RESEARCH PAPER

Protein profiling and antioxidant enzymatic activity of the ethanol extract of *Cocculus hirsutus* (L.) W. Theob. efficacy against *Rhynchophorus ferrugineus*

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ABSTRACT

The red palm weevil (*Rhynchophorus ferrugineus*) is one of the most destructive pests affecting economically important palm species worldwide. The present study investigated the insecticidal potential of the ethanol extract of *Cocculus hirsutus* and evaluated its effects on antioxidant enzyme activity and protein profiles in *R. ferrugineus* larvae. Leaves of *C. hirsutus* were collected and extracted using ethanol, and the chemical constituents of the extract were analysed through liquid chromatography–mass spectrometry (LC-MS). A total of 122 compounds were detected, including biologically active molecules such as hirsutine, gallic acid, resveratrol, ferulic acid, and quercetin derivatives. Larvae of *R. ferrugineus* were exposed to different concentrations (2–10%) of the plant extract. Higher concentrations (8% and 10%) caused rapid mortality of larvae, while the 2% treatment showed negligible effects and was excluded from subsequent biochemical analyses. Antioxidant enzyme assays revealed a marked reduction in catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and polyphenol oxidase (PPO) activities in treated larvae compared with the control, indicating a disruption of oxidative stress defence mechanisms. SDS-PAGE analysis further demonstrated alterations in protein expression, with control larvae showing multiple protein bands, whereas treated larvae exhibited progressively faint bands at higher extract concentrations. These findings suggest that *C. hirsutus* ethanol extract induces oxidative stress and protein alterations in *R. ferrugineus* larvae, contributing to their mortality. The study highlights the potential of *C. hirsutus* as a promising botanical pesticide for the sustainable management of red palm weevil infestations.

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INTRODUCTION

Cocculus hirsutus (L.) is a perennial climber distributed in tropical and subtropical areas of South Asia and Africa. In Asia, it is reported from India, Myanmar, Nepal and Pakistan, and Southern China. It comes under the phylum Spermatophyta, the class Magnoliopsida, and the family Menispermaceae (Madhavan *et al.*, 2010). Various plant parts are used to cure rheumatism, dyspepsia, skin conditions, impotence, and constipation, while the leaves are used to treat gonorrhoea, eczema, neuralgia, ophthalmia, and leucorrhea. Additionally, alcoholic leaf the leaves of the plant were extracted using alcohol)and stem extract have hypotensive and anticancer properties (Logesh *et al.*, 2020; Singh, 2015; Thavamani *et al.*, 2013). They used to cure many diseases because of their high medicinal properties.

Rhynchophorus ferrugineus, also known as red palm weevil (RPW), is a dangerous and deadly pest to many palm species. One of the major palms is known as the oil palm (*Elaeis guineensis),* which is an economically important crop throughout the world. It is mainly cultivated as a source of oil and commercially used as soap, candles, fuels, cattle feed, cosmetics and many pharmaceutical products. There are more than 193 species of pests were affect the palm worldwide. Among these, RPW is a severe and major one to affects this palm. Due to the concealed nature of their life cycle, they are unable to control. The adults and their grubs feed on the trunk and other regions of the palm tree, which leads to the wilting of the whole tree, thus having a major impact on cultivated palm tree plantations. This results in serious economic and biodiversity losses in several countries. Hence, the present study focused on the pest of oil palm (*E. guineensis)* known as *R. ferrugineus* was controlled by using *C. hirsutus* plant ethanol extract, and their protein profiling and antioxidant enzyme activity were determined.

MATERIALS AND METHODS

Collection of plants

Firstly, the plant was noticed, and the leaves were collected during the monsoon season inAugust from the Semmedu area, which is in the Coimbatore District of Tamil Nadu. The plant was authenticated by the Botanical Survey of India (BSI), Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The collected plant leaves were washed with water and shade-dried. The shade-dried plant was used for further extraction. The crude samples were successfully extracted in a Soxhlet apparatus using ethanol. The ratio of plant weight was 750:300 (fresh leaf: dry leaf), respectively. Ethanol extracts of *C. hirsutus* were analysed in Liquid chromatography-mass spectrometry (LC-MS).

Identification and isolation of compounds

LC-MS analysis of the ethanol extract of *C. hirsutus* was carried out using a Shimadzu LC-MS/MS-8030 triple quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) source. Chromatographic separation was achieved on an Acquity UPLC BEH C18 column (Shimadzu Corporation, 2019).

Rearing of *R. ferrugineus*

For the mass rearing ofRPW, the earlier stages were maintained on soft diet medium; once they attained their 5th stage, they were transferred to sugarcane stem medium because of their aggressive feeding habit. Throughout the study, *R. ferrugineus* culture was reared and maintained in the Entomology Laboratory, Department of Zoology, Government Arts College, Coimbatore, Tamil Nadu, India.

Experimental analysis

For analysis, the samples of infected and control larvae were homogenised in 2 ml of 5% trichloroacetic acid and centrifuged for twenty minutes at 10000 rpm at 4°C.

Estimation of antioxidant enzyme activity

Catalase assay

The whole larva homogenate 500 µl was mixed with 500 µl of H_2O_2 and 2 ml of phosphate buffer, and the results were compared to a control cuvette containing onlyhydrogen peroxide (H_2O_2) and phosphate buffer. At 240 nm, absorbance was measured. The change in

absorbance was recorded every 30 seconds up to 2 minutes in a spectrophotometer (Wang and Jiao, 2001).

Superoxide dismutase

The prepared reaction mixture was added to 100 µl of larvae homogenate extract and incubated at 30°C for 5 minutes. At last, 80 µl of 50 µM riboflavin was added. The tubes were exposed to a 200 W fluorescent lamp for 10 minutes. After exposure, 1 ml of Griess reagent was added. The absorbance of the colour formed was measured at 543 nm (Marklund and Marklund, 1974).

