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ORIGINAL PAPER

Molecular basis for efficacy of Guduchi and Madhuyashti feeding on different environmental stressors in *Drosophila*

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Abstract

Stressors of different kinds adversely affect life history parameters like growth, development, and reproduction. Organisms overcome the negative impact of environmental stressors and strive to reach a tolerant state through genetic and metabolic activities. Ayurvedic formulations are reported to have life trait benefitting properties which improve capacity to withstand stress and tolerate adverse conditions. Guduchi (*Tinospora cordifolia*) and Madhuyashti (*Glycirrhiza glabra*) Ayurvedic formulations are known to have immunomodulatory, intellect promoting, and adaptogenic properties, thus favoring good health and healthy aging. Present study investigates the efficacy of Guduchi and Madhuyashti in providing tolerance to different stresses and the underlying mechanisms using the *Drosophila* model. *Drosophila* larvae/flies fed on Guduchi or Madhuyashti were better thermo-protected, which correlated with increased expression of heat shock genes even without the heat shock. Guduchi or Madhuyashti feeding also increased antimicrobial peptide expression, thus providing better tolerance to pathogenic assaults. Feeding on Guduchi- or Madhuyashti- supplemented food also enhanced starvation and desiccation tolerance. However, neither of these formulations provided beneficial effects when grown under crowded conditions or when exposed to oxidative stressors.

Keywords Ayurveda · Guduchi · Madhuyashti · Heat shock proteins · ROS · Thermotolerance · Starvation · Desiccation

Introduction

Any alteration in external or internal environment of a cell that disrupts its homeostasis is termed as stress. Living systems are optimally tuned to adjust to environmental conditions in which they live and any change in these conditions, such as temperature shock, starvation, desiccation, bacterial, and/or viral infection, challenges the homeostasis of living systems. Cells, however, employ a variety of ways to combat these adverse situations which include either activation of cell survival promotion pathways or programmed cell death to eliminate damaged cells. Stress can also arise from metabolic activities which result in the production of reactive oxygen species (ROS). Most stresses are neutralized by immediate synthesis of heat shock proteins (Hsps) (Tisseries et al. 1974; Arrigo and Landry 1994; Arya et al. 2007; Maio et al. 2012) or the production of anti-oxidants. On the other hand, since starvation and desiccation or overcrowding stresses target lipid content and carbohydrate reserves of the body (Djawdan et al. 1996; Chippindale et al. 1996; Harshman et al. 1999), nutritional status at different developmental stages and body weight of the organism becomes important determinants for surviving such stresses (Service 1987). Infections cause pathogenic stress, which is countered by the immune system with the innate immune system operating as the first line of defense.

The damages inflicted by various environmental perturbations can be reduced if the organisms are rendered to cope better with various challenges. Plant products that help animals survive better under stressful conditions have been widely studied. They improve stress tolerance, fitness, and adaptability of organisms through hormesis (Calabrese and Baldwin 2003; Rattan 2008) or xenohormesis (Howitz et al. 2003; Howitz and Sinclair 2008; Hooper et al. 2010), finally activating the organism's own stress-defense pathways. Some earlier studies have shown that feeding on some of the Ayurvedic formulations can provide better stress tolerance against a variety of stressors (Dwivedi et al. 2012; Dwivedi

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and Lakhotia 2016). In the present study, we used two Ayurvedic formulations, viz., Guduchi and Madhuyashti. Guduchi plant, also known as Giloy or Amrita, belongs to family Menispermaceae and its stem is used for its medicinal properties. The Guduchi formulation is prepared by powdering the completely dried stem. Madhuyashti plant, also known as liquorices, belongs to family Fabaceae and its root is claimed to possess medicinal properties (Damle 2014). The Madhuyashti formulation is a powdered form of dried roots of this plant. Active components identified from Guduchi include Berberine, Palmatine, Tinocordiside, Tinocordifolioside, Cordifolioside A, Cordifolioside B, and Tinocordifolin (Maurya et al. 1996; Maurya and Handa 1998; Singh et al., 2003). Similarly, Madhuyashti contains Glycyrrhizin, Glycyrrhizic acid, and Glycyrrhizinic acid as its active constituents (Damle 2014; Singh et al. 2018). Both these formulations belong to the adaptogen class as they are reported to improve stress response and help the body to adapt to stress by normalizing physiological processes towards homeostasis (Mehta et al. 2015). Both the formulations have been categorized under *Medha Kamya Rasayana* in Ayurveda and are supposed to possess anti-inflammatory, anti-ulcer, laxative, anti-microbial, anti-viral, immune booster, anti-pyretic, anti-oxidant, intellect promoting, emollient, and immunomudulator properties and thus promote disease-free long life (Damle 2014).

Drosophila provides enormous advantages as a model system for many human diseases and for exploring possible therapies for the same since it shares many fundamental biological phenomena with humans (Pandey and Nichols 2011; Yamamoto et al. 2014). *Drosophila* has also been successfully used to understand the mechanisms of actions of Ayurvedic formulations (Dwivedi et al. 2012, 2015; Dwivedi and Lakhotia 2016; Balasubramani et al. 2014; Jansen et al. 2014; Singh et al. 2018). In the present study, we show that Guduchi and Madhuyashti formulations provide significant protection against thermal, desiccation, starvation stress, and pathogenic challenges while each of them made the flies more sensitive to crowding or oxidative stress.

Materials and methods

Oregon R^+ strain of *Drosophila melanogaster* was reared on standard agar-cornmeal-sugar-yeast food at 24 °C ± 1 °C. Ayurvedic formulations Guduchi and Madhuyashti (in powder form) were obtained from Arya Vaidya Sala (Kottakkal, Kerala, India). The formulations were mixed with standard food separately at 0.5% concentration (Singh et al. 2018) for rearing experimental larvae and/or flies. Controls were reared on un-supplemented regular food. In all experiments, freshly emerged first instar larvae, collected from eggs laid by *Oregon* R^+ flies, maintained on regular food, were transferred to normal or formulation (Guduchi or Madhuyashti) supplemented food. For each experimental set, same batch of food was used for rearing larvae/flies under control or formulation-supplemented condition, and likewise, all larvae/adults for a given set were derived from a common pool of eggs and reared in parallel on the regular or formulation-supplemented food.

Thermotolerance assay

Thermotolerance assay (knock down and survival) was performed with late third instar larvae (100 h after hatching (AH)), grown on regular or on either of the formulation-supplemented food, and exposing them to heat shock at 37 °C (for 60, 90, or 120 min), 38 °C (for 60 or 90 min), or at 39 °C (for 30 min) as described by Dwivedi et al. (2012). For each feeding regime, a total of 200 larvae, distributed in twenty 1.5-ml microfuge tubes containing 10 larvae each, were exposed to the desired heat shock temperature and time. Two such independent biological replicates were assayed in each case.

Similar knock down and survival assays were performed with flies of different age group (3, 15, 30, 45, and 60 days old) heat shocked at 37 °C, 38 °C, or at 39 °C for different periods of time as described in Dwivedi et al. (2012). Ten replicates of 20 flies each were examined for each experimental condition.

For starvation, desiccation, crowding, or oxidative stress and pathogenic challenges, flies were reared on regular or either of the formulation-supplemented food since first instar larval stage till eclosion at 24 ± 1 °C. Freshly eclosed virgin males and females were kept separately in bottles containing the respective food medium till day 3, and the flies subjected to the given stress on day 4.

Starvation stress

Four-day-old flies were subjected to starvation stress as described (Marron et al. 2003). For each set, 10 males and 10 females were taken and 10 such replicates were assayed (N = 200). The number of dead flies was assayed every 12 h, till all the flies were dead.

Desiccation stress

Four-day-old flies were subjected to desiccation stress by keeping them in empty vials (Marron et al. 2003). For each set, 10 males and 10 females were taken and 10 such replicates were assayed (N = 200). The number of dead flies was assayed at every 6 h, till all the flies were dead.

Crowding stress

Four-day-old flies (50 males and 50 females) were kept in food vials (2.5 cm diameter, 9.8 cm length) containing 5 ml of the respective food medium as per their earlier growth. The cotton plug was placed at a distance of 9 cm, 6 cm, or 3 cm from food surface reducing the space for the flies leading to low crowding (LC), medium crowding (MC), or high crowding (HC) conditions, respectively (Dwivedi and Lakhotia 2016). The vials were changed every day and the number of flies surviving each day was counted till all the flies were dead. For each conditions, 3 batches of 100 flies each were assayed.

Pathogenic challenges

In vivo infection with *E. coli* was achieved by adding the DH5-alpha stain bacteria from the exponential log phase (3.5×10^{10} cells/ml) to 5% sucrose solution (Verma and Tapadia 2012). Four-day-old flies reared either on formulation-supplemented food or regular food were kept in vials with filter papers soaked in *E. coli* containing 5% sucrose solution, with 20 flies in each vial and 10 replicates for each feeding regime ($N = 200$). Dead flies were counted and the surviving flies were transferred to fresh vials containing the glucose solution soaked filter paper as above every 24 h to avoid contamination. Data was recorded till 50% of the flies reared on regular food were dead.

