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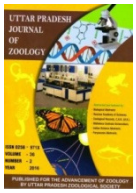

TONER INK USED IN LASER PRINTER AND COPIER MACHINES POSES TOXIC EFFECTS ON DEVELOPMENTAL STAGES OF *Sarcophaga ruficornis* Fabricius (Diptera: Sarcophagidae)

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Authors ZS and AS designed the study. Author MK performed the experiment under the guidance of authors ZS and AS. Authors ZS and MK wrote the first draft of the manuscript. Singh A gave the final edits to the draft. All authors read and approved the final manuscript.

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ABSTRACT

Printer toner is used in laser printers and photocopiers for printing text and images. Carbon powder and iron oxide are the main constituents of printer toner which are further mixed with different polymers in order to improve printing quality. Several engineered nanomaterials present in toner ink are released during the process of printing that have several adverse health effects. In the present study, we investigated toxicity of printer toner on the developmental stages of *Sarcophaga ruficornis* Fabricius (Diptera: Sarcophagidae). The fly was reared on goat flesh in the lab and 3rd instar larvae were exposed to four different concentrations of printer toner (10, 100, 1000 and 10000 ppm). Following exposure, larval mortality, pupation, pupal mortality and adult emergence were recorded. The results revealed a dose dependent toxic effect on developmental stages of *S. ruficornis* at tested concentrations. Further studies are recommended to test the toxicity of printer ink on different animal models so as improve the understanding towards its mechanisms involved in different health implications.

Keywords: Laser printer toner; carbon black; toxicity; *Sarcophaga ruficornis*; larvae; pupae; mortality.

1. INTRODUCTION

Printer toners are used for printing the text and images on the paper with the help of cartridge in laser printers and photocopiers. Laser printers and copiers are suspected to emit toner dust and volatile organic compounds and hence pose a health hazard [1]. Printer toners were earlier formulated by mixing carbon powder and iron oxides like magnetite (Fe_3O_4) [2] which was being used due to its tribocharging

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property. Now a days, toners comprise of very small particles of a thermoplastic polymers, usually a styrene-acrylate copolymer, that are fixed on the paper by fusing [3]. Carbon black is another component of printer toner. Carbon black with CAS number 1333-86-4 is the colloidal particles of pure elemental carbon resulting from incomplete combustion or thermal decomposition of liquid or gaseous hydrocarbon under controlled conditions. Though with printing advantages, printer toner also has some toxicological effects as several engineered nanomaterials (ENM) can be found in the toner-based printing equipments (TPE) which are used as toner formulations in laser printers and photocopiers. Studies have confirmed an increase in the concentration of ozone and ultrafine particle numbers in the printing processes of the printer [4]. ENMs have been reported to improve the performance and quality of the toner. During printing, these ENMs are released in the air along with some semi-volatile organic nanoparticles and gaseous co-pollutants including volatile organic compounds [5]. The released ENMs have potential adverse health effects [6]. As per one of the studies, printer toner used with external additives including titanium dioxide nanoparticles and amorphous silica nanoparticles caused pulmonary toxicity at low concentrations (1 or 2 mg) and neutrophil infiltration, inflammation and fibrosis of lung at high concentrations [7]. In another study, occupational exposure of black printer toner particles in A549 lungs cells not only damaged DNA but also induced micronuclei formation. A549 cells exposed to toner particles for 24 hours showed enhanced production of interlukin-6, interlukin-8, caspase 3/7 activity and also resulted in oxidative stress through reactive oxygen species (ROS) introduction that triggered genotoxic effects and activated pro-inflammatory pathways [8]. The effects on the structure and function of mitochondria were observed following exposure to the particulate emissions from printer toner. The mitochondrial damage was observed in the lungs of asthmatic mice by examining the levels of reactive oxygen species (ROS), lipid peroxides, reduced glutathione and activities of isocitrate dehydrogenase, alpha ketoglutarate dehydrogenase and succinate dehydrogenase. It was concluded that in the presence of tobacco smoke, printer emissions induced synergistic effect and an intense damage to the structure and functions of the mitochondrial membrane of the lungs [9]. Another study revealed bone marrow injury in lithographers exposed to the glycol ethers and organic solvents used in the multicolour offset and ultraviolet curing printing process [10]. The ultrafine particles that are produced by the thermal printer toner at high fuser heating and higher toner coverages have also shown to cause mucosal irritation symptoms in the users [11]. Even after careful search of different databases, no particular study was found to report toxic effects on *Sarcophaga sp.*

