

A comprehensive tool in recycling plant-waste of *Gossypium barbadense* L agricultural and industrial waste extracts containing Gossypin and Gossypol: Hepatoprotective, anti-inflammatory and antioxidant effects

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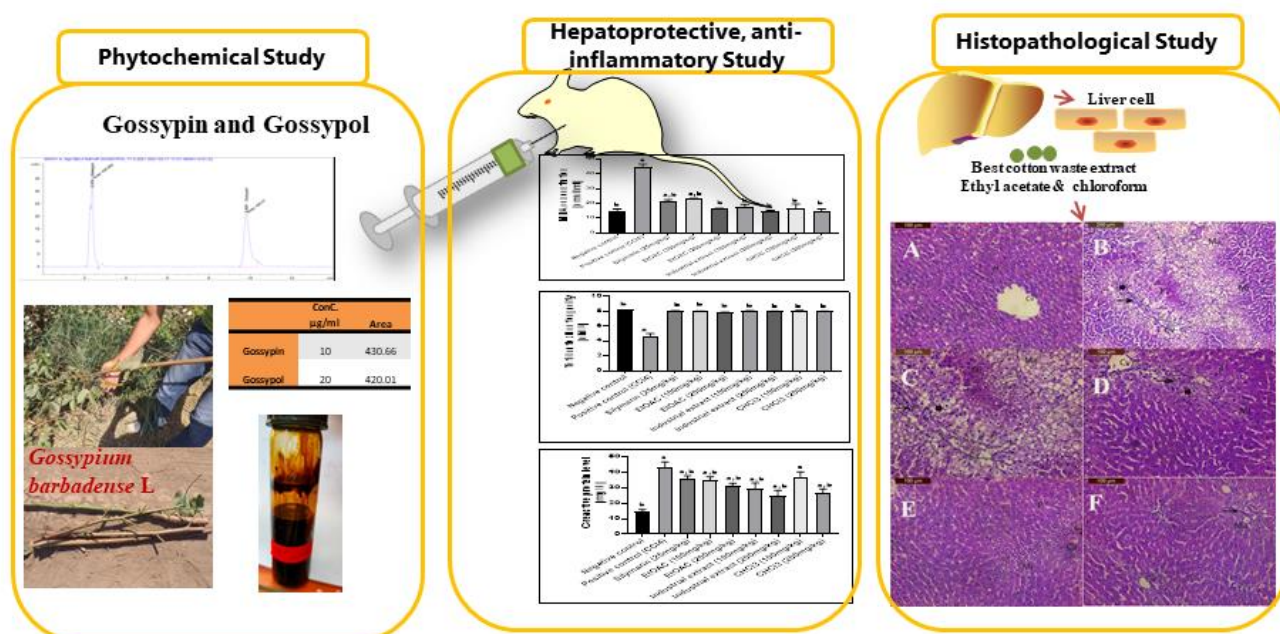
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Graphical Abstract



Abstract:

Improper management of agricultural and industrial cotton wastes causes environmental pollution and worsens the climate change challenge. Green recycling of cotton could contribute to a circular economy. One of the economic values of cotton wastes lies in their bioactive components. Two types of cotton wastes—agricultural and industrial—of the species *Gossypium barbadense* L. Giza 95 were targeted in the current study, aiming to maximize their medicinal value and investigate the anti-inflammatory, hepatoprotective, and antioxidant activities of their phytochemical extracts. Phytochemical extraction was performed using different solvents according to reported methods of extraction, followed by pharmacological studies of the therapeutic effects of some selected extracts. An anti-inflammatory effect was tested in carrageenan-induced acute edema in a rat paw model. A carbon tetrachloride chronic model of liver injury was used for the assessment of hepatoprotective potential. Liver enzymes (AST and ALT), oxidative stress markers (MDA and GSH), inflammatory biomarkers (C-reactive protein), and histopathological features were investigated. As a result, ethyl acetate proved to be the solvent of best choice to extract the gossypin polyphenolics, where the extracted amount reached 14826.2 ug/g, followed by butanol (8751.4 ug/g extract). The chloroform fraction showed the highest amounts of gossypol (190.7 ug/g extract), followed by petroleum ether. Cotton waste's composition analysis showed a wide range of components, including 33 metabolites such as gossypetin, polyphenolics, and other metabolites that possess therapeutic effects. Both chloroform extract and industrial waste extracts showed superior anti-inflammatory and hepatoprotective effects in comparison to other extracts. All tested extracts (ethyl acetate, chloroform, and industrial waste) showed proper antioxidant activities.

Keywords: Solid waste treatment, Gossypol, Gossypin, Anti-inflammatory, Hepatoprotective, Antioxidant, Bioactive components

Background

The management process of solid waste is rendered a major public health issue that invites a big deal of environmental concern especially in urban areas of developing countries. Unfortunately, the improper solid waste management may cause a substantial negative environmental impact that includes health and safety problems and prevalence of diseases that are normally associated with environmental pollution [1].

Solid waste management is a major challenge for many countries, for example the public sector in Egypt is in short of providing the adequate services for solid waste treatment due to the limited existing regulations and the inappropriate local taxation system [2]. As a consequence the illegal disposal of domestic and industrial waste is still the common practice [3-5].

Cotton fiber is considered to be one of the most important natural textile fiber worldwide. It is ranked the third among the total textile market [6] and in Egypt, the Egyptian cotton (*Gossypium barbadense* L.) is considered as an economically important cash crop. The agricultural waste in Egypt is unfortunately misused and is subjected to either burning or improper disposing. This manipulations usually results in the over consumption of potential valuable resources in addition to a non-desirable increase in the Green House Gases emission. Such a situation is inviting the search for new and innovative solutions that allow an eco-friendly and sustainable waste disposal to cotton textile solid wastes [7].

According to Johanson et al. [6] there are two types of cotton textile wastes; pre-consumer and post-consumer. The present research work is concerned with the pre-consumer cotton waste. Normally, after cultivation of cotton, the waste is left in the field where it decomposes. Afterwards, the ginning process takes place where cotton fiber is separated from the lint. The lint is used in the

spinning process and the non-lint fraction, is separated into about 85% as cotton seeds used to obtain oil or stock feed, and a remaining 15% that is classified as a waste product. In most cases, this waste product is used to produce fertilizers, oil spill clean-up, or in ethanol manufacturing. While the cotton fiber is prepared for spinning by the industrial manufacturer with 4.7% separated as waste.

According to literature [8, 9] cotton extract possess a lot of valuable bioactive components. Such bioactive components have been reported to show various activities that include relieving pain, removing phlegm, stopping bleeding, and curing chronic bronchitis. Potent biologically active components in cotton like the flavonoids gossypetin and gossypin, the sesquiterpene aldehyde gossypol, and the trisaccharide gossypose are responsible for these biological and therapeutic effects. For example, gossypol proved inhibitory effect on different cancer diseases, gossypose showed immunomodulatory effects, while gossypin became known for its bactericidal activities. However, less attention is paid to the medicinal value of cotton metabolites with a great desire for more profound and comprehensive investigation of the economic potential for bioactive components that could be extracted from cotton textile solid waste.

In such context and for maximizing the economic benefit of recycling both agricultural and industrial cotton waste, the current study aims to investigate the anti-inflammatory, hepatoprotective, and antioxidant effects of different cotton waste extracts. Phytochemical characterization of extracts that shows biological active moieties with quantitative estimation of gossypin and gossypol is another crucial objective to draw more comprehensive conclusions.

Materials and Methods

Phytochemical Study

Plant materials Egyptian cotton wastes.

The present study was carried out on two pre-consumer cotton textile solid wastes; the agricultural waste (non-lint fraction after removing the seeds), and the industrial waste produced after spinning of cotton fibers. The agricultural waste of the Egyptian cotton; *Gossypium barbadense* L. Giza 95 was obtained from a farm at Beni-suef governorate belonging to the Cotton Research Institute, Cairo, Egypt, and was identified in their laboratories by Dr. Abdel Naser, Deputy Director. Whereas, the industrial waste was obtained from a private factory for the weaving and spinning of cotton in Giza, Egypt.

Plant Extraction

Extraction is the first step to separate the desired natural products from the raw materials. successive extraction methods include different solvent extraction using petroleum ether, dichloromethane, ethylacetate, butanol, and water where pressing and sublimation are experienced according to Hamed et al.,[10].