Paraquat-induced oxidative stress

Four-day-old flies were transferred to a food-free vial (10 male and 10 female in each vial) containing a filter paper soaked in 10 mM paraquat in 5% sucrose solution and kept in a moist chamber; 10 such replicates were used ($N = 200$ for each set). The flies were monitored for survival at 6 h intervals till all the flies in a vial were dead. Similar procedure was followed for 20 mM paraquat concentration.

RNA isolation and qPCR

Internal tissues of late third instar larvae reared either on control food or formulation-supplemented food were dissected out in phosphate-buffered saline (PBS) before or after HS. For assessing levels of different AMP transcripts, fat bodies of larvae fed on regular or formulation-supplemented food were dissected. Total RNAs were isolated from different samples using Trizol as per the manufacturer's (Ambience, India) instructions. RNA pellets were re-suspended in nuclease-free water. The cDNA was synthesized and real time (qRT-PCR) was carried using appropriate primers (listed below) and SYBR-Green dye on 7500 Real Time PCR System (Applied Biosystems) with the 7500 software v2.0.4.

The primers used for Hsps (Dwivedi and Lakhotia 2016) and for AMPs were as follows:

(i) G3PDH: Forward: 5′-CCACTGCCGAGGAGGTCAACTA-3′,

Reverse: 5′-GCTCAGGGTGATTGCGTATGCA-3′,

(ii) Hsp70: Forward: 5′-AGGGTCAGATCCACGACATC-3′,

Reverse: 5′-CGTCTGGGTTGATGGATAGG-3′,

(iii) Hsp27: Forward: 5′-GTCCATGCCCACGATCTGTT-3′,

Reverse: 5′-CGACACATCCATGCACACCT-3′,

(iv) Hsp83: Forward: 5′-CCTGGACAAGATCCGCTATG-3′,

Reverse: 5′-GAAACCCACACCGAACTGAC-3′,

(v) Diptericin: Forward: 5′-CACCGCAGTACCCACTCAAT-3′,

Reverse: 5′-AATCTCGTGGCGTCCATTGT-3′,

(vi) Drosocin: Forward: 5′-TGACTCAAGCTGCCATCAGG-3′,

Reverse: 5′-TGGGAACCCCTCATTGTGTC-3′,

(vi) Attacin: Forward: 5′-AAGCATCCTAATCGTGGCCC-3′,

Reverse: 5′-CACTTTGAGTGTTTCCGGCG-3′,

(vi) Cecropin A: Forward: 5′- TCAGTCGCTCAGACCTCACT -3′,

Reverse: 5′-GATGGCCAGAATGAGAGCGA-3′,

(vii) Defencin: Forward: 5′-CTCGTGGCTATCGCTTTTGC-3′,

Reverse: 5′-CCACTTGGAGAGTAGGTCGC-3′,

(viii) Drosomycin: Forward: 5′-TACTTGTTCGCCCTCTTCGC-3′,

Reverse: 5′-CACCAGCACTTCAGACTGGG-3′,

(ix) Metchnikowin: Forward: 5′-GCATCAATCAATTCCCGCCA-3′,

Reverse: 5′-GCTCTGCCAGCACTGATGTA-3′.

Western blotting

Western blots of electrophoretically separated total proteins from late third instar larvae fed on regular food or either of the formulation-supplemented food, and subjected to desired experimental condition (with/without HS), were immunostained with the 7Fb rat anti-Hsp70 (at 1:1000 dilution; Velazquez and Lindquist 1984; Dwivedi and Lakhotia 2016) and mouse anti-β-tubulin (E7, 1:200 dilution, DSHB) as described earlier (Singh and Lakhotia 2016). The primary antibody binding was detected using alkaline phosphatase conjugated anti-rat or anti-mouse IgG (Bangalore Genei, India) secondary antibody, respectively.

Immunostaining of tissues

Desired larval tissues were dissected out in PBS and fixed in freshly prepared 4% paraformaldehyde for 20 min at RT and processed further for immunostaining as described (Prasanth et al. 2000). The different primary antibodies used were (1) rat anti-Hsp70 (7Fb, 1:100 dilution), (2) mouse monoclonal anti-Hsp27 (ab49919, 1:100 dilution, Abcam, UK), (3) mouse monoclonal anti-Hsp90 (SPA 830, 1:100 dilution, Stressgen, USA). Appropriate secondary antibodies conjugated either with Cy3 (1:200, Sigma-Aldrich) or Alexa Fluor 488 (1:200, Molecular Probes) were used to detect the given primary antibody. The tissues were counterstained with DAPI (1 μg/ml; Sigma Aldrich, India), mounted in DABCO (Sigma) and examined under LSM510 Meta Zeiss laser scanning confocal microscope and the images analyzed using LSM software. To calculate the mean fluorescence intensity, the Histo tool of LSM 510 meta was used. To measure the fluorescence intensity in projection images of 10 optical sections of the given tissue sample; projection images from 10 tissue samples were used in each case to obtain the mean fluorescence values. The images were assembled with Adobe PS 7.0.

Lipid peroxidation assay

Thirty-five-day-old 10 male flies, reared under different feeding regimes, were homogenized in 150-μl homogenization buffer (1× PBS + 1 μg/ml PMSF) at 4 °C. The homogenate was centrifuged at 10,000 RPM for 10 min at 4 °C. 100 μl of supernatant was taken out in another tube and 0.6 ml 10% SDS and 0.6 ml thiobarbituric acid were added. The reaction mixture was boiled at 100 °C for 60 min followed by incubation at 4 °C for 5 min and centrifugation at 10,000 RPM for 10 min. A blank was also kept, in parallel, with all the reagents except the sample. The OD of supernatant was taken at 532 nm by spectrophotometer. The standard Bradford's method (Kruger 1994) was used to assay protein levels in each sample. Lipid peroxidation was measured following the method of Ohkawa et al. (1979), and levels of lipid peroxides were expressed in terms of nano-moles of malondialdehyde (MDA) formed/h/mg of protein.

Statistical analysis

Sigma Plot 11.0 was used for statistical analyses. Comparisons between the groups were made using Student's *t* test.

Results

Rearing on Guduchi or Madhuyashti-supplemented food improves thermotolerance in larvae and flies

Thermotolerance following feeding on Guduchi and Madhuyashti was assessed by measuring survival of larvae and flies after HS at different temperatures for different time periods. HS at 37 °C for 60 min was not detrimental, as all larvae subjected to HS pupated and eclosed as adults, irrespective of the growth conditions. However, HS at 37 °C for 90 and 120 min resulted in emergence of only 27% or 23.5% flies, respectively, when fed on normal food. In case of Guduchi fed larvae, 50% and 46% flies eclosed after 90 min or 120 min HS at 37 °C, respectively. Similarly, Madhuyashti fed larvae also showed higher frequency of adult eclosion, 44% and 41%, respectively, after 90 or 120 min of HS at 37 °C (Fig. 1a). The significantly enhanced survival to adult stage suggests that feeding larvae on Guduchi or Madhuyashti provides better endurance to thermal stress. HS at 38 °C reduced eclosion of flies irrespective of the feeding conditions and is consistent with earlier report that sensitivity of flies increases with increasing temperatures (Huey et al. 1992). Despite the overall viability being reduced by HS at 38 °C, larvae reared on formulation-supplemented food survived the severe thermal stress better than those fed on normal food (Fig. 1b). Between the two formulations, Guduchi seemed to impart better thermotolerance than Madhuyashti.

