Suppression of induced but not developmental apoptosis in *Drosophila* by Ayurvedic *Amalaki Rasayana* and *Rasa-Sindoor*

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Earlier we showed formulation-specific beneficial effects of dietary supplement of Ayurvedic *Amalaki Rasayana* (AR, a herbal formulation) and *Rasa-Sindoor* (RS, a mercury-based organo-metallic formulation) on various biological parameters in *Drosophila*, parallel to traditional Ayurvedic literature. These formulations also suppressed cell death and pathology in fly models of neurodegeneration. To understand basis of inhibition of apoptosis, we examined effects of AR and RS on induced and developmental apoptosis in *Drosophila*. Dietary AR or RS significantly reduced apoptosis induced by *GMR-GAL4-, sev-GAL4-* or *hs-GAL4*-directed expression of Rpr, Hid or Grim (RHG) pro-apoptotic proteins or by *GMR-GAL4*-directed *DIAP1-RNAi*, resulting in significant restoration of organism's viability and eye morphology. AR or RS supplement enhanced levels of inhibitor of apoptosis proteins, DIAP1 and DIAP2, and of Bancal/Hrb57A, while the levels of RHG proteins and of initiator Dronc and effecter Drice caspases were reduced in non-apoptotic wild type as well as in RHG over-expressing tissues. Levels of Dronc or Drice remained unaffected in cells developmentally destined to die so that developmental apoptosis occurred normally. Elevated levels of DIAPs and reduced levels of RHG proteins and caspases reflect a more robust physiological state of AR or RS fed organisms allowing them to tolerate greater insults without triggering the cell-death response. Such homeostatic effects of these Rasayanas seem to contribute to 'healthy ageing', one of their effects suggested in traditional Ayurvedic practices.

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1. Introduction

With increasing burden of chronic and lifestyle diseases, various traditional medical systems, including the Ayurvedic system of India, are being more commonly utilized. However, contemporary understanding of the mechanism of actions of the traditional health care systems is very limited. In view of the antiquity and increasing popularity of Ayurveda, and realizing the need to mechanistically understand its concepts, procedures and formulations (Valiathan 2006), we are using the fruit fly model to understand cellular and molecular bases of actions of these traditional formulations (Dwivedi *et al*. 2012). We have selected two widely used Ayurvedic formulations, viz. *Amalaki Rasayana*, a herbal preparation derived primarily from fruits of *Emblica officinalis*, and *Rasa-Sindoor*, an organo-metallic sublimate of mercury, to examine if these formulations indeed affect some of the basic biological life parameters in the fly model, as claimed for human applications. *Amalaki Rasayana* (AR) is a prominent drug in Ayurvedic classics like *Charak Samhita* (Sharma 1994) and *Ashtang Hridaya* (Murthy 2000) for enhancing life expectancy, body strength, intellect and fertility, with freedom from illness. *Rasa-Sindoor* (RS), on the other hand, is utilized singly or in combination with other formulations in a wide variety of disorders including chronic and recurrent infections.

We showed earlier that dietary supplement of AR or RS improved the general well-being of flies (Dwivedi *et al.*

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2012), as claimed for their human applications (Sharma 1994; Murthy 2000). Further, either of these traditional formulations was found to substantially suppress pathogenesis in fly models of various neurodegenerative disorders (Dwivedi *et al.* 2013). One of the significant features of neurodegeneration is apoptotic death of the affected neurons, which was greatly reduced in the mutant polyQ- or the Aβ-amyloid-protein-expressing eye imaginal discs of AR- or RS-fed larvae (Dwivedi *et al.* 2013). It was, however, not clear if these Ayurvedic formulations directly suppressed apoptosis or if they reduced the load of toxic protein aggregates and thus eliminated the need for apoptosis.

Apoptosis is a genetically programmed, active and evolutionarily conserved process that allows regulated destruction and disposal of damaged or unwanted cells under physiological as well as pathological conditions (Wyllie *et al.* 1980; Raff *et al.* 1993; Baehrecke 2002; Edinger and Thompson 2004; Hongmei 2012; Ouyang *et al.* 2012). Besides eliminating damaged cells under pathological conditions, patterned apoptosis is an integral part of development in multicellular organisms (Abrams *et al.* 1993; Fuchs and Steller 2011). However, unwarranted cell death is also associated with several diseases and ageing (Higami and Shimokawa 2000; Muradian and Schachtschabel 2001; Lu *et al.* 2012). Since the two Ayurvedic formulations promote 'healthy' ageing (Sharma 1979; Singh 2009; Dwivedi *et al.* 2012) and also suppress neurodegeneration (Dwivedi *et al.* 2013), it is important to understand the effects of these formulations on the apoptotic process. In view of the enormous advantages offered by the fly model and following our earlier studies on the effects of AR and RS on fly development and neurodegeneration, we examined if AR and RS feeding indeed affects apoptosis in the fly model *Drosophila melanogaster.*

Caspase activation in response to intrinsic or extrinsic signals is the primary step of apoptosis in mammalian cells. On the other hand, inactivation of inhibitors of apoptotic proteins (IAP) or inhibitors of caspases by upstream pro-apoptotic proteins is the central event that triggers apoptosis in flies. The three major pro-apoptotic proteins in *Drosophila* are Reaper (Rpr), Hid and Grim or the RHG proteins (Kornbluth and White 2005; Hay and Guo 2006; Arya *et al.* 2007). Each of the RHG proteins are IAP antagonists, acting by binding to and inactivating the two caspase inhibitors, DIAP1 and DIAP2 (Ryoo *et al.* 2002; Fuchs and Steller 2011; Denton *et al.* 2013). The RHG proteins disrupt interaction of DIAPs with caspases or induce auto-ubiquitination of DIAPs leading to their inactivation (Ryoo *et al.* 2002; Arya *et al.* 2007; Steller 2008; Fuchs and Steller 2011; Denton *et al.* 2013). In spite of general similarities in mode of action of the RHG proteins, they show some differences in their regulation and effectiveness in inducing apoptosis (Wing *et al.* 1998; Yin and Thummel 2004; Fan and Bergmann 2010).

The initiator caspases in *Drosophila* include Dronc, Dcp-2/Dredd and Strica, while the effecter caspases are Drice, Dcp-1, Decay and Damm (Kumar and Doumanis 2000; Doumanis *et al*. 2001; Harvey *et al*. 2001; Fan and Bergmann 2010). Expression of *Drosophila* caspase Dronc (*Drosophila* Nedd2-like caspase) increases in response to developmental as well as external pro-apoptotic cues to activate the main effecter caspase Drice, which finally brings about the cell death (Meier *et al*. 2000; Ryoo *et al.* 2002; Fuchs and Steller 2011; Denton *et al.* 2013).

Induced apoptosis can be beneficial or deleterious depending upon the context and cell type involved. Since, as noted above, these two Ayurvedic formulations have been widely used for promoting 'healthy' living, and since our earlier studies (Dwivedi *et al.* 2013) indicated that these formulations may suppress polyQ- or amyloid-plaque-induced apoptosis, it is necessary to examine their effects on developmental as well as induced apoptosis and to understand the underlying mechanism. In the present report, therefore, we examined if the AR and RS Ayurvedic formulations affect apoptosis induced by directed expression of the RHG proteins using *GMR-GAL4, sev-GAL4* or *hs-GAL4* drivers. Formulation feeding substantially reduced the incidence of induced apoptosis accompanied by reduced levels of initiator as well as effecter caspases Dronc and Drice, respectively. The AR or RS feeding elevated cellular levels of the DIAP1 and DIAP2 anti-apoptotic proteins, as well as of Bancal (Hrb57A, hnRNP-K), which is believed to stabilise DIAP1 (Mallik and Lakhotia 2009). Both these formulations also countered the enhanced apoptosis following expression of *DIAP1-RNAi* transgene. Interestingly, the developmental apoptosis remains completely unaffected by these supplements.

2. Materials and methods

2.1 *Fly stocks and formulation feeding*

Amalaki Rasayana and *Rasa-Sindoor*, prepared by Arya Vaidya Sala (Kottakkal, Kerala, India), were separately mixed in fly-food (0.5% w/v) for rearing of experimental larvae and/or flies at 24°C±1°C as described earlier (Dwivedi *et al.* 2012), keeping controls on the standard agar-cornmeal-sugar-yeast food. Wild type (*Oregon* R^+), *w; GMR-GAL4* (Freeman 1996), *w; sev-GAL4* (Bailey, personal communication to *http://flybase.org*), w^{1118}*; UAS-rpr* (Aplin and Kaufman 1997), w^{1118}*; UAS-hid*^{14}*/CyO* (Oshima *et al*. 2006), w^{1118}*; UAS-grim*$^{wt9.8}$*/CyO; dco*^{2}*/TM6B* (Bloomington Stock Centre), *w; GMR-GAL4 UAS-DIAP1-RNAi/CyO* (Leulier *et al*. 2006) fly stocks were used.