Pytochemical Investigation

Phytochemical screening:

The following screening procedures have been applied; detection of volatile substances according to Guenther [11], detection of carbohydrates and/or glycosides by using α - naphthol sulphuric acid reagent as reported by Lewis. and Smith [12], detection of tannins according to Shellard [13], detection of alkaloids using Mayer's reagent where the appearance of yellow precipitate indicates the presence of alkaloids [14], detection of flavonoids according to Trease and Evans [15], determination of the presence of flavanones and/or flavonol [16], testing the possible presence of saponins [13], and finally detection of sterols and/or triterpenes as reported by Hanson [17].

Quantitative and qualitative of Gossypin and Gossypol in Cotton wastes by HPLC and LC-ESI-MS/MS technique.

The HPLC system in use was an HP 1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto-sampler (G1329B), quaternary pump, and a diode array detector [18]. The measurements were integrated by Chemstation chromatographic software interfaced to a personal computer. The analytical column was ZORBAX Eclipse XDB C-18 column (15 cm x 4.6 mm I.D., 5 µm, USA) according to Cai, Y., et al. [19].

LC-ESI-MS/MS analysis was performed using an Exion LCAC system for separation and SCIEX Triple Quad 5500+ MS/MS system equipped with an ESI for detection. The separation was performed with an Ascentis® C18 Column (4.6×150 mm, 3 µm). The mobile phases consisted of A: 0.1% formic acid; B: acetonitrile (LC grade) [20]. The mobile phase gradient was programmed as follows: 10% B at 0-1 min, 10-90% B from 1-33 min, 90% B from 33-37 min, 10% at 37.1, 10% from 37.1-40 min. The flow rate was 0.7 ml/min and the injection volume was 10 µl. For MS/MS analysis, negative ionization mode was applied with a scan (EMS-IDA-EPI) from 100 to 1000 Da for MS1 with the following parameters: curtain gas: 25 psi; IonSpray voltage: -4500; source temperature: 500°C; ion source gas 1 and 2 were 45 psi and from 50 to 800 Da for MS2 with a declustering potential: -80; collision energy: -35; collision energy spread: 15. Compounds' identification was performed using MS-DIAL software version 4.70 [21].

Antioxidants activity (DPPH[•] & ABTS^{•+} method).

DPPH[•] and ABTS^{•+} free radical-scavenging activity method was adopted to measure the in vitro antioxidant activity of extracts of the agricultural and industrial waste and their fractions water, butanolic, ethylacetate, dichloromethane, petroleum ether and total extract; at different concentrations (30, 20, 10, 5, 2.5, 1.5, 1 and 0.5 µg/mL) of the eight extracts. Ascorbic acid, trolox, Gossypin and Gossypol were used as standards for reference data. All determinations were carried out in triplicates are illustrated in fig. (4). The radical scavenging model for antioxidant activity, using 1,1-diphenyl-2-picrylhydrazyl (DPPH, 250mM), was performed according to Mohammed et al [22].

ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation (ABTS^{•+}) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate. Appropriate solvent blanks were run in each assay according to Dinkova-Kostova et al. [23]. The percentage inhibition of the DPPH and ABTS radicals was calculated using the following formula:

$$\% \text{ Inhibition} = [(A \text{ control} - A \text{ sample}) / A \text{ control}] \times 100.$$

Where A is the absorbance at 517 nm in DPPH and 734 nm in ABTS.

Pharmacological Study

Animals:

Male Wistar rats weighing between 180 and 200 g were utilized for the experiment. They were obtained from the National Research Center's animal house colony and kept in a standard environment with 12 hour light/dark cycles, a standard pellet diet, and relative humidity of 55 to 10%. The present study complies with local and national guidelines, as it was done in accordance with the guide for care and use of laboratory animals and obtained ethics committee approval certificate from National Research Centre ethics committee numbered [Ethical approval no: 2317/1022021]. Experiments were performed according to the National Regulations of Animal Welfare and the Institutional Animal Ethical Committee (IAEC) and are reported in accordance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

The rats were put to sleep with ketamine at the conclusion of the experiment, for animal anaesthesia during blood sampling from retro-orbital plexus of veins and for euthanasia under anaesthesia d) Formaldehyde (Sigma Chemical Co., St. Louis, MO, USA), for fixation of postmortem tissues dissected for histopathologic examination

Investigation of anti-inflammatory activity of cotton waste extracts in rats:

The carrageenan-induced rat hind paw edema test was used to assess the extract's acute anti-inflammatory activity [24]. Sixty-six adult male Wistar rats were divided into 11 groups, with six rats in each group. First group was given normal saline solution (10 mL/kg, p.o.) and represents the control group. Indomethacin was provided to the second group. (10mg/kg). The third group received gossypin (10 mg/kg). Groups 4-11 received the different cotton extracts; ethyl acetate, total extract, industrial sample, and choloform extracts; respectively. Each extract was given at two dose levels, 100 and 200mg/kg; respectively (according to acute toxicity study). All of the animals received an 0.1 mL injection of 1% (v/v) carrageenan solution in saline at the subplanter region of the right hind paw one hour after receiving the extracts orally. Before administering carrageenan, the volume of each rat's paws was measured using a digital caliber, and then it was checked hourly for the next four hours. The following formula was used to determine each group's percentage of edema:

$$\text{Edema (E) \%} = (V_t - V_0 / V_0) \times 100$$

Where; V_0 is the volume (in milliliters) prior to the carrageenan injection, and V_t is the volume at time t following the injection (mL).

Investigation of hepatoprotective and antioxidant effects of cotton extracts against CCl₄-induced chronic hepatotoxicity in rats:

Nine groups of 54 male Wistar rats (six rats per each group) were used in the current study. The first group was considered a control negative and given olive oil (1 mL/kg, p.o.) as a single dose. After one week, rats were administered olive oil (0.5 mL/kg, p.o.) twice/week and saline (0.5 mL/150 g, p.o.) every day. This group served as the control. The second group (control positive) was given CCl₄ to induce chronic hepatotoxicity. At the beginning, a single loading dose of CCl₄ (1 mL/kg, p.o.) was administered to rats. Then, after one week, CCl₄ (0.5 mL/kg, p.o.) was then administered for four weeks [25, 26]. Groups 3–8 were injected with CCl₄, given the same dose as the second group, and treated with cotton extracts; ethyl acetate, industrial, and CHCl₃ extracts. Silymarin (25 mg/kg), the standard hepatoprotective drug, was given to the 9th group. Each extract was administered orally at two doses of 100 and 200 mg/kg, respectively (according to acute toxicity study). All treatments were administered daily for 4 weeks. Twenty-four hours after last drug administration, animals were anaesthetized, blood samples from the retro-orbital venous plexus were taken, and blood was allowed to clot[27].

Serum was separated using a cooling centrifuge by centrifugation at 3000 rpm for 20 minutes at 4°C (Sigma Laborzentrifugen GmbH, Germany). The liver function was evaluated through measuring serum aspartate transaminase (AST), alanine transaminase (ALT), and oxidative stress indicators (malonedialdehyde (MDA) and total antioxidants (TAC)). C-reactive protein was measured for anti-inflammatory activity using commercially available kits. Then, rats were sacrificed, and livers were removed out and submerged in ice-cold saline solution. For histological analysis, samples from each liver major lobe of 3 animals were fixed in 8% saline-buffered formalin.

Biochemical analysis

Hepatotoxicity indices

Serum AST and ALT were determined using available kits (Biodiagnostic, Egypt) according to the method of **Reitman and Frankel [28]**.

Oxidative stress markers

Ohkawa et al [29] method was used to quantify lipid peroxides in liver homogenates, and Koracevic et al [30] method to measure Total antioxidant capacity (TAC).

Determination of c-reactive protein:

C-reactive protein was assessed by enzyme-linked immunoassay (ELISA) technique according to manufacturer's instructions ((MyBioSource, Inc., San Diego, USA).

Histopathological examinations

Liver samples, were fixed in formalin, treated with a standard alcohol and xylol blend, embedded in paraffin, and sectioned into 5 mm thick sections. Haematoxylin and eosin (H and E) staining were applied to the sections in order to examine the histopathological alterations under a light microscope.

Statistical analysis

Statistical analysis was done using SPSS version 22. The degree of variability in results was expressed as the mean $\pm$ standard error of the mean (SEM). Data were calculated by one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons. The significance level was set at $P < 0.05$.

Results and Discussions

Phytochemical Study

Solvent extraction of cotton waste

A total of 2537 g of powder from the agricultural waste, and 250 g of powder from the industrial waste were separately extracted by methanol (80%), and soaked at room temperature to obtain the total extract for both wastes. Both alcoholic extracts were concentrated under reduced pressure at 45 °C using rotary evaporator, and a yield of 240.811 g of residue was obtained from agricultural waste and 12.22g was obtained from industrial waste [31]. The crude residue from agricultural waste was suspended in 500 ml water, left for the overnight, and successively partitioned (V/V) with 1L pet. ether (40-60%) was gave (40.066g), 1L methylene chloride (8.5544g), 1L ethyl acetate (2.2945g) and 1L n-butanol (19.1818g). The water residue and precipitate extract were then obtained by weight as 150.459g, and 20.256g, respectively (fig (1)).

