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Toxicity and insect growth regulatory activities of medicinal plant, *Withania somnifera*, in flesh fly, *Sarcophaga ruficornis* (Diptera: Sarcophagidae)

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Abstract

Background: The flesh fly *Sarcophaga ruficornis* is well known for its medical and veterinary importance in causing myiasis in humans and animals. The conventional use of chemical pesticides for controlling insect pests has resulted in environmental pollution besides posing serious hazards to non-target organisms and the development of pest resistance against these compounds. Considering the various harmful and adverse effects of chemical pesticides, an alternative and safe approach for the pest management has to be explored. The plant extracts derived from plants are eco-friendly in nature, easily biodegradable, and can be used as botanical pesticides.

Results: Extracts from root of the medicinal plant *Withania somnifera* were assessed for their toxicity and insect growth regulatory activity when administered to larvae of *S. ruficornis*. Topical administration of root extracts of *W. somnifera* to 0–3-day-old third instar larvae of *S. ruficornis* resulted into severe disruption of development, moulting, and metamorphosis producing several detrimental effects such as toxicity, prolongation of larval life, ecdysial stasis, abnormal pupariation, reduced normal pupariation and adult emergence, and development of pupal-adult mosaics and adultoids. The LC_{50} values in topical application of root extracts of *W. somnifera* were 28.19, 43.49, 47.48, and 48.16 g/μL against *S. ruficornis* third instar day 0, 1, 2, and 3 larvae, respectively.

Conclusion: These effects are similar to those observed as a consequence of the administration of juvenile hormone analogs (juvenoids) and may be due to interference with the normal hormonal mechanism of moulting and metamorphosis. This clearly demonstrates that the root extracts of *W. somnifera* may be used as an insect growth regulator for the control and management of *S. ruficornis* along with other bio-rational approaches in integrated pest management programs.

Keywords: Ashwagandha, Larval-pupal ecdysis, Pupal-adult mosaics, Abnormal pupariation, Adultoids, Flesh fly

Background

Myiasis is a parasitic infestation in vertebrates caused by dipteran insects which deposit their eggs or neonate larvae in the host's body or body apertures (Kaya et al. 2014) and the larvae can infect living or necrotic tissues involving the skin, nasopharynx, urogenital, and gastrointestinal tracts, feeding or living in necrotic host tissue, bodily fluids, or ingested food (Ly et al. 2018). Many insect species are involved in causing myiasis in humans and other animals, and are responsible for severe damage to animal husbandry resulting into significant economic loss including reduction of milk production, weight and fertility, and reduced hide quality (Francesconi and Lupi, 2012).

Sarcophaga ruficornis (Diptera: Sarcophagidae) (F., 1794), commonly known as flesh fly, is also a causative agent of human myiasis (Ferraz et al. 2010). *Sarcophaga* sp. has been

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reported to cause aural myiasis, nosocomial myiasis (Francesconi and Lupi, 2012), cutaneous myiasis (Kaya et al. 2014), wound myiasis (Ergun et al. 2016), intestinal myiasis (Ly et al. 2018), and neonatal myiasis (Martinez-Rojano et al. 2018). *Sarcophaga* sp. has also been reported to cause external myiasis in three cyprinid species like *Cyprinus carpio*, *Barbus grypus*, and *Capoeta trutta* caught from Atatürk Dam Lake, Turkey (Oktener and Alas, 2009). Apart from larvae of *Sarcophaga* sp causing myiasis, the adults may act as vectors for mechanical transmission of pathogens causing gastrointestinal diseases (Graczyk et al. 2005).

Unscrupulous and non-judicious use of chemical pesticides for control and management of insect pests has resulted in deleterious effects on the environment, ecosystem, and public health. Contamination of air, water, and soil has already adversely affected ecosystem and human health, disrupted the dynamics of the food web in the community, destroyed beneficial organisms, and led to the development of pest resistance (Zacharia, 2011). Considering these harmful effects of chemical pesticides, a safe and environmental friendly approach for insect control is desired. For instance, botanicals or compounds derived from the plants are considered to be the most convincing tool for the control and management of various insect pests (Pant et al. 2016). These plant derivatives are eco-friendly with target specificity, biodegradable, economically feasible, devoid of toxic residues, and with the least chances of development of pest resistance (Pant et al. 2016; Rajapakse et al. 2016).

Withania somnifera, commonly known as Aswagandha, is an important medicinal plant belonging to the Solanaceae family and widely used in the Indian traditional system of Ayurveda. The root of *W. somnifera* contains alkaloids (withanine, withasomnine, somniferine), steroidal lactone (withaferins and withanolides), and steroids (stigmasterol and sitoinosides). This medicinal herb has been shown to possess anti-microbial, anti-tumor, anti-inflammatory, anti-stress, anti-diabetic, cardioprotective, and neuroprotective properties (Dar et al. 2015). The medicinal properties of root extracts of *W. somnifera* are very well known but its insecticidal and insect growth regulatory activity have been described only in a few lepidopteran and coleopteran insects (Gupta and Srivastava, 2008; Gaur and Kumar, 2017a, 2019; Yadav et al. 2019). In the present communication, the insect growth regulatory (IGR) activity of root extracts of *W. somnifera* against the dipteran flesh fly, *S. ruficornis* has been described.

Materials and methods

Insect rearing

Sarcophaga ruficornis was reared according to the method of Singh and Kumar (2015c). Adults of *S. ruficornis* were captured from wild and kept at a ratio of 1♂:2♀ in acrylic frame cages (15 × 15 × 15 cm) with gauze sides and provided with sugar powder and 10% honey-water solution on a cotton pad for feeding. The adult flies were reared in the laboratory at 28 ± 2 °C, 70 ± 5% relative humidity and 10 L:14 D photoperiod to obtain a pure line colony. Fresh pieces of goat's liver were supplied as a standard protein source and also for larviposition. First instar, neonate larvae from a single female were collected and transferred to sterilized glass beakers containing fresh pieces of goat's liver for feeding. In order to prevent infection by the decomposition and spoilage of liver pieces due to bacterial infestation, fresh pieces of goat's liver were supplied daily to developing larvae which were shifted to clean and sterilized glass beakers. When the last instar larvae reached the post-feeding stage, they were transferred to sterilized glass troughs containing sawdust for pupariation. The pupation followed a few hours later and the pupal period lasted for 9–11 days at the end of which adult flies emerged.

Plant material and extraction

A pure herbal root powder product of *W. somnifera* was purchased from Sri Jain Ayurvedic Pharmacy, Kattedan, Hyderabad, India (Mfg. License No.T-1633/Ayur). The root extract of *W. somnifera* was prepared according to the method described by Rathi and Gopalakrishnan (2006). Two hundred fifty grams of root powder was extracted with 500 mL (boiling point 40–60 °C) of acetone using a Soxhlet apparatus at 50 °C for two days and filtered through Whatman no. 1 filter paper. The acetone was removed with the help of a rotatory vacuum evaporator under reduced pressure at 22–26 mmHg and a crude extract of roots of *W. somnifera* was obtained. Known quantities of the crude extract were weighed and dissolved in 1 mL of acetone to get the desired concentrations or doses. For each treatment, fresh doses were prepared and stored at 4 °C to avoid differences in concentration due to evaporation.

