

# *Aspergillus flavus* induced oxidative stress and immunosuppressive activity in *Spodoptera litura* as well as safety for mammals

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## Research

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# Abstract

## Background

In the last few decades, considerable attention has been paid to entomopathogenic fungi as biocontrol agents, however little is known about their mode of action and safety. This study aimed to investigate the toxicity of *Aspergillus flavus* in insect *Spodoptera litura* by analyzing the effect of fungal extract on antioxidant and cellular immune defense. In antioxidant defense, the lipid peroxidation (Malondialdehyde content) and antioxidant enzymes activities (Catalase, Ascorbate peroxidase, Superoxide dismutase) were examined. In cellular immune defense, effect of *A. flavus* extract was analyzed on haemocytes using Scanning Electron Microscopy (SEM). Furthermore, mammalian toxicity was analyzed with respect to DNA damage induced in treated rat relative to control by comet assay using different tissues of rat (blood, liver, and kidney).

## Results

Ethyl acetate extract of *A. flavus* was administrated to the larvae of *S.litura* using artificial diet method having concentration 1340.84 µg/ml (LC<sub>50</sub> of fungus). The effect was observed using haemolymph of insect larvae for different time intervals (24, 48, 72 and 96). In particular, Malondialdehyde content and antioxidant enzymes activities were found to be significantly ( $p \leq 0.05$ ) increased in treated larvae as compared to control. *A. flavus* ethyl acetate extract also exhibit negative impact on haemocytes having major role in cellular immune defense. Various deformities were observed in different haemocytes like cytoplasmic leakage and surface abnormalities etc. Genotoxicity on rat was assessed using different tissues of rat (blood, liver, and kidney) by comet assay. Non-significant effect of *A. flavus* extract was found in all the tissues (blood, liver, and kidney).

## Conclusions

Overall the study provides important information regarding the oxidative stress causing potential and immunosuppressant nature of *A. flavus* against *S. litura* and its non toxicity to mammals (rat).

## Introduction:

Entomopathogenic fungi are natural pest controlling agents manifesting immense significance to be used as mycoinsecticides against wide range of insect pests. They have been reported from the phyla Ascomycota, Basidiomycota, Zygomycota, Chytridiomycota, and Deuteromycota (Goettel and Glare 2010). As synthetic pesticides wreak havoc on the environment, threatening biodiversity and human survival (Aktar et al. 2009). Thus, there is a need to explore alternative ecofriendly strategies for pest's management which protect and strengthen natural ecosystems rather than contaminate. Biological control using fungi is one of the most promising technique, due to their unique mechanism of action

while infection, low cost, specificity and safety to ecosystem (Castillo et al. 2000; Charnley and Collins 2007). Among entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* have been extensively explored as insecticidal agents and their formulations are also commercially available. They were recorded as efficient pest control agents against wide range of insect pests viz *Tetranychus cinnabarinus* (Boisduval), *Tetranychus evansi* (TETREV), *Helicoverpa armigera* (Hubner), *Galleria melonella* (Linnaeus) and *Aphis gossypii* (Glover) etc. (Sheeba et al. 2001; Samuels and Coracini 2004; Shi and Feng. 2004; Gindin et al. 2006; Bugeme et al. 2008; Prasad and Syed 2010; Hussein et al. 2012; Rashki and Shirvani 2013; Rana and Dinesh 2014).

Moreover various other fungal spp. viz *Aspergillus* sp., *Alternaria* sp. and *Nomuraea* sp. have also been disclosed to be entomopathogenic (Devi 1994; Kaur et al. 2013; Bawin et al. 2016a,b). Different *Aspergillus* spp. viz *A. ochraceus*, *A. kanagawaensis*, *A. sulphureus*, *A. flavus* and *A. ochraceus* were found to be pathogenic against several insects such as *Aedes fluviatilis* (Lutz), *Culex quinquefasciatus* (Say), *Anopheles gambiae* (Giles), *Oligonychus coffeae* (Nietner) (Lage et al. 2001; Seye et al. 2009; Mazid et al; 2015; Karthi et al; 2018). Genomic analysis suggested that fungi possess genes encoded for different secondary metabolites (Gibson 2014) which are also associated with insecticidal activity as reported earlier (Amiri et al. 1999; Kim et al. 2002; Calvo et al. 2015).

Insects possess antioxidant and cellular immune defense system to ward of infection. Antioxidant defense system comprises various antioxidants enzymes which are catalase (CAT), glutathione-S-transferases (GSTs), peroxidase (POX), and superoxide dismutase (SOD). All these play an important role in protecting cells and maintaining homeostasis by removing oxidative stress (Rudneva 1999). Previous studies have demonstrated that these enzymes are quickly upregulated in response to xenobiotics threats (Mittapalli et al. 2007; Russell 2011; Buyukguzel *et al.* 2013). Numbers of reports are available in literature revealing the altered antioxidant enzymes activity in insects after exposure to synthetic pesticides but meager information available regarding fungal exposure.

Cellular immune defense in insects is accomplished through haemocytes. They consists the mixture of cells having different morphological and biological functions and help in providing defense against parasites, pathogens and other foreign bodies enter in the hemocoel (Ratcliffe et al., 1985; Lackie, 1988; Ratcliffe and Rowley, 1987; Falleiros et al. 2003). Several studies revealed the effect of biopesticides on haemocytes count but the morphological alteration in haemocytes have not been studied.

On the basis of aforementioned discussion, the study examine the toxicity of fungal extract on insect by analyzing the effect of ethyl acetate extract of *A. flavus* on antioxidant and cellular immune defense of insect using *Spodoptera litura* (Fabricius) as a model, one of the major polyphagous pests.

However, if secondary metabolites of fungi are found to deter insects then it would be equitably important to detect whether these metabolites have any mammalian toxicity. As, various chronic diseases have been associated with pesticides exposures, including reproductive or developmental disorders, neurological disorders, cancer etc. Epidemiological studies suggested that occupationally exposed populations to pesticides like pesticide applicators, pesticide manufacturing workers and field workers

have developed the high risk of cancer which is due to genomic damage (Gangemi et al. 2016; Rodgers et al. 2018; Curl et al. 2020). So it is necessary to check genotoxicity of the agent which can be used as pesticide in order to decipher its effects on other non-target species. Various genotoxic markers are chromosomal aberrations (CA), sister chromatid exchange (SCE), micronuclei (MN), comet assay (CO). Comet assay or SCGE is one of the finest techniques for qualitative and quantitative analysis of DNA damage and repair. It was extensively explored in mammal and human cell studies (Burlinson et al. 2007; Gaivao et al. 2009; Karaman 2011; Collins and Azqueta 2013; Collins et al. 2014) and successfully applied on the cells of various animal groups (Woźniak and Blasiak 2003). So in the present study this technique was used to assess the genotoxicity of *A. flavus* on mammals using rat as an animal model to confirm its safety on mammals.

## Material And Methods:

### Insects rearing:

*Spodoptera litura* (Lepidoptera) eggs were obtained from the cauliflower fields around Amritsar (India). After hatching of eggs larvae were fed on castor leaf. Subsequent generations of culture were maintained in laboratory at 25±2°C temperature, 65±5% relative humidity and 12:12 (D: L) photoperiod (Kaur et al. 2018).