Appropriate crosses were carried out to obtain progenies of the desired genotypes.

In all formulation feeding experiments, eggs were collected from fly stocks that had always been reared on regular food. For each experiment, the regular and the formulation supplemented foods were prepared from the same batch; likewise all larvae/adults for a given experiment were derived from a common pool of eggs of the desired genotype and reared in parallel on the regular or formulation supplemented food.

2.2 *Assay for apoptosis*

To assay the extent of apoptosis in third instar eye imaginal discs of the desired genotypes, late third instar larvae, that had just everted the anterior pair of spiracles, were dissected in Poels' salt solution (PSS) (Tapadia and Lakhotia 1997), stained with 1μg Acridine Orange (AO, Sigma-Aldrich, India)/ml of PBS for 3 min, following which the discs were washed twice, mounted in phosphate buffered saline (Spreij 1971; Abrams *et al.* 1993) and immediately viewed in Nikon E800 fluorescence microscope.

Eye discs from similar age wild type larvae, reared on regular food, were also stained with AO and examined for the base level apoptosis. The AO fluorescence signal in these discs was neutralized by adjusting the exposure and image gain of the DS-Fi1c Nikon camera; the same parameters were used to image the RHG-expressing eye discs.

2.3 *Adult eye morphology*

For examining the external morphology of adult eyes, following larval feeding on formulation supplemented food or on regular food, one day old flies of the desired genotype were etherized and their eyes photographed using a Sony Digital Camera (DSC-75) attached to a Zeiss Stemi SV6 stereobinocular microscope. For a clearer visualization of ommatidial arrays, a transparent nail polish was used to obtain a replica (nail-polish imprint) of the external surface of eye (Arya and Lakhotia 2006), which was examined using 20X DIC objective on a Nikon Eclipse 90i microscope equipped with DS-QiMc Nikon camera.

2.4 *Developmental apoptosis*

To examine developmental apoptosis, wild type larvae were reared on regular or AR or RS supplemented food. Just forming white pupae were selected and allowed to age for 8–14 h following which their salivary glands were dissected out in PSS and examined for morphology. Salivary glands from 8–9 h old pupae were processed for immunostaining for Dronc and Drice as described below.

2.5 *Immunostaining of tissues*

The desired tissues were dissected out in PSS and transferred to freshly prepared 3.7% paraformaldehyde for 20 min at RT and processed further for immunostaining as described previously (Prasanth *et al.* 2000). The different primary antibodies used were: (1) 1:100 dilution of a rabbit anti-active Caspase-3 (Sigma, St. Louis), (2) 1:10 dilution of the Q18 mouse monoclonal anti-Hrb57A hnRNP (Saumweber *et al.* 1980), (3) 1:50 dilution of a rabbit polyclonal anti-Dronc (Cakouros *et al.* 2004), (4) 1:500 dilution of an affinity-purified rabbit polyclonal anti-αDIAP1 (Lisi *et al.* 2000), (5) 1:50 dilution of rabbit monoclonal anti-BIR3 domain of DIAP2 protein (Sigma-Aldrich, India) and (6) 1:100 dilution of rabbit polyclonal anti-Grim (Claveria *et al.* 2002), obtained from Dr M Torres (Centro Nacional de Biotecnologia, Madrid, Spain). Appropriate secondary antibodies conjugated either with Cy3 (1:200, Sigma-Aldrich) or with Alexa Fluor 488 (1:200, Molecular Probes) were used to detect the given primary antibody. The immunostained tissues were counterstained with DAPI, mounted in DABCO and examined under LSM510 Meta Zeiss laser scanning confocal microscope using appropriate laser, dichroic and barrier filters.

Quantitative analysis and colocalization of immunofluorescence were carried out using the Histo and Profile tools in the LSM510 Meta software.

All the images were assembled using Adobe Photoshop 7.0.

2.6 *Statistical analysis*

Sigma Plot 11.0 software was used for statistical analyses. All percentage data were subjected to arcsine square root transformation. One-Way ANOVA was performed for comparison between the control and formulation-fed samples. Data are expressed as mean ± S.E. of mean (SEM) of several replicates.

3. Results

3.1 *AR or RS feeding substantially reduces apoptosis induced by ectopic expression of UAS-rpr, UAS-grim or UAS-hid transgene in larval eye imaginal discs and improved larval/pupal survival*

With a view to examine if dietary AR or RS supplement affects apoptosis induced by directed expression of any of the three pro-apoptotic genes, viz. *rpr*, *grim* and *hid*, appropriate genetic crosses were made to ectopically express *UAS-rpr, UAS-grim* or *UAS-hid* transgene under *GMR-GAL4, sev-GAL4* or *hs-GAL4* driver. Larvae/flies of the various

genotypes were reared in parallel on 0.5% AR or RS supplemented food or on regular food (control).

Ectopic expression of any of the RHG proteins under control of the GMR promoter causes massive cell death in developing eye discs resulting in varying degree of damage to eye discs and pupal lethality (Goyal *et al.* 2000; Arya and Lakhotia 2008; Mallik and Lakhotia 2009). Effect of formulation feeding on induced apoptosis in *GMR-GAL4> UAS-rpr, UAS-grim* or *UAS-hid* expressing larval eye imaginal discs, was examined through Acridine Orange staining which identifies the dying cells (Spreij 1971). To ensure comparable developmental stage across samples, eye discs were removed from larvae that had just everted their anterior pair of spiracles. It was seen that expression of Rpr (figure 1A–C), Grim (figure 1E–G) or Hid (figure 1I–K) caused high incidence of AO stained cells when the larvae were reared on regular food (figure 1A, E, I), with the *GMR-GAL4>UAS-hid* expressing discs showing maximum AO-positive cells. Significantly, feeding on either of the formulation supplemented food reduced apoptosis in *UAS-rpr* (figure 1B–D), *UAS-grim* (figure 1F–H) or *UAS-hid* (figure 1J–L) expressing eye discs. Measurement of fluorescence intensities of AO stained larval eye imaginal discs of AR or RS fed larvae (figure 1D, H, L) confirmed the visually detected reduction in AO fluorescence and showed that the reduction was proportional to the extent of cell death seen in corresponding control eye discs. It is notable that RS feeding resulted in the most pronounced reduction in induced apoptosis in eye discs ectopically expressing *GMR-GAL4* driven *rpr, grim* or *hid* transgenes.

As reported earlier (Arya and Lakhotia 2008; Mallik and Lakhotia 2009), expression of any of the RHG genes under