Experimental procedure

The larvae of the desired age group, i.e., third instar day 0 (freshly moulted or 0–2 h old), 1, 2, and 3 were selected from the stock and grouped into batches of 15 larvae each. All experiments of one set were carried out on the larvae from a single batch to avoid any age difference. In separate set of experiments, the larvae were topically treated with different doses of root extracts of *W. somnifera* (5, 10, 15, and 20 μg/1 μL/larva) applied on the dorsum of the posterior abdomen with the help of a microapplicator. The control larvae were treated with pure acetone only in a similar manner. After treatment, both treated and control larvae were transferred to sterilized beakers and provided with fresh pieces of goat's liver for feeding and subsequently post-feeding larvae

were transferred to sawdust for pupariation. All the experiments were repeated thrice. Both the control and treated larvae were monitored regularly and dead, malformed specimens were fixed in Bouin's fluid and preserved in 70% ethyl alcohol for morphogenetic studies. Photography was done using Nikon SMZ 1000 Binocular Stereozoom microscope and NIS Software (Towa Optics, Japan).

Statistical analysis

The data regarding larval-pupal ecdysis was subjected to one way ANOVA to find out a significant difference between the mean larval duration of treated and control groups. Correlation coefficient was also calculated to determine the correlation between the doses administered and various morphological abnormalities observed. The LC_{50} values were also analyzed. All the calculations were performed with the help of GraphPad Prism 2007, 5.01 software (San Diego, CA).

Results

Topical administration of different doses of root extracts of *W. somnifera* to third instar day 0 (freshly moulted or 0–2 h old), 1, 2, and 3 larvae of *S. ruficornis* resulted into larval and pupal mortality, delay in larval-pupal ecdysis, abnormal pupariation, suppression of adult emergence, and formation of pupal-adult mosaics and adultoids.

Mortality

Topical administration of root extracts of *W. somnifera* to third instar 0–3-day-old larvae of *S. ruficornis* resulted into larval and pupal mortality in a dose-dependent manner showing a significant positive correlation in all the treated groups (Table 1). Maximum larval and pupal mortality occurred as a result of treatment of third instar day 0 larvae and third instar day 3 larvae of *S. ruficornis* respectively (Table 1). The LC_{50} values of root extracts of *W. somnifera* were 28.19, 43.49, 47.48, and 48.16 μg/μL against *S. ruficornis* third instar days 0, 1, 2, and 3 larvae, respectively (Fig. 1).

Larval-pupal ecdysis duration

Topical administration of root extracts of *W. somnifera* to 0–3-day third instar larvae of *S. ruficornis* resulted in a significant delay in larval-pupal ecdysis in a dose-dependent manner as compared to controls (Fig. 2). The larval duration was increased significantly at all the doses as a result of treatment of 0- and 1-day old third instar larvae of *S. ruficornis* (Fig. 2a, b) whereas in case of day 2- and day 3-old third instar larvae there was a significant prolongation of larval duration at higher doses of 15 and 20 μg/μL (Fig. 2c, d). There was a maximum prolongation of larval life of 1.81 days ($F = 8.664$; $R^2 = 0.2241$; df = 4, 120; $p \leq 0.001$), 1.01 days ($F = 5.875$; $R^2 = 0.1464$; df = 4, 137; $p \leq 0.001$), 0.86 days ($F = 4.165$; $R^2 = 0.1077$; df = 4, 138; $p \leq 0.01$), and 0.48 days ($F = 3.481$; $R^2 = 0.0893$; df =4, 142; $p \leq 0.05$) at a dose of 20 μg/μL when *S. ruficornis* third instar day 0, day 1, day 2, and day 3 larvae were respectively treated with root extracts of *W. somnifera* as compared to controls.

Abnormal pupariation

Topical administration of root extracts of *W. somnifera* to third instar 0–3-day-old larvae of *S. ruficornis* resulted in the formation of abnormal puparia in a dose-dependent manner (Table 1). There was a significant positive correlation between the doses applied and formation of abnormal puparia in case of treatment of day 0 ($r = 0.99$), day 1 ($r = 0.95$), day 2 ($r = 0.97$), and day 3 ($r = 0.95$) third instar larvae (Table 1). Production of abnormal puparia also increased with the increase in age of treated third instar larvae of *S. ruficornis.* Thus, abnormal puparia formation was dose-dependent as well as age-dependent.

Depending upon the abnormalities, abnormal puparia have been broadly classified into the following types:

(1) Body larval in appearance with coarse body surface and black-brown pupal pigmentation. The retraction of the anterior end has occurred but not fully complete whereas the longitudinal muscle contraction and cuticular shrinkage were inhibited (Plate 1C). In some cases, the larval body was extremely elongated with dark brown pupal pigmentation (Plate 1D).
(2) Contracted puparium showing brown pupal pigmentation. The anterior end has not fully retracted and the longitudinal muscle contraction and cuticular shrinkage adversely affected (Plate 1E).
(3) Highly deformed and twisted puparium with dark brown pupal pigmentation and coarse body surface; the anterior end partially retracted but the longitudinal muscle contraction and cuticular shrinkage adversely affected. The whole body was tanned and sclerotized but the posterior end, especially the posterior extremity of the body remained untanned (white) and unsclerotized (Plate 1F).
(4) Elongated larval body suffering from inhibition of longitudinal muscle contraction which was more conspicuous towards the anterior end. The smooth body surface with brown pupal pigmentation was suggestive of cuticular shrinkage but the anterior end of the body not fully retracted (Plate 1G).
(5) Puparium larval in appearance with light brown pupal pigmentation having coarse body surface and

Table 1 Effect of topical administration of root extracts of *Withania somnifera* to third instar day 0–3 larvae of *Sarcophaga ruficornis* (n = 15; replication = 3)

Age of larvae	Dose ($\mu g \cdot \mu L^{-1}$)	Larval mortality (%)	Abnormal pupariation (%)	Normal pupariation (%)	Pupal mortality (%)	Pupal-adult mosaics (%)	Adult emergence (%)	
							Normal adults (%)	Adultoids (%)
Third instar day 0	0	0	0	100	0	0	100	0
	5	22.22	11.11	66.66	8.88	31.11	13.33	13.33
	10	31.11	15.55	53.33	13.33	22.22	11.11	6.66
	15	37.77	26.66	35.55	17.77	6.66	4.44	6.66
	20	44.44	33.33	22.22	20.00	0.00	0.00	2.22
		(r = 0.96)[c]	(r = 0.99)[a]	(r = − 0.98)[b]	(r = 0.97)[b]	(r = − 0.28)	(r = − 0.79)	(r = − 0.07)
Third instar day 1	0	0	0	100	0	0	100	0
	5	15.55	15.55	68.88	11.11	24.44	20.00	15.55
	10	22.22	15.55	62.22	13.33	24.44	11.11	15.55
	15	28.88	22.22	48.88	17.77	20.00	4.44	6.66
	20	35.55	28.88	35.55	22.22	2.22	0.00	11.11
		(r = 0.98)[b]	(r= 0.95)[c]	(r = − 0.97)[b]	(r = 0.97)[b]	(r = − 0.00)	(r = − 0.82)	(r = 0.32)
Third instar day 2	0	0	0	100	0	0	100	0
	5	8.88	17.77	71.11	13.33	24.44	20.00	15.55
	10	15.55	17.77	66.66	20.00	20.00	11.11	15.55
	15	22.22	31.11	46.66	22.22	13.33	4.44	6.66
	20	28.88	37.77	33.33	24.44	2.22	0.00	4.44
		(r = 1.0)[a]	(r = 0.97)[b]	(r = − 0.98)[b]	(r = 0.93)[c]	(r = − 0.10)	(r = − 0.82)	(r = − 0.0002)
Third instar day 3	0	0	0	100	0	0	100	0
	5	4.44	22.22	73.33	15.55	28.88	15.55	13.33
	10	8.88	24.44	66.66	20.00	20.00	13.33	13.33
	15	15.55	35.55	48.88	22.22	6.66	8.88	11.11
	20	22.22	40.00	37.77	24.44	0.00	0.00	13.33
		(r = 0.99)[a]	(r = 0.95)[c]	(r = − 0.98)[b]	(r = 0.90)a	(r = − 0.27)	(r = − 0.80)	(r = 0.67)

