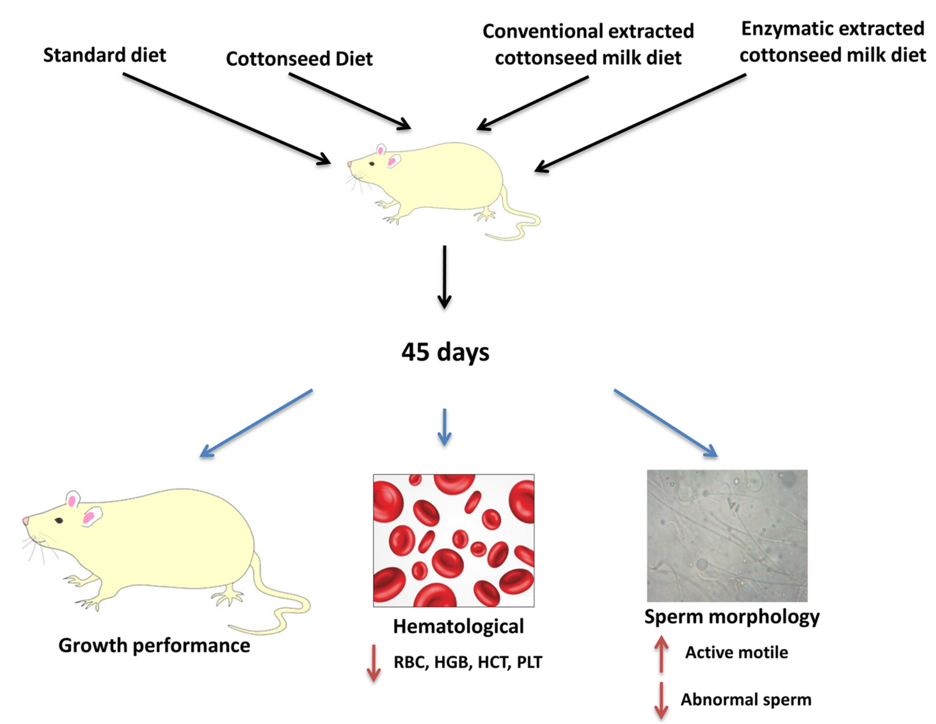
Graphical Abstract



Introduction

Cotton is one of the important cash crops and is cultivated in more than eight countries around the world. The production of cotton during 2020–2021 was 120.86 million bales and the largest cotton-producing countries were China (6.423 million metric tonnes (MMT)) followed by India (6.162 MMT), the USA (3.181 MMT), Brazil (2.341 MMT), Pakistan (0.98 MMT) and cotton account for 77% of global output in the form of fibre and animal feed (Statista 2022). Cottonseeds are the by-product of the production of fiber from the ginning process (Shahidi et al. 2020). Cottonseed contains 17–23% of protein and 18–20% of oil (Vekariya et al. 2017) and its byproducts have a high biological value (55–68%) and a safe fatty acid profile that includes 50% MUFA, 21% PUFA and 29% SFA which conforms to human health guidelines (Agarwal et al. 2003; Thirukkumar et al. 2021).

More than 60% of people worldwide are exposed to inadequate food supply accompanied by protein malnutrition and the annual production of cottonseed is estimated to meet the protein requirement of 2,40,350 million people (Henchion et al. 2017). However, the widespread consumption of cottonseed is impeded by the gossypol content with concentrations ranging from 0.02 to 6.64% in seeds among the cotton varieties. The high amount of gossypol decreases the bioavailability of lysine, arginine and cysteine (Gadelha et al. 2014). Also, gossypol interacts with iron to form a gossypol-iron complex, which could cause hematologic changes including anemia with lower red blood cell (RBC) counts, increase RBC fragility, reduce oxygen from hemoglobin (HGB), minimize oxygen transforming ability of blood cells with less HGB and hematocrit (HCT) values by the inhibitory effect on glucose-6-phosphate dehydrogenase enzyme. The inhibition of the glucose-6-phosphate dehydrogenase enzyme has resulted in enhancing the oxidant compounds in cells, and in consequence, decreasing the HGB and packed cell volume levels (Câmara et al. 2016; El-Mokadem et al. 2012; Garland 2015; Tang et al. 2017).

In addition, prolonged intake of high levels of gossypol from cottonseed-based food products inhibits the lactate dehydrogenase X enzyme, which plays an important role in the energy metabolism process in sperm and spermatogenic cells. The inhibition of lactate dehydrogenase X enzyme leads to a decrease in sperm activity in humans (Chris-Ozoko et al. 2018; Kenfack et al. 2015). Also, damaging the germinal epithelium cells through the action of an increase in glutathione peroxidase, glutathione reductase activity and oxidation of pyridine nucleotides, which resulted in decreases in the spermatogenesis, motility and spermatozoa numbers in testis tissue, which leads to a decrease in sperm count and increase in the percentage of abnormal sperm morphology (Coutinho et al. 2000; Santana et al. 2015). However, cottonseed has several important phytochemical components of interest due to the presence of several biological properties, which are used in the treatment of leukemia (Moon et al. 2008), colon carcinoma (Yan et al. 2010), breast cancer (Zhong et al. 2013), prostate cancer (Jiang et al. 2012) and other malignancies (Liu et al. 2020).

Cottonseed milk, one of the popular indigenous drinks of Tamil Nadu state, India is much preferred for its delicious taste and health benefits to humans. The traditional process for the extraction of cottonseed milk involves soaking cottonseed in water, grinding and extracting. Also, the extracted cottonseed milk is similar to the properties of milk emulsion due to the lipids in cottonseed (Kumar 2019). Nevertheless, the maximum permissible consumption level of gossypol as regulated by the USFDA (United States Food and Drug Administrative) is 450 mg/kg and by the FAO/WHO (Food and Agriculture Organization/World Health Organization) is 600 mg/kg in cottonseed-based food products deemed as safe for human consumption (Prasad & Blaise 2020).