Knock-down of flies of various ages (3, 15, 30, 45, or 60 days) at 37 °C, reared on the different food regimes since larval period, following HS was assayed (Fig. 2). As expected, sensitivity of wild-type flies to HS increased with age since while none of the 3-day-old flies reared on normal food got knocked down during 15 or 30 min HS at 37 °C (Fig. 2a); however, increasing numbers were knocked down in relation to increasing age of the flies (Fig. 2b–e). Sixty-day-old flies reared on normal food were knocked down after 15 min HS at 37 °C, 20% and 14.4% of flies reared on Guduchi or Madhuyashti-supplemented food, respectively, escaped being knocked down (Fig. 2e). Similarly, when compared with flies reared on normal food, the number of flies knocked down by HS at different temperatures, i.e., 38 °C (Fig. 3a) and 39 °C (Fig. 3b), was significantly lower in flies that were reared on Guduchi or Madhuyashti-supplemented food. Further, the

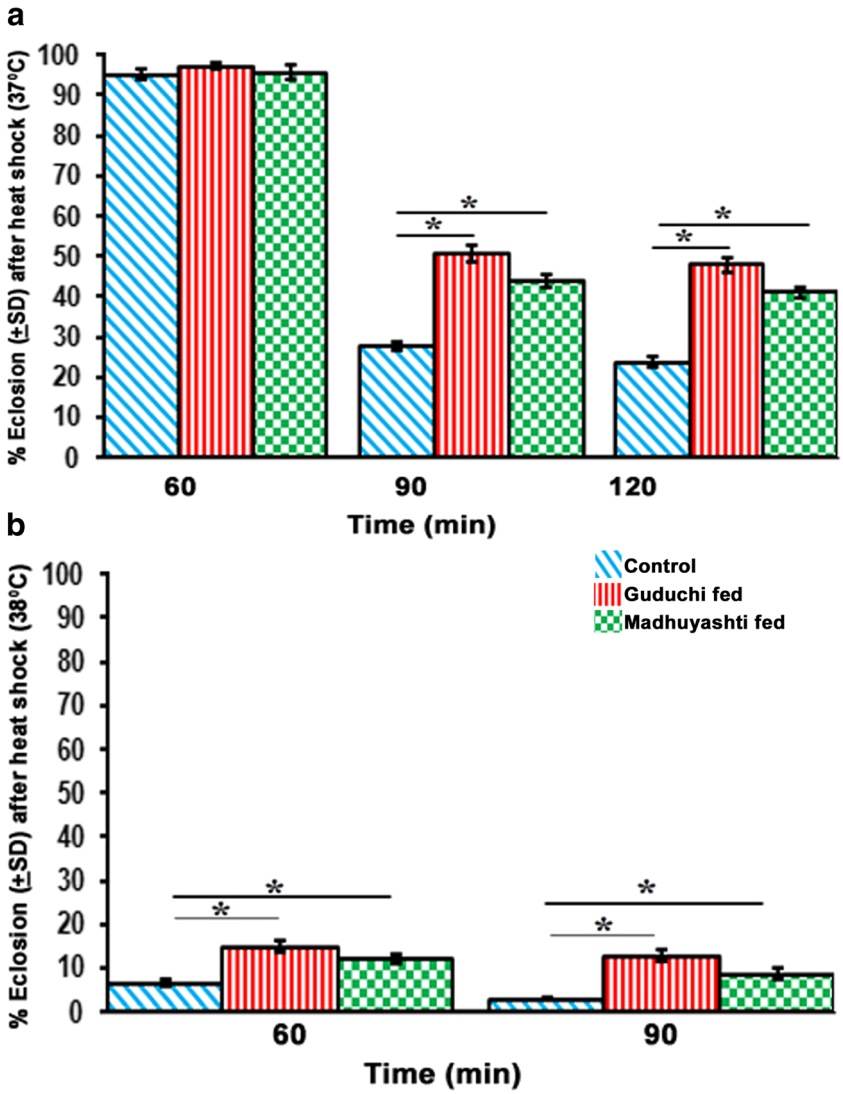

Fig. 1 Guduchi and Madhuyashti feeding improved thermotolerance of third instar *Drosophila* larvae. **a** Histogram showing mean percentage (±SD) of flies eclosed (Y-axis) after HS at 37 °C for 60, 90, or 120 min (X-axis) to third instar larvae. **b** Mean percentage eclosion after 60 min or 90 min HS at 38 °C. * indicates $P \leq 0.001$ between the compared % eclosion values (horizontal lines above the vertical bars) using Student's *t* test

proportion of flies surviving beyond 24 h after 60 min HS at 37 °C was always greater for formulation fed samples compared to those reared on the normal food (see insets in Fig. 2a–e). This also applied to survival following heat shock to 3-day-old flies at 38 °C or at 39 °C (see inset in Fig. 3a, b).

Formulation feeding differentially affected Hsp70, Hsp83, and Hsp27 expression

As thermotolerance is associated with the production of heat shock proteins (Parsell et al. 1993; Arya et al. 2007) and since the above results suggested that Madhuyashti and Guduchi improved thermotolerance, we examined expression of the three major heat shock genes, viz., *hsp70*, *hsp83*, and *hsp27* at transcript and protein levels. These genes were selected because Hsp70 is major heat shock protein in *Drosophila* while the Hsp83 and Hsp27, besides being induced by HS, are also developmentally expressed and important for normal development (Mason et al. 1984; Xiao and Lis 1989; Lakhotia and Prasanth 2002; Arya et al. 2007). Interestingly, qRT-PCR of whole third instar larvae (110 h AEL) revealed transcriptional up regulation of all the three heat shock genes in formulation fed food compared to regular food even without HS. Expression of *hsp83* and *hsp27* was several folds more elevated than of *hsp70* in larvae fed on either of the formulations (Table 1). HS at 37 °C led to further increase in transcript levels of all the three heat shock genes irrespective of feeding conditions. It is notable that while the elevation in expression of *hsp70* was least in formulation fed control larvae (without HS), its expression following heat shock in either of the feeding regimes was more elevated than *hsp83* or *hsp27* (Table 1).

Immunostaining of Malpighian tubules, salivary glands, fat bodies, and gut from control (without HS) (Fig. 4a) larvae reared on regular (a, c, e, g), Guduchi (i, k, m, o), or Madhuyashti (q, s, u, w) supplemented food showed that Hsp70 was not detectable in any of the tissues despite the above noted abundant presence of *hsp70* transcripts in formulation-fed larvae. HS at 37 °C for an hour resulted in a

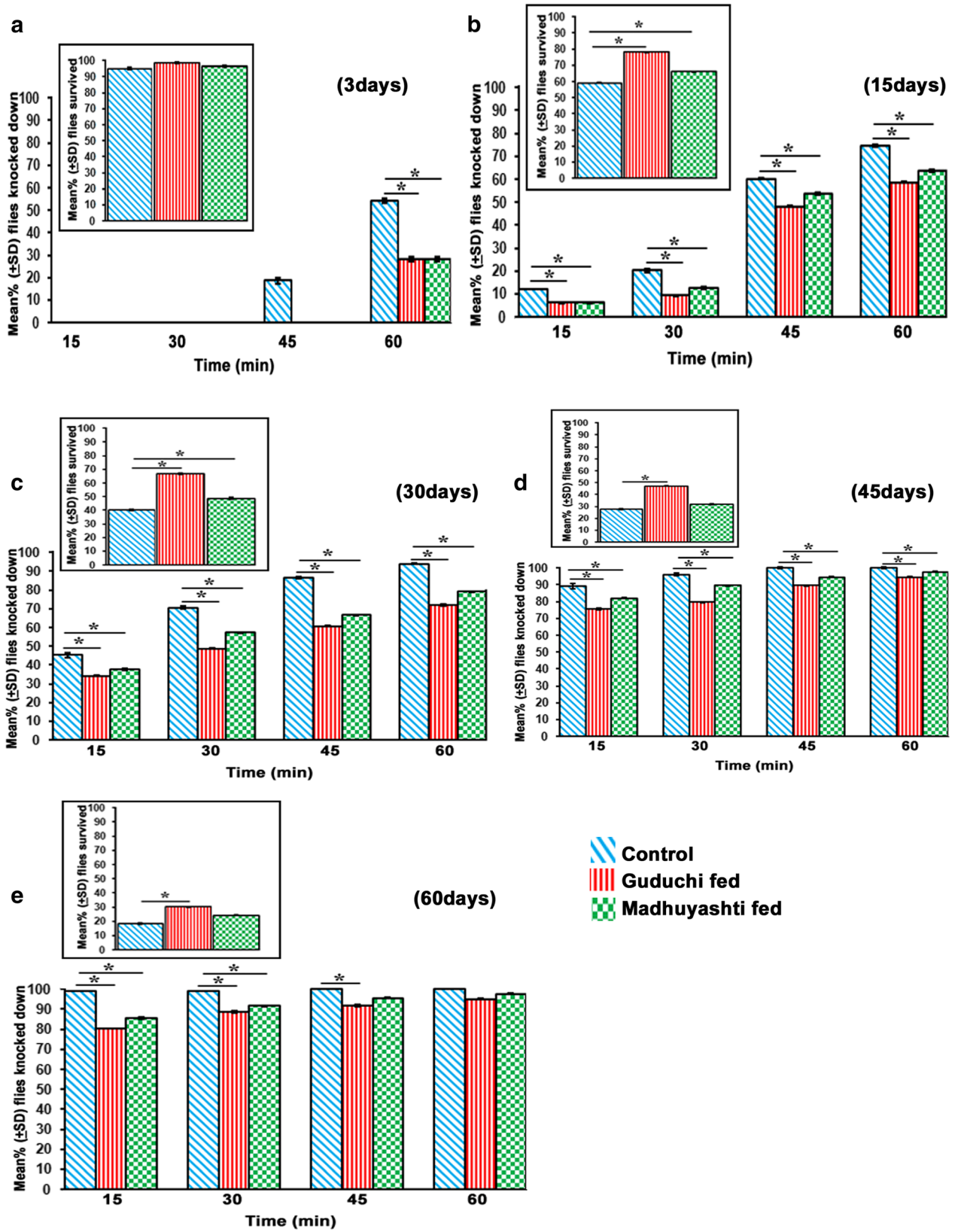

Fig. 2 Effect of formulation feeding on thermotolerance in aging flies. Bars represent mean % (±SD) 3 days (**a**), 15 days **(b)**, 30 days (**c**), 45 days (**d**), or 60 days (**e**) old flies that were knocked down (Y-axis) during HS at 37 °C. Insets in **a**–**e** show mean % of flies (Y-axis) that survived beyond 24 h after 60 min HS at 37 °C. * indicates $P \leq 0.001$ between the compared % knock down or survival values (horizontal lines above the vertical bars) using Student's *t* test