[a]Significant at $p \leq 0.001$
[b]Significant at $p \leq 0.01$
[c]Significant at $p \leq 0.05$; r =correlation coefficient

failure of retraction of anterior segments (Plate 1H and I).

(6) Abnormal puparium with smooth body surface and brown pupal pigmentation but the body was unusual in not acquiring the typical barrel-shaped structure and thus suffering from inhibition of longitudinal muscle contraction (Plate 1J).

Normal pupariation

Normal pupariation was reduced in a dose-dependent manner as a result of topical administration of root extracts of *W. somnifera* to third instar day 0–3 larvae of *S. ruficornis* as compared to controls (Table 1). Maximum reduction in normal pupariation was observed at a dose of 20 μg/μL root extracts of *W. somnifera*. At this dose, 22.22, 35.55, 33.33, and 37.77% normal puparia were formed as a result of treatment of third instar day 0, day 1, day 2, and day 3 larvae respectively (Table 1). There was a significant negative correlation between the doses administered and percentage of normal pupariation in all the age groups of treated third instar larvae viz. day 0 (r = − 0.98), day 1 (r = − 0.97), day 2 (r = − 0.98), and day 3 (r = − 0.98) (Table 1).

Pupal-adult mosaics

Topical administration of root extracts of *W. somnifera* to 0–3-day third instar larvae of *S. ruficornis* resulted into the formation of pupal-adult mosaics at all the doses except at 20 μg/μL as a result of treatment of third instar day 0 and day 3 larvae of *S. ruficornis* (Table 1).

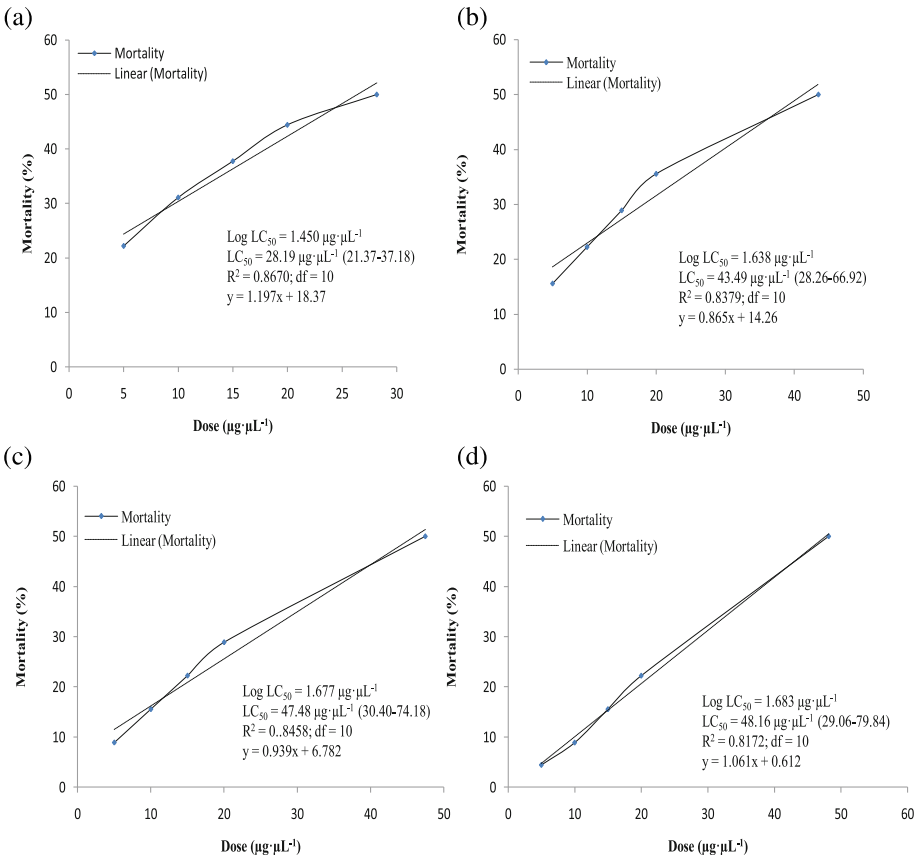

Fig. 1 Dose-mortality (LC_{50}) response to root extracts of *W. somnifera* against **a** third instar day 0, **b** third instar day 1, **c** third instar day 2, and **d** third instar day 3 larvae of *S. ruficornis*. (R^2 = coefficient of determination; df = degree of freedom)

Depending upon the development of pupal/adult characters, these have been broadly classified into the following grades;

(I) Body differentiated into head, thorax, and abdomen; whole body white, smooth, and pupal in appearance devoid of adult cuticle with hairs and bristles; mouthparts in the form of a protuberance; wings cylindrical; genitalia undeveloped (Plate 1K and L).
(II) Pupal-adult mosaic similar to grade (I) except tanning occurred partially in legs, wings, and mouthparts (Plate 1M and N).
(III) Pupal-adult mosaic with pigmented eyes, ocelli partially developed; partially tanned left pro, meso, and meta and, right metathoracic legs and, right-wing whereas the rest characters pupal in appearance (Plate 1O and P).
(IV) Pupal-adult mosaic consisting of untanned pupal head and thorax whereas the dorsal surface of abdomen highly tanned bearing small hairs and with inconspicuous adult segmentation; partially tanned legs and wings; ventral abdomen white and pupal devoid of genitalia (Plate 1Q and R).
(V) Pupal-adult mosaic with white, untanned pupal thorax; eyes pigmented; dorsal abdomen highly tanned and bearing small hairs; antennae, mouthparts, legs, and wings partially tanned; ventral abdomen white and devoid of genitalia (Plate 2A and B).
(VI) Pupal-adult mosaic with adult characteristics except for white, untanned and smooth pupal abdomen; genitalia undeveloped (Plate 2C and D).
(VII) Pupal-adult mosaic with adult characters but with less sclerotization and reduced numbers of hairs and bristles as compared to those found on the body of a normal adult; genitalia undeveloped. Pupal cuticle enveloped the entire body (Plate 2E and F).
(VIII) Pupal-adult mosaic similar to normal adult except the genitalia not fully developed and whole body enclosed in the pupal cuticle (Plate 2G-I).