Perxidase assay

2400 µl of pyrogallol solution and 100 µl of the whole larvae homogenate sample were mixed well with 0.5 ml of H_2O_2. The absorbance, which occurs in the reaction, was recorded every 30 seconds up to 3 minutes at 430 nm with a UV–Visible spectrophotometer (Reddy *et al.*, 2004).

Ascorbate peroxidase assay

1 ml of sample was added to 2.7 ml of 100 mm phosphate buffer solution (PBS) (pH 7.2), mixed well, then added with 100 µl of L-ascorbic acid and 100 µl of H_2O_2. At 290 nm, absorbance was measured (Nakano and Asada, 1981).

Polyphenol oxidase

300 µl of enzyme extract was added to 1200 µl of phosphate buffer, then mixed well, and after thorough mixing, 500 µl of Catechol was added to adjust the final volume. A UV-Visible spectrophotometer was used at 420 nm to measure the absorbance (Gulen and Eris, 2004).

Protein profiling using SDS-PAGE gel electrophoresis

SDS-PAGE was carried out (Laemmli, 1970) to profile the proteins present in the sample of infected and uninfected *R. ferrugineus*. Twelve per cent separating gel was prepared using the following components: 3 ml acrylamide solution, 1.85 ml Tris free base (pH 8.8), 2.55 ml distilled water, 100 µl of 10% SDS, 100 µl of 10% ammonium persulfate (APS), and 10 µl of Tetramethylethylenediamine (TEMED) for mini gel.

Add TEMED and APS at the end, then the solution was poured into the glass plates and leaving one-third of the space for stacking gel. A four per cent stacking gel was prepared with the following components: 1 ml of acrylamide, 1 ml of Tris free base (pH 6.8), 1 ml of distilled water, 100 µl of 10% APS and 15 µl of TEMED for mini gel. Once the stacking gel was poured, the comb was placed in the gel. Allow for polymerisation for 10-15 minutes. 50 µl of the sample was mixed with 10 µl of the sample buffer and heated for 5 minutes at a boiling temperature, and then cooled down.

The polymerised gel was mounted in the electrophoresis apparatus containing the electrode buffer, and the samples, along with a protein molecular weight marker, were loaded into the wells. Electrophoresis was conducted at 50 V until the dye front reached the separating gel, and then the voltage was increased to 100 V until completion. The power supply was turned off, gel plates were removed, and the gel was transferred to the staining solution after washing with distilled water. The staining solution was used to incubate the gel under shaking in the dark for four hours. The gel was de-stained by incubating in the de-staining solution in the dark. The blue bands were visualised under a white/fluorescent light source/gel doc.
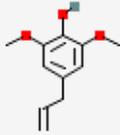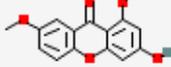
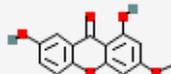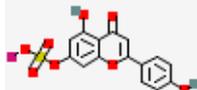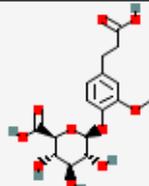**RESULTS**

***C. hirsutus* - ethanol extract against RPW**

RPW grub was infected with different dosages of 2%, 4%, 6%, 8% and 10% of *C. hirsutus* ethanol extract, respectively. In a 10 % dosage, the death of RPW larvae was observed at 14 hours and followed by 16 hours in 8% dosage. Thus, the infected larvae, along with controls, were taken to further studies. The 2% treatment showed negligible effects and was therefore excluded from further biochemical analysis.
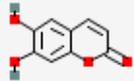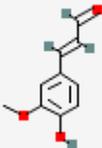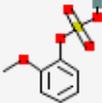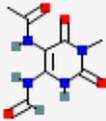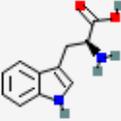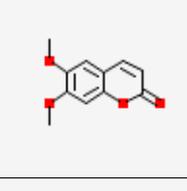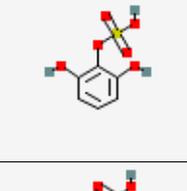
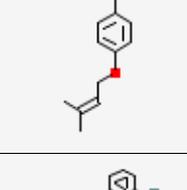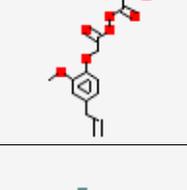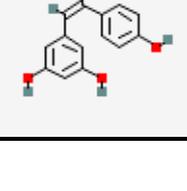**LC-MS ethanol extract of *C. hirsutus***

The following 122 compounds were identified through LC-MS, and are listed in Table 1. Among those compounds, the six compounds like hirsutine, gallic acid, resveratrol (trans-), quercetin 3'-O-glucuronide, urolithin A, and ferulic acid are previously reported to have medicinal properties.
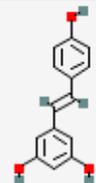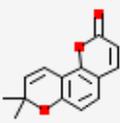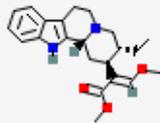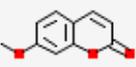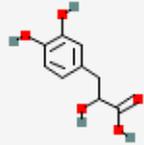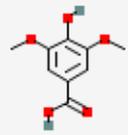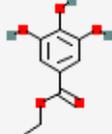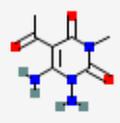
Table 1. Chemical compounds from LC-MS ethanol extract of *C. hirsutus*