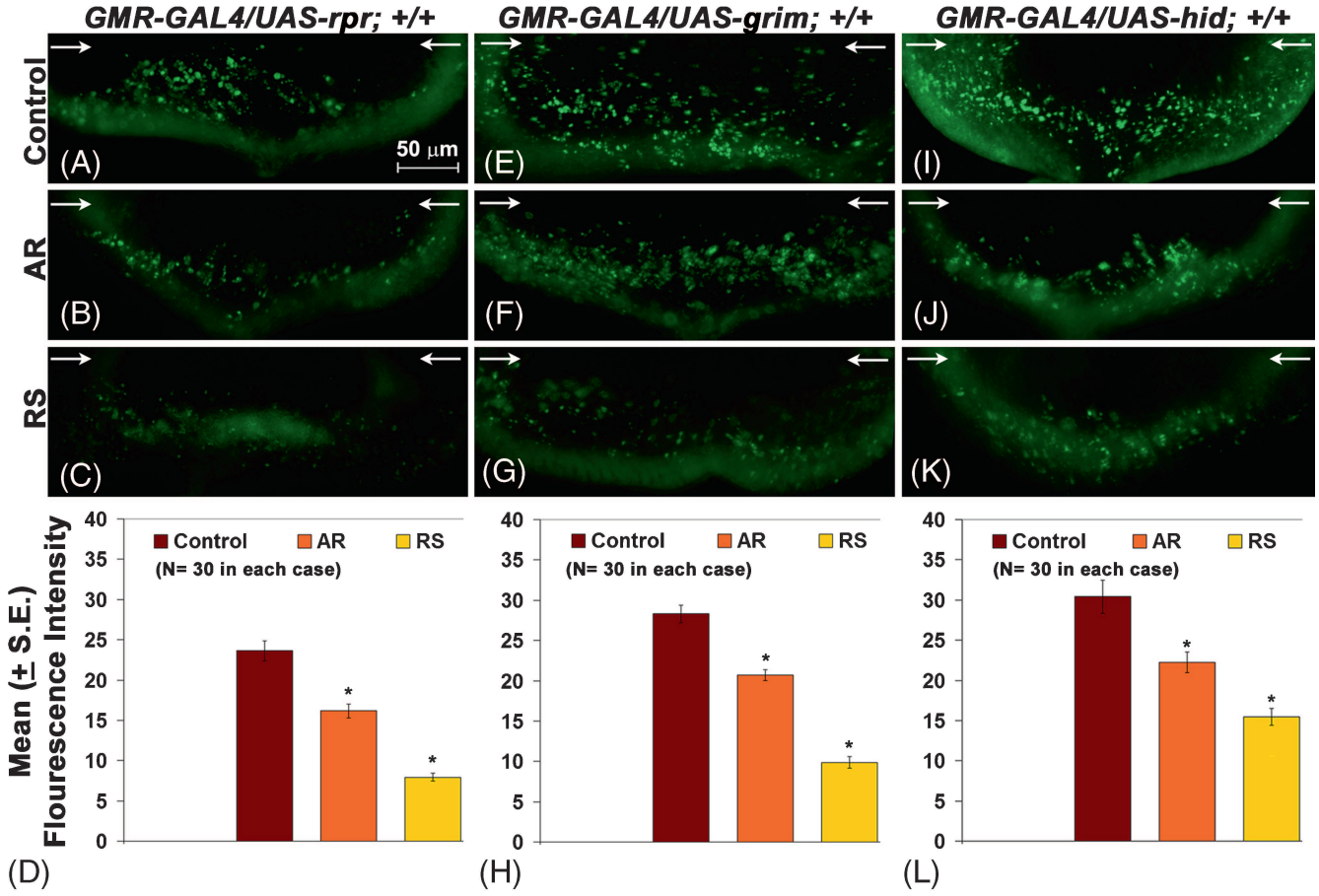


Figure 1. AR or RS feeding reduced the apoptosis induced by RHG proteins in larval eye imaginal discs. **A–C**, **E–G** and **I–K** are Acridine Orange (AO) stained live eye imaginal discs of *GMR-GAL4> UAS-rpr* (**A–C**), *UAS-grim* (**E–G**) or *UAS-hid* (**I–K**) expressing third instar larvae reared on regular food (**A**, **E**, **I**) or on AR (**B**, **F**, **J**) or RS (**C**, **G**, **K**) supplemented food. Scale bar in **A** represents 50 µm and applies to images **A–K**. Pairs of white arrows indicate the position of morphogenetic furrow in **A–K**. Histograms in **D, H** and **L** represent the mean (± S.E., N=30 in each case) AO fluorescence intensities of eye discs from *GMR-GAL4> UAS-rpr, UAS-grim* or *UAS-hid* expressing larvae. * indicates $P<0.001$ when compared with the corresponding control samples reared on regular food.

GMR-GAL4 driver resulted in complete pupal lethality. About 95% of *GMR-GAL4>UAS-rpr* expressing larvae fed either on regular or on formulation supplemented food pupated but none of them eclosed as adults. The proportion of *GMR-GAL4>UAS-grim* or *UAS-hid* expressing larvae reared on regular food that pupated was still less, more so following *hid* expression. Interestingly, a significantly higher numbers of these larvae pupated when reared on AR or RS supplemented food although none of them eclosed as flies (see table 1).

Therefore, to examine effect of formulation feeding on adult eye phenotype following apoptosis induced by the RHG proteins, we used *sev-GAL4* driver since many of the regular food reared larvae expressing *rpr*, *grim* or *hid* transgene under *sev-GAL4* driver pupated and emerged as flies (Table 1). AR or RS feeding did not result in any significant difference in the mean pupation and eclosion frequencies in *sev-GAL4>UAS-rpr* or *UAS-grim* expressing larvae reared on regular or formulation supplemented food. Interestingly, however, eclosion was significantly greater in the *sev-GAL4>UAS-hid* expressing larvae that were reared on AR or RS supplemented food when compared to those reared on regular food (Table 1). As in other instances, RS feeding improved the eclosion frequency to a greater extent (table 1).

Acridine Orange staining of *sev-GAL4> UAS-rpr, UAS-grim* or *UAS-hid* expressing eye discs of third instar larvae fed on regular food revealed high incidence of apoptosis (figure 2A–C) but this was significantly reduced in those reared on AR (figure 2E–G) or RS (figure 2I–K) supplemented food. A comparison of the fluorescence intensities (figure 2D, H, L) in the AO stained larval eye imaginal discs confirmed that compared to the control samples (regular food), AR or RS feeding significantly reduced the presence of AO-positive dying cells in *sev-GAL4> UAS-rpr* (figure 2D)*, UAS-grim* (figure 2H) or *UAS-hid* (figure 2L) expressing eye discs.

Flies emerging from *sev-GAL4>UAS-rpr, UAS-grim* or *UAS-hid* larvae reared on regular food showed a substantial degree of degeneration and disruption in ommatidial arrangement following Rpr (figure 2M), Grim (figure 2P) or Hid (figure 2S) expression but those reared on formulation supplemented food showed improved external morphology of eyes in *sev-GAL4>UAS-rpr* (figure 2N, O)*, UAS-grim* (figure 2Q, R) and *UAS-hid* (figure 2T, U) adults. Photomicrographs and nail polish imprints of 78.5% of AR fed and 80.6% of RS fed *sev-GAL4>UAS-rpr* (figure 2M–O'), 68.8% of AR fed and 72.8% of RS fed *sev-GAL4>UAS-grim* (figure 2P–R') and 60.3% of AR fed and 64.8% of RS fed *sev-GAL4>UAS-hid* (figure 2S–U') flies showed distinctly better morphology than their sib controls reared on regular food. The *sev-GAL4>UAS-hid* expression caused maximum damage to adult eyes and correspondingly least improvement.

Effects of heat shock induced global expression of the RHG proteins was also examined in *hs-Gal4>UAS-rpr, UAS-grim* or *UAS-hid* larvae. Late 3rd instar larvae of each genotype were heat-shocked for 1 hour at 37°C before assessing the incidence of apoptotic cells in their eye discs. Unlike the *GMR-GAL4* and *sev-GAL4* drivers, which cause the responder transgene to express in specific cell types in a developmentally regulated manner in eye discs, the *hs-GAL4*