However, long-term consumption of cottonseed milk or cottonseed-based food products poses the risk of gossypol toxicity and inhibits the absorption of iron, hematological characteristics and reduces sperm motility activity in monogastric animals. Overall and from our knowledge, few studies are available for supplementation of gossypol or cottonseed cake that could alter the growth performance, blood profile and sperm activity of the monogastric animal and no works related to the cottonseed aqueous milk extract has been taken up aimed at safety assurance for consumption. Therefore, studies were conducted to determine the effect of cottonseed and cottonseed milk aqueous extracts on growth performance, hematological profile and semen characteristics in male Wistar albino rats with the supplementation of standard diet feed.

Materials and methods

Animals and experimental design

The experimental procedure has been approved by the Institutional Animal Ethics Committee at the Central Animal Facility Center, SASTRA Deemed University, Thanjavur (Committee for the Purpose of Control and Supervision of Experiments on Animals, Approval Number: 648/SASTRA/IAEC/RPP), during November 2020 to August 2021.

The male Wistar albino rats of 20–25 days old (20 Nos) each weighing approximately 60 to 70 g were procured from Central Animal Facility and housed in autoclaved polypropylene cages which were filled in sterile paddy husk as the bedding material and acclimatized for 2 days with free access to a standard diet (ad libitum) and reverse osmosis (RO) water (ad libitum). The housing environmental characteristics were temperature, relative humidity and air changes per hour maintained at 23 ± 2 °C, 30 to 70%, and 12 to 15 air changes, respectively. The photoperiod for the rats is 12 and 12 hours in the light and dark, respectively. The duration of the experiment was 45 days and during that period, no instance of death in the selected experimental rats was observed.

The weight of each animal from the group was noted at zero-day and the end of the experiment day. The rats were randomly housed in groups of five per cage (Three experimental groups and one control group). The experimental diet groups were classified into STD (standard diet) 1 – standard diet, CSD (cottonseed diet) 2 - cottonseed diet, conventional aqueous extracted cottonseed milk diet (CAD) 3 - STD with conventional aqueous extracted cottonseed milk (CCM) and enzyme assisted aqueous extracted cottonseed milk diet (EAD) 4 - STD with enzyme assisted aqueous extracted cottonseed milk (ECM). The CCM and ECM were fed into the rat by oral gavage after thawing at the quantity of 1 ml/100 g of body weight/day along with STD from starting to end of the experiments respectively to the CAD and EAD (The quantity of extracted milk was fed well within the permissible level of gossypol in cottonseed based products set by FAO/WHO (Prasad & Blaise 2020). The intake of feed quantity was observed in a daily manner. At the end of the experiment day, the rats were sacrificed by 100% of CO_2 (flow rate for 30–70% cage volume per min) euthanasia (Underwood & Anthony 2020). The hematological and semen characteristics were analyzed from the five sacrificed rats in each group at the end of the experiment day.

Diet preparation and nutrient analysis

The cottonseed variety MCU 5 was procured from the Department of Cotton, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The STD was formulated from the composition of cornflour – 40 g, corn starch – 20 g, food grade casein – 20 g, wheat bran 10 g, corn oil – 5 ml, vitamins and minerals mixture – 4.5 g, methionine – 0.3 g and choline – 0.2 g and the CSD were contained cornflour – 40 g, corn starch – 15 g, food grade casein – 20 g, wheat bran 5 g, cottonseed – 10 g, corn oil – 5 ml, vitamins and minerals mixture – 4.5 g, methionine – 0.3 g and choline – 0.2 g (Ndvsu 2022). The diet was made into pellet form by using a single screw extruder (M/s La Monferrina, Italy) and packed in air-tight plastic bags. All the feeds were stored at refrigeration conditions (4 °C) until the commencement of the study.

The CCM and ECM were prepared per the method (Subramani et al. 2021). The extracted cottonseed milk was packed in an Eppendorf tube containing 1 ml and stored in freezing (−18 °C) conditions until it was used.

The crude protein, crude fat, iron and gossypol content were analyzed in the developed feed and cottonseed milk samples. The crude protein (Nitrogen ×6.25) was estimated as per the Kjeldahl method (AOAC 2000). Crude fat was estimated with hexane by soxhlet apparatus based on the method (AOAC 2000). The gossypol content was estimated by the spectrophotometer method (Sadasivam & Manickam 2018). The concentration of iron was analysed in the method described by (AOAC 2000) using atomic absorption spectrophotometer (AA-7000; Shimadzu, Tokyo, Japan).

Analysis of hematological parameters

At the end of the experiment, the blood samples were collected from the jugular vein puncture or retro-orbital sinus of the rats in ethylenediaminetetraacetate coated and heparinized vials between 8.00 AM to 9.00 AM after overnight fasting. The collected blood samples were used for assessing the hematological parameters namely white blood cell (WBC) count which included leukocyte counts (neutrophils (NEU), lymphocytes (LYM), monocyte (MON), eosinophil (EOS), basophil (BAS)) and red blood cells (RBC) count, hemoglobin (HGB), hematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean cell hemoglobin concretion (MCHC), platelet (PLT) count, mean platelet volume (MPV), platelet distribution width (PDW) and plateletcrit (PCT) which were measured by hematology analyzer (M/S Mythic 22, Orphee, Switzerland).

Analysis of semen characteristics

The testis was collected from three sacrificed rats in each group at the end of the experiment day and used for determining the total sperm count, progressive motility (active, sluggish and nonmotile), pus cells /HPF (high power fluid) in minimum and maximum, epithelial cells /HPF in minimum and maximum, RBC /HPF in minimum and maximum, normal and abnormal structure of the sperms were done in the automated semen analyser along with the microscope (M/S Prime Sperm, India). The semen was spread over the pre-warmed slide with the addition of two drops of 2.9% sodium citrate solution. This was covered with a covered slip and examined under the 40X optical microscope with reduced light (Kadir et al. 2018).

Statistical analysis

The values are expressed in the mean ± standard deviation of the mean. The significance of the difference ($P < 0.05$) was evaluated by analysis of variance (ANOVA) with the least significant difference (LSD) by using SPSS software version 17.0.