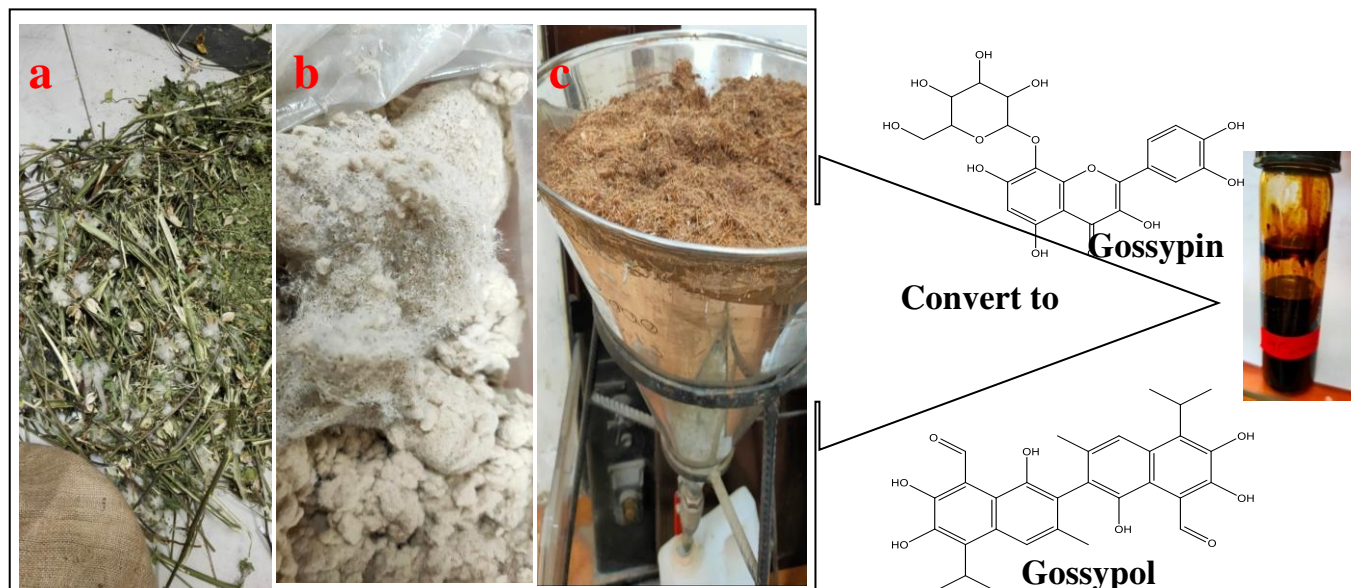


Fig 1. a) Agriculture waste, b) Industrial waste, c) percolator of agriculture waste extraction

Phytochemical screening

The results of the preliminary phytochemical screening of cotton waste extracts are shown in Table (1). The results show the presence of flavonoids, carbohydrates, tannins, triterpenoids and/or steroids, alkaloids, and saponin. Volatile oil was not detected in all of the eight samples. These results agree with the results obtained by Kumar et al (2013) [32] who stated the presence of flavonoids, carbohydrates, saponins, phenols, coumarins, and Cardiac glycosides. However alkaloid, triterpenes, and tannins were detected in our samples.

Table 1. Phytochemical screening of agriculture and industrial wastes

roups	Agricultural waste							Industrial waste
	Total Agri_waste	Pet. ether	CH ₂ CL ₂	EtOAc	Butanol	H ₂ O	Pre_H ₂ O	
Volatile Oils	-	-	-	-	-	-	-	-
Carbohydrate	+++	-	-	+	+++	+++	+	+++
Tannins	+++	-	-	-	+++	+++	+	++
Flavonoids, NaOH	+++	-	+	++	+++	++	+	+++
Flavonoids (Shinoda test)	+++	-	+	++	+++	++	++	+++
Saponin	+++	+++	-	++	+++	+++	-	++
Sterol and / or triterpenes	++	+++	+++	-	-	-	-	+
Coumarins	+++	+++	++	++	++	+	-	++
Alkaloids	++	+	++	++	+	-	-	-

(++), (+), and (-) refer to high, low, and absente amounts, respectively

Quantitative estimation of Gossypin and Gossypol in Cotton waste extracts as determined by HPLC technique.

Determination of gossypol (polyphenolic aldehyde) and gossypin (a glycosyloxyflavone) was done using single different binary gradients for the analysis. Gossypol and gossypin in the seven different agricultural extracts, and the industrial extract followed the following HPLC binary gradient program that was found to be optimum: 0–10 min isocratic with 80:20 aqueous: acetonitrile 0.1% content. The flow rate was 1 mL min and total run time 15 min. The retention time for gossypol was found to be 9.808 min and gossypin 2.374 min. (Fig. S1). This method has proven to be highly reproducible. The maximum detection of both; gossypin and gossypol was detected in chloroform extract as 190.7 ug, and 3539.3 ug, respectively. This method in use is a simple, fast, and cost-effective method for quantification of gossypin and gossypol.

Ethyl acetate showed to be the solvent of best choice to extract the gossypin polyphenolics where the amount obtained reached 14826.2 ug/g which is higher than that extracted by butanol (8751.4 ug/g) as shown in Table (2). Dichloromethane fraction contains the highest amounts of gossypol (190 ug/g extract), followed by petroleum ether that goes in agreement with Clark (1927) [33].

The choice of the proper solvents; CHCl₃ for gossypol, and EtOAc for gossypin may depend on the solubility of the phenolic nature of these components [33]. These findings are in agreement with Meyer et al. (2004) [34] who indicated that the methanol extract is the best solvent to be used for the quantitative HPLC of these phenolics. The industrial waste showed to contain only gossypin and free from gossypol because this waste contain some seed as appear in (fig 1c) that findings are agreements with European Food Safety Authority [35]

Table 2. Quantitative of gossypin and gossypol in agriculture and industrial Cotton waste by HPLC technique

Extracts (conc. µg/g)	Gossypin	Gossypol
1. Total Agricultural waste	5837.4	0.0
2. Petroleum. Ether	1107.1	105.9
3. Chloroform	3539.3	190.7
4. Ethylacetate	14826.2	0.0
5. Butanol	8751.4	0.0
6. Water	811.3	0.0
7. Precipitate _H ₂ O	264.5	0.0
8. Industrial waste	998.4	0.0

LC-ESI-MS/MS technique of cotton waste extracts.

Cotton waste extracts contain a wide range of 33 primary metabolites that function mostly as essential factors in cell biosynthetic pathways. The primary metabolites were identified based on comparison of mass spectra to the MS-Dial library. In this study, we successfully identified by LC-MS a list of various secondary metabolites in two cotton waste extracts which are: polyphenolic acids (Gossypetin, Laricitrin, Ellagic acid, Quinic acid, Isorhamnetin, Vergosin, Deoxyhemigossypol, Kaempferol, and Astragalin), some plant hormones (Dihydrozeatin, and Estrone), vitamins (Threonic acid, and Pantothenic acid), amino acid and other compounds (Table (3)). Secondary metabolites are providing protective mechanisms with an expected hepatoprotective effect. The chemical profiles share compositional similarity with Park et al. [36].