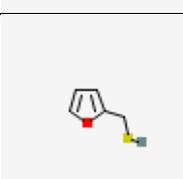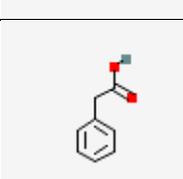| Sl	Compound name	IUPAC name	Molecular formula	Structure
1.	2,4,6-Trihydroxybenzoic acid	2,4,6-trihydroxybenzoic acid	$C_7H_6O_5$	
2.	Gallic acid	3,4,5-trihydroxybenzoic acid	$C_7H_6O_5$	
3.	3,4-Dihydroxyphenylglycol	4-(1,2-dihydroxyethyl)benzene-1,2-diol	$C_8H_{10}O_4$	
4.	Scopoletin	7-hydroxy-6-methoxychromen-2-one	$C_{10}H_8O_4$	
5.	Myristicin	4-methoxy-6-prop-2-enyl-1,3-benzodioxole	$C_{11}H_{12}O_3$	
6.	Isoferulic acid	(E)-3-(3-hydroxy-4-methoxyphenyl)prop-2-enoic acid	$C_{10}H_{10}O_4$	
7.	Ferulic acid	(E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic acid	$C_{10}H_{10}O_4$	
8.	Isobergapten	5-methoxyfuro[2,3-h]chromen-2-one	$C_{12}H_8O_4$	
9.	Xanthotoxin	9-methoxyfuro[3,2-g]chromen-7-one	$C_{12}H_8O_4$	
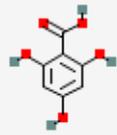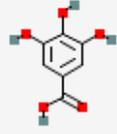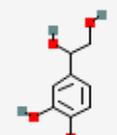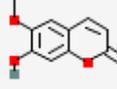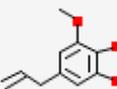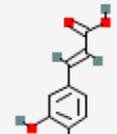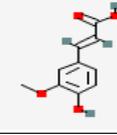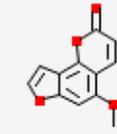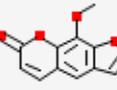
10.	Sphondin	6-methoxyfuro[2,3-h]chromen-2-one	$C_{12}H_8O_4$	
11.	Bergapten	4-methoxyfuro[3,2-g]chromen-7-one	$C_{12}H_8O_4$	
12.	Caffeine	1,3,7-trimethylpurine-2,6-dione	$C_8H_{10}N_4O_2$	
13.	Methoxyeugenol	2,6-dimethoxy-4-prop-2-enylphenol	$C_{11}H_{14}O_3$	
14.	5-(3'-hydroxyphenyl)valeric acid	5-(3-hydroxyphenyl)pentanoic acid	$C_{11}H_{14}O_3$	
15.	Isogentisin	1,3-dihydroxy-7-methoxyxanthen-9-one	$C_{14}H_{10}O_5$	
16.	Gentisin	1,7-dihydroxy-3-methoxyxanthen-9-one	$C_{14}H_{10}O_5$	
17.	Apigenin 7-O-sulfate	potassium [5-hydroxy-2-(4-hydroxyphenyl)-4-oxochromen-7-yl] sulfate	$C_{15}H_9KO_8S$	
18.	Dihydroferulic acid-4'-O-glucuronide	(2S,3S,4S,5R,6S)-6-[4-(2-carboxyethyl)-2-methoxyphenoxy]-3,4,5-trihydroxyoxane-2-carboxylic acid	$C_{16}H_{20}O_{10}$	
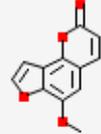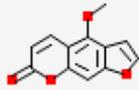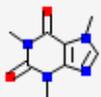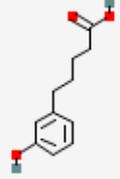
19.	Methyl allyl disulfide	3-(methyldisulfanyl)prop-1-ene	$C_4H_8S_2$	
20.	Allyl methyl sulfone	3-methylsulfonylprop-1-ene	$C_4H_8O_2S$	
21.	4-Vinylphenol	4-ethenylphenol	C_8H_8O	
22.	Diallyl trisulfide	3-(prop-2-enyltrisulfanyl)prop-1-ene	$C_6H_{10}S_3$	
23.	Esculetin	6,7-dihydroxychromen-2-one	$C_9H_6O_4$	
24.	Ferulaldehyde	(E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enal	$C_{10}H_{10}O_3$	
25.	Guaiacol sulfate	(2-methoxyphenyl) hydrogen sulfate	$C_7H_8O_5S$	
26.	5-acetylamino-6-formylamino-3-methyluracil	N-(6-formamido-3-methyl-2,4-dioxo-1H-pyrimidin-5-yl)acetamide	$C_8H_{10}N_4O_4$	
27.	Tryptophan	(2S)-2-amino-3-(1H-indol-3-yl)propanoic acid	$C_{11}H_{12}N_2O_2$	