Results and discussion

Nutrients and gossypol content of the developed feed

The nutrients namely, crude protein, crude fat, iron and toxic principle such as gossypol content were analysed in supplied feed and the values are presented in Table 1. The crude protein was 22.95 and 25.10 g/100 g, crude fat was 6.45 and 6.68 g/100 g and iron content were 1.532 and 1.58 mg/100 g respectively in the STD and CSD. The cottonseed milk extract contained 2.69 and 3.42 g/100 ml of protein, 2.31 and 2.95 g/100 ml of crude fat and 0.0118 and 0.0482 mg/100 m of iron, respectively in CCM and EAAECM. Gossypol content was detected as 2.22 mg/100 g in the CSD feed, 5.76 mg/100 ml in CCM and 4.20 mg/100 ml in EAAECM. The proximate content of the developed feed was similar to the millet or corn or groundnut incorporated feed (Olufunke & Toyin 2012). Whereas, the quantity of the gossypol content varied between the different varieties of cottonseed and ranged between 0.02 to 6.64%. Gossypol compound was linked with NH_3 (Ammonia), −SH (Sulfhydryl), SCH_3 (sulfide) and OH (Hydroxyl) containing groups of amino acids

Table 1 Nutrient content for developed feed and cottonseed milk aqueous extracts

Nutrient parameters	Feed (100 g)		Cottonseed aqueous extract (100 ml)	
	STD	CSD	CCM	ECM
Crude protein (g)	22.95 ± 0.86b	25.10 ± 0.65a	2.69 ± 0.019c	3.42 ± 0.078c
Crude fat (g)	6.45 ± 0.21a	6.68 ± 0.05a	2.31 ± 0.039c	2.95 ± 0.080b
Iron (mg)	1.532 ± 0.026a	1.58 ± 0.057a	0.0118 ± 0.001b	0.0482 ± 0.008b
Gossypol (mg)	ND	2.22 ± 0.11c	5.76 ± 0.531a	4.20 ± 0.043b

Values mentioned as Mean ± SD (standard deviation) of three replicate analysis ($n = 3$); The values mentioned in superscripts are significantly different at $P < 0.05$ in the same row

STD Standard diet, *CSD* Cottonseed diet, *CCM* conventional aqueous extracted cottonseed milk, *ECM* Enzyme assisted aqueous extracted cottonseed milk, *ND* Nondetectable

(Gandhi 2015; Kenar 2006). Hence, gossypol is associated with the protein content of cottonseed-based food products. The concentration of the gossypol varied between the nature of the seed, cultivation practices, and storage practices of the seeds and processing factors in the cottonseed.

Growth performance

The growth performance data related to food, water, protein and fat intake, and average weight gain of the experimental groups are given in Table 2. The selected rats had an average initial weight that ranged from 64.64 to 67.18 g/rat and after the end of the experiment, the final weight of the animal ranged from 168.98 to 182.55 g/rat. The mean value of the increased weight was found in the following order of groups: EAD (116.17 g/rat) > CAD (111.17 g/rat) > STD (106.54 g/rat) > CSD (103.67 g/rat). This indicated that the components presented in the CSM, CAD and EAD had significantly affected the weight of the rodents. Statistical analysis revealed a non-significant difference between cottonseed milk-fed groups with the other two groups (STD and CSD) of animals as reflected in the average daily weight gain which varied between 2.20 and 2.47 g/rat which is augmented by the intake of feed quantity and presence of nutrient in the feed. The weight gain of the rodents was agreed with the cottonseed meal with or without tryptophan supplementation to rabbit bucks (Alaba et al. 2021). The daily food intake was significantly different in each group and was highest in group EAD (9.86 g/rat/day) and lowest in the group CSD (9.26 g/rat/day). The mean values for the intake of protein and fat ranged between 2.19 to 2.51 g/rat/day and 0.618 to 0.635 g/rat/day respectively in the experimented groups. These results were correlated with the continuous supply of lysine-incorporated cottonseed meal that had significantly affected the weight gain, feed intake and feed conversion ratio of the broiler chicks (Henry et al. 2001).

Hematological characteristics

The results of the hematological characteristics of experimented rats are given in Table 3. The WBC count of treated groups (CSD, CAD and EAD) was non-significant in comparison with control STD and values ranged from 11.50 to 12.46×10^3/μl. The animal groups fed with CSD (12.32×10^3/μl) and both the cottonseed milk extracts diet (CAD (12.46×10^3/μl) and EAD (11.98×10^3/μl)) fed groups had the highest WBC count compared with STD fed group (11.50×10^3/ml). The leukocytes viz., MON, EOS and BAS (except in NEU and LYM) had non-significant effects between the groups. However, the parameters were not significantly reflected in the quantity of the leukocyte counts including NEU, LYM, MON, EOS and BAS ranging from 2.80 to 5.27×10^3/μl, 7.04 to 9.48×10^3/μl, 0.01 to 0.05×10^3/μl, 0.03 to 0.04×10^3/μl and 0.00 to 0.07×10^3/μl respectively in the experimented groups. Among the WBC components, BAS was not presented in the CSD fed group and LYM was presented at high levels (9.48×10^3/μl) in the CSD fed group compared with other experimented groups. The modification of cellular immunity has reflected the changes in WBC and leukocytes via inhibition of the activity of adenylate cyclase c-AMP, which is responsible for WBC formation, and the reduction of arachidonic acid via inhibition of the lipoxygenase enzyme (association of chemotactic releasing factor) (Mahmood et al. 2022; Nwoha 1995). Our results were matched with (Mahmood et al. (2022) by the cotton seed cake-fed rabbit groups.