### Fungal culture isolation, production and identification

Fungus was isolated from the surface of dead insect (Kaur et al. 2019). The production was carried out in 50ml malt extract (malt extract=20g/l, dextrose=20g/l, peptone = 1g/l, pH=5.5) broth in 250ml Erlenmeyer flask by inoculating one plug (1 cm square) taken from the periphery of an actively growing culture. The flasks were incubated at 30°C and 250rpm for 10 days. After 10 days extraction was carried out twice using ethyl acetate at 120rpm and 40°C. The extracts were concentrated by using rotavapor and dissolved in 1ml DMSO and stored at 4°C.

The fungus was identified as *Aspergillus flavus* on morphological and molecular basis as indicated in our previous study (Kaur et al. 2019) by using ITS1 and ITS4 primer to amplify ITS1-5.8S- rDNA- ITS2 region. Amplified ITS region was Purified and sequenced at first base sequencing (Malaysia). The sequence similarity was matched with other available databases retrieved from NCBI using BLAST (Sharma *et al.* 2008).

### Toxicity test of fungus and LC50 value against *S.litura*:

Toxicity of fungus was tested by checking mortality rate. For this different concentrations (125, 250, 500, 1000 and 2000µg/ml) of fungal extract were made in 0.5% DMSO and added in artificial diet. The Second instar larvae (6 days old) were reared on fungal extract amended diets as well as with control diet (0.5% DMSO) at controlled temperature 25±2°C and relative humidity 70±5% conditions. The experiment was replicated six times with five larvae per replication. Each larva was put in separate container (4×6cm) and

the diet was changed daily till pupation. Dead larvae were checked daily till pupation. The total numbers of dead larvae were counted. The toxic effect of fungal extract on *S.litura* was calculated using the probit analysis LC<sub>50</sub> (lethal concentration) determination method.

### **Effect on Malondialdehyde (MDA) content and antioxidant enzymes activity:**

To evaluate the effect of fungal extracts on lipid peroxidation and antioxidant enzymes, the third instar larvae (12 days old) were fed with fungal extracts supplemented diet having concentration 1340.84µg/ml (LC<sub>50</sub> of fungus). The MDA content and enzymes activities [Superoxide dismutase (SOD), catalase (CAT), Ascorbate peroxidase (APOX)] were analyzed in haemolymph of third instar (12days) larvae.

Larvae were divided into two groups, treatment and control. Treatment group was treated with LC<sub>50</sub> of fungus at controlled temperature 25±2°C and relative humidity 65±5%. The second group was treated with control diet (0.5% DMSO) at same conditions of temperature and relative humidity. The effect of fungal extract has been recorded after different time intervals (24hr, 48hr, 72hr and 96hr) in lipid peroxidation and enzyme activities. The experiment was replicated three times. For each treatment and control there are 10 larvae per replication were taken.

### **Tissue collection**

Haemolymph was collected by cutting proleg with microscissor from 10 different larvae fed with same concentration and then it was pooled. Pooled haemolymph (10%) was mixed with PBS (Phosphate Buffer Saline pH 7.0) containing 0.01% phenylthiourea and centrifuged for 20 min at 10000g, 4°C and supernatant obtained was used for enzyme activities studies

The extraction procedure was same for lipid peroxidation and all enzymes.

### **Malondialdehyde (MDA) content:**

MDA content was measured according to Jain and Levine (1995) with slight modifications. MDA content as an indicator of lipid peroxidation was determined after incubation of 0.5ml of sample (supernatant) at 95 °C with Trichloroacetic acid (TCA) (20% w/v), Thiobarbituric acid (TBA) (1 % w/v). Absorbance was taken at 532 nm against the blank. MDA content was expressed as nanomole/ml by using  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  extinction coefficient.

### **Catalase (CAT) activity:**

Enzyme activity was estimated according to methodology given by Aebi (1984) with slight modifications. 0.1ml of supernatant was added into 2.9ml of H<sub>2</sub>O<sub>2</sub> in a cuvette. Decrease in absorbance was read at 240nm for 5min at 1min interval (25°C). The enzyme activity was expressed as µM/ml (haemolymph).

### **Ascorbate peroxidase (APOX) activity:**

The enzyme activity was calculated according to methodology given by Asada (1984) with slight modifications. 0.1ml of sample, 0.6ml extraction buffer and 0.125ml of 0.3% H<sub>2</sub>O<sub>2</sub> were taken in cuvette. The decrease in absorbance was recorded at 290nm for 5min at 30sec interval (25°C). The enzyme activity was expressed as µM/ml (haemolymph).

#### **Superoxide dismutase (SOD) activity:**

The enzyme activity was calculated according to methodology given by Kono (1978) with slight modifications. 0.05ml sample, 1.5ml sodium carbonate buffer, 0.5ml NBT (Nitroblue tetrazolium), 0.1ml TritonX-100, 0.1ml hydroxylamine hydrochloride were taken in cuvette and increase in absorbance was recorded at 540nm. The enzyme activity was expressed as µM/ml (haemolymph).

#### **Effect on haemocytes:**

Haemolymph was collected and pooled from 10 larvae fed with same concentration. Effect on haemocytes was studied by scanning electron microscopy (SEM).

#### **Scanning electron microscopy (SEM):**

SEM was done according to methodology of Wang *et al.* (2012) with slight modifications. Haemolymph was bled on termanox discs after cutting proleg of larvae. It was allowed to dry and fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for two hours. After this sequential dehydration was done by using graded series of ethanol i.e 25% followed by 50%, 70%, 90% and at the end with absolute (100%) alcohol. Then discs were placed in dry chamber for proper drying. At the end silver coating was done by mounting samples on aluminium stubs and haemocytes were observed under SEM at magnification of 10.00KX operated at 10KV after 96hr of treatment with fungal extracts.

#### **Mammalian toxicity study:**

Sexually mature male wistar albino rats having weight 120±20gm were used in study. Animals were reared on commercial pellet diet and water adds libitum and housed in cages at particular temperature (25±2°C) and humidity conditions (50-60%). All experiments were performed according to guidelines provided of Institutional Animal Ethics Committee (IAEC). The application for permission for animal experiments was submitted to the CPCSEA, New Delhi and after approval of Institutional Animal Ethics Committee (IAEC) got Registration number: 226/PO/Re/S/2000/CPCSEA. The animals were acclimatized 5days before experiments. Two concentrations 100mg/kg b.wt and 200mg/kg b.wt of fungal extracts were selected for experiments and effects were studied after 24hr and 96hr of exposure. The *A. flavus* fungal extract dissolved in 0.5%DMSO was injected intraperitoneally to the rat. The experiment was replicated thrice and DNA damage was assessed according to methodology given by Ahuja and Saran (2001) in different tissues viz. blood, liver and kidney. Blood samples (1ml) were taken directly from heart and used as such after adding anticoagulant however two tissues i.e. liver, kidney were homogenized in PBS and centrifuged at 10,000g for 10min. Cell suspension was taken and used for DNA damage study.