Table 3. LC-MS metabolites of agriculture waste and industrial waste

No	RT (min)	Metabolite name	m/z [M-H] ⁻	Mass fragments	Molecular Formula	Agriculture waste	Industrial waste	Gaussian	S/N	Ref.
1	1.50	2-Hydroxyisobutyric acid	103.10	76	C ₄ H ₈ O ₃		*	0.868897	6.012494	MS-dial
2	1.63	N-Methyllysine	159.25	130/111	C ₇ H ₁₆ N ₂ O ₂	*	*	0.656398	6.071359	MS-dial
3	1.92	Gluconic acid	195.20	-	C ₆ H ₁₂ O ₇		*	0.961515	65.01656	[36]
4	2.10	Cysteic Acid	167.90	152	C ₃ H ₇ NO ₅ S		*	0.719606	26.75691	[37]
5	2.41	4-Isopropylbenzoic acid	162.95	-	C ₁₀ H ₁₂ O ₂	*		0.678502	74.67952	[38]
6	2.73	Dihydrozeatin	220.2	184/167	C ₁₀ H ₁₅ N ₅ O	*		0.817118	12.09927	[39]
7	2.79	Gossypetin	317	221/176/96	C ₁₅ H ₁₀ O ₈	*	*	0.712954	71.22651	[40]
8	2.95	Laricitrin	331.1	191/111	C ₁₆ H ₁₂ O ₈	*	*	0.93064	329.4837	[41]
9	3.23	Equol	241.2		C ₁₅ H ₁₄ O ₃		*	0.702379	14.47749	MS-dial
10	9.64	Ellagic acid	301.15	272/165	C ₁₄ H ₆ O ₈	*	*	0.931935	19.82557	MS-dial
11	9.76	2'-O-Methyl-5-methyluridine	271.1	-	C ₁₁ H ₁₆ N ₂ O ₆		*	0.736028	49.46224	MS-dial
12	10.23	Sucrose	341.1	-	C ₁₂ H ₂₂ O ₁₁	*	*	0.647778	238.7748	[36]
13	12.21	beta-Hydroxymyristic acid	243.3		C ₁₄ H ₂₈ O ₃		*	0.942568	19.94858	[42]
14	12.77	Estrone	269.2		C ₁₈ H ₂₂ O ₂		*	0.72722	5.531574	[43]
15	12.66	Threonic acid	134.9	-	C ₄ H ₈ O ₅	*		0.854257	2.89147	[44]
16	13.6	Pantothenic acid	218.15	-	C ₉ H ₁₇ NO ₅		*	0.908312	82.81514	
17	14.24	Inosine-5-monophosphate	347.1		C ₁₀ H ₁₃ N ₄ O ₈ P	*		0.789652	207.5004	[36]
18	14.38	Malic acid	133.05	-	C ₄ H ₆ O ₅		*	0.949185	10.3574	[36]
19	14.50	Linoleic acid	279.2	-	C ₁₈ H ₃₂ O ₂		*	0.852049	43.42567	[45]
20	15.20	Quinic acid	191		C ₇ H ₁₂ O ₆	*		0.871278	6.95587	[46]
21	15.31	Isorhamnetin	315.1	219/152/108	C ₁₆ H ₁₂ O ₇	*	*	0.726716	26.68773	[47]
22	15.99	6,7-Dihydroxycoumarin	177.15	-	C ₉ H ₆ O ₄	*		0.690777	16.96198	MS-dial
23	16.26	Adenine	133.9	-	C ₅ H ₅ N ₅	*		0.805316	7.804921	[36]

24	16.26	Hypoxanthine	134.95	-	C ₅ H ₄ N ₄ O	*	*	0.845876	5.005095	[36]
25	16.45	Retinoic acid	299.3	-	C ₂₀ H ₂₈ O ₂		*	0.837278	36.04725	MS-dial
26	16.55	Kaempferol	285.2	223	C ₁₅ H ₁₀ O ₆	*		0.840564	124.0466	[40]
27	16.89	N-Acetyltryptophan	245	-	C ₁₃ H ₁₄ N ₂ O ₃	*		0.652122	41.79542	MS-dial
28	16.82	Docosatetraenoic acid	331.35	-	C ₂₂ H ₃₆ O ₂		*	0.936923	10.88153	MS-dial
29	18.57	Octadecanedioic acid	313.3		C ₁₈ H ₃₄ O ₄	*		0.828433	69.35403	[45]
30	20.48	Heneicosanoic acid	325.3	-	C ₂₁ H ₄₂ O ₂		*	0.789929	16.40839	[28]
31	22.18	beta-Bisabolol	221.15	-	C ₁₅ H ₂₆ O	*	*	0.808356	549.4781	[48]
32	22.48	Vergosin	331.3	147	C ₁₆ H ₁₈ O ₃	*	*	0.832863	27.44086	[49]
33	37.03	Deoxyhemigossypol	243.06	153	C ₁₅ H ₁₆ O ₃	*		0.630911	530.6302	[49]
34	38.60	Astragalin	447.11	295/279	C ₂₁ H ₂₀ O ₁₁	*		0.617828	667.2794	[40]

Ref. mean identification compare to literature and MS-Dial database.

Antioxidant activity

DPPH[•] scavenging radical

In the present study, results of the antioxidant activity reflected by the DPPH assay has demonstrated that extracts is relatively the best antioxidant active agent when compared with the other sample but still showed semi to lower antioxidant activity than Vit C, Trolox, gossypol, gossypin at the tested concentrations used (Table (S1) and Fig (2)).

In vitro antioxidant evaluation of total agricultural waste, petroleum ether fraction, dichloromethane fraction, ethylacetate fraction, butanol fraction, water fraction, pre_H₂O fraction and industrial fraction for are presented in Table (S2). All extracts examined against vitamin C and trolox as a standard. The results revealed that from strong to low IC₅₀ gradient as follow 4>5>1>6>2>8>3>7 as (IC₅₀ 0.6612, 0.8908, 1.056, 1.094, 1.894, 2.892, 10.12, and 42.05, respectively). It was also noticed that the ethylacetate and butanol both extracts showed the highest antioxidant effect than other extracts and four standards through the inhibition of the DPPH free radicals Fig 2C [50].

ABTS^{•+} radical cation.

The results give a measure of the antioxidant activity of the ABTS^{•+} through measuring the reduction of the radical cation as percentage inhibition of absorbance at 734 nm. Table (S3) presents the effects of the duration of interaction of specific antioxidants on the suppression of the absorbance of the ABTS^{•+} radical cation at 734 nm for trolox, ascorbic acid, gossypin and gossypol the standard reference compounds. The values of the four standards are compared with those of total agricultural waste, pet ether fraction, dichloromethane fraction, ethylacetate fraction, butanol fraction, water fraction, pre_H₂O fraction and industrial fraction [50].

The antioxidant activity is concentration dependent on the increases as the concentration increase. This is true with the four standards as used. This pattern is also repeated with all eight samples. Considering the antioxidant activity level from high to low IC₅₀ as followed: 5>2>4>1>6>8>3>7 as values (0.9706, 1.252, 1.366, 1.519, 1.927, 4.461, 18.18 and 34.78, respectively) Fig 2D.

The two methods adopted in this part for measuring the antioxidant activity semi confirmed each other, when taking in consideration all fractions. This means that all extracts studied may contain similar chemical group that their effect are attributed to them [23]. In this respect, determination and characterization of such polyphenolics may be useful in developing natural antioxidant substances from a cotton waste material which may add an economic value to beneficial character of the decrease environmental pollution.

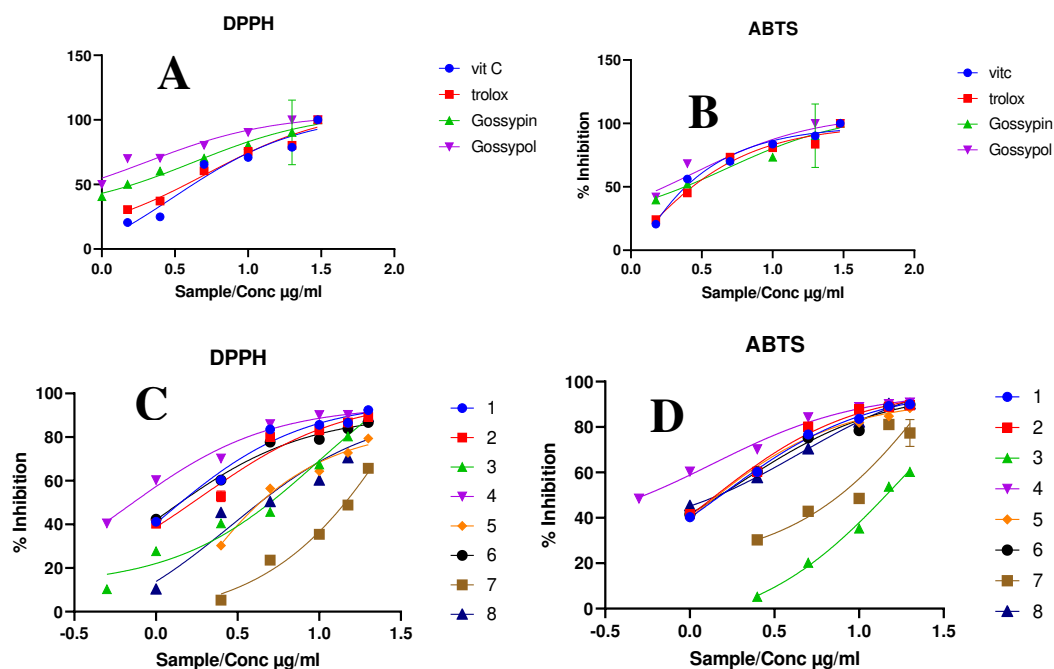


Fig. 2. A) antioxidant activity of Vit C, Trolox, gossypol & gossypin Standard in DPPH & B) ABTS. C) In vitro DPPH: antioxidant activity of different extracts cotton waste as (1=total agricultural waste , 2=pet ether fraction, 3=dichloromethane fraction, 4=ethylacetate fraction, 5=butanol fraction, 6= water fraction, 7=pre_H₂O fraction and 8=industrial fraction) D) and in ABTS⁺. Graphs are constructed to show IC₅₀ using graph prism.