The RBC count of the experimented groups varied between 6.92 and 8.50×10^6/μl and the RBC count of

Table 2 Effect of cottonseed and cottonseed aqueous extracts diet on growth performance of male wistar albino rats

Particulars	Diets			
	STD	CSD	CAD	EAD
Initial weight (g)	64.64 ± 12.89	65.31 ± 10.01	67.18 ± 10.64	66.54 ± 10.81
Final weight (g)	171.18 ± 20.14b	168.98 ± 25.46b	178.35 ± 17.21a	182.55 ± 12.28a
Increased weight (g)	106.54 ± 7.25c	103.67 ± 15.45c	111.17 ± 6.57b	116.01 ± 1.47a
Average daily gain (g)	2.26 ± 0.07	2.20 ± 0.19	2.40 ± 0.07	2.47 ± 0.00
Food intake (g/day)	9.67 ± 1.37bc	9.26 ± 1.51c	9.72 ± 1.618a	9.86 ± 1.59bc
Water intake (ml/day)	18.18 ± 3.69c	20.80 ± 5.86a	17.98 ± 5.06d	19.38 ± 6.02b
Protein intake (g/day)	2.22 ± 0.37c	2.51 ± 0.48a	2.19 ± 0.43c	2.29 ± 0.44b
Fat intake (g/day)	0.623 ± 0.002b	0.618 ± 0.003c	0.626 ± 0.002b	0.635 ± 0.005a
Iron intake (mg/day)	0.147 ± 0.00b	0.146 ± 0.001b	0.148 ± 0.001b	0.151 ± 0.002a

STD Standard diet, *CSD* Cottonseed diet, *CAD* standard diet with conventional aqueous extracted cottonseed milk for 1 ml/100 g of animal weight/day, *EAD* standard diet with enzyme assisted aqueous extracted cottonseed milk for 1 ml/100 g of animal weight/day; Values are mean ± SD of five observations from the experimental animals; The values mentioned in superscripts are significantly different at $P < 0.05$ in the same row

Table 3 Effect of cottonseed and cottonseed aqueous extracts diet on hematological characteristics of male wistar albino rats

Parameters	Diets			
	STD	**CSD**	**CAD**	**EAD**
WBC (10^3/µl)	11.50 ± 0.72	12.32 ± 1.62	12.46 ± 0.61	11.98 ± 1.84
NEU (10^3/µl)	3.58 ± 0.30ab	2.80 ± 0.28b	5.27 ± 0.46a	3.75 ± 2.09ab
LYM(10^3/µl)	7.81 ± 0.87ab	9.48 ± 1.38a	7.04 ± 1.08b	8.10 ± 0.39ab
MON (10^3/µl)	0.05 ± 0.05	0.01 ± 0.01	0.04 ± 0.03	0.03 ± 0.03
EOS (10^3/µl)	0.03 ± 0.01	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.02
BAS (10^3/µl)	0.02 ± 0.03	0.00 ± 0.00	0.06 ± 0.11	0.07 ± 0.12
RBC (10^6/µl)	8.50 ± 0.32ab	8.34 ± 0.14b	6.92 ± 1.20c	7.75 ± 0.39bc
HGB (g/dL)	15.37 ± 0.29a	14.33 ± 0.21ab	12.53 ± 1.53b	14.20 ± 0.95ab
HCT (%)	61.27 ± 1.21a	61.53 ± 1.21a	56.33 ± 0.64b	59.93 ± 2.75a
MCV (fL)	71.95 ± 2.70b	73.77 ± 1.81ab	82.83 ± 12.68a	77.37 ± 0.38ab
MCH (ρg)	18.13 ± 0.83	17.23 ± 0.06	18.20 ± 0.92	18.33 ± 0.47
MCHC (g/dL)	25.10 ± 0.26a	23.37 ± 0.57ab	22.23 ± 2.58b	23.73 ± 0.74ab
PLT (10^3/µl)	792.67 ± 197.88	659.00 ± 17.35	733.67 ± 203.49	732.00 ± 16.09
MPV (fL)	6.90 ± 0.30	7.03 ± 0.06	6.80 ± 0.66	6.90 ± 0.20
PDW (%)	15.57 ± 0.06a	15.63 ± 0.15a	15.47 ± 0.38ab	15.60 ± 0.20a
PCT (%)	0.55 ± 0.14	0.46 ± 0.01	0.49 ± 0.09	0.51 ± 0.02

STD Standard diet, *CSD* Cottonseed diet, *CAD* standard diet with conventional aqueous extracted cottonseed milk for 1 ml/100 g of animal weight/day, *EAD* standard diet with enzyme assisted aqueous extracted cottonseed milk for 1 ml/100 g of animal weight/day; Values are mean ± SD of five observations from the experimental animals; The values mentioned in superscripts are significantly different at $P < 0.05$ in the same row; *WBC* white blood cells, *RBC* red blood cells count, *HGB* hemoglobin, *HCT* hematocrit, *PLT* platelet, *NEU* neutrophils, *LYM* lymphocytes, *MON* monocyte, *EOS* eosinophil, *BAS* basophil, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean cell hemoglobin concretion, *MPV* mean platelet volume, *PDW* platelet distribution width and *PCT* plateletcrit

STD (8.50×10^6/µl) was significantly increased compared to the CSD (8.34×10^6/µl), EAD (7.75×10^6/µl) and CAD (6.92×10^6/µl) groups. The same trend was reflected in the HGB content and MCHC in the experimented groups. The HCT, MCV and MCH values ranged from 56.33 to 61.53%, 71.95 to 82.83 fL and 17.23 to 18.33 ρg respectively in the experimented groups. The PLT count was decreased in the following order as STD (792.67×10^3/µl) > CAD (733.67×10^3/µl) > EAD (732.00×10^3/µl) > CSD (659.00×10^3/µl). Whereas the MPV, PDW and PCT percentages of CSD and both the cottonseed milk fed groups (CAD and EAD) were on par with the control group (STD).

The hematological characteristics were influenced by the binding of gossypol with iron in the CSD, CAD and EAD groups compared to STD. The gossypol reacts with the iron and reduces the iron bioavailability which leads to decreased biosynthesis of hemoglobin through the formation of an iron insoluble complex in the intestine and results in reduced synthesis of HGB (Gadelha et al. 2014). Similar changes were observed in the consumption of cottonseed cake which had increased the erythron characteristics and was reported in the sheep feed which contained 40% of cottonseed cake and had significantly increased the packed cell volume, erythrocyte counts and HGB concentration (Câmara et al. 2016; El-Mokadem et al. 2012). The reason for these changes is augmented the high consumption of gossypol for prolonged periods, which leads to inhibition of the activity of glucose-6-phosphate dehydrogenase enzyme has resulted in enhancing the oxidant compounds in cells, and in consequence, decreasing the hemoglobin and packed cell volume levels (El-Mokadem et al. 2012).