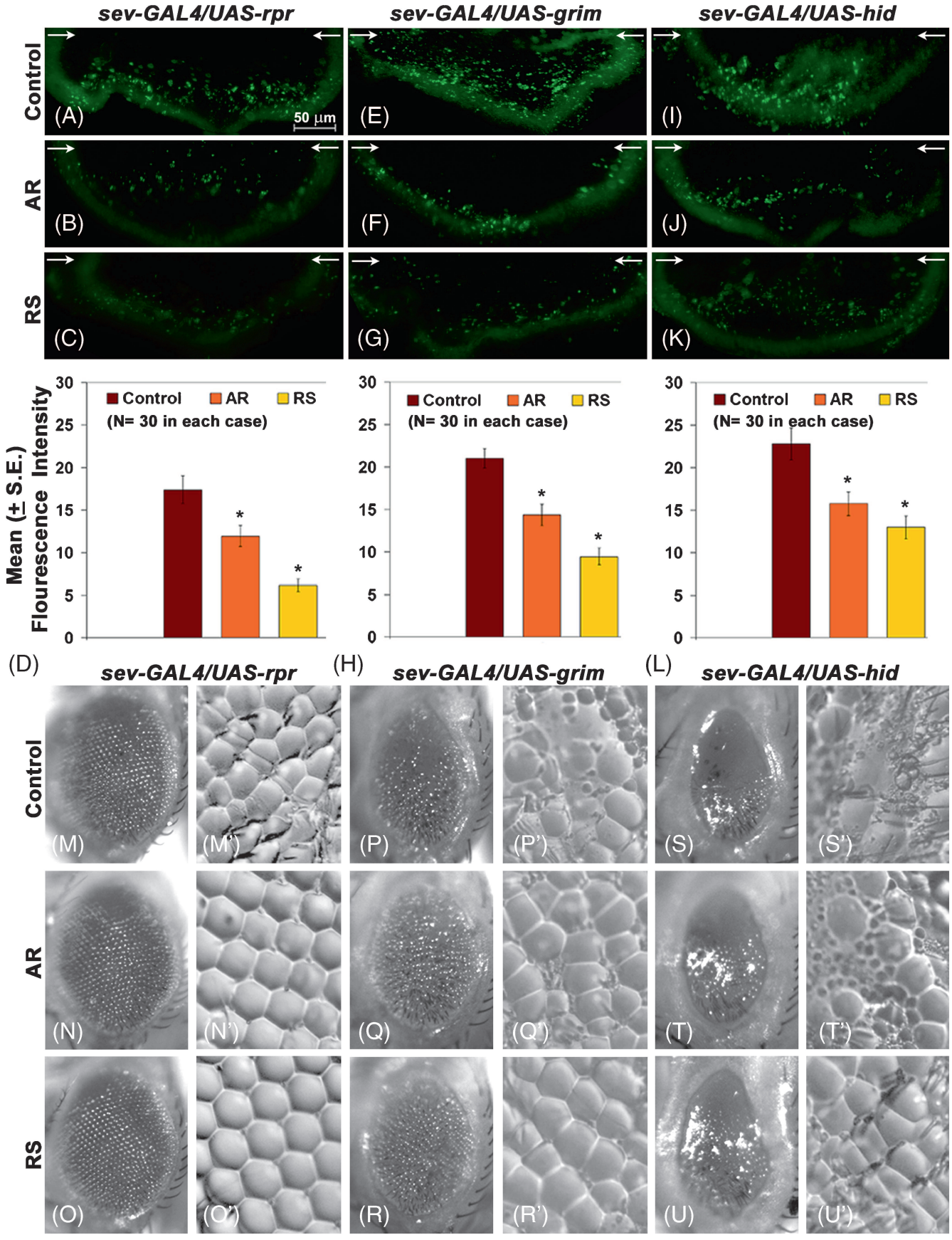
Table 1. Pupation and eclosion frequencies of *GMR-GAL4, sev-GAL4* and *hs-GAL4>UAS-rpr, UAS-grim* or *UAS-hid* expressing larvae reared on regular or formulation supplemented food

		Mean (±S.E) Pupation Frequency			Mean (±S.E) Eclosion Frequency		
Driver	Responder	Control	AR	RS	Control	AR	RS
GMR-GAL4	*UAS-rpr*	94.0±1.7 (8)	95.0±2.0** (8)	93.5±1.8** (8)	0 (8)	0 (8)	0 (8)
	UAS - (UAS-grim) grim	75.6±2.5 (9)	89.8±2.0* (11)	91.3±2.0* (12)	0 (11)	0 (11)	0 (11)
	UAS-hid	19.1±1.8 (14)	41.1±3.0* (14)	45.7±0.0* (14)	0 (14)	0 (14)	0 (14)
sev-GAL4	*UAS-rpr*	92.0±1.8 (8)	92.5±2.2** (8)	90.0±1.5** (8)	83.5±1.9 (8)	84.5±1.9** (8)	82.5±1.5** (8)
	UAS-grim	91.5±2.6 (8)	93.5±1.6** (8)	92.0±2.5** (8)	87.5±2.1 (8)	89.0±1.5** (8)	88.5±2.1** (8)
	UAS-hid	93.5±1.7 (8)	95.5±2.5** (8)	94.0±1.8** (8)	51.5±2.8 (8)	63.0±1.8* (8)	68.5±1.6* (8)
hs- GAL4	*UAS-rpr*	56.7±2.3 (4)	78.3±2.6* (4)	80.6±2.2* (4)	51.3±3.5 (4)	67.3±2.3* (4)	72.6±2.2* (4)
	UAS - (UAS-grim) grim	54.4±3.5 (4)	70.3±3.2* (4)	75.7±3.4* (4)	44.6±1.3 (4)	64.6±1.9* (4)	70.8±2.6* (4)
	UAS-hid	50.2±2.3 (4)	67.3±1.3* (4)	73.7±2.3* (4)	44.4±3.5 (4)	62.3±2.3* (4)	67.6±3.5* (4)

Numbers in parentheses indicate the total number of replicates of 25 larvae each reared on different feeding regimes (Control = regular food; AR = AR supplemented food; RS = RS supplemented food).

**P>0.05 when compared with the corresponding control.

*P<0.001 when compared with the corresponding control (reared on regular food) set of flies.


sev-GAL4/UAS-rpr
sev-GAL4/UAS-grim
sev-GAL4/UAS-hid
Control
AR
RS
(A)
(B)
(C)
(E)
(F)
(G)
(I)
(J)
(K)
50 µm
Mean (± S.E.) Flourescence Intensity
Control AR RS
(N= 30 in each case)
(D)
(H)
(L)
(M)
(M')
(N)
(N')
(O)
(O')
(P)
(P')
(Q)
(Q')
(R)
(R')
(S)
(S')
(T)
(T')
(U)
(U')

◀**Figure 2.** AR or RS feeding reduced apoptosis in larval eye imaginal discs and improved the eye morphology in *sev-GAL4>UAS-rpr, UAS-grim* or *UAS-hid* transgene expressing flies. **A–C**, **E–G** and **I–K** are Acridine Orange (AO) stained eye imaginal discs from *sev-GAL4>UAS-rpr* (**A–C**), *UAS-grim* (**E–G**) or *UAS-hid* (**I–K**) expressing third instar larvae reared either on regular food (**A**, **E**, **I**) or on AR (**B**, **F**, **J**) or RS (**C**, **G**, **K**) supplemented food. Pairs of white arrows indicate the position of morphogenetic furrow in **A–K**. Scale bar in **A** represents 50 µm and applies to all the images in panel. Histograms in **D, H** and **L** represent the mean (± S.E., N=30 in each case) fluorescence intensities of eye discs from *sev-GAL4>UAS-rpr, UAS-grim* or *UAS-hid* expressing larvae, respectively. * indicates $P<0.001$ when compared with the corresponding control (reared on regular food). **M–O, P–R** and **S–U** are photomicrographs of *sev-GAL4>UAS-rpr* (**M–O**), *sev-GAL4>UAS-grim* (**P–R**) and *sev-GAL4>UAS-hid* (**S–U**) eyes of one day old flies while **M'-O', P'-R'** and **S'-U'** are nail-polish imprints of eyes of corresponding genotypes.

driver causes only a transient but global expression of the responder transgene after heat shock. AO staining of eye discs of these larvae showed that while the incidence of AO-positive cells in eye discs was not enhanced immediately after heat shock, it gradually increased with increasing duration of the recovery from heat shock (data not presented). The observed low frequency of AO positive cells immediately after the heat shock but their increasing abundance during recovery is obviously related to the time required for the heat shock induced synthesis of GAL4 and subsequent synthesis of RHG proteins to trigger cell death. Significantly, rearing on AR or RS supplemented food reduced the incidence of AO-positive cells during recovery in all the three genotypes, proportionate to that in corresponding control samples (data not shown).

Since heat shock results in expression of the RHG proteins in all tissues of the *hs-GAL4>UAS-rpr* or *UAS-grim* or *UAS-hid* late third instar larvae, many of those reared on regular food died before pupation and some more died during the pupal period (Table 1). On the other hand, as the data in table 1 show, significantly higher proportion of the AR or RS fed larvae pupated and subsequently emerged as healthy adults, in spite of the *hs-GAL4* driven global expression of *UAS-rpr* or *UAS-grim* or *UAS-hid* transgene.

3.2 *Dietary supplement of AR or RS reduces level of Grim in GMR-GAL4>UAS-grim expressing larval eye discs*

Since formulation feeding substantially reduced the frequencies of AO positive apoptotic cells following RHG protein expression, we examined levels of Grim, one of the members of RHG family, by immunostaining of *GMR-GAL4>UAS-grim* expressing larval eye discs. It was seen that AR (figure 3B) and RS (figure 3C) feeding detectably reduced the levels of Grim when compared with corresponding controls (figure 3A). Measurement of fluorescence intensities in the eye imaginal discs immunostained for Grim confirmed that the RS feeding resulted in greater reduction in the abundance of Grim (figure 3D). This is in agreement with the above noted greater suppression of the induced apoptosis by RS feeding.