Pharmacological Study

Acute toxicity study

Total, chloroform, EtOAc, and industrial extracts were given orally to male albino mice of both sexes in graduated doses up to 4 g/kg. The equal amount of distilled water was given to the control group. After 24 h, the animals were checked for mortality. Additionally, mice's skin, hair, respiratory, circulatory, and behavioral patterns were examined for any changes. This was performed by dissolving the extracts in physiological saline.

When the plant extract was given to animals at a dose of 4 g/kg, neither mortalities nor toxic symptoms were seen. So, the plant can be utilized safely in accordance to the Organization for Economic Co-operation and Development (OCED) guideline no. 423 (acute class method) because the dose used is larger than 2 g/kg. These findings show that the various cotton extracts are safe and the pharmacological doses were determined accordingly.

Investigation of anti-inflammatory activity

Treatment with gossypin (10mg/kg), industrial extract (200mg/kg), and chloroform extract (100 and 200mg/kg) significantly decreased paw edema as compared to the control group starting from the first hour after carrageenan injection. Their effect was comparable to the indomethacin (standard anti-inflammatory drug) group. This indicates the superior anti-inflammatory properties of these agents over the other groups. The EtOAc (200mg/kg) and Industrial extract (100mg/kg) also induced certain level of anti-inflammatory activity, but, only at the 4th hour after carrageenan injection (Table (4)). The anti-inflammatory activity for gossypin was previously stated in literature [51] and was suggested to be due to its cyclooxygenase-2 inhibitory activity. Being a flavone present as a major constituent in the agricultural cotton waste extract and also in the industrial one, gossypin is thought to be responsible for the currently demonstrated anti-inflammatory activity for these extracts.

Table 4. Effect of different cotton extracts and gossypin on percent carrageenan-induced paw edema (E %):

Groups	AST	ALT
Negative control	93.70 ^b ± 3.71	37.14 ^b ± 3.22
Positive control	237.41 ^a ± 3.62	81.53 ^a ± 4.23
Silymarin (25mg/kg)	129.80 ^{a, b} ± 9.08	53.35 ^b ± 3.21
EtOAc (100mg/kg)	240.30 ^a ± 3.43	75.65 ^a ± 5.67
EtOAc (200mg/kg)	229.5 ^a ± 13.82	74.30 ^a ± 2.31
Industrial (100mg/kg)	186.9 ^{a, b} ± 7.37	70.92 ^a ± 4.93
Industrial (200mg/kg)	159.61 ^{a, b} ± 2.66	61.46 ^{a, b} ± 2.59
CHCl ₃ (100mg/kg)	160.80 ^{a, b} ± 2.15	74.30 ^a ± 2.99
CHCl ₃ (200mg/kg)	131.90 ^{a, b} ± 4.54	43.89 ^b ± 1.91

Every value corresponds to the mean% volume of paw edema (E%) plus SEM (n=6). One-way ANOVA was used for statistical analysis, and Dunnett's post hoc test was then performed. Changes recorded in paw diameter for each group (at t h) from its parallel basal value (at 0 h) were compared to the control group.* Significantly different from the control group (p<0.05) at the relevant time interval. *EtOAc*: ethyl acetate; *CHCl₃*: chloroform.

Effect of cotton extracts on hepatotoxicity induced by CCl₄

In comparison to the negative control group CCl₄ injection (p<0.05) was able to increase serum AST and ALT levels by around 2.5 times [52]. However, when compared to the group that had only received CCl₄, the treatment of rats with the industrial extract (200 mg/kg) and chloroform (CHCl₃) extract (200 mg/kg) considerably lowered liver enzymes. Both extracts had a similar lowering effect on ALT levels to the standard drug, silymarin, and only CHCl₃ effect on AST level was equivalent to silymarin. In addition, industrial extract (100 mg/kg) and chloroform extract (100 mg/kg) induced a significant reduction on AST level as compared to CCl₄-treated group. It is worthy to note that the level of ALT in both silymarin and CHCl₃ groups was not significantly different from that of the negative control group indicating normalization of ALT level. However, ethyl acetate (EtOAc) extract induced non-significant decrease in liver enzymes as compared to the positive control group (Table 5). Similar hepatoprotective effect of another plant species, *Gossypium hirsutum* L., was shown by Batur et al. [53]. This cotton honeydew extracts decreased acute liver injury as reflected by decreased transaminases and enhanced liver antioxidant status.

Table 5: Effect of different cotton extracts on liver enzymes in CCl₄-intoxicated rats:

Groups	1 st h	2 nd h	3 rd h	4 th h
Control	43.55 ± 2.2	48.82 ± 4.56	51.25 ± 3.89	58.66 ± 3.40
Indomethacin (10 mg/kg)	28.01* ± 2.62	37.74* ± 2.35	38.00* ± 3.24	33.56* ± 1.04
Gossypin (10 mg/kg)	29.89* ± 1.98	36.28* ± 3.51	29.13* ± 1.06	27.66* ± 1.92
EtOAc (100mg/kg)	38.99 ± 3.19	43.57 ± 2.80	49.33 ± 0.86	54.17 ± 2.76
EtOAc (200mg/kg)	42.66 ± 1.41	48.86 ± 2.2	49.58 ± 1.4	39.63* ± 3.16
Total extract (100mg/kg)	45.47 ± 1.70	65.96 ± 5.37	66.84 ± 2.03	56.42 ± 4.44
Total extract (200mg/kg)	40.95 ± 1.55	43.42 ± 2.39	49.52 ± 3.74	50.62 ± 4.35
Industrial 100mg/kg)	48.76 ± 2.94	49.23 ± 1.39	54.57 ± 3.21	47.51* ± 3.09
Industrial (200mg/kg)	35.01* ± 1.20	39.80* ± 3.51	40.06* ± 2.04	37.99* ± 2.48
CHCl ₃ (100mg/kg)	26.27* ± 0.58	35.87* ± 2.44	40.05* ± 1.59	30.67* ± 2.26
CHCl ₃ (200mg/kg)	27.12* ± 1.62	33.39* ± 1.11	41.72* ± 0.48	29.16* ± 1.06

Each value represents mean $\pm$ SEM (n = 6). Statistical analysis was carried out by One-way ANOVA followed by Tukey post hoc test. ^asignificantly different from negative control. ^bsignificantly different from positive control (CCl₄) at p < 0.05.

3.2.3.1. Antioxidant activity of cotton extracts in CCl₄-intoxicated rats:

CCl₄ injection induced a significant increase in MDA level (about 3 fold) when compared to the negative control group indicating increased lipid peroxidation (**fig 3**). Treatment with different cotton extracts decreased MDA significantly as compared to the CCl₄ group in a dose-dependent manner. The MDA level in groups treated with EtOAc extract (200mg/kg), industrial extract (100 & 200mg/kg), and CHCl₃ extract (100 & 200mg/kg) did not differ significantly from that of the normal value and was better than the standard drug, silymarin.

Additionally, CCl₄ induced a depression of total antioxidant capacity (TAC) in comparison to the negative control group. However, administration of all investigated cotton extracts significantly enhanced TAC as compared to the CCl₄-treated group. The TAC for all extracts was comparable to silymarin (**Fig 4**).