Semen characteristics

The semen characteristics and morphology of sperm from the experimented rats are presented in Table 4 and Fig. 1 respectively. The total sperm count was comparatively high in CSD and both CAD and EAD fed groups had 70, 45 and 55 million sperms/ml respectively with 31.65 million sperms/ml in the STD group. The actively motile, sluggish motile and nonmotile percentages of sperms were presented as 38.35, 21.65 and 40.0% respectively in CSD, 41.65, 26.65 and 31.65% respectively in CAD, 46.65, 21.65 and 31.65% respectively in EAD and 30.0, 25.0 and 45.0% respectively in STD.

The minimum value of pus cells, epithelial cells and RBC ranged from 7.65 to 11.65 /HPF (High Power Field), 4.65 to 5.68/HPF and 1.35 to 2.35/HPF respectively and the maximum value of pus cells, epithelial cells and RBC ranged from 9.65 to 15.00/HPF, 7.00 to 7.65/HPF and 3.00 to 4.35/HPF, respectively among the experimented groups. The morphology of semen includes normal and abnormal or defective sperm. All the experimented

Table 4 Effect of cottonseed and cottonseed aqueous extracts diet on semen characteristics of male wistar albino rats

Parameters	Diet			
	STD	CSD	CAD	EAD
Total sperm count (Million Sperms/ml)	31.65 ± 10.41c	70.00 ± 10.000a	45.00 ± 5.00b	55.00 ± 5.00b
Actively Motile (%)	30.00 ± 8.66b	38.35 ± 2.89a	41.65 ± 14.45a	46.65 ± 11.55a
Sluggish Motile (%)	25.00 ± 8.66	21.65 ± 10.41	26.65 ± 11.55	21.65 ± 7.65
Non Motile (%)	45.00 ± 15.00	40.00 ± 10.00	31.65 ± 2.90	31.65 ± 7.65
Pus Cells /HPF (Minimum)	11.65 ± 3.51ab	7.65 ± 2.52b	9.65 ± 2.52ab	11.35 ± 1.15ab
Pus Cells /HPF (Maximum)	15.00 ± 5.00ab	9.65 ± 2.52c	12.65 ± 3.21bc	14.00 ± 1.75bc
Epithelial Cells /HPF (Minimum)	5.00 ± 1.00	4.65 ± 0.58	5.00 ± 0.00	5.68 ± 0.60
Epithelial Cells /HPF (Maximum)	7.00 ± 1.00	7.00 ± 1.00	7.00 ± 0.00	7.65 ± 0.60
RBC /HPF (Minimum)	1.35 ± 0.58	2.35 ± 1.55	1.35 ± 0.58	2.00 ± 0.0
RBC /HPF (Maximum)	3.00 ± 1.00	4.35 ± 1.55	3.65 ± 1.15	4.00 ± 0.0
Normal Structure (%)	48.35 ± 20.20	56.65 ± 5.75	53.35 ± 5.75	60.00 ± 10.0
Abnormal (%)	51.65 ± 20.20	43.35 ± 5.75	46.65 ± 5.75	40.00 ± 10.0

STD Standard diet, *CSD* Cottonseed diet, *CAD* standard diet with conventional aqueous extracted cottonseed milk for 1 ml/100 g of animal weight/day, *EAD* standard diet with enzyme assisted aqueous extracted cottonseed milk for 1 ml/100 g of animal weight/day, *HPF* High power fluidm, *RBC* Red blood cells; Values are mean ± SD of five observations from the experimental animals; The values mentioned in superscripts are significantly different at $P < 0.05$ in the same row

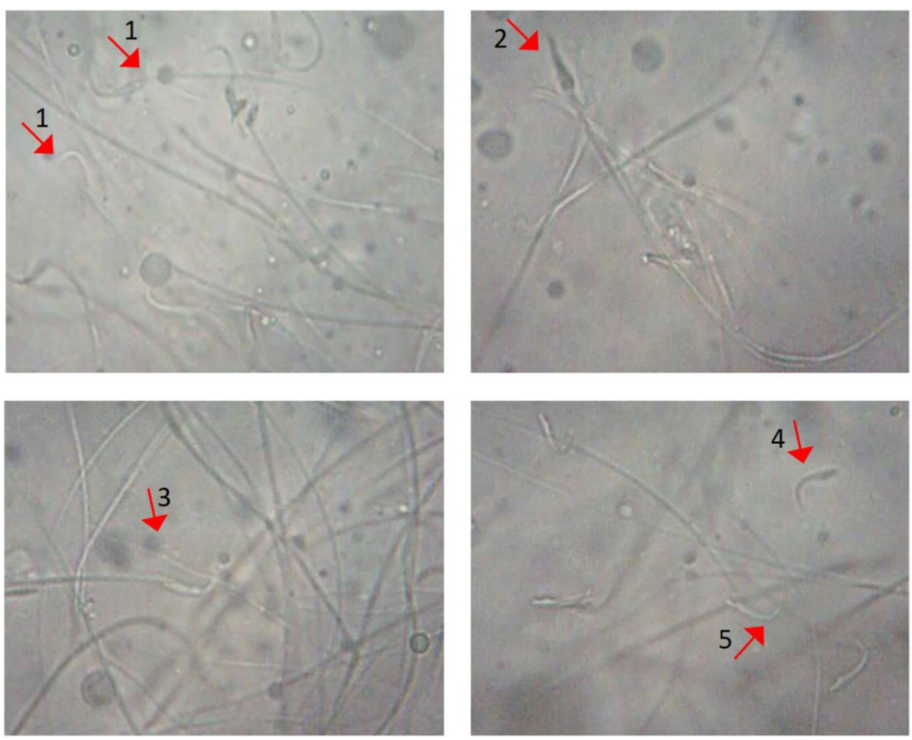


Fig. 1 Microphotographs of semen morphology from experimented male wistar albino rats