Sarcophaga ruficornis Fabricius is a flesh fly belonging to the family Sarcophagidae. These flies resemble houseflies in appearance but are larger in size. Unlike houseflies, they have three black strikes running along the top surface of their thorax. Their abdomen is bristled and have reddish brown tip at the end. Flesh flies infest animal carcasses, decaying material, carrion and open wounds of an animal. They feed on liquid substances. Unlike other flies, they deposit their hatched or hatching maggots on the decaying material and open wounds of an animal and thus are ovoviviparous [12]. These are well known myiasis causing agents. The infestation generally occurs on the skin, in the eyes or ears. Some studies used different cell lines to find out the toxicological effects of printer toner or its constituents. In the present study, *S. ruficornis* is used as an invertebrate experimental model to investigate upon the toxic effects of printer toner ink on its development cycle. *S. ruficornis* was selected as a model because of its short life cycle, in which the larvae hatch out within 24 hours and develop into 3rd instar forms after 3-4 days. Adult flies emerge out after 10-14 days afterwards. Apart from short life cycle, the rearing of fly is easy as large number of larvae can be procured under controlled lab conditions. The temperature and humidity range do not affect the developmental cycle of the fly much. Another reason for choosing *S. ruficornis* was that this model has not been explored much in research studies in comparison to other dipteran flies as *Drosophila melanogaster* [13,14]; *Musca domestica* [15,16]; *Chironomus riparius* [17] and *Chrysomya megacephala* [18]. Thus, in the present study, *S. ruficornis* has been presented as a new model to assess the toxicological effects of printer toner. This study will surely add to the existing knowledge about the toxic features of printer toner and printer inks.

2. MATERIALS AND METHODS

2.1 Larval Source

The present study was conducted in the department of Zoology, Khalsa College Amritsar, Punjab, India. The larvae of *S. ruficornis* were obtained from the goat flesh which was procured from local market, Amritsar (Fig. 1A). It was kept in petri dishes inside insect cages measuring 50x50x50 cm. Insect cages were kept open for a day in order to allow the oviposition. Flies visited the insect cages and laid eggs or larvae over the goat flesh. First instar larvae were

transported to BOD incubator at 28^0C with relative humidity of 70% till they matured into third instars. Fully grown 3rd instar larvae (Fig. 1B) were used for the experiment.

2.2 Larval Identification

In the mass of larvae formed at 5th day, a mixture of different larvae was found. In order to identify and separate the larvae of *S. ruficornis* from the mass, permanent mount of taxonomically important larval regions was prepared in which the structures of anterior and posterior spiracles were observed and compared. After observing the slides it was found that the number of lobes present in the anterior spiracles of the larvae varied and was of three types. It was 4-6 lobed as in case of *C. bezziana*, 5-7 lobed in *M. domestica* and 12-13 lobed in *S. ruficornis* (Fig. 1E). The structure of posterior spiracles also varied and showed three distinct types as in case of *M. domestica*, tortuous slits were present and the membrane of peritreme was closed. Peritremes were found open, number of inter-peritremal plates was 1 and oblique slits were present in case of *C. bezziana*. In case of *S. ruficornis*, peritremes were hidden in concavity, number of inter-peritremal plates was 2 and slits were more or less vertical (Fig. 1F). After getting these observations, the larvae of *S. ruficornis* were separated out from the bulk for the present experiment.