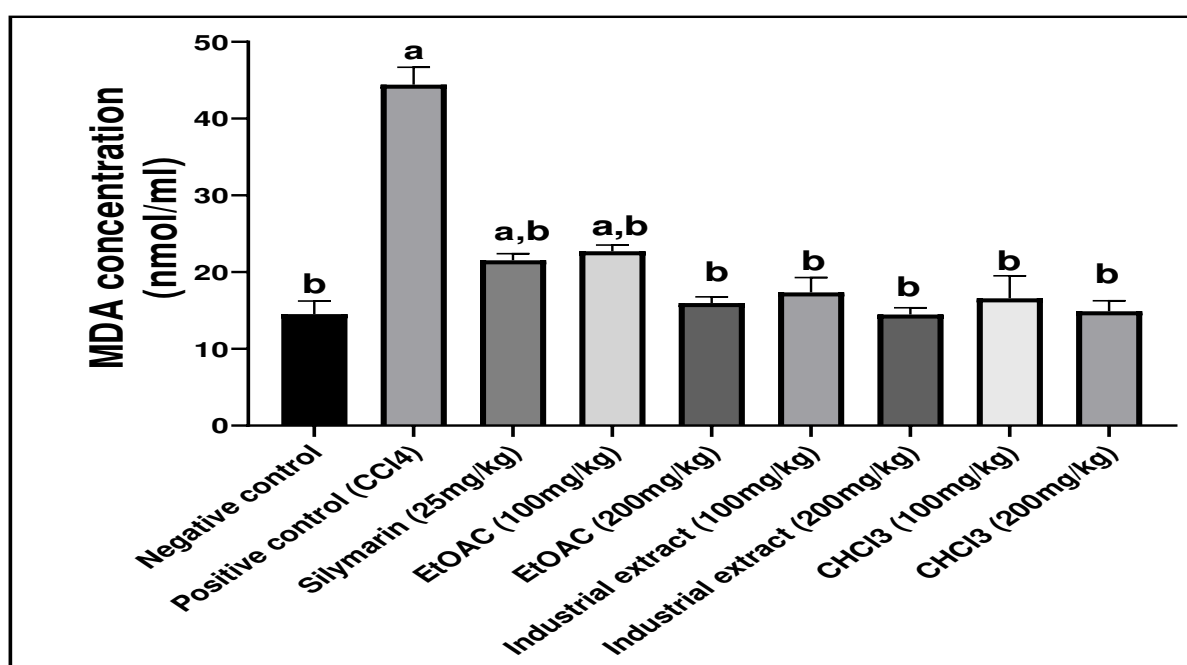


Fig 3: Effect of different cotton extracts on MDA level in CCl₄-intoxicated rats.

Each number corresponds to the mean $\pm$ SEM (n = 6). One-way ANOVA was used for statistical analysis, followed by the Tukey post hoc test. ^asignificant difference from negative control. ^bsignificant difference from positive control (CCl₄) at p < 0.05.

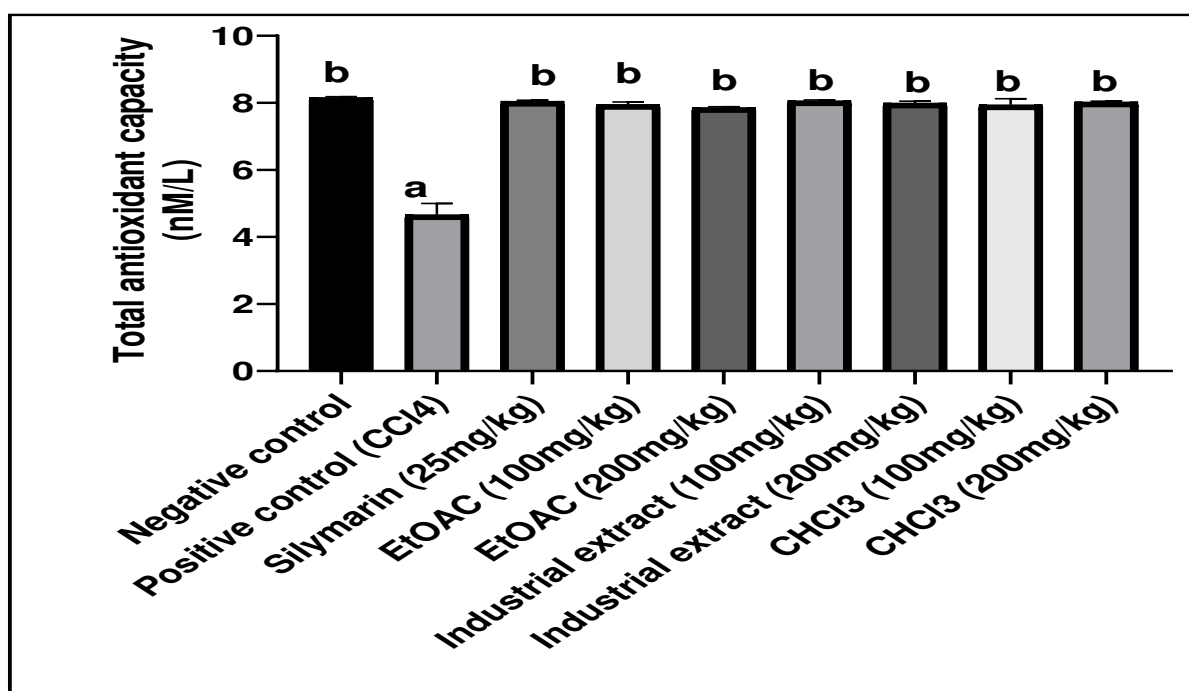


Fig 4: Effect of different cotton extracts on TAC level in CCl₄-intoxicated rats. Each number corresponds to the mean $\pm$ SEM (n = 6). One-way ANOVA was used for statistical analysis, followed by the Tukey post hoc test.^asignificant difference form negative control. ^bsignificant differencefrom positive control (CCl₄) at p< 0.05. TAC: total antioxidant capacity.

By causing irreversible changes to the lipids, proteins, DNA contents and, more importantly, by altering the pathways that regulate normal biological functions, oxidative stress causes hepatic damage and progression of a variety of liver diseases. These include chronic viral hepatitis, alcoholic liver diseases, and non-alcoholic steatohepatitis [54]. In the CCl₄-induced liver injury model, oxidative stress is triggered, causing lipid peroxidation to damage the hepatocellular membrane. Subsequently, a significant amount of pro-inflammatory chemokines and cytokines may be released, which may result in more liver injury [55].

Antioxidants have been applied to the treatment of liver disease, thus, showed hepato-protection partially via improving oxidative damage [56]. In the current study, EtOAc, industrial, and CHCl₃ extracts of *Gossypium barbadense* L showed better antioxidant activities than the standard drug, silymarin. Similarly, *Gossypium herbaceam* was previously found to possess good antioxidant properties due to flavonoid-rich part and was capable to be used in prevention of Alzheimer disease [47].

Effect on the inflammatory marker; C-reactive protein:

CCl₄ injection significantly (P < 0.05) increased inflammatory indicator, C-reactive protein, as compared to the negative control group indicating increased lipid peroxidation (**fig 5**). Administration of different cotton extracts except CHCl₃ (100mg/kg) significantly decreased serum level of C-reactive protein as compared to the CCl₄-intoxicated group. Both industrial extract (200mg/kg) and CHCl₃ extract (200mg/kg) showed the best anti-inflammatory activity which was superior to the standard hepato-protective drug; silymarin.

Hepatic inflammation, which is thought to be the primary cause of hepatic tissue destruction, is a frequent trigger of liver disease. Oxidative stress damage initiates the inflammatory response by

activation of the innate immunity. This, in turn, aggravates the consequent processes of fibrosis, cirrhosis [57].

In parallel, the anti-inflammatory activity for other *Gossypium* species was previously shown. *Gossypium herbaceum* could inhibit Nuclear factor kappa-B activity (NF-kappaB) [58]. Another cotton extract flavone component, Gossypetin, also showed anti-inflammatory properties after it had been isolated from the flowers of *Hibiscus sabdariffa* [59]. Gossypin, isolated from *Hibiscus vitifolius* and also present in cotton extract, prevented carcinogenesis progression via its anti-inflammatory properties. It suppressed tumor necrosis factor and NF-kappaB ligand, thus, down-regulated gene products involved in cellular invasion and osteoclastogenesis [60].