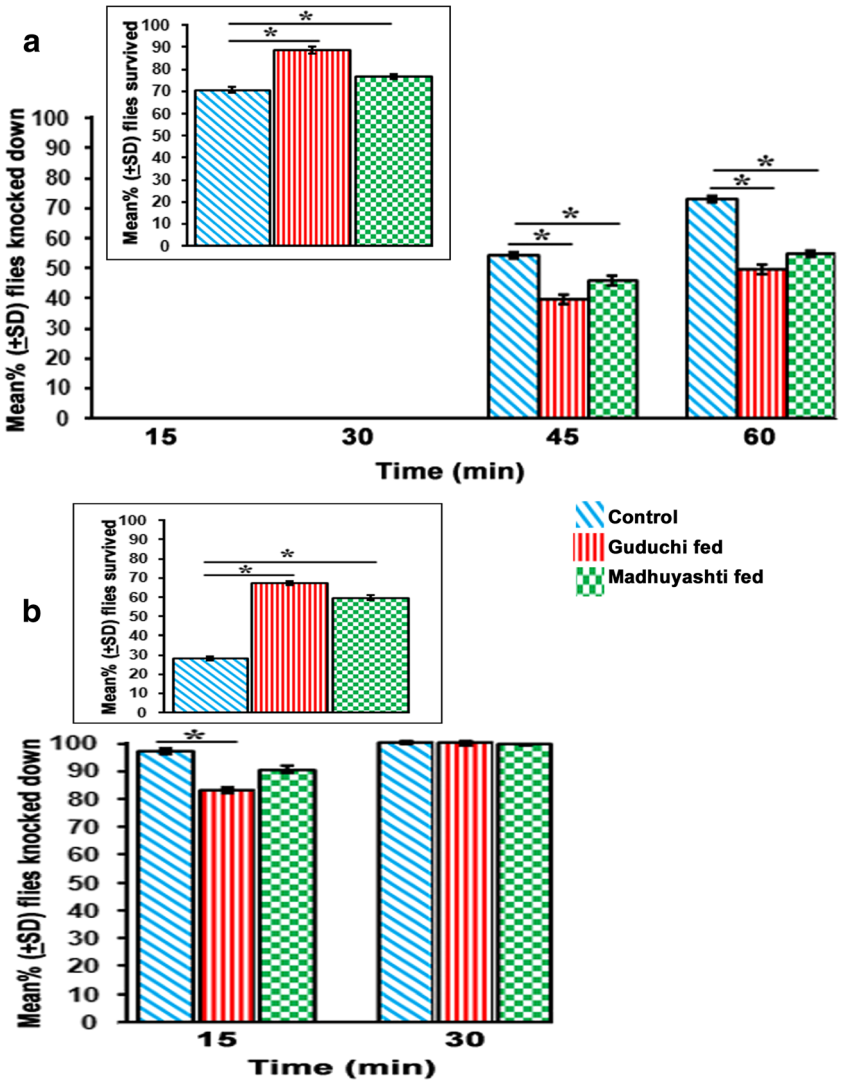

Fig. 3 Guduchi and Madhuyashti feeding improved thermotolerance of flies at higher temperatures. Histograms showing mean percentage (±SD) of 3-day-old flies knocked down (Y-axis) after HS for different durations (X-axis) at 38 °C (**a**) or at 39 °C (**b**). Insets in **a**, **b** show mean % of flies (Y-axis) that survived beyond 24 h after 60 min HS at respective temperatures. * indicates $P \leq 0.05$ between the compared % knock down or survival values (horizontal lines above the vertical bars) using Student's *t* test

robust presence of Hsp70 in all the tissues in all feeding regimes (Fig. 4a), viz., control (b, d, f, h), Guduchi (j, l, n, p), or Madhuyashti (r, t, v, x). Expression of Hsp70 following HS was highest in Guduchi as revealed by the mean fluorescence intensity obtained using LSM meta software (Fig. 4b). Western blotting of proteins from whole larvae also revealed that after heat shock, expression of Hsp70 in formulation fed samples was significantly greater than in those from larvae reared on regular food, with greater increase in Guduchi fed samples (Fig. 4c).

As expected on the basis of known developmental expression of Hsp83 (Mason et al. 1984; Arya et al. 2007),

Table 1 Hsp transcript levels are enhanced following formulation feeding

Samples	Mean ΔΔCT values (±SD; $N = 3$ biological replicates for each sample) for expression of various Hsps upon formulation feeding without or with heat shock, when compared to control larvae reared on regular food (> 2 ΔΔCT value is considered significant and is marked with *)		
	hsp70	*hsp83*	*hsp27*
Guduchi without HS	2.88 ± 0.05*	7.78 ± 0.16*	8.06 ± 1.6*
Madhuyashti without HS	2.87 ± 0.08*	7.21 ± 0.21*	7.21 ± 0.5*
Regular food HS	14.8 ± 0. 3*	9.8 ± 0.05*	9.18 ± 2.3*
Guduchi HS	16.8 ± 0. 4*	12.8 ± 0.04*	12.11 ± 1.6*
Madhuyashti HS	16.26 ± 0.17*	10.8 ± 0.05*	12.81 ± 2.6*