28.	Butin	(2S)-2-(3,4-dihydroxyphenyl)-7-hydroxy-2,3-dihydrochromen-4-one	$C_{15}H_{12}O_5$	
29.	Histamine-betaxanthin	(2S,4E)-4-[2-[2-(1H-imidazol-5-yl)ethylimino]ethylidene]-2,3-dihydro-1H-pyridine-2,6-dicarboxylic acid	$C_{14}H_{16}N_4O_4$	
30.	Quercetin 3'-O-glucuronide	(2S,3S,4S,5R,6S)-6-[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxochromen-3-yl]oxy-3,4,5-trihydroxyoxane-2-carboxylic acid	$C_{21}H_{18}O_{13}$	
31.	Scoparone	6,7-dimethoxychromen-2-one	$C_{11}H_{10}O_4$	
32.	Urolithin A	3,8-dihydroxybenzo[c]chromen-6-one	$C_{13}H_8O_4$	
33.	Pyrogallol 2-O-sulfate	(2,6-dihydroxyphenyl) hydrogen sulfate	$C_6H_6O_6S$	
34.	Valencic acid	4-(3-methylbut-2-enoxy)benzoic acid	$C_{12}H_{14}O_3$	
35.	Acetyl eugenol	(2-methoxy-4-prop-2-enylphenyl) acetate	$C_{19}H_{18}O_7$	
36.	Resveratrol (cis-)	5-[(Z)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol	$C_{14}H_{12}O_3$	
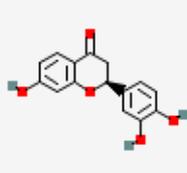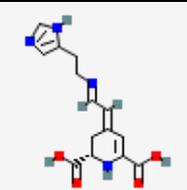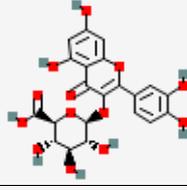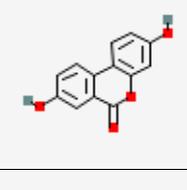
37.	Resveratrol (trans-)	5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol	$C_{14}H_{12}O_3$	
38.	Seselin	8,8-dimethylpyrano[2,3-f]chromen-2-one	$C_{14}H_{12}O_3$	
39.	Hirsutine	methyl (E)-2-[(2S,3R,12bR)-3-ethyl-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-a]quinolizin-2-yl]-3-methoxyprop-2-enoate	$C_{22}H_{28}N_2O_3$	
40.	Herniarin	7-methoxychromen-2-one	$C_{10}H_8O_3$	
41.	3,4-dihydroxyphenyllactic acid	3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid	$C_9H_{10}O_5$	
42.	2-hydroxy-3-(3',4'-dihydroxyphenyl)propanoic acid	3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid	$C_9H_{10}O_5$	
43.	Syringic acid	4-hydroxy-3,5-dimethoxybenzoic acid	$C_9H_{10}O_5$	
44.	Gallic acid ethyl ester	ethyl 3,4,5-trihydroxybenzoate	$C_9H_{10}O_5$	
45.	5-acetyl-amino-6-amino-3-methyluracil	5-acetyl-1,6-diamino-3-methylpyrimidine-2,4-dione	$C_7H_{10}N_4O_3$	
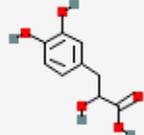
46.	Serotonin	3-(2-aminoethyl)-1H-indol-5-ol	$C_{10}H_{12}N_2O$	
47.	(+)-Epicatechin	(2S,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	$C_{15}H_{14}O_6$	
48.	(-)-Epicatechin	(2R,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	$C_{15}H_{14}O_6$	
49.	Furfuryl mercaptan	furan-2-ylmethanethiol	C_5H_6OS	
50.	Diallyl sulfide	3-prop-2-enylsulfanylprop-1-ene	$C_6H_{10}S$	
51.	Phenylacetic acid	2-phenylacetic acid	$C_8H_8O_2$	
52.	4-Methoxybenzaldehyde	4-methoxybenzaldehyde	$C_8H_8O_2$	
53.	Anisaldehyde (p-)	4-bromo-N-[(E)-(4-methoxyphenyl)methylideneamino]aniline	$C_{14}H_{13}BrN_2O$	
54.	4-Methylumbelliferone sulfate	(4-methyl-2-oxochromen-7-yl) hydrogen sulfate	$C_{10}H_8O_6S$	
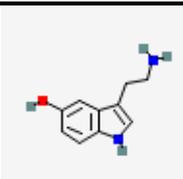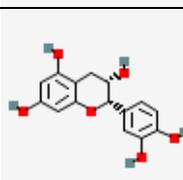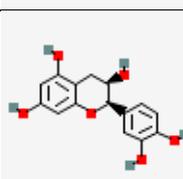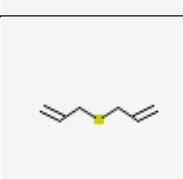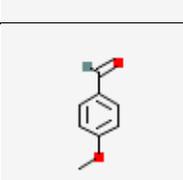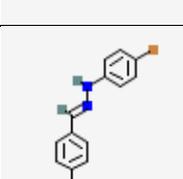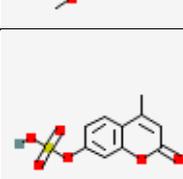
55.	Hydroxytyrosol 4'-sulfate	[2-hydroxy-4-(2-hydroxyethyl)phenyl] hydrogen sulfate	$C_8H_{10}O_6S$	
56.	Hydroxytyrosol 3'-sulfate	[2-hydroxy-5-(2-hydroxyethyl)phenyl] hydrogen sulfate	$C_8H_{10}O_6S$	
57.	3,5-Dihydroxybenzoic acid sulfate	3-hydroxy-5-sulfooxybenzoic acid	$C_7H_6O_7S$	
58.	Protocatechuic acid-4-O-sulfate	3-hydroxy-4-sulfooxybenzoic acid	$C_7H_6O_7S$	
59.	Protocatechuic acid-3-O-sulfate	4-hydroxy-3-sulfooxybenzoic acid	$C_7H_6O_7S$	
60.	Isoliquiritigenin	(E)-1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one	$C_{15}H_{12}O_4$	
61.	Liquiritigenin	(2S)-7-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one	$C_{15}H_{12}O_4$	
62.	Pratensein	5,7-dihydroxy-3-(3-hydroxy-4-methoxyphenyl)chromen-4-one	$C_{16}H_{12}O_6$	
63.	Diosmetin	5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chromen-4-one	$C_{16}H_{12}O_6$	