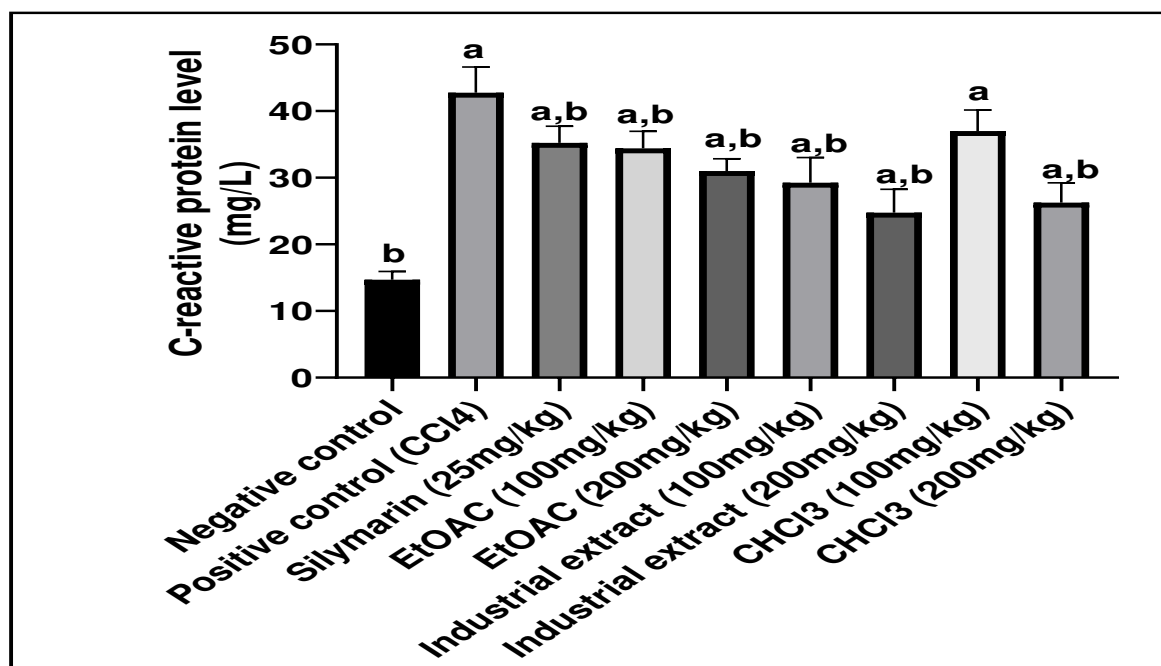


Fig 5: Effect of different cotton extracts on c-reactive protein level in CCl4-intoxicated rats. Each number corresponds to the mean $\pm$ SEM (n = 6). One-way ANOVA was used for statistical analysis, followed by the Tukey post hoc test. ^asignificant difference from negative control. ^b significant difference from positive control (CCl4) at p < 0.05.

Histopathological examination:

Sections of the liver from the control group showed the hepatocytes' typical architecture to be composed of branching cords that extended outward from the central veins. These cords were separated by blood sinusoids, acidophilic cytoplasm with single central rounded vesicular nuclei (**Fig.6A**).

Examination of sections of the rat liver received CCL4 injections for four weeks showed microvesicular and macrovesicular fatty changes of hepatocytes, centrilobular necrosis in most cases, ballooning degeneration and broad mononuclear cells infiltration mostly around central veins and in portal areas with mild fibrous tissues. Also, loss of the usual hepatic architecture, deep acidophilic cytoplasm and deeply stained nuclei and many blood vessels were obviously congested (**Fig. 6B & 6C**). Examination of the CCL4 and chloroform extract (100mg/kg) treated group showed moderate improvement in hepatic structure with microvesicular fatty changes of hepatocytes, mild inflammatory cells infiltration and pyknotic nuclei (**Fig.6D**). The liver section of CCl4 and chloroform extract 200 treated group showed ameliorative effect with congestion of central vein, microvesicular fatty changes of hepatocytes, and pyknotic nuclei (**Fig.6E**). In the group treated with CCl4 and ethyl acetate extract 100 showed more or less normal hepatic structure with microvesicular fatty changes of hepatocytes mild inflammatory cells infiltration (**Fig.6F**).

In the group treated with CCl₄ and ethyl acetate extract (200mg/kg) showed more or less normal hepatic structure with congestion of central vein, macrovesicular fatty changes of hepatocytes, ballooning degeneration and mild inflammatory cells infiltration (**Fig.7A**). Liver sections of CCl₄ with industrial (100mg/kg) group showed reduced the development of histopathological damage except microvesicular fatty changes of hepatocytes mild inflammatory cells infiltration and pyknotic nuclei (**Fig.7B**). Liver sections of CCl₄ with industrial (200mg/kg) group showed improved hepatic structure except little congestion of central vein, microvesicular fatty changes of hepatocytes and pyknotic nuclei (**Fig.7C**). Silymarin and CCl₄ group section showed moderate improvement with less inflammation and less ballooning and moderate microvesicular fatty changes of hepatocytes was observed (**Fig.7D**). In consistence with the biochemical results, the histopathological finding confirmed the hepatoprotection exhibited by EtOAC, CHCl₃, and industrial cotton waste extracts. The reduction in hepatic injury was superior in rats treated by CHCl₃, and industrial cotton waste extracts at high doses.

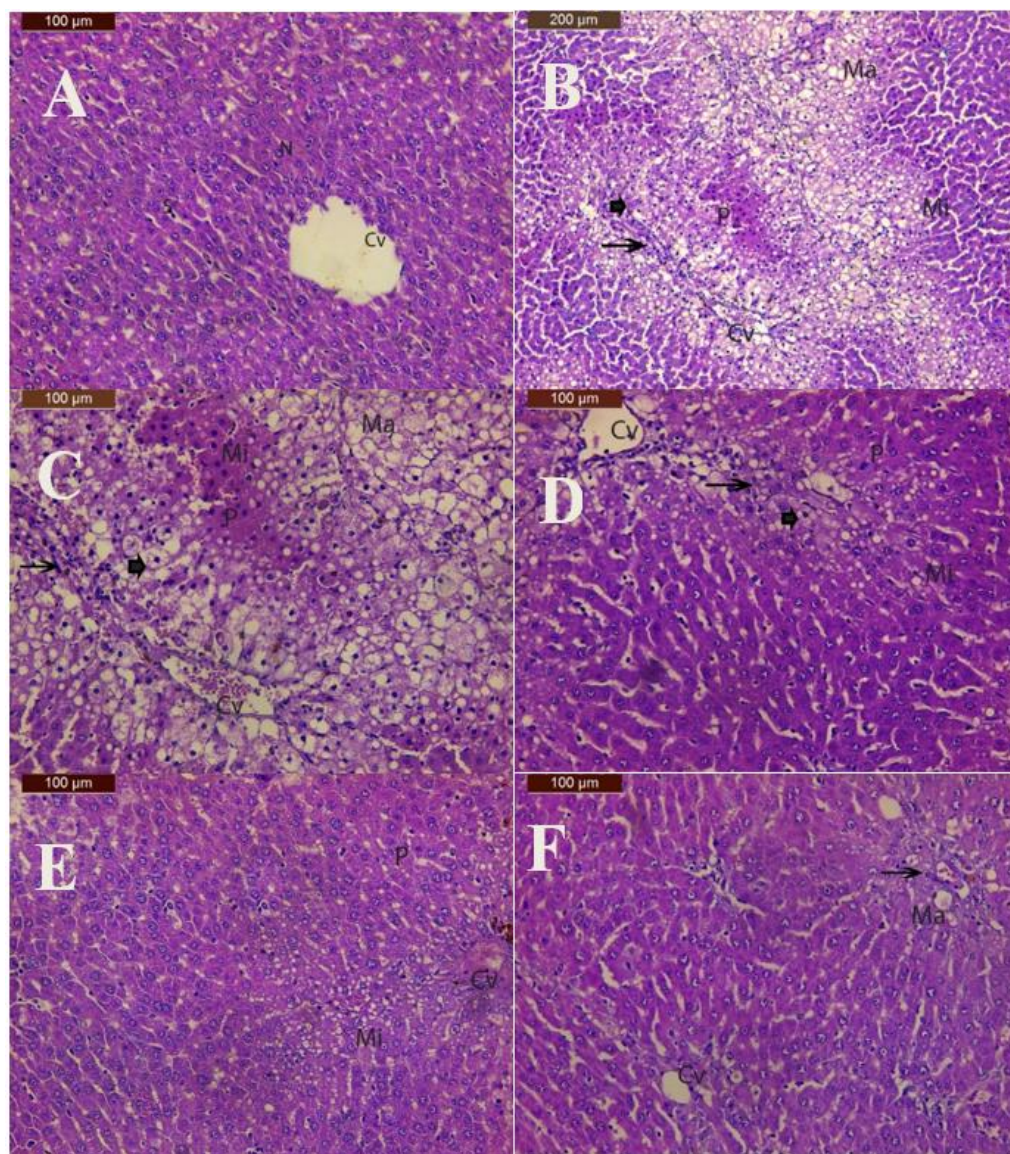


Fig. 6 A: A photomicrograph of liver of control group showing normal histological architecture with central vein (CV), the hepatic cells with nucleus (N) and the sinusoids (S). **B:** A photomicrograph of liver of CCl₄ group showing microvesicular (Mi) macrovesicular (Ma) fatty changes of hepatocytes, centrilobular necrosis in most cases, ballooning degeneration (arrowhead) broad mononuclear cells infiltration mostly around central veins and in portal areas with mild