Different grades of pupal-adult mosaics produced as a result of treatment of 0–3-day-old third instar larvae with different doses of root extracts of *W. somnifera* have been shown in Fig. 3. Pupal-adult

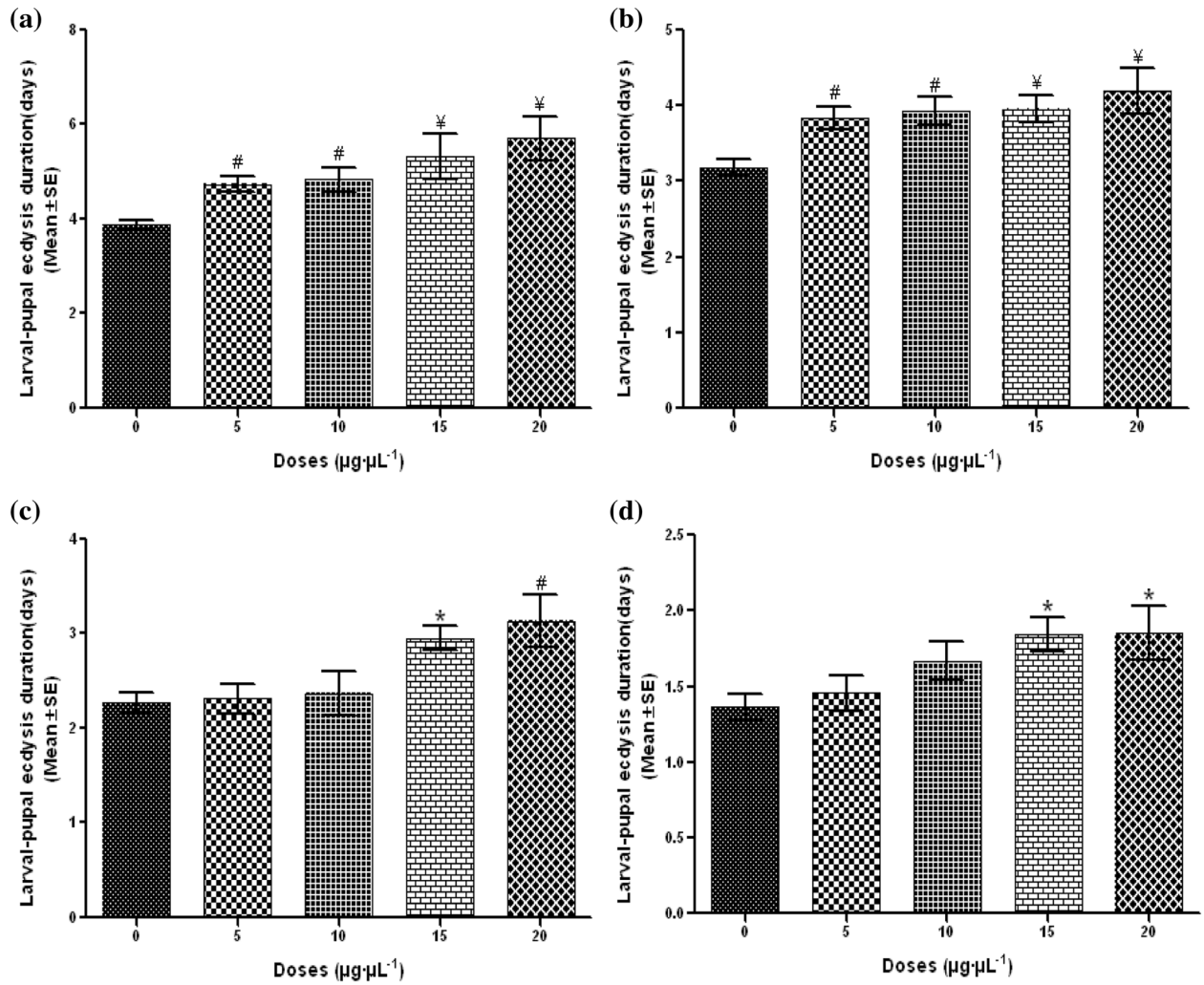

Fig. 2 Larval-pupal ecdysis duration (days) after topical administration of root extracts *W. somnifera* to **a** third instar day 0 larvae, **b** third instar day 1 larvae, **c** third instar day 1 larvae, and **d** third instar day 3 larvae of *S. ruficornis*.*Significant at $p \leq 0.05$; #Significant at $p \leq 0.01$; ¥Significant at $p \leq 0.001$

mosaics of grades I, II, III, and IV were produced in case of treatment of 0–1-day-old last instar larvae (Fig. 3a, b), whereas those of grades V, VI, VII, and VIII were produced when 2–3-day-old third instar larvae were treated (Fig. 3c, d).

Adult emergence

Topical administration of *W. somnifera* root extracts to third instar 0–3-day larvae of *S. ruficornis* resulted into a reduction in adult emergence showing a negative correlation between the doses administered and the percentage of adult emergence in a dose-dependent manner (Table 1). The normal adult emergence was totally suppressed at a dose of 20 µg/µL of root extracts in all the cases of treatment (Table 1).

Adultoids

Topical administration of root extracts of *W. somnifera* to third instar 0–3-day-old larvae of *S. ruficornis* resulted in the emergence of adultoids.

Depending upon the morphological abnormalities, the adultoids have been classified into the following types:

(1) Adultoid with deformed wings, unable to stretch; deformed and partially tanned legs; abdomen reduced with black pigmentation as compared to light and dark square dots present on the abdomen of a normal adult; scar of genitalia developed (Plate 2K and L).
(2) Adultoid with deformed wings unable to inflate; ptilinum everted; legs deformed, twisted and not suitable to sit or walk; a few hairs and bristles