1-Normal morphology; Abnormal morphology includes 2-Pairing phenomenon; 3-Headless tail; 4-Detached head and 5-Bend neck

groups had normal and abnormal morphology of sperm structure. However, the percentage of normal morphology of sperm was presented in CSD (56.65%), CAD (53.35%) and EAD (60%), was more than the STD at 48.35% and a reverse trend was presented in terms of the abnormality or defective morphology of sperm in CSD

(43.35%), CAD (46.65%) and EAD (40%) compared with STD (51.65%). The normal morphology of the semen structure was significantly affected by the bend necks of the sperm, without a head or tail in the sperm and the attachment of two heads in single sperm (Fig. 1). These changes were observed as a percentage of active, sluggish and nonmotile sperm and normal and abnormal morphology of the sperm.

The pus cells had an inverse relationship between sperm motility and count and this observation was noted in infertile patients (Khan et al. 2012). Among the experimented groups, the group fed the EAD showed the highest percentage of active motile sperms, the normal structure of the sperms and less percentage of sluggish, nonmotile and abnormal structure of the sperms. However, the other experimented groups that contained gossypol in CSD and CAD had better performance in terms of total sperm count, active motility of sperm and normal structure of the sperm compared with STD fed group.

The results from the previous study present that the gossypol content of the MCU 5 cottonseed variety, CCM and EAAECM was 22.81 mg/100 g, 5.76 mg/100 ml in CCM and 4.20 mg/100 ml, respectively (Subramani et al. 2021). The gossypol content of the selected feed samples was within the safe level for consumption and met the permissible intake level of gossypol in food products as set by USFDA and FAO/WHO (Prasad & Blaise 2020). However, male fertility characteristics may be influenced by dose and time-dependent factors. The prolonged intake of a large quantity of cottonseed (as gossypol) may affect fertility characteristics due to the inhibition of lactate dehydrogenase X enzyme activity, an increase in the 17b-HSD and 17-ketosteroid reductase activity, which leads to an increase in the degradation of testosterone, damaging the germinal epithelium and disturbing both the structural and functional characteristics of the epididymis (Amini & Kamkar 2005; El-Sharaky et al. 2010). Also, gossypol interferes with steroidogenic enzymes viz., 5α-reductase and 3α-hydroxysteroid dehydrogenase in the rat testis (Hu et al. 2009). Various studies have reported that prolonged intake of high levels of cottonseed-based food products viz., cottonseed meal, cottonseed cake, cottonseed oil and cottonseed extract caused a significant decrease in weight of the reproductive organs, sperm count and changes in sperm morphology (Babatunde et al., 2021; Chenoweth et al. 1994; Chris-Ozoko et al. 2018; Cunha et al. 2012; Singla & Garg 2013). However, intake of gossypol at 10 mg/kg of body weight/day for 9 weeks in the male rats showed a not significant effect on sperm characteristics (Beaudoin 1985). Kenfack et al. (2015) reported that the spermatozoa concentration in the cauda epididymis was higher, although not significant in the cottonseed cake (up to 12.5% inclusion of cottonseed) compared with the control diet, as well as the adverse effect was observed in the 18% cottonseed included diet fed in rabbits. In another study, feeding over 30% of the whole cottonseed for up to 90 days showed an adverse effect on the semen characteristics and testicular profile in the Red Sokoto Bucks. Moreover, testicular and epididymis had higher concentrations of sperms in animals fed with up to 30% of the whole cottonseed (Itodo et al. 2020). Therefore, the above studies related to semen characteristics confirmed that the intake of the highest gossypol for a prolonged time causes an antagonistic effect on sperm characteristics. However, our results show that the intake quantity of the treated diet has improved the semen characteristics compared with the STD diet in the rats and that cottonseed-based products had the permissible intake level of gossypol as mentioned by USFDA and FAO/WHO.

Conclusion

The consumption of below or permissible intake level of cottonseed, CCM and ECM by male Wistar albino rats during the period of 45 days had a significant effect on the growth performance, whereas increases in the WBC include leukocyte characteristics and induce decreases in the RBC count, HGB, HCT and PLT content compared with STD fed group due to the formation of gossypol-iron complex mechanism. The semen characteristics such as total sperm count, active motility of sperm and normal structure of the sperm had better performance in the cottonseed-based products fed groups and were significantly on par with STD fed group. Further studies may be required by increasing the concentration of cottonseed or cottonseed aqueous extract or the duration of the dose supplied period for safe consumption of cottonseed-based products for human consumption.

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Authors' contributions

TS has contributed to carrying out this research and the preparation of this manuscript. HG has designed and coordinated the study and revised the manuscript. VS has helped in analysis of parameters. DRC has performed sample collection, and preparation and conducted the morphological analysis. All authors have equally contributed and approved the manuscript.

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Availability of data and materials
The data used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval
This investigation was carried out with the relevant guidelines and regulations of animal studies of the Institutional Animal Ethics Committee of SASTRA Deemed University.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no conflict of interest.

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References

Agarwal, D., Singh, P., Chakrabarty, M., Shaikh, A., & Gayal, S. (2003). Cottonseed oil quality, utilization and processing. *CICR Technical Bulletin*, *25*, 1–16.

Alaba, O., Adenuga, B., Odu, O., & Adejumo, D. (2021). Effect of cottonseed meal supplemented with tryptophan on growth performance and organ development of rabbit bucks. *International Journal of Animal Science, Husbandry and Livestock Production*, *7*(5), 398–401.

Amini, A., & Kamkar, F. (2005). The effects of gossypol on spermatogenesis in NMRI mice. *Iranian Journal of Science and Technology*, *29*(1), 123–133.

AOAC (2000). *Official methods of analysis*. Gaithersburg: The Association of Official Analytical Chemists.