## Statistical analysis:

To study the effect of duration one way analysis of variance (ANOVA) with Tukey's test was performed and to study the effect of treatment student's t-test was applied

## Results:

### Toxicity test of fungus and LC50 value against *S.litura*:

The entomopathogenic fungus, *A. flavus* was tested at different concentrations for toxicity against larvae of *S. litura*. The mortality percentages were proportional to extract concentration as shown in Fig. 1. Different concentrations of the extract caused 16.66-56.66% mortality as compared to 10% in control. The concentrations ranging between 500- 2000µg/ml resulted in a significantly higher mortality with respect to control ( $F=8.38$ ,  $p\leq 0.01$ ) (Table.1). The LC50 value of ethyl acetate extract of *A. flavus* as calculated by probit analysis using SPSS software was found to be 1340.84µg/ml.

### Effect on Malondialdehyde (MDA) content and antioxidant enzymes:

Larvae treated with ethyl acetate extract of *A. flavus* showed hike in level of lipid peroxidation as indicated by MDA content which significantly increased in all treated groups as compared to control in haemolymph of *S. litura* (Student's t-test) except for 24hr group in which non-significant increase was observed. MDA content was maximum at 96hr ( $8.76\pm 0.16$ nmol/ml) which was significantly higher from control ( $5.75\pm 0.13$ nmol/ml) ( $t=14.80$ ,  $p\leq 0.01$ ). One Way ANOVA reveals the effect of duration was found to be significant ( $F=76.14$ ,  $p\leq 0.01$ ). Further Tukey's test reveals the significant difference between 48, 72 and 96hr exposure groups (Fig. 2a).

Statistically significant (t test,  $p\leq 0.05$ ) increase in activities of all enzymes with respect to control of the exposed *S.litura* larvae was observed. Fig. 2b reflected the significant (t test,  $p\leq 0.05$ ) hike in catalase (CAT) activity of treated larvae as compared to control. A measured value of CAT was 3 fold higher than control one, for 96hr exposure time group. The difference between all exposure time groups was statistically significant (ANOVA) and significant difference observed between 48hr, 72hr and 96hr groups (Tukey's test) (Fig. 2b).

An upsurge in Ascorbate peroxidase (APOX) activity was noticed in haemolymph of *S. litura* after treatment with *A.flavus* ethyl acetate extract (Table.4). The level of enzyme activity was found to be  $67.77\pm 0.34$ ,  $82.20\pm 3.23$ ,  $87.42\pm 0.56$ ,  $125.80\pm 4.59$ µmole/ml following treatment up to 24hr, 48hr, 72hr and 96hr respectively. Highest activity was found in 96hr exposure group where almost 3.74 fold increase was observed in treated groups as compared to control ( $t=19.60$ ,  $p\leq 0.01$ ). Time dependent significant effect was observed (ANOVA) ( $F=76.81$ ,  $p\leq 0.01$ ) however significant changes were observed between 24hr and 48hr, 72hr and 96hr exposure groups (Tukey's test) (Fig. 2c).

Superoxide dismutase (SOD) activity also found to be increase in larvae fed with diet amended with ethyl acetate extract of *A. flavus* (Fig. 2d). Significant (t test,  $p\leq 0.05$ ) rise in SOD activity was occurred in all

exposure groups as compared to control however highest activity was obtained at 72hr and 96hr exposure groups where activity increased from  $28.19 \pm 0.17 \mu\text{mol/ml}$  (control) to  $41.54 \pm 0.87 \mu\text{mol/ml}$  (exposed group) and  $28.33 \pm 0.18 \mu\text{mol/ml}$  (control) to  $51.68 \pm 0.88 \mu\text{mol/ml}$  (exposed group) respectively. With increase in time duration the enzyme activity was significantly increased (ANOVA) ( $F=202.57$ ,  $p \leq 0.01$ ). Significant difference observed between 48hr, 72hr and 96hr exposure groups (Tukey's test) (Fig. 2d).

### **Effect on haemocytes:**

The scanning electron microscopy studies revealed that the haemocytes of *S.litura* were changed very apparently after treatment with *A.flavus* ethyl acetate extract (Fig. 3, 4, 5). After 96 hr various morphological deformities were observed in different types of haemocytes. As compared to normal plasmatocytes treated ones showed cell perforation and cytoplasmic leakage (Fig.3). Similarly normal granulocytes not shown any deformity but treated ones showed strumae and surface abnormalities (Fig.4). Prohaemocytes showed surface abnormalities after treatment with *A.flavus* (Fig.5). Overall SEM studies revealed that morphology of haemocytes was highly disrupted after treatment with ethyl acetate extract of *A.flavus* for 96hr which might be leads to cytotoxicity.

### **Mammalian toxicity:**

Comet assay was conducted to assess genotoxicity on rat using parameters, Tail length, % Tail DNA, Tail Moment and Olive Tail Moment. The obtained data in Table 6, 7, 8 showed that there were no significant differences in all the parameters after 24 and 96hr of rat's administration with *A.flavus* extract at dose level of 100 mg/kg body weight and 200 mg/kg body weight relative to control (ANOVA). In case of rat blood non-significant increase was observed for all the parameters except in case of tail length of 48hr group where significant increase was observed from  $14.78 \pm 0.13$  (control) to  $15.51 \pm 0.11$  (200mg/kg b.wt). In all other groups non-significant increase was observed after treatment with different concentrations of *A. flavus* (Table. 6). Similarly in rat liver non-significant effect was obtained for all the parameters after treatment with both concentrations ethyl acetate extract of *A. flavus* (ANOVA) (Table. 7). In rat kidney all parameters showed non-significant increase except tail length of 24hr group and %tail DNA of 96hr group where significant increase was observed (Table. 8). Effect of duration was also found to be non-significant in all the tissues as revealed by student's t-test. Overall non-significant effect was observed. Photomicrographs showing DNA damage in different tissues of rat after treatment with *A. flavus* fungal extracts are shown in fig. 6.

## **Discussion:**

Under stress conditions, large quantities of reactive oxygen species (ROS) produced by insects (Ding et al. 2015) leads to oxidative stress, ultimately damage to the organism itself (Li *et al.* 2006). There are free radical scavenging systems in insects including various protective enzymes viz superoxide dismutase (SOD), catalase (CAT), Ascorbate peroxidase (APOX). All work co-coordinately to maintain the state of dynamic equilibrium in organism, keeping ROS low level to prevent the cells from damage (Gao et al.

1995; Zhang et al. 2001; Kontogiannatos *et al.* 2011). Increase in malondialdehyde (MDA) content and antioxidant enzymes activity is an important indicator of oxidative stress.

So, in this study, the MDA content and activities of antioxidant enzymes were determined in *Spodoptera litura* larvae at different times after treatment with *A. flavus* in order to speculate *A. flavus* induced oxidative stress and effect on antioxidant defense. The effect on insect survival was also studied which showed that *A. flavus* ethyl acetate extract has negative impact on larval survival. Previously various species of *Aspergillus* are found to be entomopathogenic like *A. flavus*, *A. oryzae*, *A. tamarii* and *A. versicolor*, *A. parasiticus* etc. (Senthilkumar et al. 2014; Bawin et al. 2016a,b; Bhan et al. 2015; Karthi et al. 2018).