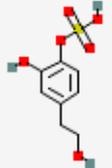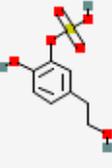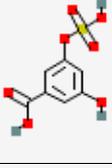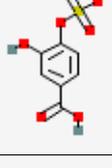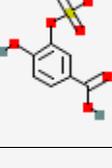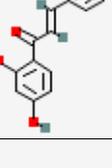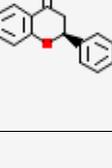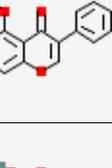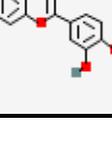
64.	Chrysoeriol	5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)chromen-4-one	$C_{16}H_{12}O_6$	
65.	Cirsimaritin	5-hydroxy-2-(4-hydroxyphenyl)-6,7-dimethoxychromen-4-one	$C_{17}H_{14}O_6$	
66.	4-Caffeoylquinic-1,5-lactone	[(1S,3R,4R,5R)-1,3-dihydroxy-7-oxo-6-oxabicyclo[3.2.1]octan-4-yl] (E)-3-(3,4-dihydroxyphenyl)prop-2-enoate	$C_{16}H_{16}O_8$	
67.	Shikimate	(3R,4S,5R)-3,4,5-trihydroxycyclohexene-1-carboxylate	$C_7H_9O_5^-$	
68.	trans-Cinnamyl alcohol	(E)-3-phenylprop-2-en-1-ol	$C_9H_{10}O$	
69.	Chavicol	4-prop-2-enylphenol	$C_9H_{10}O$	
70.	N-(2-furoyl)glycine	2-(furan-2-carbonylamino)acetic acid	$C_7H_7NO_4$	
71.	Juglone	5-hydroxynaphthalene-1,4-dione	$C_{10}H_6O_3$	
72.	5-Hydroxyindole acetic acid	2-(5-hydroxy-1H-indol-3-yl)acetic acid	$C_{10}H_9NO_3$	
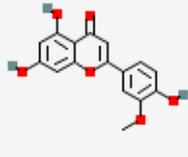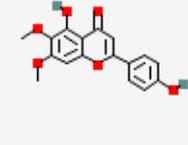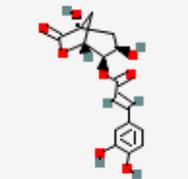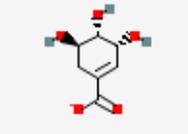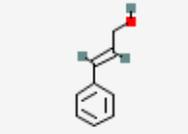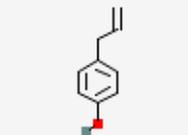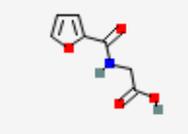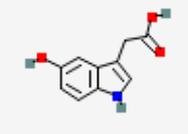
73.	3-methylhistidine	(2S)-2-amino-3-(3-methylimidazol-4-yl)propanoic acid	$C_7H_{11}N_3O_2$	
74.	Equol	(3S)-3-(4-hydroxyphenyl)-3,4-dihydro-2H-chromen-7-ol	$C_{15}H_{14}O_3$	
75.	4-Vinylsyringol	4-ethenyl-2,6-dimethoxyphenol	$C_{10}H_{12}O_3$	
76.	Perlolyrine	[5-(9H-pyrido[3,4-b]indol-1-yl)furan-2-yl]methanol	$C_{16}H_{12}N_2O_2$	
77.	Urolithin C	3,8,9-trihydroxybenzo[c]chromen-6-one	$C_{13}H_8O_5$	
78.	Piceatannol	4-[(E)-2-(3,5-dihydroxyphenyl)ethenyl]benzene-1,2-diol	$C_{14}H_{12}O_4$	
79.	Piceatannol (cis-)	4-[(Z)-2-(3,5-dihydroxyphenyl)ethenyl]benzene-1,2-diol	$C_{14}H_{12}O_4$	
80.	Hippuric acid	2-benzamidoacetic acid	$C_9H_9NO_3$	
81.	Umbelliferone	7-hydroxychromen-2-one	$C_9H_6O_3$	
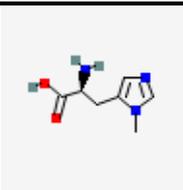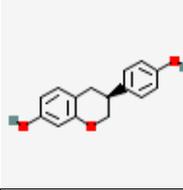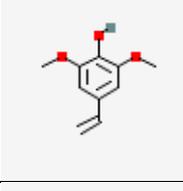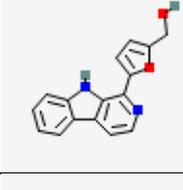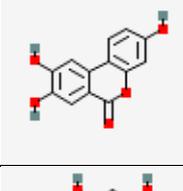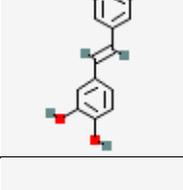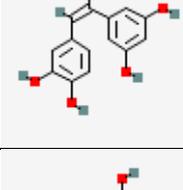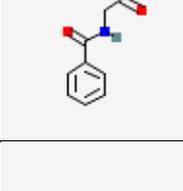
82.	3,4-dihydroxyphenyllactic acid	3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid	$C_9H_{10}O_5$	
83.	2-hydroxy-3-(3',4'-dihydroxyphenyl)propanoic acid	3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid	$C_9H_{10}O_5$	
84.	Syringic acid	4-hydroxy-3,5-dimethoxybenzoic acid	$C_9H_{10}O_5$	
85.	Gallic acid ethyl ester	ethyl 3,4,5-trihydroxybenzoate	$C_9H_{10}O_5$	
86.	5-acetyl-amino-6-amino-3-methyluracil	5-acetyl-1,6-diamino-3-methylpyrimidine-2,4-dione	$C_7H_{10}N_4O_3$	
87.	Pelargonidin	2-(4-hydroxyphenyl)chromenylium-3,5,7-triol	$C_{15}H_{11}O_5$	
88.	Dihydroisoferulic acid-3'-O-sulfate	3-(4-methoxy-3-sulfooxyphenyl)propanoic acid	$C_{10}H_{12}O_7S$	
89.	Dihydroferulic acid-4'-sulfate	3-(3-methoxy-4-sulfooxyphenyl)propanoic acid	$C_{10}H_{12}O_7S$	
90.	Bergaptol	4-hydroxyfuro[3,2-g]chromen-7-one	$C_{11}H_6O_4$	
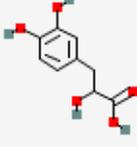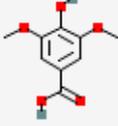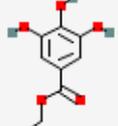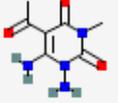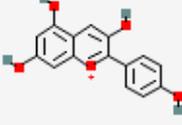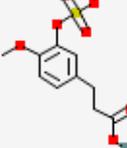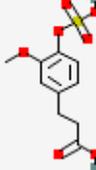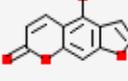
91.	Xanthotoxol	9-hydroxyfuro[3,2-g]chromen-7-one	$C_{11}H_6O_4$	
92.	Pyrogallol 2-O-sulfate	(2,6-dihydroxyphenyl) hydrogen sulfate	$C_6H_6O_6S$	
93.	4'-O-Methylgallic acid	3,5-dihydroxy-4-methoxybenzoic acid	$C_8H_8O_5$	
94.	2-aminophenol sulfate	(2-aminophenyl) hydrogen sulfate	$C_6H_7NO_4S$	
95.	Scoparone	6,7-dimethoxychromen-2-one	$C_{11}H_{10}O_4$	
96.	Psoralen	furo[3,2-g]chromen-7-one	$C_{11}H_6O_3$	
97.	Angelicin	furo[2,3-h]chromen-2-one	$C_{11}H_6O_3$	