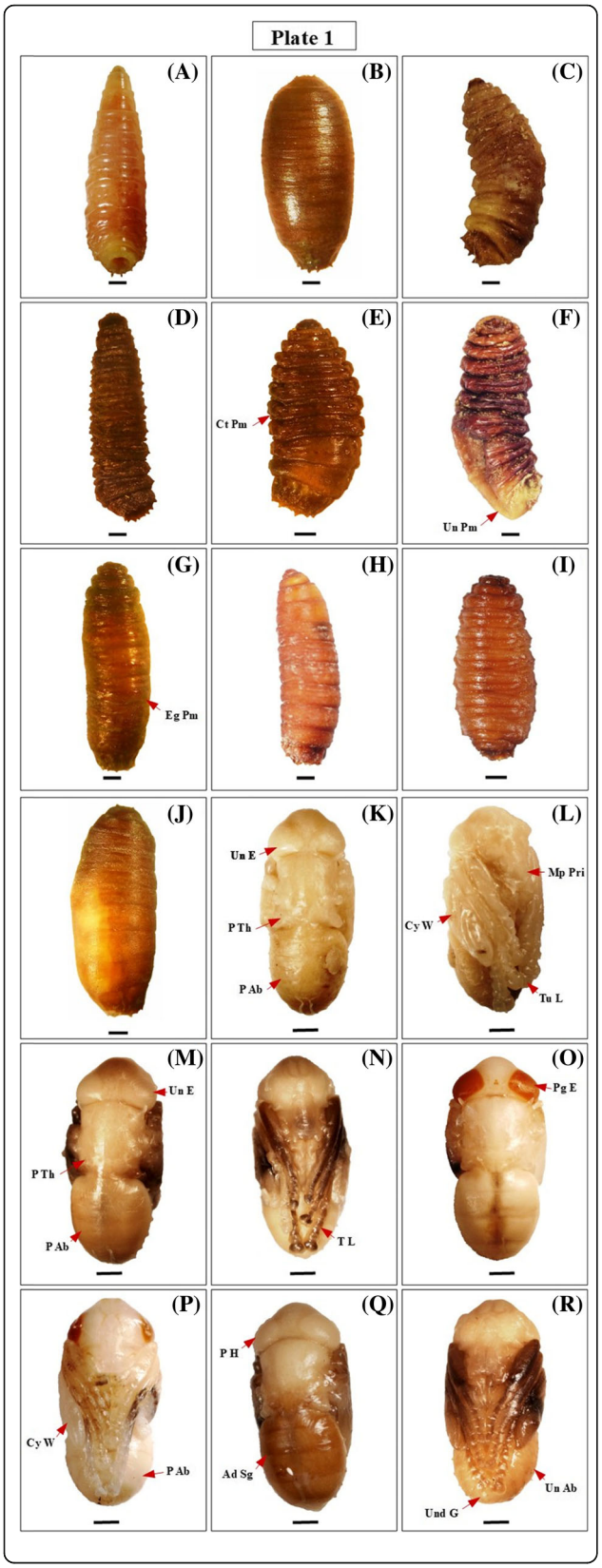

Plate 1 Types of deformities produced after topical administration of root extracts of *Withania somnifera* to 0–3-day-old third instar larvae of *Sarcophaga ruficornis*. (A) Normal third instar larva. (B) Normal puparium. (C) Abnormal puparium, 10 μg/μL, third instar day 0. (D) Abnormal elongated puparium, 5 μg/μL, third instar day 1. (E) Abnormal contracted puparium, 20 μg/μL, third instar day 2. (F) Twisted puparium with unsclerotized posterior part of body, 15 μg/μL, third instar day 3. (G) Abnormal elongated puparium, 10 μg/μL, third instar day 2. (H) Abnormal puparium with light brown pupal pigmentation, 15 μg/μL, third instar day 3. (I) Abnormal puparium with light brown pupal pigmentation, 20 μg/μL, third instar day 0. (J) Abnormal puparium, 5 μg/μL, third instar day 2. (K, L) Dorsal and ventral view of pupal-adult mosaic grade I, 15 μg/μL, third instar day 0. (M, N) Dorsal and ventral view of pupal-adult mosaic grade II, 20 μg/μL, third instar day 1. (O, P) Dorsal and ventral view of pupal-adult mosaic grade III, 5 μg/μL, third instar day 0. (Q, R) Dorsal and ventral view of pupal-adult mosaic grade IV, 10 μg/μL, third instar day 1. (Bar = 1 mm; red arrow pointing out the deformities. Ad Sg = adult segmentation, Ct Pm = contracted puparium, Cy W = cylindrical wings, Eg Pm = elongated puparium, Mp Pri = mouthparts primordial, P Ab = pupal abdomen, P H = pupal head, P Th = pupal thorax, Pg E = pigmented eyes, T L = tanned legs, Tu L = tubular legs, Un Ab = untanned abdomen, Un E = untanned eyes, Un Pm = untanned puparium, Und G = undeveloped genitalia)

present on the posterior part of dorsal abdomen; genitalia deformed (Plate 2M and N)

(3) Adultoid with highly malformed legs and crumpled wings (Plate 2O)

(4) Adultoid with deformed body unable to eclose from the puparium (Plate 2P and Q). In some cases, the puparium remained attached to the metathoracic leg (Plate 2R).

Discussion

Topical administration of different doses of root extracts of *W. somnifera* to 0–3-day-old last instar larvae of *S. ruficornis* resulted into larval and pupal mortality, delay in larval-pupal ecdysis, abnormal pupariation, suppression of adult emergence, and formation of pupal-adult mosaics and adultoids.

Topical treatment of 0–3-day-old larvae of *S. ruficornis* with different doses of root extracts of *W. somnifera* produced larval and pupal mortality in a dose-dependent manner. The toxic effect of root extracts of this medicinal plant has also been observed when the larvae of dipteran insects such as *Anopheles stephensi* (Diptera: Culicidae), *Aedes aegypti*, and *Culex quinquefasciatus* are treated (Bansal et al. 2011). Apart from dipterans, the larvae and pupae of coleopterans and lepidopterans are also susceptible to root extracts of *W. somnifera* (Arora et al. 2011; Gaur and Kumar, 2017a, 2019) showing its broad-spectrum insecticidal activity. In the present study, it has been observed that freshly moulted third instar larvae (0-day old) are highly susceptible to treatment with root extracts as compared to older larvae as maximum toxicity has been observed in case of