In our study, oxidative stress was determined by measuring the amount of MDA via thiobarbituric acid color reaction and antioxidant enzymes activities. Results showed significant increase in MDA level in response to fungal extract as compared to control. At 96hr value increased 1.5 times as compared to control. This finding is in corroboration with the finding of Karthi et al. (2018) revealing the toxic effects of *Aspergillus flavus* spores on *S. litura*. MDA is the product formed during lipid peroxidation (LP). LP decreases the membrane fluidity by attacking on polyunsaturated fatty acids. Lipidhydroperoxides (LOOH) generated during lipid peroxidation also cause DNA damage by inducing single and double strand break in DNA and cell death (Ayala et al. 2014).

The activities of enzymes significantly increased after exposure to *A. flavus* extract, showing influence of fungus on antioxidant defense of insect. This might be due to activation of host response after toxicity induced by *A. flavus*, in which enzymes activities were remarkably accelerated to metabolize the ROS, reaching the maximum value at 96hr. Previous researches have documented the activation of insect protection system after fungal infection, to ward off infection and to maintain the normal physiological activities (Song et al. 2002; Zhang et al. 2003; Ding et al. 2015).

In *A. flavus* treated larvae SOD activity increased 1.82 fold as compared to control at 96hr, suggesting the increase in O<sub>2</sub> consumption lead to substantial increase in the free radical HO<sup>-</sup>. This might be activated SOD to clear the excess free radicals from the insect body. CAT and APOX activities were also found to be increased to rid the effects of the toxins. *in vivo* metabolism produced large amount of H<sub>2</sub>O<sub>2</sub>. This led to increased CAT and APOX activity. The production of H<sub>2</sub>O<sub>2</sub> was so high, both CAT and APOX helps in eliminating as consequences activities of enzymes increases. CAT and APOX activities increased 3.00 and 3.74 fold at 96hr in haemolymph of *S. litura* respectively. Similar increase in antioxidant enzymes activities [superoxide dismutase (SOD), catalase (CAT), peroxidases (POX)] was observed by Karthi et al. (2018) under the influence of *A. flavus* spores.

Scarce reports recorded the effect of fungal agents on protective enzymes activities. Ding et al. (2015) documented the effect of *B. bassiana* on various protective enzymes in *Xylotrechus rusticus* (Linnaeus). *M. anisopliae* also found to alter the antioxidant and detoxifying enzymes activities in *Periplaneta americana* (Linnaeus) and *Locusta migratoria* (Linnaeus) (Mutyalala *et al.* 2013; Narenbabu *et al.* 2013,

2014; Jia et al. 2016). Chaurasia et al. (2016) observed variable activity of antioxidant enzymes in *P. americana* under the influence of entomopathogenic fungus *Hirsutella thompsonii*. However alteration in enzymes activities due to different stress factors in insects has been reported by many studies (Hyrsl et al. 2007; Aslanturk et al. 2011). Barata et al. (2005) recorded the increase in CAT activity along with intensify lipid peroxidation in larvae of *Hydropsyche exocellata* (Dufour) after exposure to cadmium. Sezer and Ozalp (2015) observed increase in antioxidant enzyme activity in *Galleria mellonella* (Linnaeus) due to pyriproxyfen. Similarly the effect of UV radiations on antioxidants defense system has been documented by Karthi et al. (2014) and Ali et al. (2017).

Cellular response in insect immune system acts as an important barrier to the infection process (Hoffmann, 1995, 2003). Haemocytes types and their specific responses while insect-pathogen interaction act as a good indicators of insect defense reactions (Da Silva et al. 2000; Gillespie et al. 2000). There are different types of haemocytes which have been morphologically and functionally characterized in various insects. (Lavine and Strand 2002; Giulianini et al. 2003; Costa et al. 2005; Giglio et al. 2008). The most common types of haemocytes reported in the literature are prohemocytes, granulocytes, plasmatocytes, and oenocytoids. Multifunctional role of haemocytes are phagocytosis, encapsulation, cell agglutination, detoxification etc. Change in number and configuration was observed in haemocytes under different stresses which ultimately affect the health of insects. Consequently these cells have been used to ascertain the cytogenetic damage by toxic chemicals (Begum and Gohain 1996; Gayfullina et al., 2006).

So, in our investigation effect on cellular immune response was also judged by analyzing effect on haemocytes by scanning electron microscopy. SEM results showed various cellular deformities in different haemocytes (Plasmatocytes, Granulocytes, Prohaemocyte) like cell perforation, cytoplasmic leakage, strumae and surface abnormalities after treatment with ethyl acetate extract of *A. flavus* as compared to control. The results are in corroboration with study of Fan et al. (2013) which observed the cell perforation and rupturing with cytoplasmic leakage in haemocytes of *Bombyx mori* (Linnaeus) after treatment with destruxin A. There are very few studies which reveal the abnormalities of insect haemocytes using SEM, however technique has been used by various researchers to observe and characterize the different types of haemocytes in insects (Silva et al. 2002; Falleiros et al. 2003) and to observe spores accumulation in insect's body after fungal infection (Bawin et al. 2016; Baggio et al. 2016). Recently Duan et al. (2017) observed the infection of *B. bassiana* to *Leptinotarsa decemlineata* (Say) via scanning electron microscopy. However the morphological changes observed in present study were demonstrated by various workers due to entomopathogenic fungi and insecticides under light microscopy (Ferrarese et al. 2005; Habeeb and Abou El-Hag 2008; Kaur et al. 2011; Thakur et al. 2014; Kaur et al. 2015).

Mammalian toxicity of *A. flavus* was carried out by assessing DNA damage in blood, liver and kidney of rat. Non-significant effect was observed in all the tissues of fungal extracts treated rats as compared to control. Similarly negligible toxicity of azadirachtin, a neem biopesticides was earlier reported in rats (Raizada et al. 2001) and human (Boeke et al. 2004). There are few other botanical extracts which were

also checked for their toxicity on rat such as *Cassia senna*, *Caesalpinia gilliesii*, *Thespesia populnea*, *Chrysanthemum frutescens*, *Euonymus japonicus*, *Bauhinia purpurea*, and *Cassia fistula* extracts (Derbalah *et al.* 2012), *Dichaetanthera africana* extract (Kognou *et al.* 2018). Toxicity of fungal extracts on mammals was not checked previously however, Sprando *et al.* (2017) reported the safety of bacterial species *Paenibacillus alvei* to rat.

## **Conclusion:**

The study highlights the adverse effect of *A. flavus* on physiology of *S.litura* which might be due to negative impact on antioxidant and cellular immune defense of insect. The study helps to identify the insect defenses that could be manipulated to accelerate host death in biological control scenario. The study also showed its safety for mammals as it showed negligible toxicity on rat.

## **Declarations**

### **Acknowledgement:**

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### **Conflict of Interest:**

All authors declare that they have no conflict of interest.

### **Consent for publication:**

Not applicable.