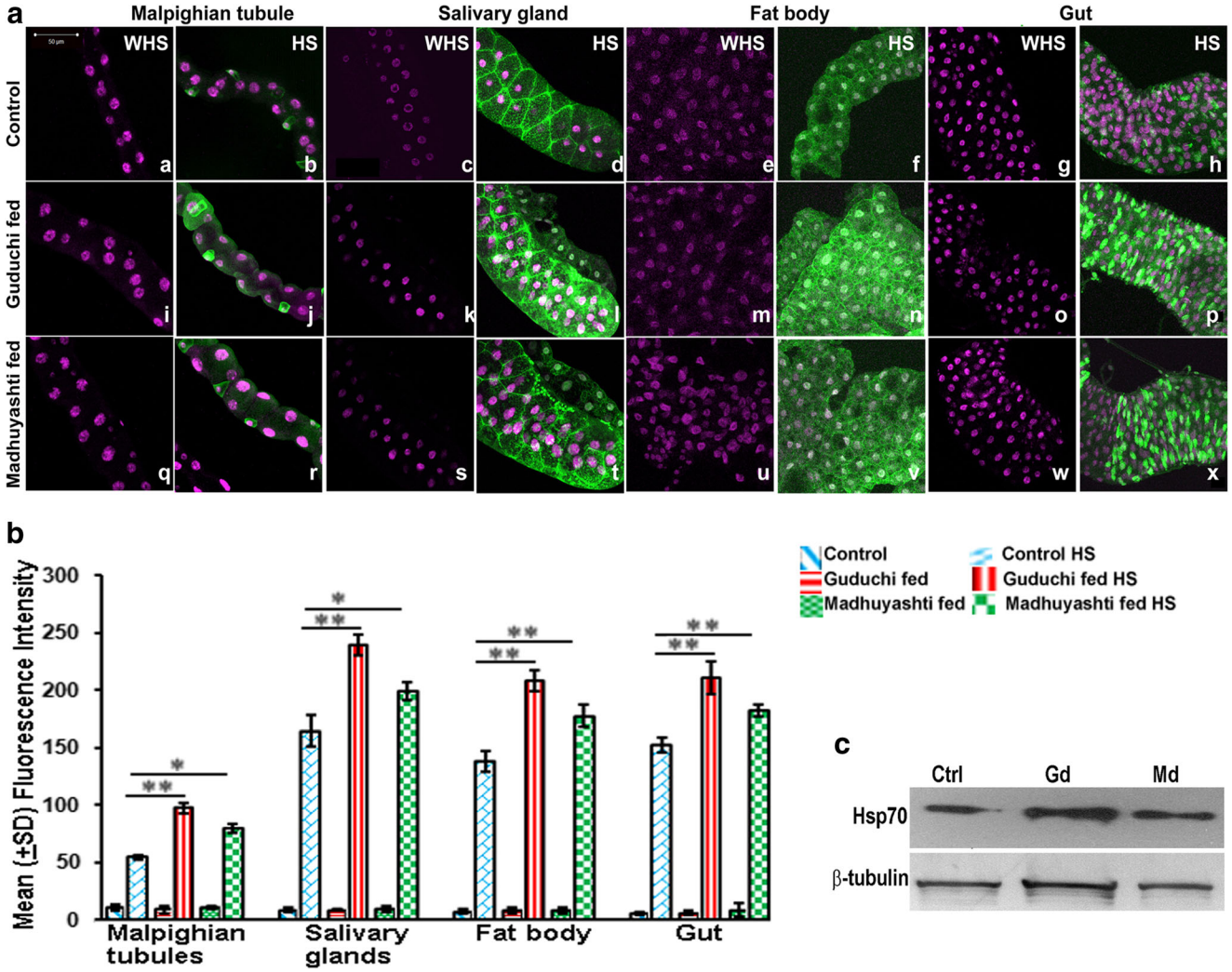

Fig. 4 Expression of Hsp70 after HS in different feeding regimes. **a** Hsp70 expression (green) after 1 h HS in various tissues from differently fed larvae (noted on left of each row). Confocal projection images show immunostaining for Hsp70 (green) in Malpighian tubules (**b**, **j**, **r**), salivary glands (**d**, **l**, **t**), fat bodies (**f**, **n**, **v**), and gut (**h**, **p**, **x**) without (WHS) or after heat shock (HS). Scale barin **a** represents 50 μm and applies to all images (**a** to **x**). DAPI stained nuclei are shown in pink color. **b** Bars represent mean (±SD) fluorescence intensities of Hsp70 (Y-axis) in various tissues (X-axis) under different feeding regimes after without or with HS. * and ** marks above the horizontal bars indicate $P < 0.05$ and $P < 0.001$, respectively, when compared with the corresponding control. **c** Western blot shows levels of Hsp70 (detected by the 7Fb antibody) from differently fed (control, Guduchi, and Madhuyashti lanes) late third instar larvae after HS; β-tubulin was used as internal loading control

immunostaining revealed its presence in cytoplasm as well as nucleus in all the tissues even without HS (Fig. 5a) in larvae fed on control (a, c, e, g), Guduchi (i, k, m, o), or Madhuyashti (q, s, u, w) supplemented food. Following HS, Hsp83 levels increased comparably in larval tissues from all the three feeding regimes (Fig. 5a), viz., control (b, d, f, h), Guduchi (j, l, n, p), or Madhuyashti (r, t, v, x), as confirmed through mean fluorescence intensity comparison using the LSM 510 Meta software (Fig. 5b).

Hsp27 expression was also present without HS (Fig. 6a) in larvae fed on control (a, c, e, g), Guduchi (i, k. m, o), or Madhuyashti (q, s, u, w) supplemented food in all the examined tissues. Following HS, Hsp27 levels increased comparably in samples from all the three feeding regimes (Fig. 6a), viz., control (b, d, f, h), Guduchi (j, l, n, p), or Madhuyashti (r, t, v, x). This was also confirmed by mean fluorescence intensity analysis using the LSM 510 Meta software (Fig. 6b).

Guduchi or Madhuyashti feeding improved tolerance to desiccation or starvation stress

Starvation and desiccation stress results when organisms face food deficiency and deficiency of food as well as water, respectively. Since Guduchi and Madhuyashti are classified as adaptogens (Mehta et al. 2015), we examined

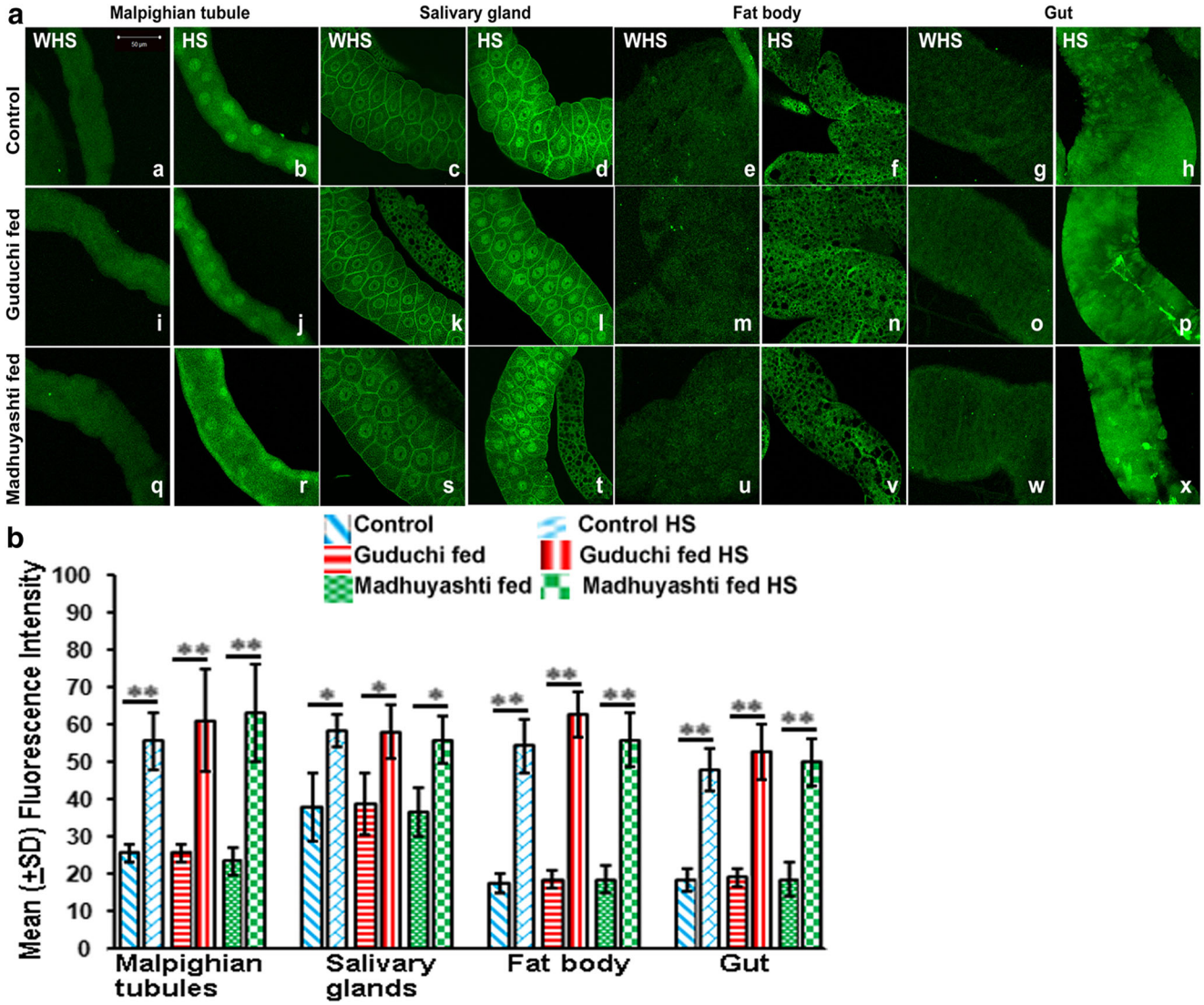

Fig. 5 Formulation feeding did not affect constitutive or HS induced levels of Hsp83. Confocal optical sections of different third instar larval tissues (noted on top), without (WHS) or after heat shock (HS), immunostained with anti-Hsp83 antibody (green) under the three feeding regimes (noted on left). Scale bar in **a** represents 50 μm and applies to **a**–**x**. **b** Histogram bars represent mean (±SD) fluorescence intensities of Hsp83 (Y-axis) in various tissues (X-axis) under the three feeding regimes with or without HS. * and ** marks above the horizontal bars indicate $P < 0.05$ and $P < 0.001$ respectively when compared with the corresponding control

if feeding on these formulations affected tolerance to starvation or desiccation stress. Results presented in Fig. 7 show that flies reared since larval stages on either of the formulation-supplemented food survived desiccation (Fig. 7a) or starvation (Fig. 7b) stress better than those reared on the regular food. Flies grown on Madhuyashti and Guduchi could survive up to 84 h and 78 h, respectively, while those grown on control food survived only for 66 h following desiccation stress. Similarly, when they were subjected to starvation condition, flies reared on Madhuyashti and Guduchi could survive up to 204 h which was significantly higher than that of controls, which failed to survive beyond 156 h. Interestingly, while Guduchi elicited better thermotolerance than Madhuyashti, the latter was more effective in conferring tolerance for starvation or desiccation stress.