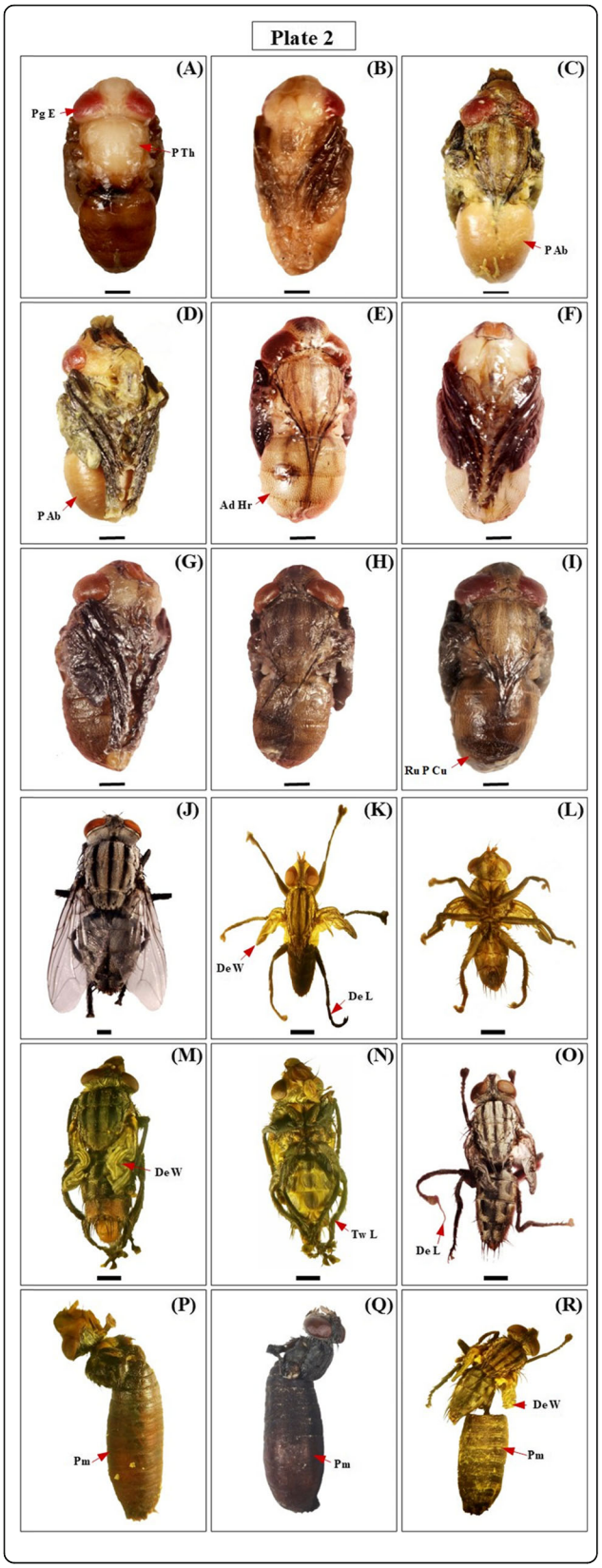

Plate 2 Types of deformities produced after topical administration of root extracts of *Withania somnifera* to 0–3-day-old third instar larvae of *Sarcophaga ruficornis*. (A, B) Dorsal and ventral view of pupal-adult mosaic grade V, 5 μg/μL, third instar day 2. (C, D) Dorsal and ventral view of pupal-adult mosaic grade VI, 10 μg/μL, third instar day 3. (E, F) Dorsal and ventral view of pupal-adult mosaic grade VII, 20 μg/μL, third instar day 2. (G, H) Dorsal and ventral view of pupal-adult mosaic grade VIII, 15 μg/μL, third instar day 3. (I) Dorsal view of pupal-adult mosaic grade VIII, 5 μg/μL, third instar day 2. (J) Dorsal view of normal adult. (K, L) Dorsal and ventral view of adultoid with deformed wings and legs, 20 μg/μL, third instar day 0. (M, N) Dorsal and ventral view of adultoid with deformed wings and twisted legs, 5 μg/μL, third instar day 1. (O) Dorsal view of adultoid with crumpled wings, 15 μg/μL, third instar day 2. (P) Lateral view of adultoid unable to eclose from the puparium, 10 μg/μL, third instar day 3. (Q) Lateral view of adultoid unable to eclose from the puparium, 15 μg/μL, third instar day 0. (R) Doral view of adultoid with puparium, 20 μg/μL, third instar day 1. (Bar = 1 mm; red arrow pointing out the deformities. Ad Hr = adult hairs, De L = deformed legs, De W = deformed wings, P Ab = pupal abdomen, P Th = pupal thorax, Pg E = pigmented eyes, Pm = puparium, Ru P Cu = ruptured pupal cuticle, Tw L = twisted legs)

treatment of younger larvae. This is comparable with similar findings when *Aedes albopictus* larvae are treated with an insect growth regulator and a chitin synthesis inhibitor, dimilin (Ho et al. 1987). This toxicological effect could be due to the presence of certain phyto-active ingredients like withaferins and withanolides in the root extracts of *W. somnifera* (Dar et al. 2015).

Topical administration of root extracts of *W. somnifera* to last instar day 0–3 larvae of *S. ruficornis* significantly increased the life span of last instar larvae in a dose-dependent manner causing postponement of pupariation. The younger larvae (day 0 or 1) are highly susceptible in this regard as compared to older larvae (day 2 or 3) as the maximum effect has been observed in the former case. Plant extracts from different families of angiosperm plants have been observed to increase larval and pupal duration when administered to developmental stages of dipteran insects such as leaves extracts of *Pelargonium citrosa* (Geraniaceae) to fourth instar larvae of *A. stephensi* (Jeyabalan et al. 2003); leaves extracts of *Dysoxylum malabaricum* (Meliaceae) to eggs of *A. stephensi* (Nathan et al. 2006a); leaves and seed extracts of *Melia azedarach* (Meliaceae) to eggs of *A. stephensi* (Nathan et al. 2006b); leaves extracts of *Artemisia annua* L. (Asteraceae) to eggs of *A. stephensi* (Sharma et al. 2006b); leaves extracts of *Carica papaya* (Caricaceae) to fourth instar larvae of *C. pipiens pipiens* (Olayemi et al. 2013).

Such prolongation in the life span of the developmental stages of dipteran insects is similar to those that have been observed by the administration of insect growth regulators (IGRs)/juvenile hormone analogs (JHAs). For instance, IGRs buprofezin, hexaflumuron, lufenuron

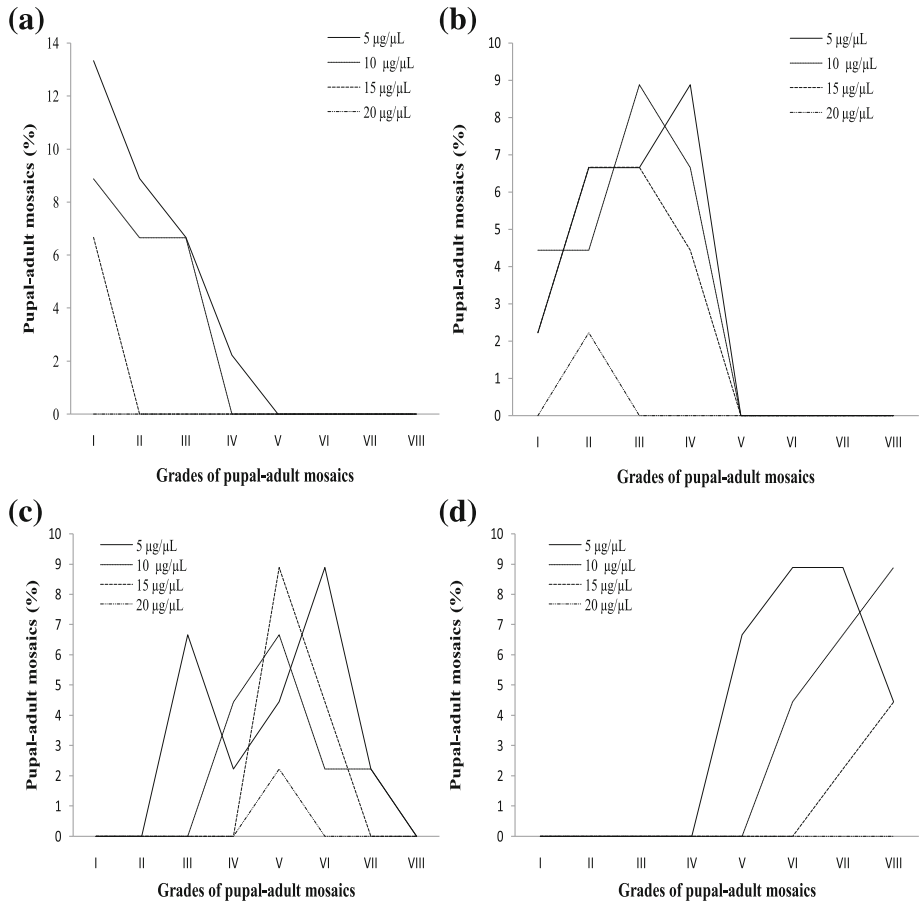

Fig. 3 Different grades of pupal-adult mosaics produced by the treatment of **a** 0-day-old third instar larvae, **b** 1-day-old third instar larvae, **c** 2-day-old third instar larvae, and **d** 3-day-old third instar larvae of *S. ruficornis* with root extracts of *W. somnifera*