### **Ethical approval:**

This article contains studies involving animals. Rats were used after getting permission from animal ethical committee

### **Availability of data and materials:**

All data generated or analyzed during this study are included in this article and its additional files.

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### Authors' contributions:

PC, AK and SK designed the study and analyzed the content. MK performed the experiments and analyzed the content related to it. All authors have read and approved the manuscript.

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## Tables

Table 1

Effect of different concentrations of ethyl acetate extract of *A. flavus* on different parameters of comet assay in rat blood

	Tail length		% Tail DNA		Tail Moment		Olive Tail Moment	
	24	96	24	96	24	96	24	96
<b>Control</b>	14.57 ± 0.18 <sup>a</sup>	14.78 ± 0.13 <sup>a</sup>	4.73 ± 0.41 <sup>a</sup>	5.13 ± 0.09 <sup>a</sup>	1.54 ± 0.27 <sup>a</sup>	1.64 ± 0.32 <sup>a</sup>	2.01 ± 0.07 <sup>a</sup>	2.14 ± 0.15 <sup>a</sup>
<b>100 mg/kg b.wt</b>	14.84 ± 0.21 <sup>a</sup>	14.99 ± 0.10 <sup>a</sup>	4.84 ± 0.02 <sup>a</sup>	5.19 ± 0.23 <sup>a</sup>	1.65 ± 0.11 <sup>a</sup>	1.44 ± 0.11 <sup>a</sup>	2.11 ± 0.05 <sup>a</sup>	2.45 ± 0.06 <sup>a</sup>
<b>200 mg/kg b.wt</b>	15.14 ± 0.04 <sup>a</sup>	15.51 ± 0.11 <sup>b</sup>	4.99 ± 0.45 <sup>a</sup>	5.52 ± 0.18 <sup>a</sup>	2.02 ± 0.10 <sup>a</sup>	2.33 ± 0.15 <sup>a</sup>	2.25 ± 0.01 <sup>a</sup>	2.78 ± 0.29 <sup>a</sup>
<b>F value</b>	NS	14.73**	NS	NS	NS	NS	NS	NS

The values represented as mean ± standard error. Different letters a, b within the columns are significantly different (Tukey's test,  $p \leq 0.05$ ) and signify the effect of concentration.

Table 2

Effect of different concentrations of ethyl acetate extract of *A. flavus* on different parameters of comet assay in rat liver

	Tail length		% Tail DNA		Tail Moment		Olive Tail Moment	
	24	96	24	96	24	96	24	96
<b>Control</b>	12.44 ± 0.15 <sup>a</sup>	12.69 ± 0.23 <sup>a</sup>	4.07 ± 0.29 <sup>a</sup>	4.22 ± 0.23 <sup>a</sup>	1.16 ± 0.15 <sup>a</sup>	1.34 ± 0.14 <sup>a</sup>	1.46 ± 0.19 <sup>a</sup>	1.44 ± 0.18 <sup>a</sup>
<b>100 mg/kg b.wt</b>	12.67 ± 0.20 <sup>a</sup>	12.84 ± 0.33 <sup>a</sup>	4.62 ± 0.22 <sup>a</sup>	4.78 ± 0.11 <sup>a</sup>	1.47 ± 0.13 <sup>a</sup>	1.44 ± 0.21 <sup>a</sup>	1.52 ± 0.07 <sup>a</sup>	1.57 ± 0.06 <sup>a</sup>
<b>200 mg/kg b.wt</b>	13.03 ± 0.27 <sup>a</sup>	12.92 ± 0.36 <sup>a</sup>	4.79 ± 0.25 <sup>a</sup>	4.64 ± 0.21 <sup>a</sup>	1.31 ± 0.10 <sup>a</sup>	1.70 ± 0.15 <sup>a</sup>	1.51 ± 0.20 <sup>a</sup>	1.85 ± 0.03 <sup>a</sup>
<b>F value</b>	NS	NS	NS	NS	NS	NS	NS	NS

The values represented as mean ± standard error. Different letters a, b within the columns are significantly different (Tukey's test,  $p \leq 0.05$ ) and signify the effect of concentration.

Table 3

Effect of different concentrations of ethyl acetate extract of *A. flavus* on different parameters of comet assay in rat kidney

	Tail length		% Tail DNA		Tail Moment		Olive Tail Moment	
	24	96	24	96	24	96	24	96
Control	14.95 ± 0.22 <sup>a</sup>	15.16 ± 0.39 <sup>a</sup>	4.74 ± 0.14 <sup>a</sup>	4.53 ± 0.04 <sup>a</sup>	1.70 ± 0.15 <sup>a</sup>	1.72 ± 0.16 <sup>a</sup>	1.93 ± 0.32 <sup>a</sup>	2.01 ± 0.31 <sup>a</sup>
100 mg/kg b.wt	15.18 ± 0.37 <sup>ab</sup>	15.21 ± 0.15 <sup>a</sup>	4.96 ± 0.09 <sup>a</sup>	5.07 ± 0.16 <sup>b</sup>	1.92 ± 0.05 <sup>a</sup>	1.91 ± 0.31 <sup>a</sup>	1.99 ± 0.01 <sup>a</sup>	1.96 ± 0.35 <sup>a</sup>
200 mg/kg b.wt	15.38 ± 0.02 <sup>b</sup>	15.57 ± 0.04 <sup>a</sup>	5.05 ± 0.30 <sup>a</sup>	5.06 ± 0.02 <sup>b</sup>	2.12 ± 0.13 <sup>a</sup>	2.31 ± 0.09 <sup>a</sup>	2.10 ± 0.15 <sup>a</sup>	2.17 ± 0.17 <sup>a</sup>
F value	0.71*	NS	NS	9.92*	NS	NS	NS	NS

The values represented as mean ± standard error. Different letters a, b within the columns are significantly different (Tukey's test, p ≤ 0.05) and signify the effect of concentration.