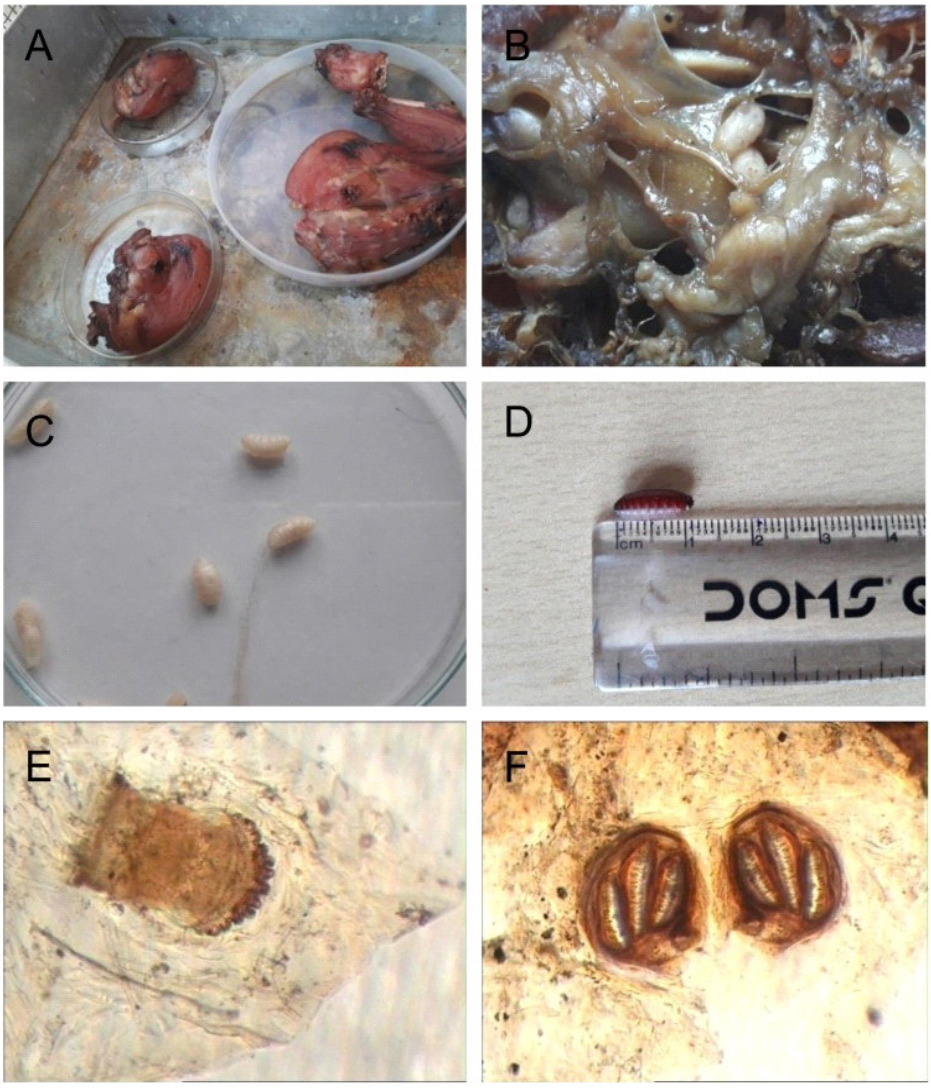

Fig. 1. Goat flesh kept in petri dishes for flies to lay eggs (A); 3rd instar larvae of *Sarcophaga ruficornis* on goat flesh (B) and in a petri dish (C); A live pupa of *S. ruficornis* (Average length=1 cm) (D); Permanent Mounts of anterior (E) and posterior spiracles (F) of 3rd instar larvae *S. ruficornis* (Reprinted from previous work [8])

2.3 Exposure Treatment

1 mg, 10 mg, 100 mg and 1 g of analytical grade of printer toner (Image Star Pvt. Ltd., India) was dissolved in 100 ml distilled water so as to formulate treatment concentrations. Different concentrations of 10, 100, 1000 and 10000 ppm were prepared to investigate the effects on the separated 3rd instar larvae of *S. ruficornis*.