Guduchi and Madhuyashti provided better resistance against pathogenic challenges

The humoral immune response in *Drosophila* is associated with production of anti microbial peptides (AMPs) either by the IMD pathway in response to Gram –ve bacteria or through the Toll pathway in response to Gram +ve bacteria or fungi (Hoffmann and Reichhart 2002). Since Guduchi and Madhuyashti are known immunomodulators (Damle 2014),

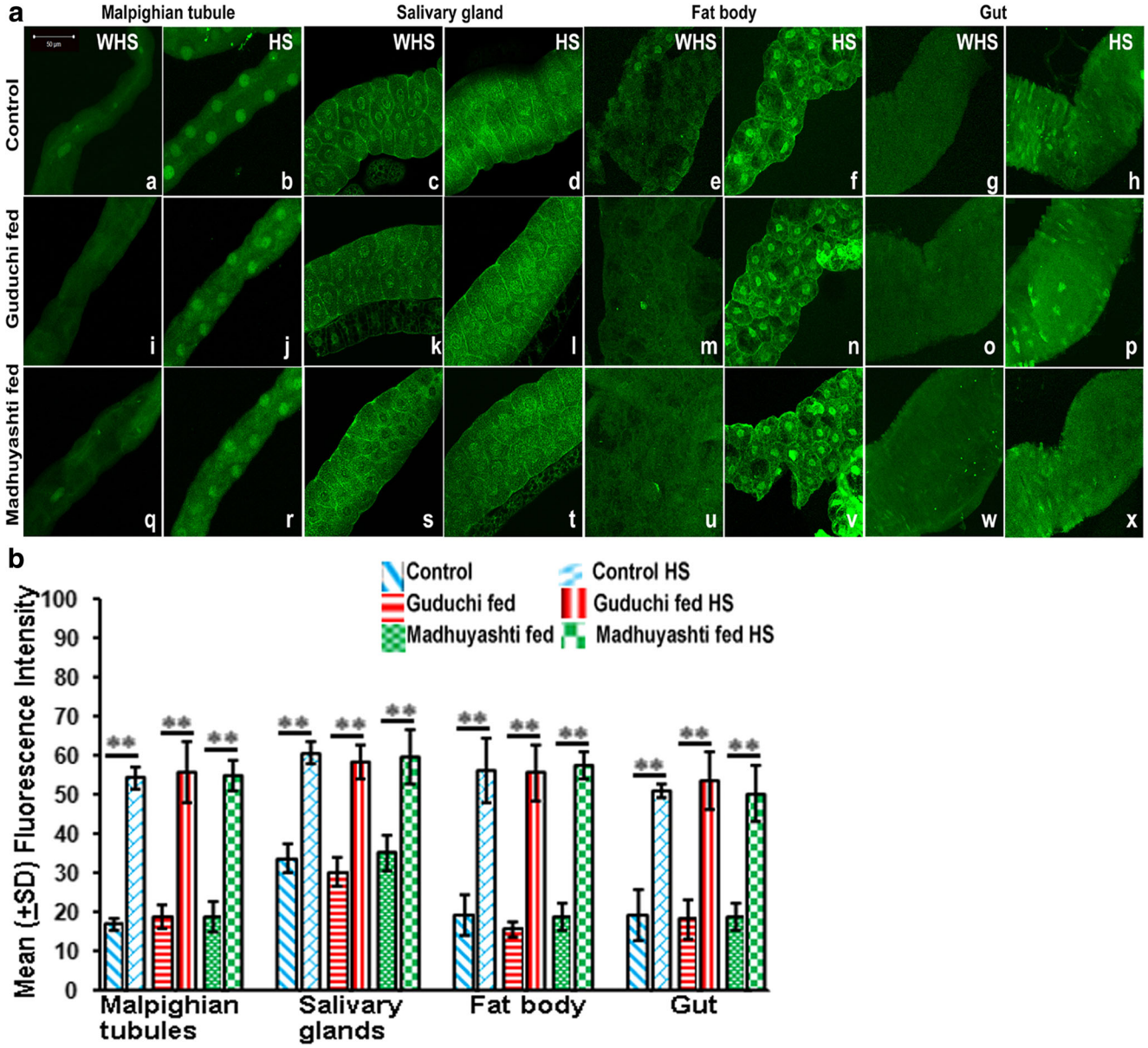

Fig. 6 Formulation feeding did not affect constitutive or HS induced levels of Hsp27. Confocal sections of different third instar larval tissues (noted above columns), without (WHS), or after heat shock (HS) immunostained with anti-Hsp27 antibody (green) under the three feeding regimes (noted on left of rows). Scale bar in **a** represents 50 μm and applies to **a**–**x**. **b** Graph represents the mean (±SD) fluorescence intensities of Hsp27 (Y-axis) in different tissues (X-axis) under the three feeding regimes with or without HS. ** marks above the horizontal bars indicate $P < 0.001$ when compared with the corresponding control

we checked expression of different AMPs upon formulation feeding by qRT-PCR using AMP specific primers. A significant variation was seen in expression of the different AMPs in flies reared on formulation supplemented food (Table 2). Highest expressing AMP was *Drosocin*, followed by *Metchnikowin* and then *Diptericin*. Formulation specific differences in the expression of AMPs in uninfected larvae was also noted. *Cecropin* levels increased only in case of Guduchi feeding while *Attacin* and *Metchnikowin* expression was enhanced by Guduchi as well as Madhuyashti. *Drosocin* and *Diptericin* expression also increased in both the feeding regimes, but unlike *Attacin* and *Metchnikowin*, their expression was significantly higher in Guduchi than Madhuyashti fed larvae. Expression of *Drosomycin* and *Defencin* was not affected by either formulation. The differential response of AMPs to the two formulations is consistent with the known varied responses of different AMPs (Verma and Tapadia 2012).

Pathogenic challenges cause cellular stress (Morimoto 1993) which results in production of AMPs (Wu et al. 2012). Since formulation feeding enhanced expression of several AMPs, we also examined tolerance of these flies to *E. coli* infection for 20 consecutive days and counted the number of

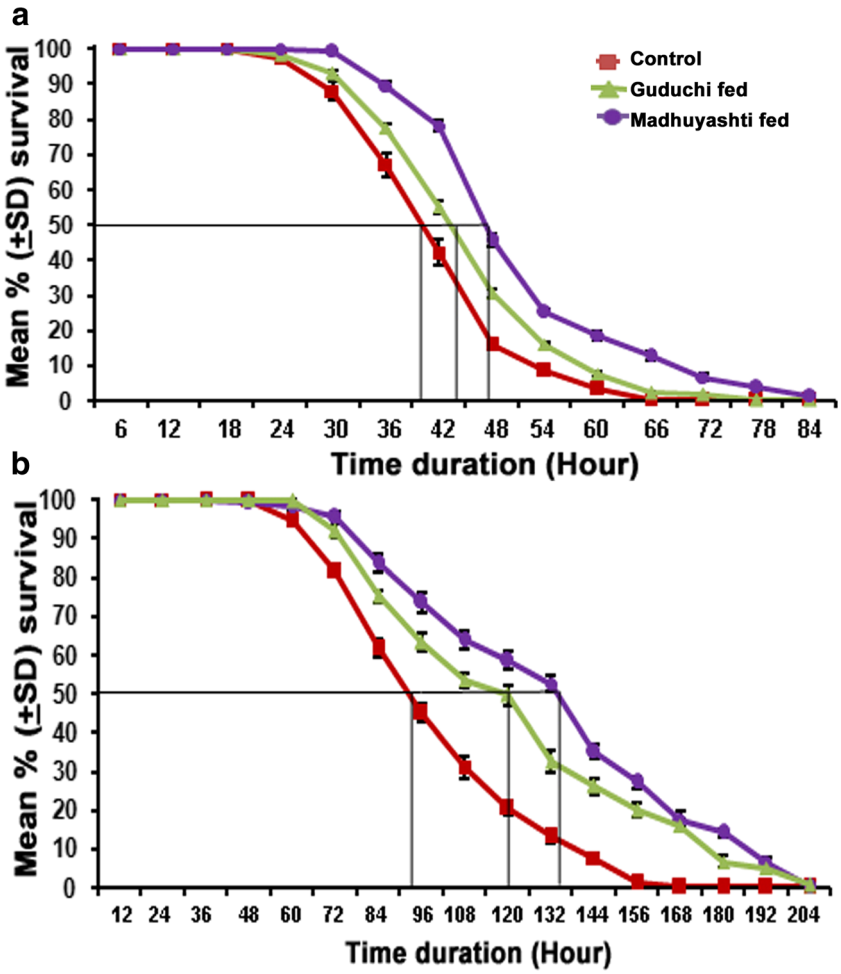

Fig. 7 Formulation feeding improved starvation or desiccation tolerance in flies. Mean % survival (Y-axis) of flies reared on different feeding regimes at different times (X-axis) after **a** desiccation or **b** starvation stress

flies that succumbed to infections. It was seen that 50% flies reared on normal food died due to bacterial infection between 9th and 10th day (Fig. 8) while nearly 60% of those fed on Guduchi or Madhuyashti supplemented food continued to survive even at the end of 20 days (Fig. 8). Together, these results showed that the Guduchi or Madhuyashti feeding protected against the pathogenic challenge, which is directly correlated with the elevated production of AMPs.