## Figures

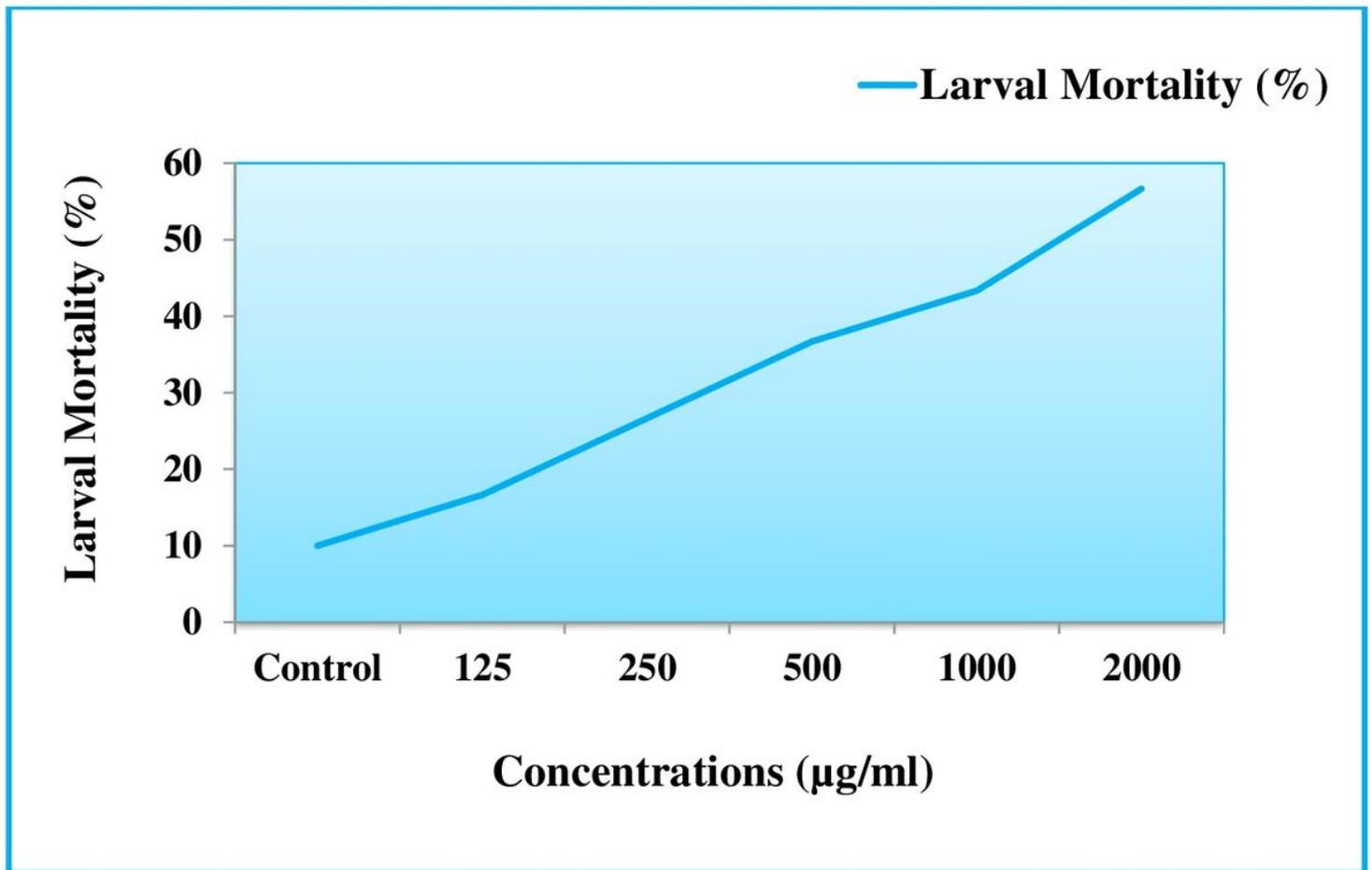


Figure 1

Mortality of *S. litura* larvae fed on diet supplemented with different concentrations of *A. flavus*.

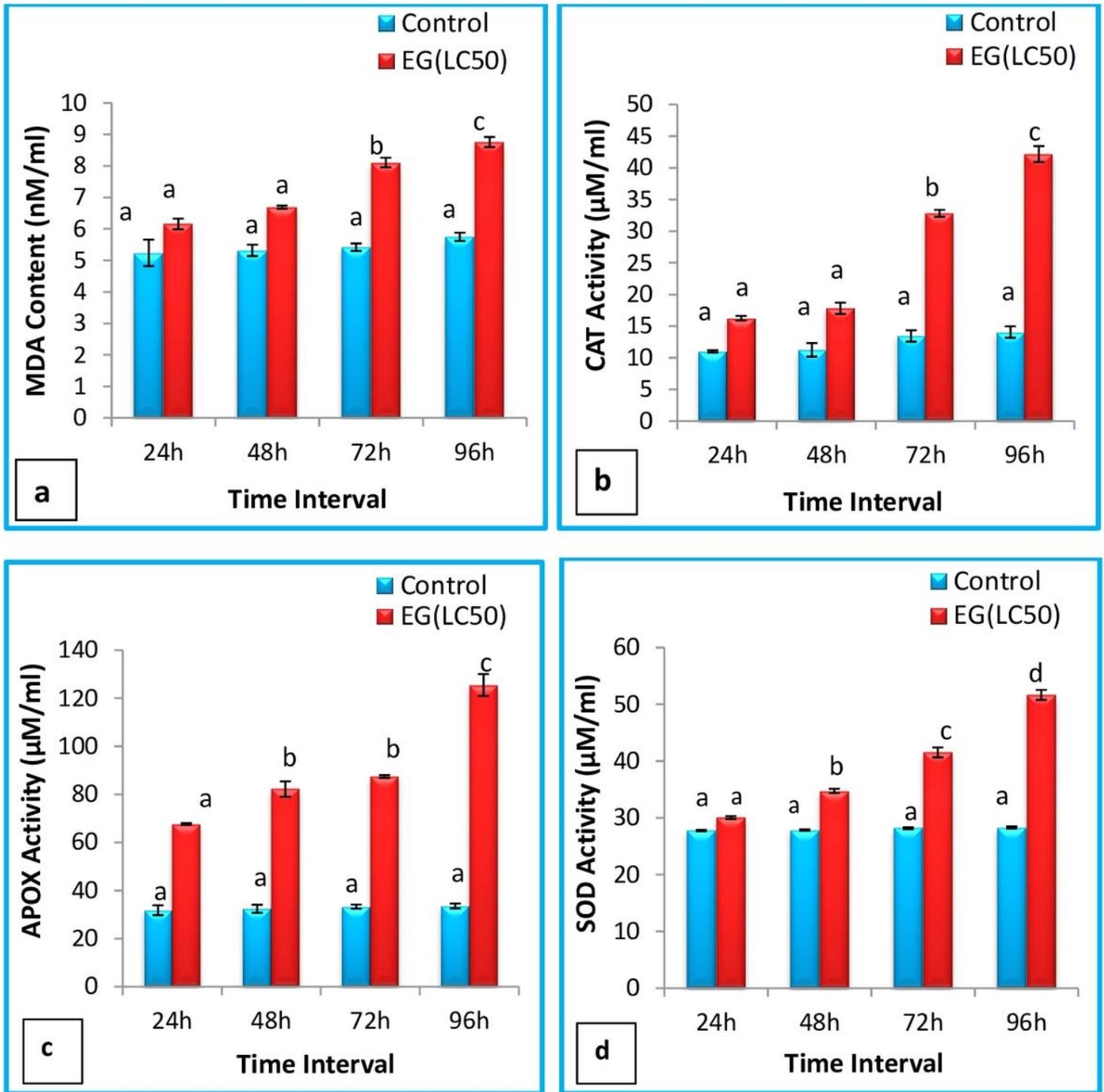
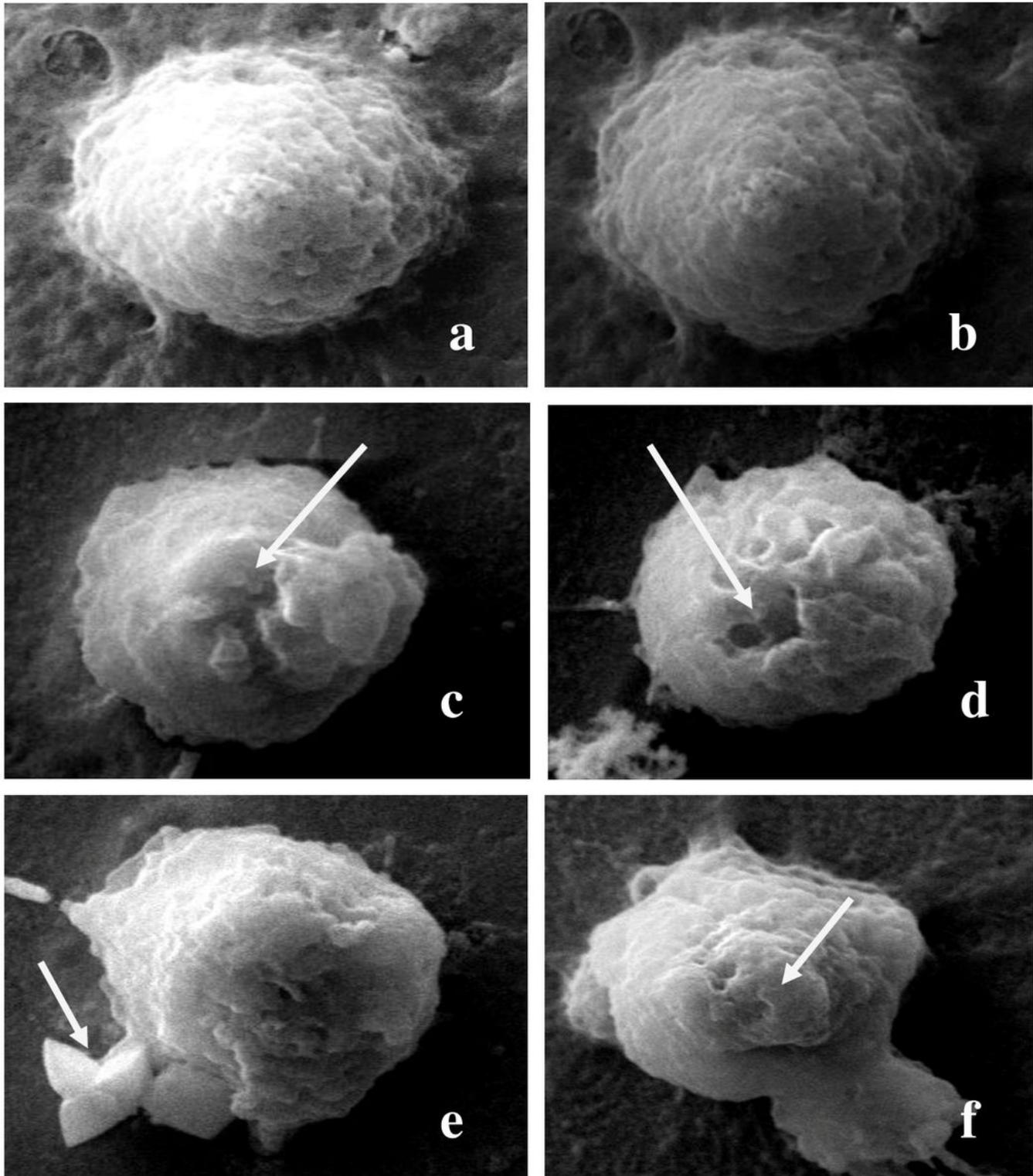