3.3 *AR or RS feeding decreases levels of Dronc and Active-Drice in WT and RHG protein expressing larval eye imaginal discs*

The primary pro-apoptotic function of the RHG proteins is to liberate caspases like Dronc from DIAP inhibition and thus lead to activation of the effecter caspase, Drice (Arya *et al.* 2007; Fuchs and Steller 2011; Denton *et al.* 2013). Accordingly, over-expression of Dronc in *Drosophila* eye discs induces cell death and results in an ablated eye phenotype (Meier *et al.* 2000; Quinn *et al.* 2000). Therefore, we

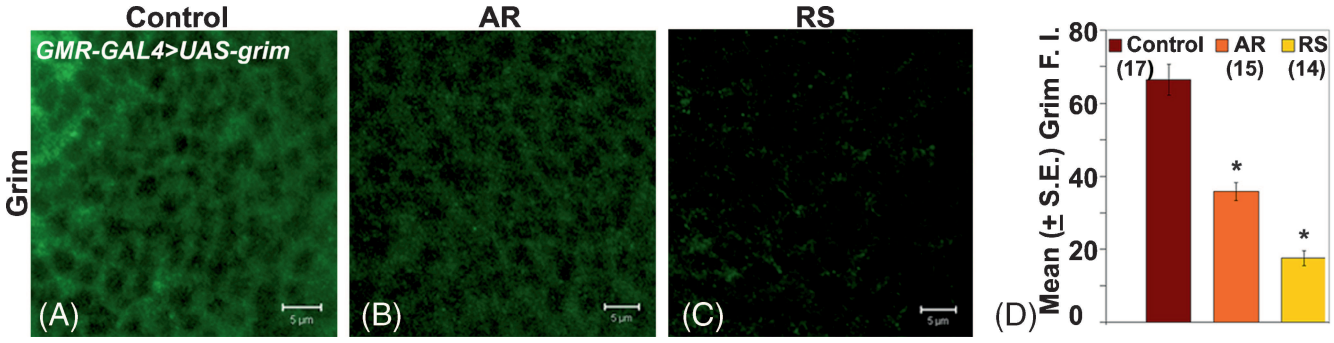


Figure 3. Formulation feeding reduced levels of Grim in *GMR-GAL4>UAS-grim* expressing larval eye imaginal discs. **A–C** are Confocal projections of four consecutive medial optical sections of third instar larval eye imaginal discs of control and formulation (AR or RS) fed larvae showing distribution of Grim (green). Bars in **D** represent the mean (±S.E.) fluorescence intensities of Grim in different feeding regimes; numbers in parentheses after the bar legends indicate the number of eye discs examined for each data point. * indicates $P<0.001$ when compared with the corresponding control (reared on regular food).

examined levels of Dronc and the *Drosophila* effecter caspase Drice in WT as well as in *GMR-GAL4* driven *UAS-rpr* expressing eye discs from larvae reared either on formulation supplemented food or on regular food. Interestingly, AR or RS feeding reduced levels of Dronc (figure 4A–C, E–G) and active Drice (figure 4I–K, M–O) in WT as well as in *GMR-GAL4>UAS-rpr* expressing eye discs as evident from the confocal images and immuno-fluorescence intensities (figure 4D, H, L, P). The peripodial cells in eye discs of regular food reared *GMR-GAL4>UAS-rpr* larvae were abnormal and showed nuclear fragmentation (inset in figure 4E, M). AR or RS formulation feeding substantially rescued the damage to these cells so that they appeared similar to those in wild type (insets in figure 4F, G, N, O).

3.4 *Formulation feeding enhances levels of DIAPs and Bancal in wild type and GMR-GAL4> UAS-rpr, grim and hid expressing larval eye discs*

Since the pro-apoptotic proteins regulate DIAPs, cellular levels of DIAP1 and DIAP2 were examined in wild type as well as *GMR-GAL4>UAS-rpr, grim* and *hid* larval eye imaginal discs. It has been reported earlier from our laboratory that DIAP1 and Hrb57A (Bancal), an hnRNP, show significant colocalization (Mallik and Lakhotia 2009) and that AR or RS feeding enhances levels of various hnRNPs, including Bancal (Dwivedi *et al.* 2012). Therefore, third instar larval eye imaginal discs were co-immunostained with antibodies against DIAP1 and Bancal.

AR and RS feeding significantly enhanced levels of DIAP1 as well as Bancal in eye disc cells of WT (figure 5B, C) and in *GMR-GAL4>UAS-rpr* (figure 5F, G), *>UAS-grim* (figure 5J, K) or *>UAS-hid* expressing discs (figure 5N, O) when compared with their corresponding controls (figure 5A, E, I, M). Quantification of total fluorescence intensities in WT (figure 5D), *GMR-GAL4>UAS-rpr* (figure 5H), *>UAS-grim* (figure 5L) and *>UAS-hid* (figure 5P) larval eye imaginal discs confirmed elevation of levels of both the proteins in all genotypes upon formulation feeding.

DIAP1 was seen to be present in third instar larval eye discs cells as distinct cytoplasmic and nuclear granules, besides in a diffuse manner in the cytoplasm. Bancal too was present in nuclei of the photoreceptor cells in a diffuse and speckled pattern. In addition, distinct cytoplasmic granules of Bancal were present in these cells and, significantly, many of the cytoplasmic Bancal and DIAP1 granules were often adjacent to each other or they overlapped partially or fully (insets in figure 5A–C). A careful examination of the eye discs co-immunostained for DIAP1 and Bancal and analyses of the colocalization coefficients (insets in figure 5D, H, L and P) revealed that elevation in levels and colocalization of these two proteins were inversely proportional to the severity of induced apoptosis following *GMR-GAL4* driven expression of Rpr (figure 5E–H), Grim (figure 5I–L) or Hid (figure 5M–P), so that the Hid expressing discs, which showed highest incidence of apoptosis in larvae reared on regular food (figure 1), displayed least increase in levels of these two proteins and their colocalization following AR or RS supplemented diet (figure 5I–K).

In order to further assess the role of DIAP1 in formulation feeding mediated rescue of the induced apoptosis, the *UAS-DIAP1-RNAi* transgene was expressed under *GMR-GAL4* driver. Two different genotypes were used: in one case only *DIAP1-RNAi* transgene was expressed under the *GMR-GAL4* driver (*GMR-GAL4 UAS-DIAP1-RNAi/CyO;+/+*) while in the other, the *GMR-rpr* transgene was also co-expressed (*GMR-GAL4 UAS-DIAP1-RNAi/CyO; GMR-rpr/TM6B)* to further down-regulate the DIAP1 levels by over-expression of Rpr and the larvae were reared on regular or formulation-supplemented food. As expected, AO staining of larval eye discs revealed high incidence of apoptosis following DIAP1 depletion in *GMR-GAL4 DIAP1-RNAi/CyO* larvae; this was further enhanced in Rpr over-expressing background (*GMR-GAL4 UAS-DIAP1-RNAi/CyO; GMR-rpr/TM6B)*. AR or RS feeding significantly reduced cell death in *GMR-GAL4 DIAP1-RNAi/CyO* as well as in *GMR-GAL4 UAS-DIAP1-RNAi/CyO; GMR-rpr/TM6B* eye discs. Quantification of the AO fluorescence intensities (data not shown) confirmed the reduction in incidence of AO positive dying cells in imaginal discs of formulation fed larvae of either genotype.

Since many of the *GMR-GAL4> UAS-DIAP1-RNAi* and *GMR-GAL4> UAS-DIAP1-RNAi, GMR-rpr* expressing larvae reared on regular food died during pupal stage, a lethality assay was also performed. *GMR-GAL4* driven expression of *UAS-DIAP1-RNAi* caused a substantial reduction in pupation and eclosion. DIAP1 depletion through RNAi in Rpr over-expression background caused even greater lethality (table 2). Interestingly, formulation feeding significantly improved the pupation and eclosion frequencies in both cases (table 2).

Eyes of one day old *GMR-GAL4 UAS-DIAP1-RNAi/CyO* and *GMR-GAL4 UAS-DIAP1-RNAi/CyO; GMR-rpr/TM6B* flies showed that, as expected from the above noted massive apoptosis in larval eye discs, DIAP1 depletion led to substantial disruption of ommatidial units in eyes so that only a few ommatidia were discernible. AR or RS feeding, on the other hand, detectably improved the eye morphology in both the genotypes (not shown).