98.	Caffeic acid ethyl ester	ethyl (E)-3-(3,4-dihydroxyphenyl)prop-2-enoate	$C_{11}H_{12}O_4$	
99.	Kynurenine	(2S)-2-amino-4-(2-aminophenyl)-4-oxobutanoic acid	$C_{10}H_{12}N_2O_3$	
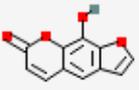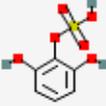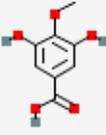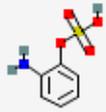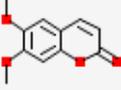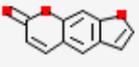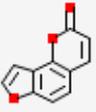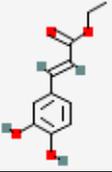
100.	5-Hydroxyindole acetic acid	2-(5-hydroxy-1H-indol-3-yl)acetic acid	$C_{10}H_9NO_3$	
101.	Benzoic acid-3-sulfate	5-[2-[[(4R)-4-[(3R,5R,8R,9S,10S,13R,14S,17R)-10,13-dimethyl-3-sulfooxy-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]ethylcarbamothioylamino]-2-(3-hydroxy-6-oxoxanthen-9-yl)benzoic acid	$C_{47}H_{57}N_3O_{10}S_2$	
102.	Benzoic acid-4-O-sulfate	3-hydroxy-4-sulfooxybenzoic acid	$C_7H_6O_7S$	
103.	Tyrosol 4-sulfate	[4-(2-hydroxyethyl)phenyl] hydrogen sulfate	$C_8H_{10}O_5S$	
104.	Xanthohumol	(E)-1-[2,4-dihydroxy-6-methoxy-3-(3-methylbut-2-enyl)phenyl]-3-(4-hydroxyphenyl)prop-2-en-1-one	$C_{21}H_{22}O_5$	
105.	Sesamin	5-[(3S,3aR,6S,6aR)-3-(1,3-benzodioxol-5-yl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-6-yl]-1,3-benzodioxole	$C_{20}H_{18}O_6$	
106.	Scopolin	6-methoxy-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-2-one	$C_{16}H_{18}O_9$	
107.	Phenylacetic acid	2-phenylacetic acid	$C_8H_8O_2$	
108.	Persenone B	[(E)-2-hydroxy-4-oxononadec-5-enyl] acetate	$C_{21}H_{38}O_4$	
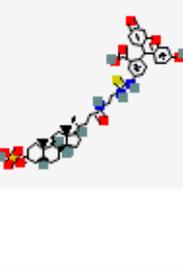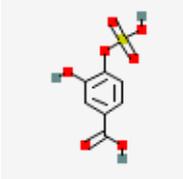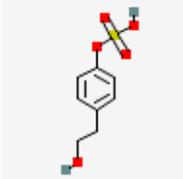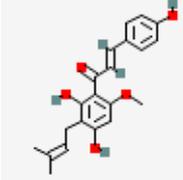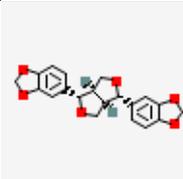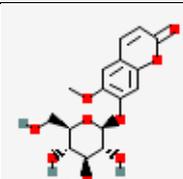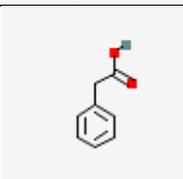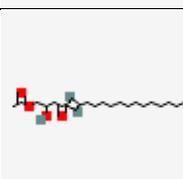
109.	Neochlorogenic acid	(1R,3R,4S,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid	$C_{16}H_{18}O_9$	
110.	Methyl allyl thiosulfinate	3-methoxysulfinylsulfanylprop-1-ene	$C_4H_8O_2S_2$	
111.	Lubimin	(3R,5S,6R,8S,10S)-8-hydroxy-6-methyl-3-prop-1-en-2-ylspiro[4.5]decane-10-carbaldehyde	$C_{15}H_{24}O_2$	
112.	Licoisoflavone A	3-[2,4-dihydroxy-3-(3-methylbut-2-enyl)phenyl]-5,7-dihydroxychromen-4-one	$C_{20}H_{18}O_6$	
113.	Isoxanthohumol	7-hydroxy-2-(4-hydroxyphenyl)-5-methoxy-8-(3-methylbut-2-enyl)-2,3-dihydrochromen-4-one	$C_{21}H_{22}O_5$	
114.	Farnesoic acid	(2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienoic acid	$C_{15}H_{24}O_2$	
115.	Cyclokievitone	3-(2,4-dihydroxyphenyl)-5-hydroxy-8,8-dimethyl-2,3-dihydropyrano[2,3-h]chromen-4-one	$C_{20}H_{18}O_6$	
116.	Capsidiol	(1R,3R,4S,4aR,6R)-4,4a-dimethyl-6-prop-1-en-2-yl-2,3,4,5,6,7-hexahydro-1H-naphthalene-1,3-diol	$C_{15}H_{24}O_2$	
117.	Anisaldehyde (p-)	4-bromo-N-[(E)-(4-methoxyphenyl)methylideneamino]aniline	$C_{14}H_{13}BrN_2O$	
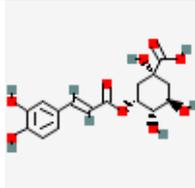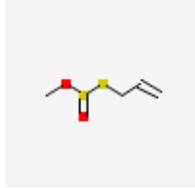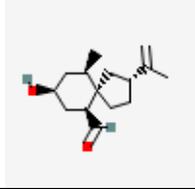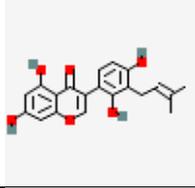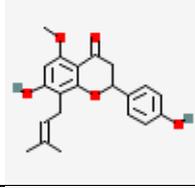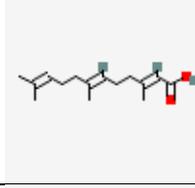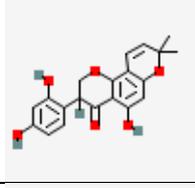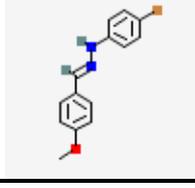
118.	5-O-Caffeoylquinic acid	(1R,3R,4S,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid	$C_{16}H_{18}O_9$	
119.	4-Methoxybenzaldehyde	4-methoxybenzaldehyde	$C_8H_8O_2$	
120.	3-O-Caffeoylquinic acid	1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid	$C_{16}H_{18}O_9$	
121.	3,4-Dihydroxystyrene	4-ethenylbenzene-1,2-diol	$C_8H_8O_2$	
122.	1-O-Caffeoylquinic acid	trans-(3R,5R)-1-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-3,4,5-trihydroxycyclohexane-1-carboxylic acid	$C_{16}H_{18}O_9$	
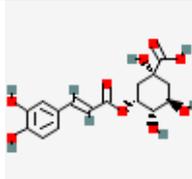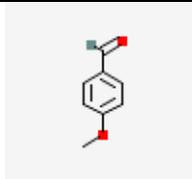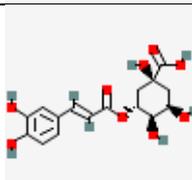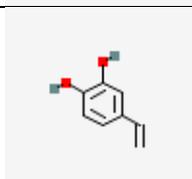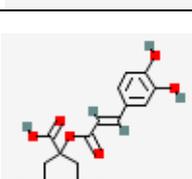
Antioxidant enzyme activity in the grub of RPW-infected ethanol extract of *C. hirsutus*