2.4 Experimental Setup

For carrying out the experiment, third instar larvae procured from the goat meat kept in insect cages were used. A single replicate glass petri dish (Table 1, R) containing 15 larvae was set up for all the four concentrations along with the original experiment (Table 1, O) for calculating averages. The larvae were dipped one by one in each concentration for 30 seconds. After the treatment, the larvae were shifted to another petri dish covered with lid. These petri dishes were marked with an appropriate number and were kept in a dark place. These were examined for a month and the observations were recorded till the emergence of the adult flies.

2.5 Parameters Used

The effects of different concentrations of printer toner on the larvae of *S. ruficornis* were evaluated. Larval mortality, pupation, pupal mortality and adult emergence were recorded following all the treatments. For recording the observations, larvae were touched with fine zero grade brush. Pupation was recorded by counting the number of viable, turgid and brown coloured puparia at all the four treatment concentrations.

3. RESULTS AND DISCUSSION

The present study used *S. ruficornis* as a model to access the toxicity of printer toner. The effect of printer toner on the development of *S. ruficornis* in the form of larval mortality, pupation, pupal mortality and adult emergence is shown in Table 1. Our results showed a dose dependent toxic effect of laser printer toner on the observed life stages of *S. ruficornis.*

The first life stage taken to be observed under the experiment was larval stage. Fig. 3A shows the average larval mortality observed in the 3rd instar larvae of *S. ruficornis* on exposure to different test concentrations. No larval mortality was observed in lowest concentrations viz. 10 ppm. An average of 10% and 16.6% larval mortality was observed at 1000 and 10000 ppm toner concentration respectively. Fig. 2a shows a dead larva of *S. ruficornis* following higher toner concentrations. After observing the larval stage, second stage was the pupal stage. Number of larvae that successfully went to the pupal stage was observed. Fig. 3B shows the average number of larvae that pupated. Under test concentrations, it was found that all larvae exposed to 10 ppm concentration pupated normally (100% pupation; Table 1). However, at higher concentration of 100 ppm, 96.6% pupation was observed. A minimum of 83.3% pupation was found at highest concentration of 10000 ppm. Fig. 1D shows a successful pupation by a larva of *S. ruficornis.* The average size of the pupae was recorded to be 1 cm.

Third observation in the present study was to record the number of mortality in case of pupal stages. Some larvae successfully pupated but died at this stage and were unable to reach the adult stage. Fig. 3C shows average pupal mortality. The average number of pupae that were unable to emerge was recorded to be 10%, 13.3%, 20% and 26.6% at 10, 100, 1000 and 10000 ppm respectively. Fig. 2d shows a dead pupa following a 10000 ppm treatment. The fourth and the last stage observed was the adult emergence. The pupae that escaped death in the pupal stage emerged as adults. Fig. 3D shows average adult emergence. Maximum fly emergence was recorded at lowest concentration of 10 ppm followed by an average of 83.3% at 100 ppm, 70% at 1000 ppm and only 56.6% at 10000 ppm. Fig. 2b shows an emerging adult fly of *S. ruficornis* and Fig. 2c shows a free adult fly which escaped the toxicity effects of even the highest concentration of printer toner. On the basis of overall mortality of the fly following treatment concentrations, a trendline was drawn on the basis of percentage decrease in the adult emergence viz. 10%, 16.7%, 30% and 43.4% in all the four treatment concentrations. The linear trendline for overall % mortality gave the value for LC50 to be very close to 10000 ppm.

On the same line, exposure of human monocytes to nano- and micron- sized carbon black particles resulted in decrease in cell viability and an increase in pro-inflammatory cytokines IL-1β, TNF-α and chemokine IL- 8. An alteration of phagocytic capacity of monocytes was also observed on exposure to nano-sized carbon black particles [19]. Sub-chronic inhalation of carbon black particles caused lung inflammation and injury in rats as compared to mice and hamsters which were also chosen for another study. No observable adverse effects level (NOAEL) doses were chosen. The effects were more severe and prolonged in rats than mice and hamster [20]. In another study, female BALB/c mice were put in $1x10^6$pfu (plaque forming unit) respiratory syncytial