Babatunde, D. J., Tope, O. O., Fidelis, O. E., Enevwo, O. G., & Olugbenga, E. (2021). Aqueous extract of adansonia digitata reversed cotton seed extract-induced testicular damage in wistar rats. *JBRA Assisted Reproduction*, *25*(2), 257–265. https://doi.org/10.5935/1518-0557.20200092.

Beaudoin, A. R. (1985). The embryotoxicity of gossypol. *Teratology*, *32*(2), 251–257.

Câmara, A. C. L., do Vale, A. M., Mattoso, C. R. S., Melo, M. M., & Soto-Blanco, B. (2016). Effects of gossypol from cottonseed cake on the blood profile in sheep. *Tropical Animal Health and Production*, *48*(5), 1037–1042. https://doi.org/10.1007/s11250-016-1039-0.

Chenoweth, P., Risco, C., Larsen, R., Velez, J., Tran, T., & Chase Jr., C. (1994). Effects of dietary gossypol on aspects of semen quality, sperm morphology and sperm production in young Brahman bulls. *Theriogenology*, *42*(1), 1–13. https://doi.org/10.1016/0093-691X(94)90657-5.

Chris-Ozoko, L. E., Okpe, O., Iju, W. J., & Oyem, J. C. (2018). Histomorphological effects of cottonseed oil on testes in adult male wistar rats. *Galician Medical Journal*, *25*(4), 2–5. https://doi.org/10.21802/gmj.2018.4.2.

Coutinho, E. M., Athayde, C. I., Atta, G., Gu, Z.-P., Chen, Z.-W., Sang, G.-W., … Otubu, J. (2000). Gossypol blood levels and inhibition of spermatogenesis in men taking gossypol as a contraceptive: A multicenter, international, dose-finding study. *Contraception*, *61*(1), 61–67. https://doi.org/10.1016/s0010-7824(99)00117-1.

Cunha, M. G. G., Gonzalez, C. M., Carvalho, F. F. R., & Soares, A. T. (2012). Effect of diets containing whole cottonseed on the quality of sheep semen. *Acta Scientiarum Animal Sciences*, *34*(3), 305–311. https://doi.org/10.4025/actascianimsci.v34i3.12963.

El-Mokadem, M., Taha, T., Samak, M., & Yassen, A. (2012). Alleviation of reproductive toxicity of gossypol using selenium supplementation in rams. *Journal of Animal Science*, *90*(9), 3274–3285. https://doi.org/10.2527/jas.2011-4545.

El-Sharaky, A. S., Newairy, A. A., Elguindy, N. M., & Elwafa, A. A. (2010). Spermatotoxicity, biochemical changes and the histological alteration induced by gossypol in testicular and hepatic tissues of male rats. *Food and Chemical Toxicology*, *48*(12), 3354–3361. https://doi.org/10.1016/j.fct.2010.09.004.

Gadelha, I. C. N., Fonseca, N. B. S., Oloris, S. C. S., Melo, M. M., & Soto-Blanco, B. (2014). Gossypol toxicity from cottonseed products. *The Scientific World Journal*, *2014*, 1–11. https://doi.org/10.1155/2014/231635.

Gandhi, K. D. (2015). *Biochemical characterization of cotton (Gossypium herbaceum L.) for seed quality traits*. Anand: Anand Agricultural University.

Garland, T. (2015). Overview of gossypol poisoning. https://www.msdvetmanual.com/toxicology/gossypol-poisoning/overview-of-gossypol-poisoning. Accessed 23 Aug 2021.

Henchion, M., Hayes, M., Mullen, A. M., Fenelon, M., & Tiwari, B. (2017). Future protein supply and demand: Strategies and factors influencing a sustainable equilibrium. *Foods*, *6*(7), 1–21. https://doi.org/10.3390/foods6070053.

Henry, M. H., Pesti, G. M., Bakalli, R., Lee, J., Toledo, R. T., Eitenmiller, R. R., & Phillips, R. D. (2001). The performance of broiler chicks fed diets containing extruded cottonseed meal supplemented with lysine. *Poultry Science*, *80*(6), 762–768. https://doi.org/10.1093/ps/80.6.762.

Hu, G. X., Zhou, H. Y., Li, X. W., Chen, B. B., Xiao, Y. C., Lian, Q. Q., … Ge, R. S. (2009). The (+)- and (−)-gossypols potently inhibit both 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase 3 in human and rat testes. *The Journal of Steroid Biochemistry and Molecular Biology*, *115*(1–2), 14–19. https://doi.org/10.1016/j.jsbmb.2009.02.004.

Itodo, J. I., Ibrahim, R. P., Rwuaan, J. S., Aluwong, T., Shiradiyi, B. J., Owoicho, A. K., … Agbi, K. A. (2020). The effects of feeding graded levels of whole cottonseed on semen characteristics and testicular profiles of red Sokoto bucks. *Acta Scientiarum. Animal Sciences*, *43*, 1–9. https://doi.org/10.4025/actascianimsci.v43i1.50990.

Jiang, J., Slivova, V., Jedinak, A., & Sliva, D. (2012). Gossypol inhibits growth, invasiveness, and angiogenesis in human prostate cancer cells by modulating nf-κb/ap-1 dependent-and independent-signaling. *Clinical & Experimental Metastasis*, *29*(2), 165–178. https://doi.org/10.1007/s10585-011-9439-z.

Kadir, E. R., Ojulari, L. S., Ibrahim, A., Ekundayo, O. J., Jaji-Sulaimon, R., & Jimoh-Abdulghaffaar, H. O. (2018). Testicular morphology and seminal fluid parameters of adult Wistar rats following honey administration. *Tropical Journal of Pharmaceutical Research*, *17*(7), 1331–1335. https://doi.org/10.4314/tjpr.v17i7.15.

Kenar, J. A. (2006). Reaction chemistry of gossypol and its derivatives. *Journal of the American Oil Chemists' Society*, *83*(4), 269–302. https://doi.org/10.1007/s11746-006-1203-1.