Figure 2

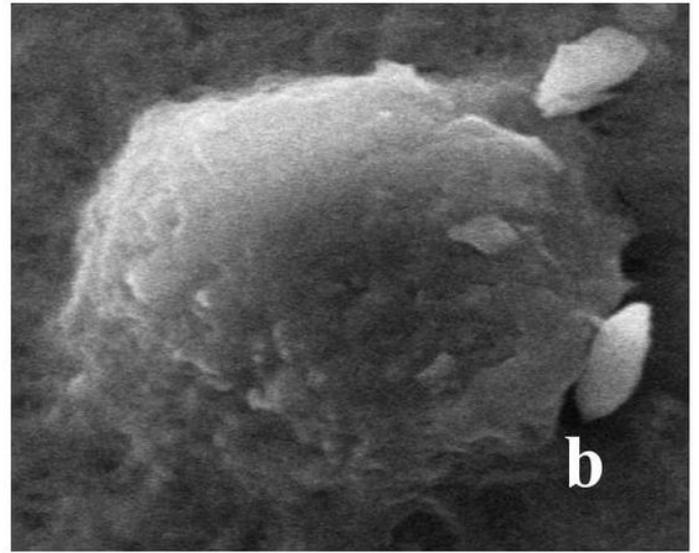
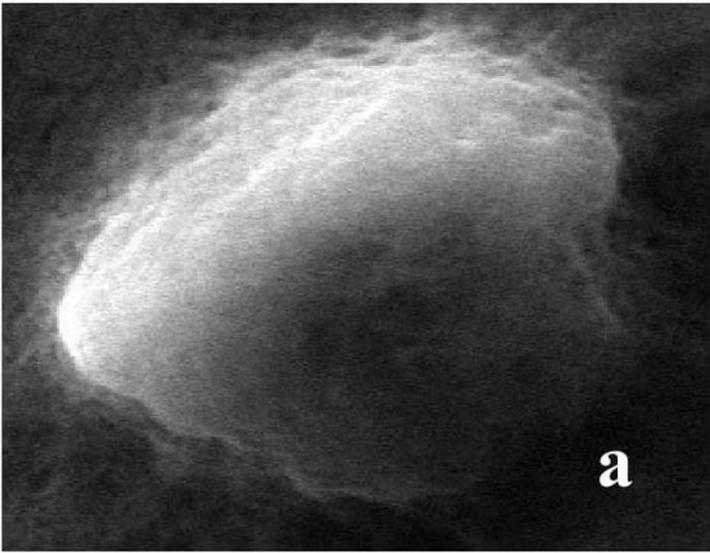
Malondialdehyde (MDA) content, Catalase (CAT), Ascorbate peroxidase (APOX) and Superoxide dismutase (SOD) activity in haemolymph of *S. litura* after treatment with ethyl acetate extract of *A. flavus* for different time intervals. EG = Exposed group. Bars represent mean  $\pm$  S.E. \*Ascribes the significant

difference between exposed group and control group (t-test,  $p \leq 0.05$ ). Different letters a, b, c, d are significantly different (Tukey's test,  $p \leq 0.05$ ) and signify the effect of duration.