Catalaseassay

The CAT activity in the control *R. ferrugineus* larvae was 113.83 μmol/min, whereas CAT activity decreased markedly in larvae treated with *C. hirsutus* extract. At 4%, the activity declined to 0.631 μmol/min, followed by 0.999 μmol/min at 6%, 0.202 μmol/min at 8%, and 0.125 μmol/min at 10%, respectively (Fig. 1).

Fig. 1. Catalase enzyme activity in the grub of RPW

SOD assay

The SOD activity in the RPW control was 4.80 μmol/min, while *C. hirsutus*-treated grub exhibited markedly reduced levels. At 4% concentration, the activity declined to 0.789 μmol/min, and at 6%, it increased slightly to 1.136 μmol/min. This was followed by further reductions at higher doses, with 8% showing 0.420 μmol/min and 10% recording 0.392 μmol/min.
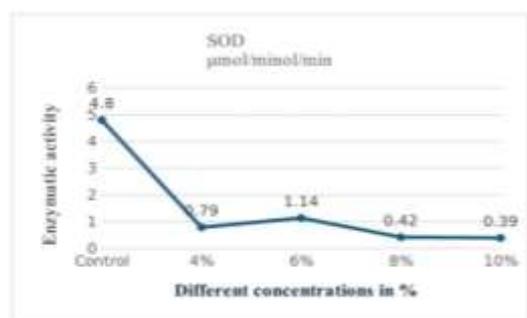
Fig. 2. SOD enzyme activity in the grub of RPW

Peroxidase assay

The POD of RPW control was 16.802 µmol/min, *C. hirsutus* infected with the dosage of 4% was 1.382 µmol/min, the dosage of 6% RPW was 0.426 µmol/min,0.424 µmol/min followed by 8% dosage, infected RPW was 0.420 µmol/min, and the dosage of 10% was 0.294 µmol/min, respectively. The POD activity in the RPW control was 16.802 µmol/min, whereas the *C. hirsutus*–treated grub showed a sharp decline. At 4% concentration, the activity dropped to 1.382 µmol/min. Further reductions were observed at higher doses: 6% recorded 0.426 µmol/min, followed closely by 8% with 0.424 µmol/min, and 10% showing the lowest value of 0.294 µmol/min.
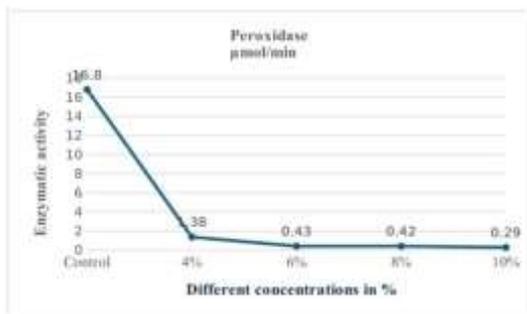
Fig. 3. Peroxidase enzyme activity in the grub of RPW

Ascorbate peroxidase assay

The APX activity in RPW control was found to be 564.55 µmol/min, When infected with a dosage of 4% the APX activity was found to be 232.186 µmol/min, When treated with a dosage of 6% the APX activity was found to be 71.583 µmol/min, When treatedwith 8% dosage the APX activity was found to be 70.625 µmol/min, When treated witha dosage of 10% the activity was found to be 49.392 µmol/min, respectively.
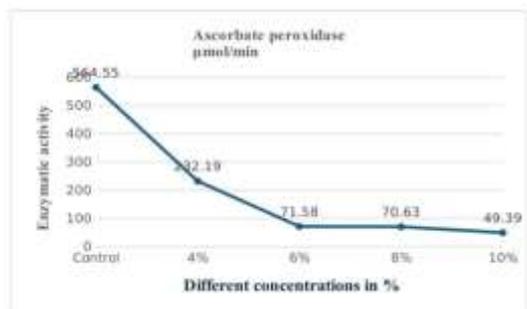
Fig. 4. Ascorbate peroxidase activity in the grub of RPW