Examination of levels of DIAP2 protein in wild type, and in *GMR-GAL4* driven RHG expressing eye discs from larvae reared on regular or formulation

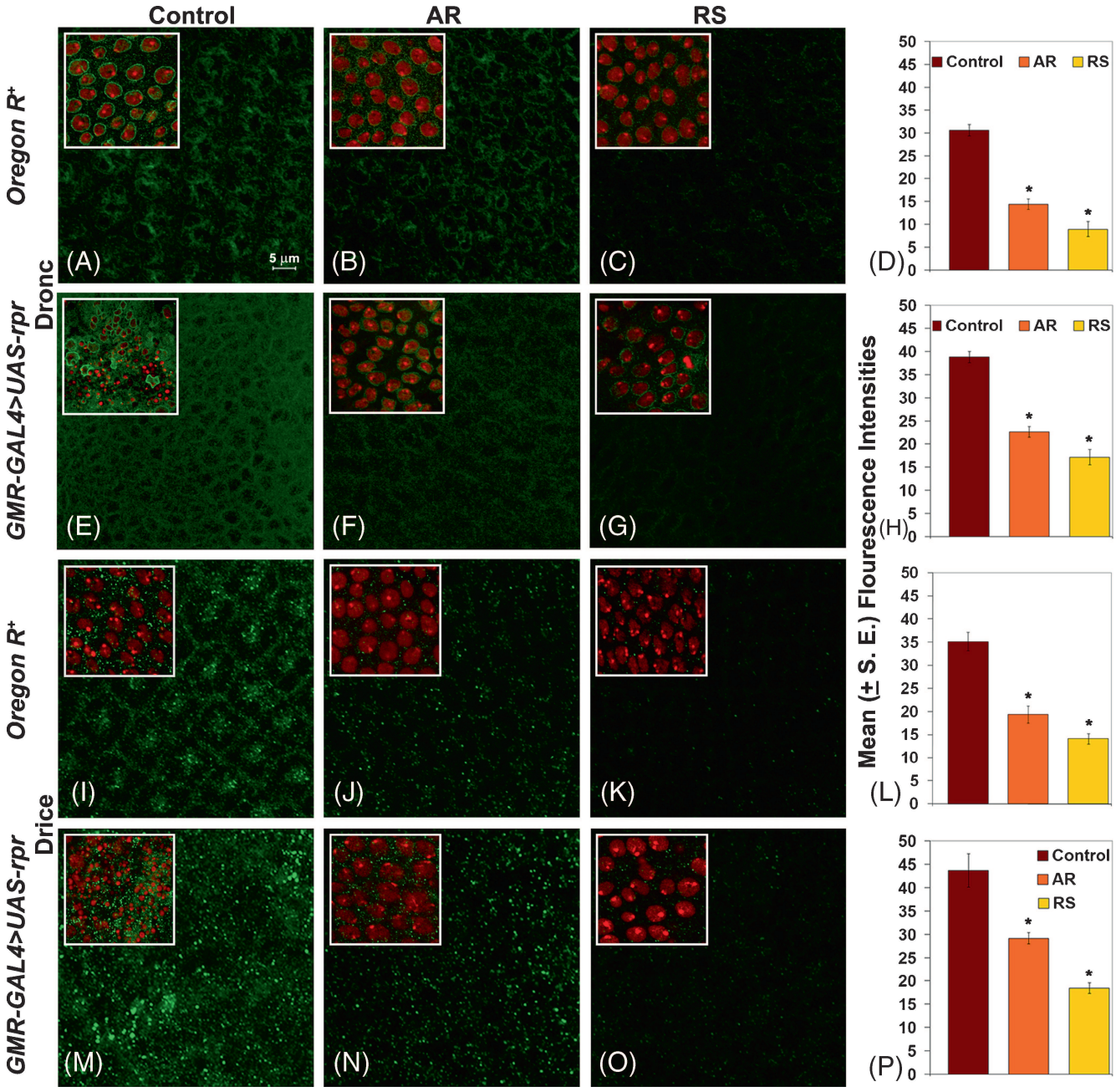


Figure 4. Formulation feeding reduced accumulation of Dronc and Drice in WT and *GMR-GAL4>UAS-rpr* expressing larval eye imaginal disc cells. **A–C** and **E–G** are confocal projections of four medial optical sections from eye discs of WT (**A–C**) and *GMR-GAL4>UAS-rpr* (**E–G**) expressing larval eye imaginal discs immunostained with anti-Dronc (green). **I–K** and **M–O** are confocal projections of WT (**I–K**) and *GMR-GAL4>UAS-rpr* expressing eye discs (**M–O**) immunostained with anti-Drice (green). Scale bar in **A** represents 5µm and applies to images **A–O**. Insets in **A–G** and **I–O** show the peripodial cells of eye discs immunostained for Dronc and Drice (green), respectively, in WT (**A–C, I–J**) and *GMR-GAL4>UAS-rpr* (**E–G, M–O**) expressing larvae; nuclei are counterstained with DAPI (red). Histograms in **D, H, L** and **P** show the mean (±S.E.) fluorescence intensities of Dronc (**D, H**) and Drice (**L, P**). * indicates $P<0.001$ when compared with the corresponding control (reared on regular food).

supplemented food by immunostaining, revealed that dietary supplement of AR or RS enhanced the levels of DIAP2 protein in wild type (figure 6A–C) and in *GMR-GAL4>UAS-rpr* (figure 6E–G), *>UAS-grim*