virus and after 3 days, they were exposed to 40µg ultrafine carbon black particles. This resulted in airway hyper-responsiveness, pulmonary inflammation and chemokine and cytokine expression in mice [21]. Toxicity of carbon black (Printex 90) was observed in mice on gestation days 7, 10, 15 and 18 which affected the health of the offsprings. Female offspring which were prenatally exposed to 268µg printex 90 showed altered habituation pattern during open field test [22]. High concentrations of heavy metals, vanadium (V), molybdenum (Mo) and nickel (Ni) present in carbon black waste decreased the viability of three human cell lines (HepG2 cells, MRC-5 cells and MDA-MB-231 cells) and an increase in the level of apoptosis [23]. In a study, the endothelial toxicity of carbon black nanoparticles aggregates formed by crosslinking of carbon black nanoparticles was investigated. It was observed in Human EA.hy926 vascular cells that nanoparticles induced cytoskeleton damage, dysfunction of endothelial barrier and expression of inflammatory factors [24]. The toxicity of single-walled carbon nanotubes was studied in rodents where acute and chronic inflammation, granuloma formation, collagen deposition, fibrosis and genotoxic effects were observed in lungs [25]. In another study, the effect of black carbon and ozone-oxidized black carbon was observed in mice. Oxidation increased the toxicity of black carbon and the results showed an enhanced infiltration of inflammatory cells pronounced in lungs and tissues exposed to ozone black carbon as compared to black carbon [26]. Similar effects were observed in mice exposed to black carbon and 1,4-naphthoquinone coated black carbon particles [27]. Carbon black and metal ions were found to cause autophagy and lysosomal dysfunction that accounted for the synergistic pulmonary toxicity [28]. Some of the studies on *S. ruficornis* mentioned the toxic effects of certain compounds like sodium azide [29] and thiourea [30]. No study was available presenting the toxic effects of printer toner or its components on *Sarcophaga*. Hence this paper is first of its kind to present *S. ruficornis* as a model for assessing toxicity of printer toner. Further studies are needed to evaluate the full toxic profile of printer toner using various other invertebrate models.