fibrous tissues (arrow), loss of the usual hepatic architecture, deep acidophilic cytoplasm and deeply stained nuclei (P) and many central vein were obviously congested (CV). **C:** High magnification of pervious figure of liver of CCL₄ group showing microvesicular (Mi) macrovesicular (Ma) fatty changes of hepatocytes, centrilobular necrosis in most cases, ballooning degeneration (arrowhead) broad mononuclear cells infiltration mostly around central veins and in portal areas with mild fibrous tissues (arrow), loss of the usual hepatic architecture, deep acidophilic cytoplasm and deeply stained nuclei (P) and many central vein were obviously congested (CV). **D:** A photomicrograph of liver of CCL₄ and chloroform extract (100mg/kg) group showing moderate improvement in hepatic structure with microvesicular fatty changes of hepatocytes (Mi), ballooning degeneration (arrowhead), mild inflammatory cells infiltration (arrow) and pyknotic nuclei (P). **E:** A photomicrograph of liver of CCL₄ and chloroform extract (200mg/kg) group showing microvesicular fatty changes of hepatocytes (Mi), ballooning degeneration (arrowhead), mild inflammatory cells infiltration (arrow), congestion of central vein (CV) and pyknotic nuclei (P). **F:** A photomicrograph of liver of CCL₄ and ethyl acetate extract (100mg/kg) group showing more or less normal hepatic structure with macrovesicular fatty changes of hepatocytes (Ma) mild inflammatory cells infiltration (arrow).

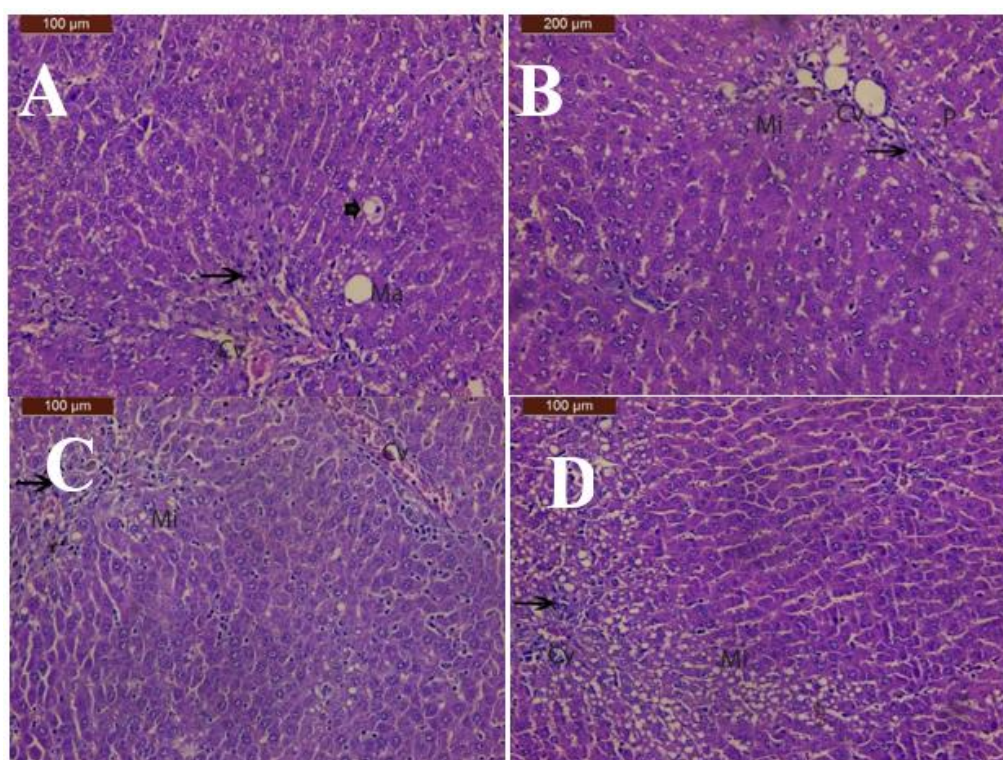


Fig. 7A: A photomicrograph of liver of CCL₄ and ethyl acetate extract (200mg/kg) group showing more or less normal hepatic structure with congestion of central vein (CV), macrovesicular fatty changes of hepatocytes (Ma), ballooning degeneration (arrowhead) and mild inflammatory cells infiltration (arrow). **7B:** A photomicrograph of liver of CCL₄ and industrial (100mg/kg) group showing Liver reduced the development of histopathological damage except microvesicular fatty changes of hepatocytes (Mi) mild inflammatory cells infiltration (arrow) and pyknotic nuclei (P). **7C:** A photomicrograph of liver of CCL₄ and industrial (200mg/kg) group showing Liver decrease the development of histopathological damage except congestion of central vein (CV), microvesicular fatty changes of hepatocytes (Mi) and pyknotic nuclei (P). **7D:** A photomicrograph of liver of CCL₄ and silymarin group showing moderate improvement with less inflammation and less ballooning and moderate microvesicular fatty changes of hepatocytes (Mi).

Conclusion

Comparative analysis of gossypin and gossypol content was performed on different fractions of cotton solid waste extracts. The results indicated ethyl acetate containing higher gossypin levels than butanol fraction. Gossypol was also found with greater amounts in chloroform rather than petroleum ether. The choice of the proper solvents; CHCl₃ in case of gossypol and EtOAc in case of

gossypin may depend on the solubility desired by their phenolic nature. Chloroform extract and industrial waste extract showed superior anti-inflammatory and hepatoprotective effects against CCl₄ induced hepatotoxicity in a dose-dependent manner, as demonstrated by the biochemical indicators' considerable recovery. Ethyl acetate, chloroform, and industrial waste extracts showed proper antioxidant activities. The hepatoprotective efficacy of these extracts was further supported by the histopathological analysis. The presence of the flavonoid gossypin, as well as the other active ingredients, is thought to be associated with the hepatoprotective effect of cotton waste extracts. The extraction of bioactive substances with biological activity using the proper extraction method as demonstrated by the present work could help in green economy due to the commercial value of the outcome. Besides, this may contribute to climate change mitigation efforts as it introduces an ecofriendly alternative way of disposal of cotton solid wastes both in the farm and in industry.

Abbreviations List:

HPLC	High Performance Liquid Chromatography
LC-ESI-MS/MS	Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry
ESI	Electrospray Ionization
EtOAc	Ethyl Acetate
CHCl₃	Chloroform
TAC	Total Antioxidant Capacity.
DPPH	2,2-Diphenylpicrylhydrazyl
ABTS	2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
CCl₄	Carbon Tetrachloride
NF-kappa B	Nuclear Factor-kappa B activity

Supplementary Information

The online version contains supplementary material available at https://docs.google.com/document/d/1NJWX-UOqMJyevjslPrRJwhHkwSHN9ren/edit?usp=drive_link&ouid=111245179703153265112&rtpof=true&sd=true

Additional file 1: **Fig S1 HPLC of standard Gossypin and Gossypol.**

Additional file 2: **Fig S2 Determination of gossypin and gossypol by HPLC in eight samples.**

Additional file 3: **Table S1. *In vitro* ABTS+ and DPPH antioxidant activity of Standards.**

Additional file 4: **Table S2. *In vitro* DPPH antioxidant activity of different extracts cotton waste.**

Additional file 5: **Table S3. *In vitro* ABTS+ antioxidant activity of different extracts cotton waste.**

Author contributions

Mona A. Mohammed¹: Designed the plan of the plant material, phytochemical study, and were responsible for performing the extraction of plant extract as well as identification and characterization part and in vitro studies, they shared in writing and revising the phytochemical section and all manuscripts; **Heba M. I. Abdallah**³: was responsible for designing, performing, writing and revising the pharmacological study including the acute toxicity, subchronic toxicity and efficacy studies of the extracts, biochemical analysis, Histopathological study, they shared in writing and revising their section and all manuscripts; **Nagat M. Amer**² and **Mai S. Saleh**²: were principal investigator and co-investigator of the project under Title 'prediction of health risk among textile workers and obtaining commercially valuable compounds from cotton industry waste' and they shared in revising all manuscripts. All authors contributed to improving the paper and approved the final manuscript.

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Data availability

The datasets used during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval

Authors declare that the study complies with current ethical considerations.

Ethical issues including: Plagiarisms, misconduct, data fabrication, falsification, double publication or redundancy were considered by the authors.

All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). Experiments were performed according to the National Regulations of Animal Welfare and the Institutional Animal Ethical Committee (IAEC).

The present study was done in accordance with the guide for care and use of laboratory animals and obtained ethics committee approval certificate from National Research Centre ethics committee numbered [Ethical approval no: 2317/1022021].

Competing interests

The authors declare that they have no conflict of interest to the publication of this article.

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