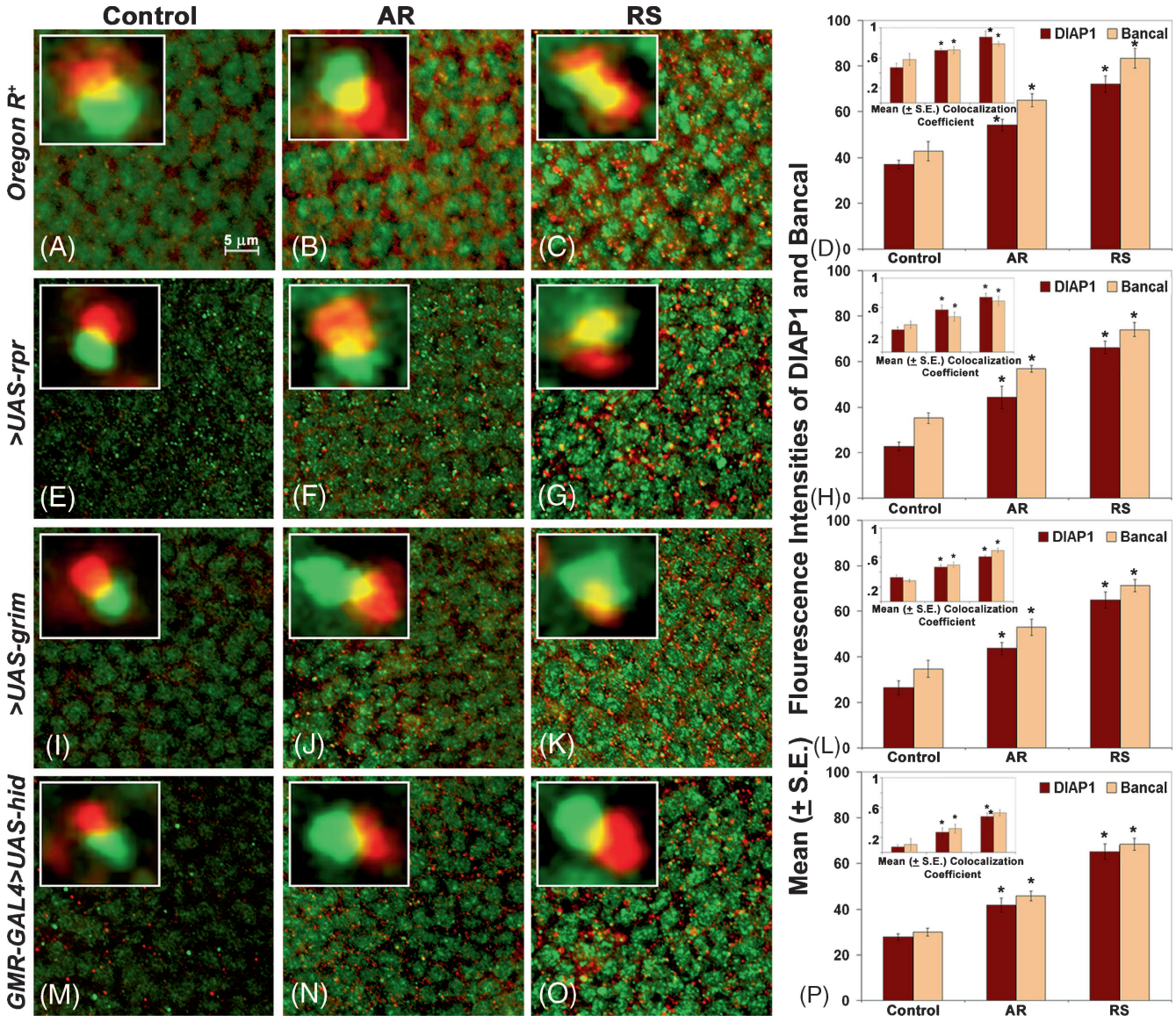


Figure 5. Formulation feeding enhanced the levels of DIAP1 and Bancal and enhanced their colocalization in wild type and *GMR-GAL4>UAS-rpr, UAS-grim* or *UAS-hid* expressing larval eye imaginal discs. **A–C, E–G, I–K** and **M–O** are confocal projections of four medial optical sections of late third instar larval eye discs of WT (**A–C**), *GMR-GAL4>UAS-rpr* (**E–G**), *GMR-GAL4>UAS-grim* (**I–K**) and *GMR-GAL4>UAS-hid* (**M–O**) immunostained for DIAP1 (red) and Bancal (green). Scale bar in **A** corresponds to 5µm and applies to all the other images (excluding the insets, which are at a higher magnification). Images in insets show association and colocalization (yellow) of DIAP1 (red) and Bancal (green) in wild type (**A–C**), *GMR-GAL4>UAS-rpr* (**E–G**), *>UAS-grim* (**I–K**) and *>UAS-hid* (**M–O**) expressing larval eye discs. Histograms in **D, H, L** and **P** represent the mean (± S.E.) fluorescence intensities of DIAP1 and Bancal in different genotypes (noted on left of the corresponding row). Histograms in insets in **D, H, L** and **P** represent mean ± S.E. colocalization coefficients of Bancal and DIAP1 in wild type (**D**), *UAS-rpr* (**H**), *UAS-grim* (**L**) and *UAS-hid* (**P**) expressing larval eye imaginal discs. * indicates $P<0.001$ when compared with the corresponding control (reared on regular food).

(figure 6I–K) or *>UAS-hid* (figure 6M–O) expressing larval eye discs. Remarkably, the RS feeding resulted in greater increase in levels of DIAP2 transcripts and protein, as evident from quantification of fluorescence intensities (figure 6D, H, L, P).

3.5 *Formulation feeding does not affect developmental apoptosis*

It is known from our earlier studies (Dwivedi *et al.* 2012) that AR or RS supplemented food does not have any adverse

Table 2. Formulation feeding enhanced viability of *GMR-GAL4::UAS-DIAP1 RNAi* larvae

	Mean Pupation ± S.E			Mean Eclosion ± S.E		
	Control	AR	RS	Control	AR	RS
GMR-GAL4::UAS-DIAP1 RNAi/CyO; +/+	76.8±2.1 (8)	84.5±2.4* (8)	89.2±2.2* (8)	65.4±2.9 (8)	77.8±2.5* (8)	81.4±3.1* (8)
GMR-GAL4::UAS-DIAP1 RNAi/CyO; GMR-rpr/TM6B	60.0±2.3 (8)	73.5±2.6* (8)	75.0±3.0* (8)	25.5±2.3 (8)	45.0±3.1* (8)	51.5±2.6* (8)

Numbers in parentheses indicate the total number of replicates of 25 larvae each reared on different feeding regimes (Control = regular food; AR = AR supplemented food; RS = RS supplemented food).

*$P<0.001$ when compared with the corresponding control (reared on regular food) set of flies.

effect on normal development and thus the developmental apoptosis is expected to occur as scheduled. To further confirm the occurrence of scheduled developmental apoptosis, we selected larval salivary glands which undergo programmed cell death soon after pupal metamorphosis utilizing apoptotic as well as autophagic pathways (Farkas and Mechler 2000; Yin and Thummel 2006). Morphology of salivary glands in control and AR or RS supplemented food reared larvae was examined in late 3rd instar larvae and in 8, 12 and 14 h old pupae. As seen in figure 7A–L, the typically expected histolytic changes in salivary glands occurred during pupal stages of larvae that were reared on AR or RS supplemented food. Morphological examination of other tissues in pupae also revealed the expected developmental changes irrespective of the larval feeding regime (not shown).

We examined expression of Dronc and Drice, the initiator and effecter caspases, respectively, in early pupal salivary glands. It has been reported that during normal development, Dronc expression in salivary glands is elevated during late 3rd instar larval stage (Dorstyn *et al.* 1999). We immunostained 8–9 h old pupal salivary glands when their developmental cell death programme is being initiated (Daish *et al.* 2004; Berry and Baehrecke 2007). Comparison of Dronc or Drice immunofluorescence in salivary glands (figure 7M–R) and other tissues (not shown) from 8–9 h old pupae revealed comparable immunostaining irrespective of the larval feeding regime. Unlike in non-apoptotic cells (see above), the levels of Dronc and Drice were not reduced in salivary glands of pupae which were reared during the larval period on AR or RS supplemented food (compare figure 7N, O, Q and R with 7M and O). Thus, while the AR or RS supplement, greatly suppress RHG induced apoptosis, the normal developmental apoptosis is not affected.

4. Discussion

Current Ayurvedic practices mostly rely on the observed effects of a given formulation without a clear mechanistic understanding of the underlying events. As has been widely emphasized in recent years (Valiathan 2006; Lakhotia 2013; Patwardhan 2014), it is necessary to gain deeper insights into the underlying cellular and molecular processes that culminate in the observed beneficial effects. Keeping this in view, the present study was directed to understand the basis of our earlier observed (Dwivedi *et al.* 2013) reduced death of the affected neuronal cells in fly models of neurodegeneration by dietary AR or RS.

Present studies clearly show that dietary supplement of AR or RS significantly suppressed apoptosis induced by *GMR-GAL4-* or *sev-GAL4*-directed expression of the pro-apoptotic RHG proteins in eye discs and in all body cells when these proteins were expressed globally using the *hs-GAL4* driver. Our results further show that the two Ayurvedic formulations act at multiple levels to inhibit induced apoptosis since they not only reduced the levels of pro-apoptotic proteins (exemplified by Grim) but also elevated the levels of anti-apoptotic proteins like DIAP1 and DIAP2 and reduced the effecter and active caspases, Dronc and Drice, respectively. In agreement with our earlier findings that RS is more effective than AR in most of its beneficial effects (Dwivedi *et al.* 2012, 2013), the present study also revealed RS to be more effective in suppressing the induced apoptosis as well. Traditional Ayurvedic literature indeed considers *Rasa-Sindoor* as the most potent Rasayana and is designated as '*Maharasa*' (Sharma 1979; Sitaram 2006).

A few earlier studies have examined effects of some Ayurvedic and other herbal formulations on apoptosis. For example, a herbo-mineral Ayurvedic formulation, *Manasamitra vatakam*, was found to inhibit neuronal apoptosis and improve cognitive functions against aluminium-induced toxicity in animal models (Thirunavukkarasu *et al.* 2013). On the other hand, Bullatacin, an acetogenin isolated from fruits of *Annona atemoya* (Chih *et al.* 2001), Gugulipid extract of *Commiphora mukul* (Xiao *et al.* 2011) and phenolic extracts of *Emblica officinalis* (Rajeshkumar *et al.* 2003) have been reported to induce apoptosis in Dalton's Lymphoma Ascites, CeHa cell lines and mouse,