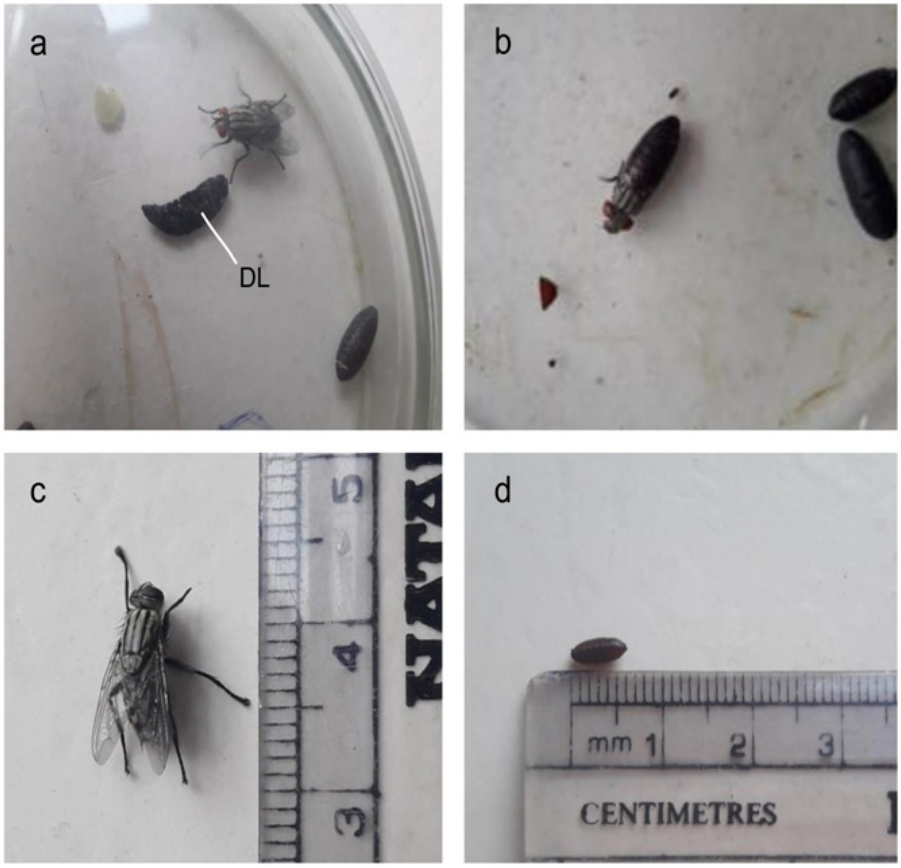

Fig. 2. A dead larva of *Sarcophaga ruficornis,* DL (a); Fly emerging from pupa under controlled conditions (b); An emerged adult fly *Sarcophaga ruficornis* (c); A dead pupa after larval exposure to 10000ppm (Average length=5.6mm) (d) (Reprinted from previous work [8])

Table 1. Larval mortality, pupation, pupal mortality and adult emergence in *S. ruficornis* following 30 second dip treatment at different concentrations

Treatment concentration (ppm)	Larval mortality		Pupation		Pupal mortality		Adult emergence	
Experiment →	**O**	**R**	**O**	**R**	**O**	**R**	**O**	**R**
10	0 0%	0	15 100%	15	1 10.0%	2	14 90.0%	13
100	0 3.3%	1	15 96.6%	14	2 13.3%	2	13 83.3%	12
1000	1 10.0%	2	14 90.0%	13	4 20.0%	2	10 70.0%	11
10000	3 16.6%	2	12 83.3%	13	5 26.6%	3	7 56.6%	10

O, Original experiment; R, Replicate experiment; Number of larvae per experiment: 15