Polyphenol oxidase assay

The PPO activity in the RPW control was 12.961 µmol/min, whereas *C. hirsutus*–infected larvae showed a substantial decline across all concentrations. At 4%, the activity dropped to 1.184 µmol/min, followed by 0.852 µmol/min at 6%. Further decreases were observed at higher doses, with 8% recording 0.630 µmol/min and 10% showing 0.588 µmol/min.
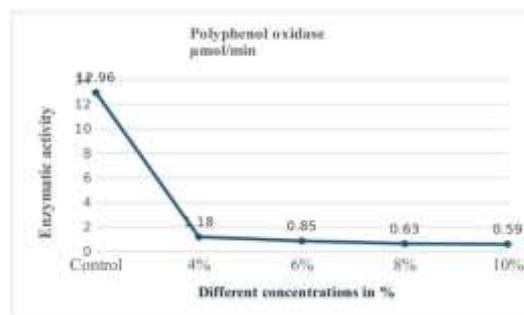
Fig. 5. Polyphenol oxidase activity in the grub of RPW

Protein profiling by SDS Page of the grub of *R. ferrugineus* infected with *C. hirsutus* ethanol extract

The protein profiling by SDS-PAGE was done in both the control and infected *R. ferrugineus*. Control shows 11 bands, noted as 200kDa, 190kDa, 180kDa, 170kDa, 140kDa, 130kDa, 135kDa, 125kDa, 120kDa, 95kDa and 90kDa and *C. hirsutus* infected with the dosage of 4% and 6% shows the bands above as control, followed by the dosage of 8 % and 10% shows the fade bands especially in Lane 4-shows the bands were lite noted as 125kDa, 120kDa, 90kDa and 95kDa. All showed multi-band patterns (Fig. 6).
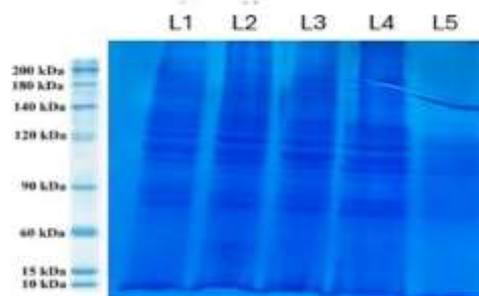
Fig. 6. SDS-PAGE of C. hirsutus-infected RPW
L1- Control, L2- 4% *C. hirsutus*- RPW, L3- 6% *C. hirsutus*- RPW, L4- 8% *C. hirsutus*- RPW, L5- 10% *C. hirsutus*- RPW

DISCUSSION

Botanical pesticides, called botanicals, are derived from complete plants or specific plant parts. The chemically active element dissolves in solvents used to extract or derive it from the plant or plant part. Nowadays, botanical pesticides are employed in agriculture to control insects and other pests instead of synthetic chemical pesticides (Ngegba *et al.*, 2022). The study (Dalavayi Haritha *et al.*, 2021) has shown that over the past 25 years, hundreds of secondarymetabolites, or phytochemicals, that demonstrate toxic or repulsive effects of insect pest feeding have been described and documented in the scientific literature. Hence, the secondary metabolites of plants play a crucial role in controlling pests. This study was planned to control the pest RPW by using the *C. hirsutus* plant ethanol extract (Singh, 2015).

This study examined the antioxidant enzymes, like CAT, SOD,POD, APX, and PPO, in all the infected compared to the control, which were very low. This may be due to the insecticidal activity, and it may affect the insect's innate immune signalling pathway. This leads to the damageof cellular components and impairs insect physiology (Abdabd-Allah *et al.*, 2024).

Insects will produce an oxidative stress response to prevent the damaging effects of excessive Reactive oxygen species (ROS) on DNA, lipids, proteins, and other biomolecules when they encounter the invasion of foreign substances, because an excess of ROS in their bodies will upset the equilibrium between the oxidative and antioxidant mechanisms within cells and organisms (Sule *et al.*, 2022). By controlling the activity of antioxidant enzymes and the levels of antioxidant substances, such as reduced GSH, CAT, POD, and SOD, the oxidative stress mechanism primarily balances the amount of ROS in cells (Apel and Hirt, 2004).

Proteins and nucleic acids are the most common biological macromolecules that may be separated using polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (Laemmli, 1970).

The protein bands in the present study were of high to low molecular weight, and all showed a multibanding pattern. When compared to the control, the infected samples showed lite bands. The *C. hirsutus*-infected at a dosage of 10% have shown 125kDa, 120kDa, 90kDa and 95kDa, respectively. The bands are 200kDa, 190kDa, 180kDa, 170kDa, 140kDa, 130kDa, and 135kDa, which are unseen in the infected larvae but present in the control. This may be the toxic protein that has caused the death of the pest. This protein can be isolated and formulated as a biopesticide.

SDS-PAGE was used to qualitatively evaluate hemolymph protein, and the results showed changes in protein bands across different treatments. Certain protein bands may be produced to satisfy acute physiological needs based on their appearance and disappearance during larval development. Furthermore, these modifications show that the larvae are under stress or are changing metabolically (Abdabd-Allah *et al.*, 2024). This may be the reason forthe present finding also.

CONCLUSION

The *C. hirsutus* plant extract showed insecticidal activities, which cause interference with insect defence mechanisms and immune suppression and exhibits oxidative damage and cytotoxicity increases after infection of insect *R. ferrugineus* larvae, and this may be the cause of its mortality. Since the chemical and pheromone trap methods are highly recommended for controlling this pest, knowledge of natural pesticide awareness is lacking. Therefore, this study concluded that the pest RPW can be able to control by using the plant extraction of *C. hirsutus*.

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