Guduchi or Madhuyashti feeding reduced survival of flies maintained under crowded condition

We examined effects of Guduchi or Madhuyashti feeding on the ability of flies to survive under crowding conditions. It is known that the density at which a population is reared or maintained has important consequences for several life history traits (Chiang and Hodson 1950; Bakker 1959; Joshi and

Table 2 Mean fold change in the expression of AMPs upon formulation feeding

AMPs	Mean ΔΔ-CT values (±SD; $N = 3$ biological replicates in each) of expression of various AMPs with respect to larvae fed on control (> 2 ΔΔCT value is considered significant and is marked with *)	
	Guduchi	Madhuyashti
Attacin	4.87 ± 0.40*	5.30 ± 0.26 *
Cecropin	2.72 ± 0.06*	1.46 ± 0.06
Defencin	1.73 ± 0.02	1.32 ± 0.04
Drosomycin	1.1425 ± 0.04	1.11 ± 0.01
Diptericin	6.45 ± 0.32*	3.30 ± 0.49 *
Drosocin	12.05 ± 0.67*	9.88 ± 0.75 *
Metchnikowin	8.78 ± 0.54*	9.86 ± 0.67 *

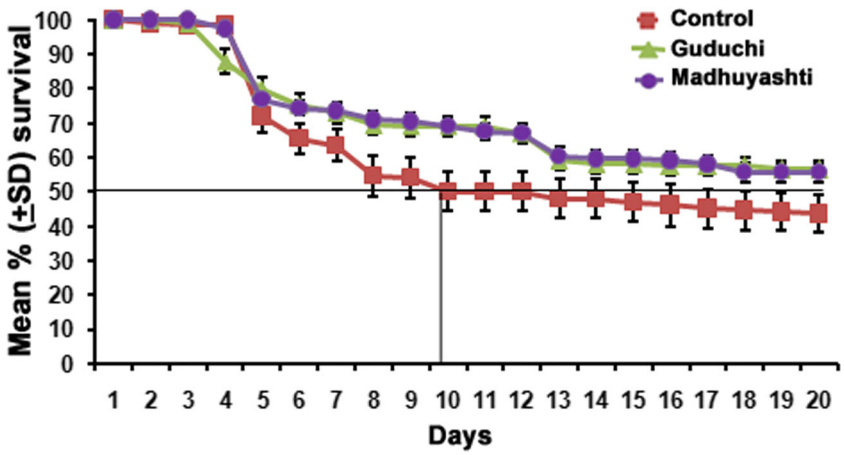

Fig. 8 Formulation feeding improves survival against pathogenic insults. Graphical representation of mean % (±SD) survival of flies (Y-axis) on each day (X-axis) following feeding on the pathogenic *E. coli*. ($N = 200$ from 10 replicates)

Mueller 1996; Joshi 1997; Sewell et al. 1975; Sokolowski et al. 1997; Borash and Ho 2001). Measurement of life spans of flies subjected to low, medium, and high crowding conditions revealed that feeding on either of the formulations made them more vulnerable to crowding since their median life spans were significantly reduced than in flies reared under comparable crowding (low, medium, or high) conditions on regular food (Fig. 9). Guduchi proved to be more detrimental than Madhuyashti under all the three crowding conditions. As expected, flies were more sensitive to high crowding under all the three feeding regimes.

Formulation feeding enhanced sensitivity of flies to ROS

Another important source of stress is the ROS levels which increase as flies age (Peng et al. 2014). Mutants deficient in ROS metabolism display reduced longevity and acute sensitivity to stress (Raamsdonk and Hekimi 2012). Therefore, we examined if Guduchi or Madhuyashti affected sensitivity of flies to ROS levels. Three-day-old flies grown on regular or formulation-supplemented food were exposed to 10 mM paraquat (N, N′-dimethyl-4,4′-bipyridinium dichloride) and number of flies surviving each day were observed (Fig. 10a). Intriguingly, flies reared on either of the formulation-supplemented food were more sensitive to paraquat since their survival was significantly less than those reared on normal food. As seen above for starvation, desiccation, and crowding stresses, Guduchi fed flies were more sensitive to paraquat than those fed on Madhuyashti. Increase in paraquat concentration to 20 mM further enhanced sensitivity of the formulation fed flies (Fig. 10b). At this concentration, none of the flies reared on Guduchi or Madhuyashti survived beyond 90 h or 96 h, respectively, while some of those reared on regular food survived beyond 108 h.

We also measured lipid peroxidation levels as lipids are probably the first easy target of free radicals (Kasapoglu and Ozben 2001; Holmbeck and Rand 2015). Lipid peroxidation measurement in 35-day-old flies reared on normal or formulation-supplemented food revealed significant increase in the levels of lipid peroxidation in the experimental samples. The MDA level in flies reared on normal food was 1.5 nmol/μg while the levels were significantly ($P < 0.05$) higher at 2.5 nmol/μg and 2 nmol/μg in samples from Guduchi or Madhuyashti fed flies, respectively

Discussion

This study aims to understand molecular mechanisms underlying effects of Guduchi and Madhuyashti Rasayanas, which are used in Ayurvedic health-care system to promote wellness and disease-free life in a holistic manner (Lakhotia 2013). Our results suggest that the beneficial effects of Guduchi and Madhuyashti are mediated through multiple paths such that each formulation uniquely modifies tolerance to various stresses that affect life.

A significant finding was that levels of heat shock gene transcripts were elevated in larvae fed on Guduchi or Madhuyashti even in absence of HS. The enhanced activity of the heat shock genes in Guduchi or Madhuyashti fed larvae and flies seems to be the primary reason for their enhanced thermo-tolerance as found in this study since these major Hsps are known to be important in alleviating the thermal damage (Lindquist 1980; Krebs and Feder 1998; Young et al. 2004; Mayer and Bukau 2005; Craig et al. 2006; Arya et al. 2007; Richter et al. 2010; Lin et al. 2014; Stetina et al. 2015). It is interesting that although the *hsp70* transcripts are completely absent in unstressed *Drosophila* cells (Lindquist 1980; Krebs and Feder 1998; Lakhotia and Prasanth 2002), present results revealed that Guduchi or Madhuyashti feeding activated the *hsp70* gene transcription even in absence of any obvious cell stress. Intriguingly, despite the presence of *hsp70* transcripts, the stress-inducible Hsp70 protein was not seen in the formulation-fed but unstressed larvae. On the other hand, heat shock not only further enhanced the major Hsp gene