Kenfack, A., Chombong, J. K., Ngoula, F., Vemo, N. B., Tsambou, A. M. M., Zeukeng, G. M. Z., … Tchoumboué, J. (2015). Effect of feeding cottonseed cake on male fertility in rabbit. *Bangladesh Journal of Animal Science*, *44*(1), 16–20. https://doi.org/10.3329/bjas.v44i1.23123.

Khan, M. S., Mohammad, S. H., Deepa, F., & Tahir, F. (2012). Association between pus cells and semen parameters in infertile Pakistani males. *Sultan Qaboos University Medical Journal*, *12*(4), 479. https://doi.org/10.12816/0003174.

Kumar, M. (2019). Paruthi paal, a nutrient-rich healthy drink from cottonseed: An Indian delicacy. *Journal of Ethnic Foods*, *6*(32), 1–6. https://doi.org/10.1186/s42779-019-0035-1.

Liu, Y., Ma, Y., Li, Z., Yang, Y., Yu, B., Zhang, Z., & Wang, G. (2020). Investigation of inhibition effect of gossypol-acetic acid on gastric cancer cells based on a network pharmacology approach and experimental validation. *Drug Design, Development and Therapy*, *14*, 3615–3622. https://doi.org/10.2147/DDDT.S256566.

Mahmood, A., Khan, M. A., Parveen, S., Hussain, T., & Azad, A. (2022). Studies on haematological and some serum biochemical changes by oral administration of gossypol from cotton seed cake in rabbits. *Sarhad Journal of Agriculture*, *38*(2), 417–421. https://doi.org/10.17582/journal.sja/2022/38.2.417.421.

Moon, D. O., Kim, M. O., Lee, J. D., & Kim, G. Y. (2008). Gossypol suppresses nf-κb activity and nf-κb-related gene expression in human leukemia u937 cells. *Cancer Letters*, *264*(2), 192–200. https://doi.org/10.1016/j.canlet.2008.01.030.

Ndvsu (2022). *Nutrient requirement and feed formulations for laboratory animals*. Madhya Pradesh: University NDVS.

Nwoha, P. U. (1995). The blood constituents of gossypol-treated, protein-malnourished wistar rats. *Contraception*, *52*(4), 249–254. https://doi.org/10.1016/0010-7824(95)00188-G.
Olufunke, D., & Toyin, O. (2012). Animal feeding trial on formulated rat diet. *Advances in Life Science and Technology*, *4*, 42–50.
Prasad, R., & Blaise, D. (2020). Low gossypol containing cottonseed: Not only a fibre but also a food crop. *National Academy Science Letters*, *43*(7), 599–602. https://doi.org/10.1007/s40009-020-00931-1.
Sadasivam, S., & Manickam, A. (2018). *Biochemical methods: Estimation of gossypol*. India: New Age International Publishers.
Santana, A. T., Guelfi, M., Medeiros, H. C., Tavares, M. A., Bizerra, P. F., & Mingatto, F. B. E. (2015). Mechanisms involved in reproductive damage caused by gossypol in rats and protective effects of vitamin E. *Biological Research*, *48*(1), 1–8. https://doi.org/10.1186/s40659-015-0026-7.
Shahidi, F., Oh, W. Y., Wan, P. J., & Wakelyn, P. J. (2020). Cottonseed oil. In F. Shahidi (Ed.), *Bailey's industrial oil and fat products*, (7th ed., pp. 1–95). USA: Wiley. https://doi.org/10.1002/047167849x.bio022.pub2.
Singla, N., & Garg, M. (2013). Effect of crude cottonseed oil containing gossypol on fertility of male and estrous cycle of female *Bandicota bengalensis* gray and hardwicke. *Journal of Applied Animal Research*, *41*(2), 156–165. https://doi.org/10.1080/09712119.2012.738230.
Statista, (2022). Cotton production worldwide by top countries. https://www.statista.com/statistics/263055/cotton-production-worldwide-by-top-countries/. Accessed on 24 Apr 2022.
Subramani, T., Ganapathyswamy, H., Sampathrajan, V., & Sundararajan, A. (2021). Optimization of extraction parameters to improve cottonseed milk yield and reduce gossypol levels using response surface methodology (RSM). *Journal of Food Processing and Preservation.*, *46*, e15945. https://doi.org/10.1111/jfpp.15945.
Tang, M. Q., Zhang, K., Zhan, T., Zhao, Q., Zhang, S., & Zhang, J. (2017). Multi-omics analyses of red blood cell reveal antioxidation mechanisms associated with hemolytic toxicity of gossypol. *Oncotarget*, *8*(61), 103693. https://doi.org/10.18632/oncotarget.21779.
Thirukkumar, S., Hemalatha, G., Vellaikumar, S., Amutha, S., & Murugan, M. (2021). Studies on selected cotton seed (*Gossypium* sp) varieties nutrient profile for human consumption in Tamil Nadu. *Journal of Cotton Research and Development*, *35*(1), 79–87.
Underwood, W., & Anthony, R. (2020). AVMA guidelines for the euthanasia of animals: 2020 edition. Retrieved on March, 2013(30), 2020–1.
Vekariya, R., Nimbal, S., Batheja, A., Sangwan, R., & Mandhania, S. (2017). Combining ability and gene action study for seed cotton yield and its related traits in diploid cotton (*Gossypium arboreum* L.). *Electronic Journal of Plant Breeding*, *8*(4), 1159–1168.
Yan, F., Cao, X. X., Jiang, H. X., Zhao, X. L., Wang, J. Y., Lin, Y. H., & Liu, Q. L. (2010). A novel water-soluble gossypol derivative increases chemotherapeutic sensitivity and promotes growth inhibition in colon cancer. *Journal of Medicinal Chemistry.*, *53*(15), 5502–5510. https://doi.org/10.1021/jm1001698.
Zhong, S., Leong, J., Ye, W., Xu, P., Lin, S. H., Liu, J. Y., & Lin, Y. C. (2013). (−)-gossypol-enriched cottonseed oil inhibits proliferation and adipogenesis of human breast pre-adipocytes. *Anticancer Research*, *33*(3), 949–955.

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