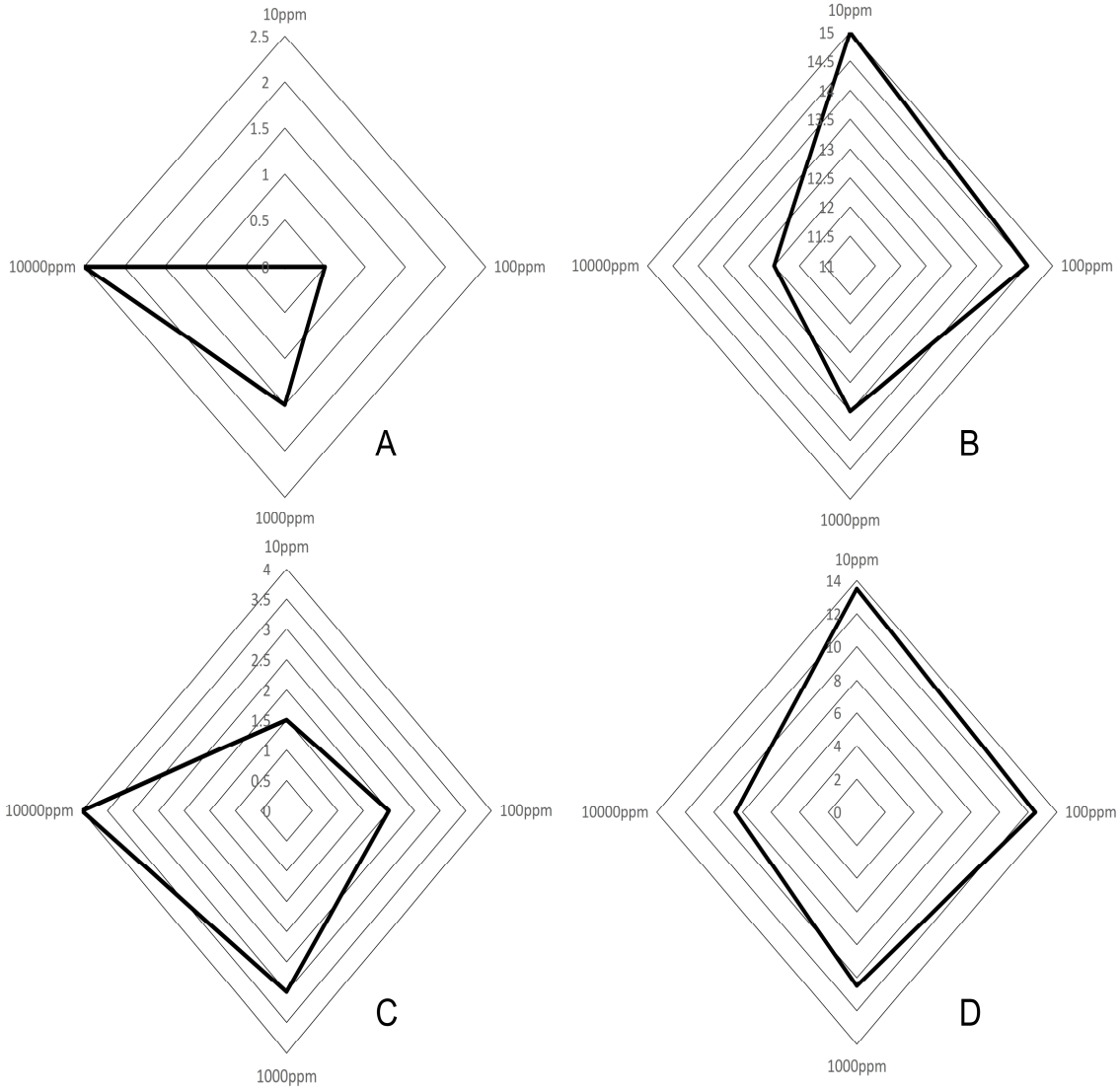

Fig. 3. Average larval mortality at different concentrations of printer toner (A); Average pupation at different concentrations of printer toner (B); Average pupal mortality at different concentrations of printer toner (C); Average adult emergence at different concentrations of printer toner (D)

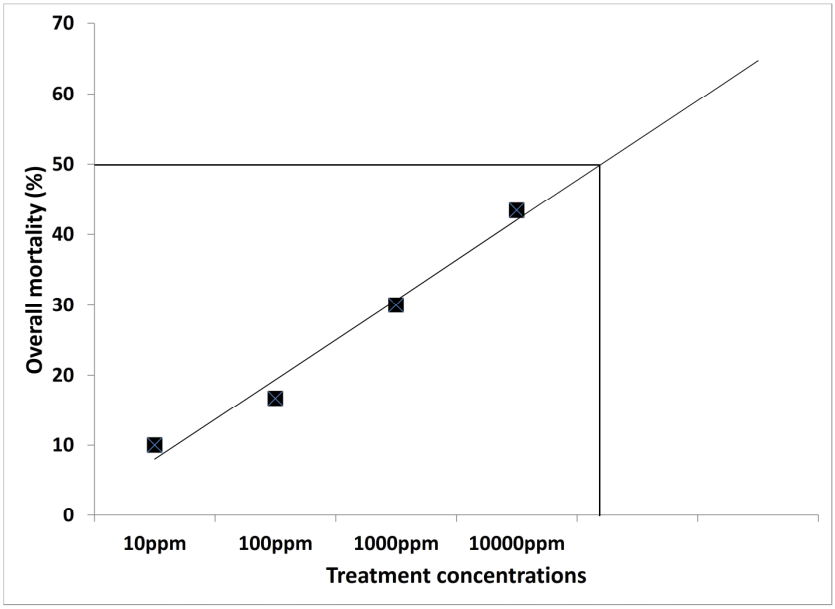

Fig. 4. Average adult emergence at different treatment concentrations with linear trendline forecast for LC50

4. CONCLUSION

Printer toner used in laser printer and photocopier has several adverse health effects including pulmonary inflammations and oxidative stress. In the present study, the effect of different concentrations of laser printer toner on 3rd instar larvae of *Sarcophaga ruficornis* was observed. Though statistical analysis was not possible for 2 groups (O and R) yet the results revealed a dose dependent toxic effect of printer toner on the developmental stages of the fly with an average of 90% adult emergence at lowest (10 ppm) and 56.6% at highest (10000 ppm) concentrations. Further studies on the toxicity of laser toner are recommended so as to highlight the mechanisms involved in the toxic effects.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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