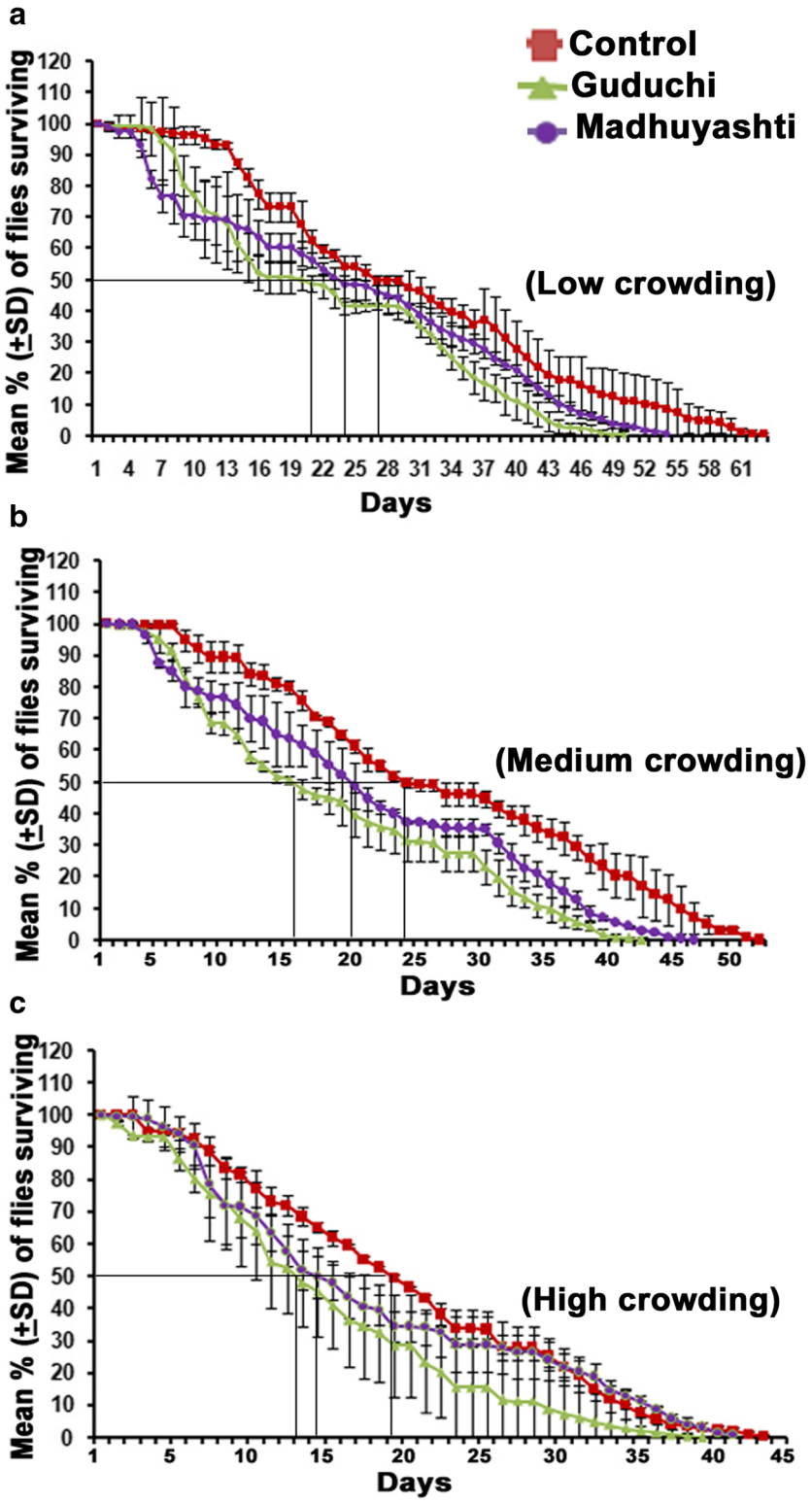

Fig. 9 Formulation feeding increased sensitivity to crowding. Graphical representation of mean % (±SD) survival of flies (Y-axis) on different days (X-axis) kept under **a** low, **b** medium, or **c** high crowding conditions. Horizontal line in each corresponds to 50% survival and the connecting vertical drop shows the median survival time

transcripts but their protein levels also showed greater elevation in formulation-fed larvae. It appears that the increased transcription of *hsp70*, *hsp83*, and *hsp27* in formulation fed but unstressed larvae provides for their faster translation as and when the cells face thermal stress (Neal et al. 2006). Together, the changes in activation and levels of the different

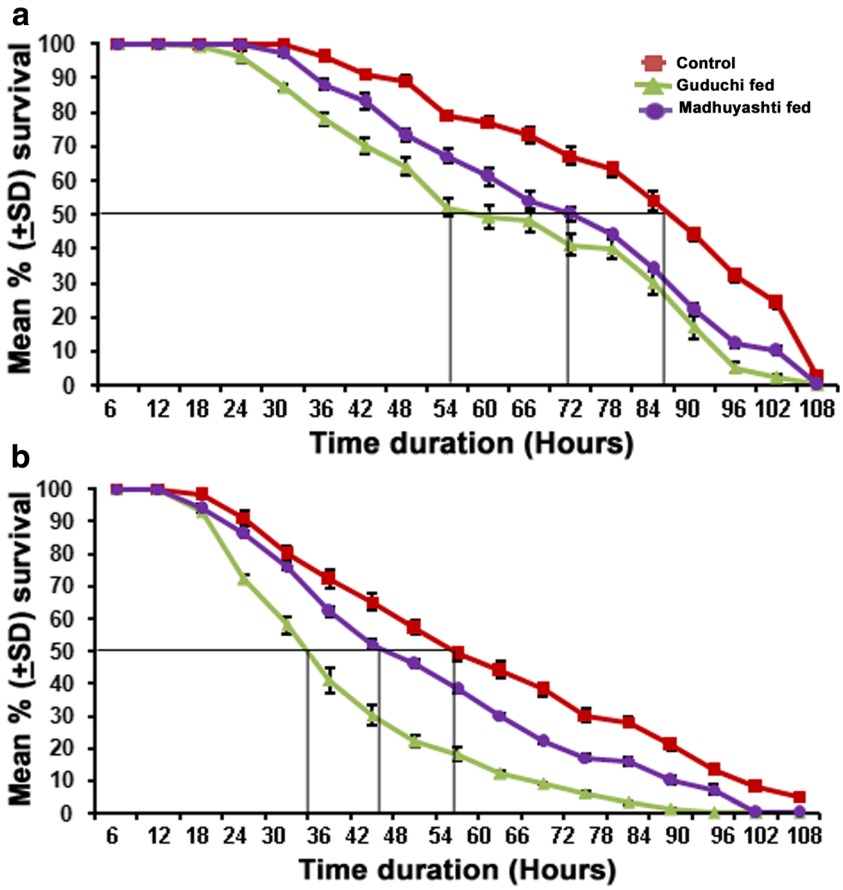

Fig. 10 Formulation feeding reduced survival of flies when exposed to paraquat. Graphical presentation of mean % survival of flies (Y-axis) reared on different feeding regimes at different times (X-axis) following exposure to **a** 10 mM or **b** 20 mM paraquat. Horizontal line in each corresponds to 50% survival and the connecting vertical drop shows the median survival time

Hsps brought about by Guduchi or Madhuyashti feeding seem to be the major factors that underlie the observed improved tolerance against thermal, desiccation, or starvation stresses. Increased expression of Hsp27 has been reported earlier (Hao et al. 2007) to provide better tolerance to starvation or desiccation stress. Increased Hsp83, besides being associated with better thermotolerance, may also be responsible for the earlier reported (Singh et al. 2018) increased fecundity of flies fed on either of these formulations.

Our finding that several AMPs were elevated following Guduchi or Madhuyashti feeding agrees with the earlier reported immunomodulatory activities of Guduchi and Madhuyashti (Mittal et al. 2014; Goel et al. 2014; Patel et al. 2009; Gupta and Sharma 2011; Damle 2014). The finding that levels of different AMPs were differently affected in Guduchi or Madhuyashti-fed larvae agrees with earlier reports that expression of the various AMPs is differentially affected by ecdysone signaling and stage of development (Meister and Richards 1996; Dimarcq et al. 1997; Verma and Tapadia 2015).

In view of the advantage conferred by Guduchi or Madhuyashti feeding against thermal, starvation, desiccation, or pathogenic stress, the elevated sensitivity of formulation-fed flies to crowding or high exogenous ROS appears surprising. ROS, synthesized normally as part of cellular metabolism, is important for cell signaling. However, excess of ROS is detrimental as they target lipids, nucleic acids, polypeptides etc. (Halliwell and Chirico 1993; Halliwell and Gutteridge 1999; Feng and Stockwell 2018) and thus destabilize membranes as well as cell functioning. Elevated lipid peroxidation following feeding on either of these two formulations indicates that Guduchi as well as Madhuyashti enhance ROS levels. This was also confirmed by DCFH-DA staining (data not presented). Apparently, exposure of the Guduchi or Madhuyashti fed larvae to paraquat or crowding stress further enhances ROS levels which cannot be effectively neutralized making them more sensitive to crowding or paraquat stress (Gupta et al. 2007; Singh et al. 2009). It may be noted in this context that feeding of *Drosophila* larvae on Ayurvedic Rasa-Sindoor (a mercury and sulfur-based sublimate preparation) supplemented food was also reported to significantly elevate theormo-tolerance but make the larvae more sensitive to oxidative stress (Dwivedi and Lakhotia, 2016).

Our results suggest that some of the beneficial effects of Guduchi and Madhuyashti for which these formulations are used in Ayurvedic health-care system relate to the improved dynamics of major Hsps and improved innate immune response. Both these conditions are expected to reduce the diverse stress-induced damages in aging cells. ROS is an important signaling molecule and 'cross-talk' involving ROS and

heat shock response and immune pathways may be important for maintaining the homeostasis. At present, it is difficult to elucidate the cause and consequence relation between formulation feeding, ROS production, heat shock response, and immune response, but it is possible that formulation feeding elevates ROS level, which in turn signals the heat shock response and immune response (Domenico et al. 1982; Buchon et al. 2014).

Ayurvedic medicines have been used since ages largely as experience-based system of health care. A good understanding of their mechanisms of actions at cell and molecular levels is essential for more specifically defining their therapeutic usages. Thus, the present findings that while both the formulations have generally similar consequences for cell stress tolerance, there are small but significant differences between the two. Thus, while Guduchi provides better thermotolerance, Madhuyashti is more effective in improving starvation and desiccation stress tolerance, and yet, both improve immune response, although through different AMPs. Another important finding of the present study is that despite improving tolerance to thermal, starvation, desiccation, and pathogenic stress, both the formulations make the organism more sensitive to crowding and oxidative stress. Such differences between biological actions of Guduchi and Madhuyashti obviously would have implications for their therapeutical applications.

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