(chitin synthesis inhibitors), and JHA (pyriproxyfen) caused prolongation of larval and pupal duration in *Musca domestica* (Diptera: Muscidae) (Abo El-Mahasen et al. 2010). Similarly, JHAs have been reported to cause prolongation of larval and pupal duration in dipteran insects such as kinoprene in *C. pipiens* (Hamaidia and Soltani, 2014), pyriproxyfen in *Drosophila melanogaster* (Diptera: Drosophilidae) (Bensebaa et al. 2015); methoprene in *Parasarcophaga argyrostoma* (Diptera: Sarcophagidae) (Bakr and Tanani, 2018). This clearly demonstrates that root extracts of *W. somnifera* act as insect growth regulators and mimic the action of JHAs/juvenoids. Such kind of prolongation of the life span of last instar larvae or postponement of pupariation/pupation may be due to inhibition of moulting process caused by an increased titre of juvenile hormone in the insect body (Lapcharoen et al. 2005). Administration of JHAs like DL-juvenile hormone (DL-methyl trans, trans, cis-10-epoxy-7-ethyl-3,11 dimethyl-2,6-tridecadienoate) and C_{17} methyl ester (methyl trans, trans, cis-7-ethyl-3, 11-dimethyl-2,6,10-tridecatrienoate) to last larval instar of *S. bullata* caused prolongation of larval life and subsequent delay in puparium formation. These effects may be due to presence of juvenile hormone in the insect body at an inappropriate time and it exerts inhibitory effect on the secretion/synthesis of moulting hormone (ecdysone) either by directly inhibiting the ecdysial glands (prothoracic glands) or by indirectly preventing the secretion of brain hormone (Srivastava and Gilbert, 1969). During the normal development, JH is responsible to regulate the action of ecdysone and 20-hydroxyecdysone (20E) and a high titre of JH prevents these two hormones in causing a switch from the larval to pupal or pupal to adult differentiation (Riddiford, 1994, 1996). If the JH is exogenously applied during this critical period, this switch in the differentiative program will not take place (Riddiford et al. 2003). Thus, prolongation in the life span of developmental stages may be due to alteration in ecdysteroids titer and interruption of 20E action (Suzuki et al. 2010). There are two JH-sensitive periods during the last instar larvae, first controls larval-pupal and second controls pupal-adult development. If JH is present during these sensitive periods, the current developmental state will be maintained

and no developmental switch or differentiation to the next developmental stage will take place (Nijhout, 1998).

Topical administration of root extracts of *W. somnifera* to last instar 0–3-day-old larvae of *S. ruficornis* inhibited the puparium formation in varying degrees. This kind of effect has also been observed with various plant extracts and oils when applied to dipteran insects such as plant oils from *Trigonella foenum-graecum*, *Apium graveolens*, *Raphanus sativus*, *Brassica compestris* to third instar larvae of *Lucilia sericata* (Diptera: Calliphoridae) (Khater and Khater, 2009) and leave extracts from the plants *Aloe zebrine* and *Erythrina lysistemon* to second or third instar larvae of *Lucilia cuprina* and *Chrysomya marginalis* (Diptera: Calliphoridae) (Mukandiwa et al. 2012a). It is remarkable that the abnormal pupariation in *S. ruficornis* as a result of the treatment of third instar larvae with root extracts of *W. somnifera* and that observed with plant extracts and plant oils are similar to those observed with the administration of IGR/JHAs. It has been observed that the administration of IGR/JHAs produces similar effects as reported in the present case, for example, IGR cyromazine [larvadex (n-cyclopropyl-1,3,5-triazine-2,4,6-triamine)] induces abnormal pupariation as a result of treatment of third instar larvae of *M. domestica* (Awad and Mulla, 1984). JHAs such as methyl 10,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoate, isopropyl 11-chloro-3,7,11-trimethyl-2-dodecanoate, and isopropyl 11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate produce similar effect in *Musca*, *Sarcophaga*, *Ceratitis*, *Calliphora* spp (Sehnal and Zdarek, 1976). Besides JH agonist, pyriproxyfen application to eggs of *C. megacephala* also caused the formation of abnormal puparia (Singh and Kumar, 2015b).

In cyclorrhaphous diptera, the larval cuticle of the last larval instar, unlike the lepidopteran insects, is not shed at the larval-pupal moult and rather it is retained as a hard protective covering called puparium. There are several steps involved in the puparium formation such as retraction of anterior larval segments, longitudinal body contraction and cuticular shrinkage leading to a smooth, barrel-shaped and sclerotized body. The hormones involved in puparium formation are anterior segment retraction factor (ARF), puparium-immobilizing factor (PIF), and puparium tanning factor (PTF). These are proteinaceous neurohormones simultaneously secreted by the neurosecretory cells of the central nervous system (Sivasubramanian et al. 1974). ARF is responsible for retraction of anterior larval segments shaping the larval integument into barrel-shaped puparium. PIF causes the larvae to become immobile and the larvae become more and more sensitive to this factor as the time of normal pupariation approaches and finally, PTF is responsible for the sclerotization of the puparium (Nijhout, 1998). In the present study, all the events related with the puparium formation are adversely affected and this demonstrates that secretion/activity of all the pupariation factors such as ARF, PIF, and PTF is either suppressed or inhibited in varying degrees.

The development of pupal-adult mosaics is another prominent and conspicuous effect observed as a consequence of administration of root extracts of *W. somnifera* to last instar larvae of *S. ruficornis*. It is worthwhile to mention that younger larvae are more susceptible to *W. somnifera* root extract treatment as compared to older larvae as greater suppression of imaginal differentiation or retention of more pupal characters occurs when the former is treated. This is similar to pupal treatment of *Spodoptera litura* (Lepidoptera: Noctuidae) with a juvenoids (6, 7-epoxy-3-ethyl-1 (p-ethyl phenoxy)-7 methylnonane) where more pupal characters are retained in pupal-adult intermediates when younger pupae are treated (Srivastava and Prasad, 1982). Formation of mosaics (larval-pupal/pupal-adult) has also been observed as a result of treatment of developmental stages of lepidopteran insects with root extracts of *W. somnifera*, for instance, treatment of sixth instar larvae and freshly moulted 0–2-h-old pupae of *S. litura* (Gaur and Kumar, 2017a, 2019) and treatment of seventh instar larvae of *Pericallia ricini* (Lepidoptera: Arctiidae) (Gaur and Kumar, 2017b). The root extracts of a medicinal plant, *Ecbolium viride* contain a lignin compound, ecbolin B and this has been reported to produce larval-pupal and pupal-adult mosaics/intermediates in the dipteran insect, *A. aegypti* (Reegan et al. 2016). Apart from root extracts, the extracts from other parts of the plants also produce similar effects such as seed extracts of *Argemone mexicana* administration to second instar larvae of *A. aegypti* (Sakthivadivel and Thilagavathy, 2003), leaves extracts of *Artemisia annua* to third instar larvae of *A. stephensi* (Sharma et al. 2006a) and leaves extracts of *Artemisia annua* to eggs of *C. quinquefasciatus* (Sharma et al. 2006b).