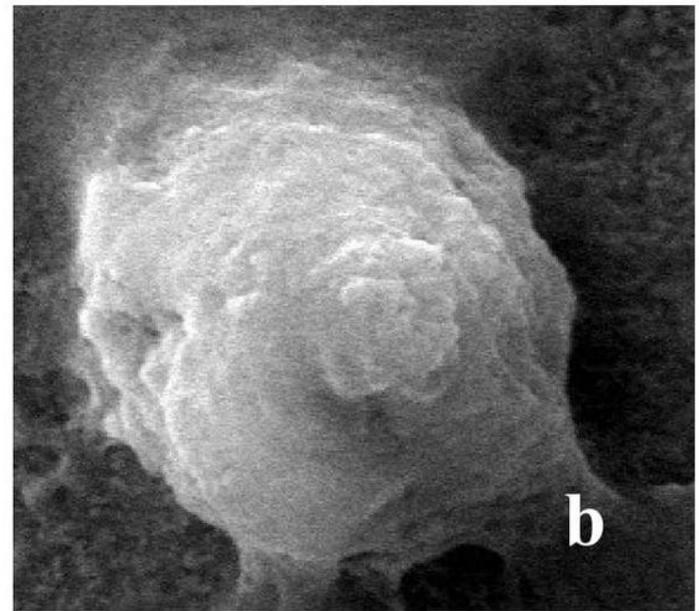
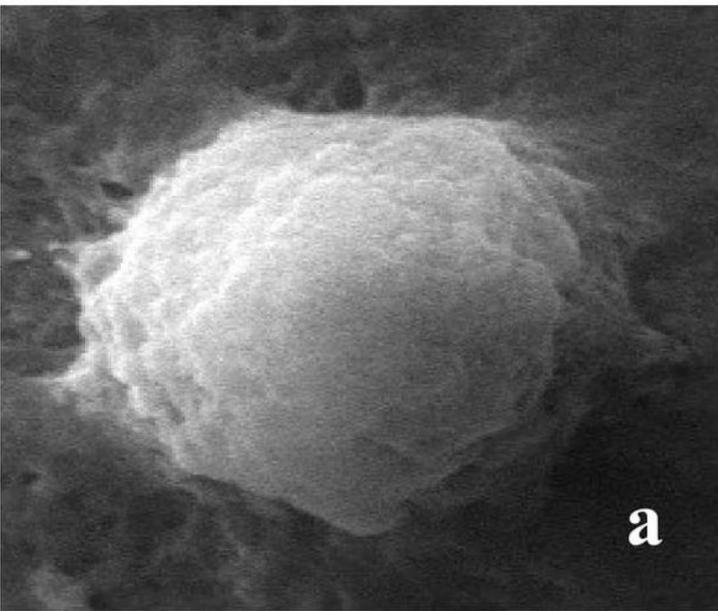
**Figure 3**

Microphotographs showing (a-b). Normal haemocytes (Plasmatocytes) (c-f). Various deformities observed in haemocytes after treatment with ethyl acetate extract of *A. flavus* (c-d). Cell perforation (e-f). Cytoplasmic leakage



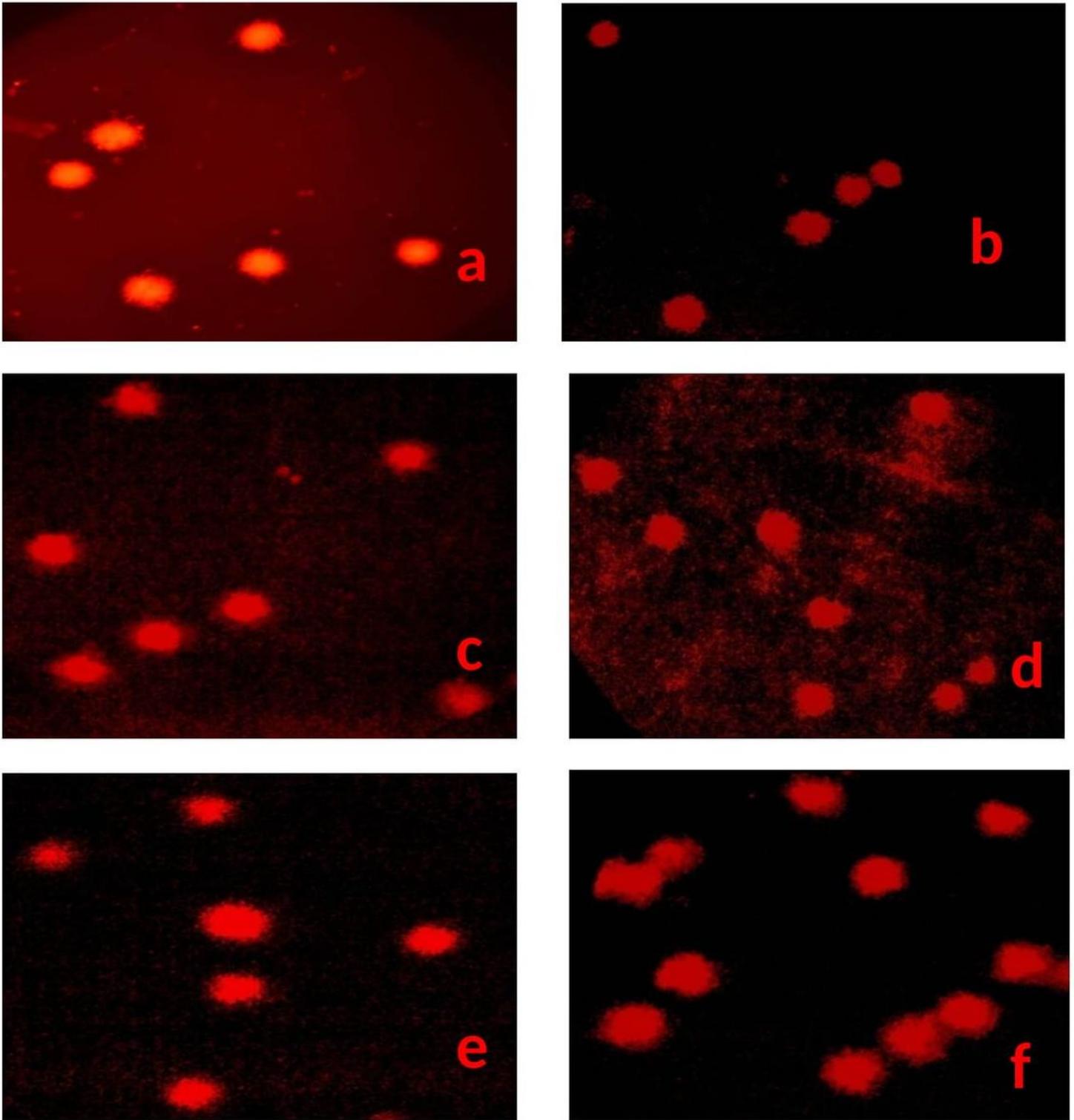
**Figure 4**

Microphotographs showing haemocytes (Granulocytes) (a). Normal haemocyte; (b). Strumae and surface abnormalities in haemocytes after treatment with ethyl acetate extract of *A.flavus*



**Figure 5**

Microphotographs showing haemocytes (prohaemocyte) (a). Normal haemocytes (b) surface abnormalities in haemocytes after treatment with ethyl acetate extract of *A.flavus*



**Figure 6**

Photomicrographs showing DNA extracted from (a, b) rat blood cells (a) Control; (b) After treatment with *A.flavus* ethyl acetate extract (c, d) rat kidney cells (a) Control; (b) After treatment with *A.flavus* ethyl acetate extract (e, f) rat liver cells (a) Control; (b) After treatment with *A.flavus* ethyl acetate extract

## Supplementary Files

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- [graphicalabstrac.jpg](#)