Surprisingly, administration of IGRs/JHAs has also been observed to produce such forms of mosaics/intermediates in several dipteran insects, as observed in the present study, such as treatment with IGRs like altosid^{TM} (isopropyl 11-methoxy-3,7,11-trimethylododeca-2, 4-dienoate) in *C. tarsalis* (Arias and Mulla, 1975); PH 60-40 [N-(4-chlorophenyl)-N′-(2,6-diflurobenzoyl) urea] in *Stomoxys calcitrans* (Diptera: Muscidae) (Hayakawa, 1976); pyriproxyfen, novaluron and methoxyfenozoide in *Bactrocera zonata* (Diptera: Tephritidae) (Fahmy et al. 2013) and JHAs such as methoprene in *C. moletsus* and *C. quinquefasciatus* (Gelbic et al. 2002); pyriproxyfen in *S. ruficornis* and *C. megacephala* (Singh and Kumar, 2015b, c). The development of pupal-adult mosaics/intermediates may be considered as a true juvenilizing

effect (Wright 1970; Wright and Spates, 1971). It has been suggested that the plants produce phytochemicals which may act as JH mimics (juvenoids) and interfere with the endocrine control of insects (Bede and Tobe, 2000) leading to disruption of normal hormonal activity of the metabolic processes in the developing stage (Sakthivadivel and Thilagavathy, 2003). There are two different developmental switches in the holometabolous insects, first during larval-pupal and second during pupal-adult moult. The developmental switches that occur during JH sensitive period are activated by the ecdysteroids and progress to the next developmental stage takes place when either JH is absent or very low during JH sensitive period (Nijhout, 1998). Thus the presence of certain bioactive plant compounds during JH sensitive period may interfere with the normal endocrine regulation of moulting and metamorphosis producing non-viable larval-pupal or pupal-adult mosaics/intermediates in the same way as natural JH/juvenoids do (Kabir et al. 2013). In fact, the presence of JH during the critical period of development when it is not required may disrupt the hierarchy of ecdysone-mediated metamorphic change resulting in developmental aberrations (Wilson, 2004).

The pupal-adult mosaics formed as a consequence of the treatment of root extracts of *W. somnifera* show ecdysial stasis. The ecdysial failure has also been reported by the administration of other plant extracts (fruit and leaves extracts of *Azadirachta indica* and *Melia azedarach*) to dipteran insects, *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae) (Andrade-Coelho et al. 2009), and IGR (diflubenzuron) to *C. quinquefasciatus* (Mulla, 1995). In an orthopteran insect, *Locusta migratoria* (Orthoptera: Acrididae), a phytochemical, azadirachtin induced moulting inhibition as a result of suppression of ecdysteroid titre (Sieber and Rembold, 1983). In rice weevil, *Sitophils oryzae* (Coleoptera: Curculionidae), treatment with JHA, hydroprene, and IGR, R-20458 caused the formation of pupal-adult intermediates which suffered from ecdysial failure and ultimately died and the effect could be due to interference with the endogenous level of JH during metamorphosis (Gupta and Mkhize, 1983). Moult inhibition has also been caused by a phytochemical, podophyllotoxin, which may interfere with the neuroendocrine system as a result of blocking the synthesis/release of PTTH and ecdysone (Garcia and Azambuja, 2004).

The significant reduction in normal pupariation and adult emergence as a result of treatment of last instar larvae of *S. ruficornis* with root extracts of *W. somnifera* is similar to those induced by other plant extracts (Mukandiwa et al. 2012a, b) or oils in dipteran insects (El-Khateeb et al. 2003; Khater and Khater, 2009). It has been observed that exogenous administration of JHA, pyriproxyfen to freshly laid eggs of *C. megacephala* or adults of *S. ruficornis* produced similar effects in ensuing postembryonic development or F1 generation (Singh and Kumar 2015b, c). This clearly demonstrates that the plant extracts mimic the action of JH/JHA as the juvenoids are well known to inhibit/suppress the pupation (Eto, 1990; Nakakita, 1982, 1990) and adult emergence due to reduction or interference with the secretion of eclosion hormone (Ghoneim et al. 2007).

The formation of adultoids is another very prominent effect induced by *W. somnifera* root extracts, and it is a manifestation of hormonal imbalance as a result of interference with the endocrine system. This has also been observed as a result of the treatment of dipteran larvae with others plant extracts (Mwangi and Mukiama, 1988; Kabir et al. 2013)/their secondary metabolites (Sharma and Sohal, 2013, 2015) and active ingredients (Reegan et al. 2016). It is interesting that similar effect has also been observed as a result of administration of JHA, pyriproxyfen to dipteran insects, *D. melanogastor* (Bensebaa et al. 2015), *C. megacephala* (Diptera: Calliphoridae) (Singh and Kumar, 2015b), and *S. ruficornis* (Singh and Kumar, 2015c). It has been suggested that plant extracts mimic the action of JH (Munoz et al. 2013) which may affect the development of imaginal structures (Riddiford and Ashburner, 1991) as the JH/JHA are well known to inhibit proliferation of imaginal disc cells (Oberlander et al. 2000). There is an additional JH sensitive period during the initial phase of the pupal stage during which JH must be absent to allow the epidermal cells to proceed for imaginal development and differentiation and if the JHA (pyriproxyfen) is present during this critical period this may result into deformed adults or reduction in adult emergence (Nijhout, 1998; Bensebaa et al. 2015). In the present study, some of the adultoids were unable to extricate from the puparium and this shows that the secretion/activity of the eclosion hormone is interfered with as it is responsible for freeing the adult body from the puparium. Such kind of detrimental effect on the eclosion behavior has also been observed as a result of administration of plant oils from two medicinal plants, *Cymbopogon citrates* and *Syzygium aromaticum* to dipteran insects, *A. aegypti* and *A. dirus* (Soonwera and Phasomkusolsil, 2016). Besides IGRs such as cyromazine (Moreno-Mari et al. 1996) and novaluron (Fahmy et al. 2013) and JHA, pyriproxyfen produce similar effect in dipteran insects (Singh and Kumar, 2015c). This demonstrates that root extracts of *W. somnifera* possess IGR activity and mimic the action of JH/JHA that adversely affect the secretion/release of a neuropeptide eclosion hormone responsible for the eclosion of an adult from the puparium and bursicon responsible for the sclerotization and tanning and the effects can be compared by the administration of JHAs,

pyriproxyfen, and diofenolan, to last instar larvae of *S. litura* (Singh and Kumar, 2015a).

Conclusion

Therefore, root extracts of *W. somnifera* may be used as a potential insect growth regulator for the control and management of myiasis causing flesh fly *S. ruficornis* along with other bio-rational approaches.

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