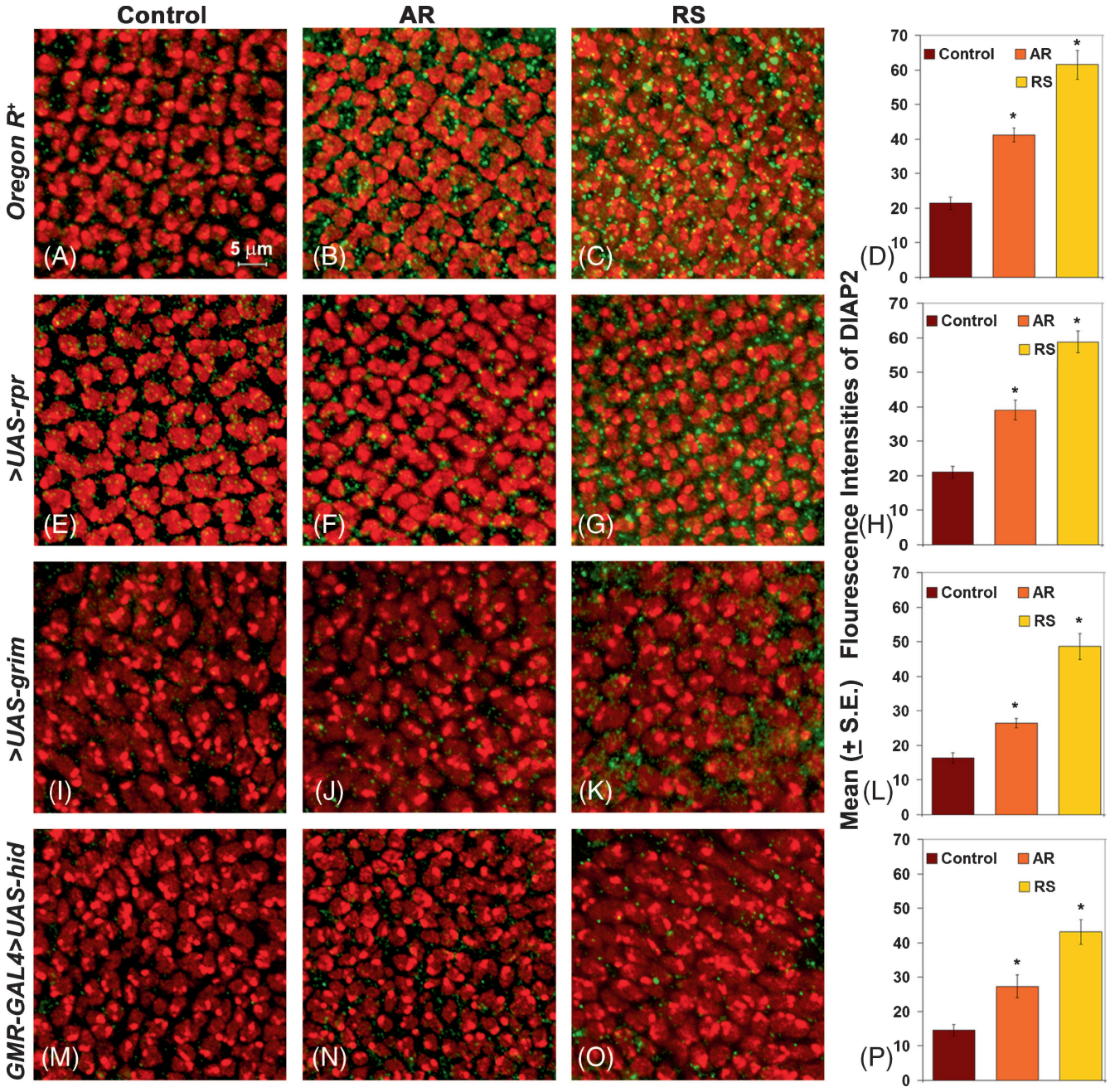


Figure 6. AR or RS feeding elevated cellular levels of DIAP2 in wild type and *GMR-GAL4>UAS-rpr, GMR-GAL4>UAS-grim* and *GMR-GAL4>UAS-hid* larval eye discs. **A–C, E–G, I–K** and **M–O** are confocal projections of four medial optical sections of eye discs of WT (**A–C**), *GMR-GAL4>UAS-rpr* (**E–G**), *>UAS-grim* (**I–K**) and *>UAS-hid* (**M–O**) late third instar larvae immunostained with anti-DIAP2 (green) and counterstained with DAPI (red). Scale bar in **A** corresponds to 5µm and applies to all other images. Histograms in **D, H, L** and **P** show the fluorescence intensities of DIAP2 in eye discs from larvae reared on regular or formulation feeding. * indicates $P<0.001$ when compared with the corresponding control (reared on regular food).

respectively. These studies, however, did not examine the cellular and/or molecular basis of the observed effects on cell death.

Expression of the pro-apoptotic RHG proteins triggers the apoptotic cascade by removing inhibitory actions of DIAP1 and DIAP2 so that effecter caspases can convert pro-

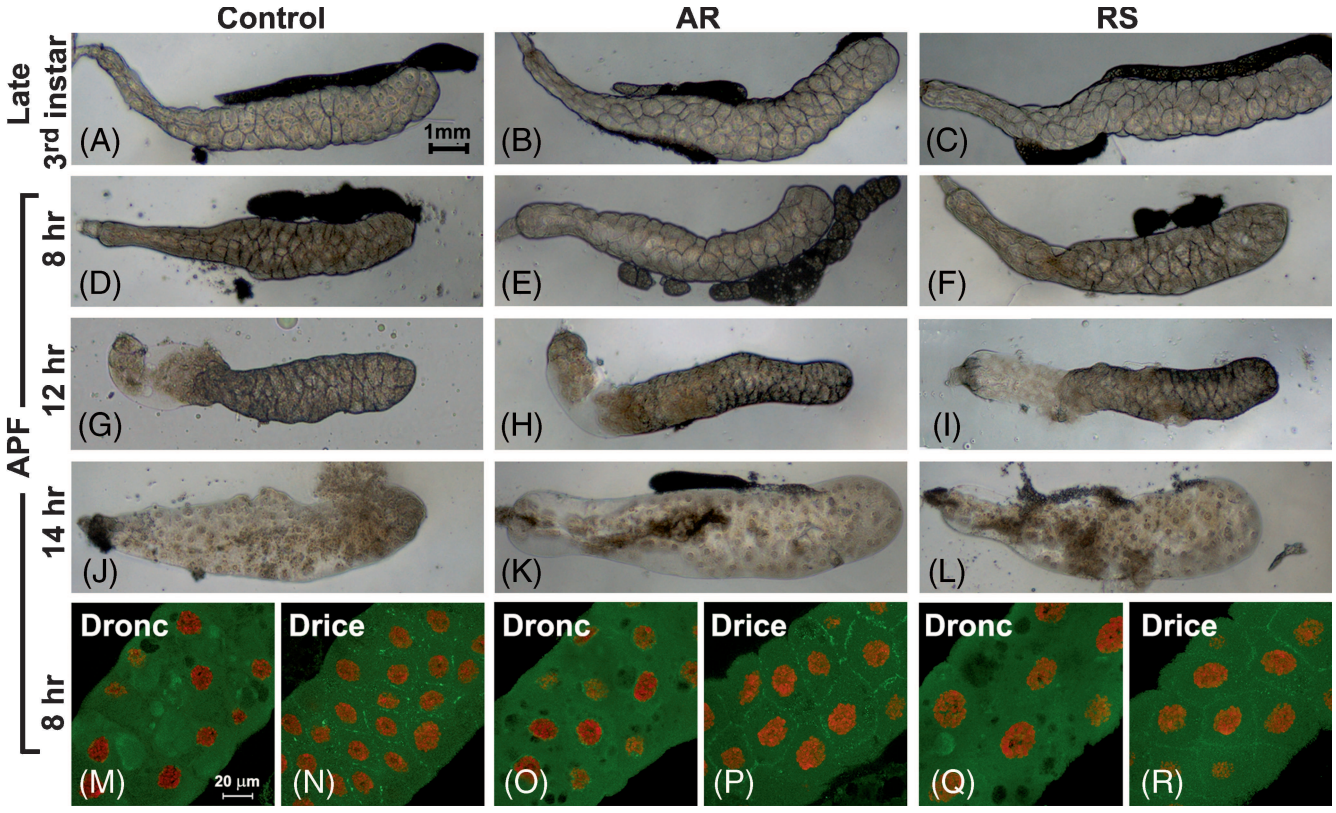


Figure 7. Unlike induced apoptosis, developmental histolysis of larval salivary glands is not affected by dietary AR or RS. **A–L** are phase-contrast images of salivary glands from late 3rd instar larvae (**A–C**), 8 h (**D–F**), 12 h (**G–I**) and 14 h (**J–L**) old pupae that were reared on control (**A**, **D**, **J**) or AR (**B**, **E**, **K**) or RS (**C**, **F**, **L**) supplemented food; note comparable histolytic changes in pupal salivary glands, irrespective of the larval feeding regime (noted on top of each column). **M–R** are confocal images of salivary glands from 8 h old pupae, reared during larval period on control (**M**, **N**) or AR (**O, P**) or RS (**Q**, **R**) supplemented food, immunostained for Dronc (**M, O, Q**) or Drice (**N, P, R**); each image is a projection of 4 medial optical sections. Note the similar pattern and immunofluorescnce intensity for Dronc as well as Drice in each of the three samples. The scale bar in **A** applies to **A** through **L** while that in **M** applies to **M** through **R**.

caspases into active caspases (Ryoo *et al.* 2002; Steller 2008; Fuchs and Steller 2011; Denton *et al.* 2013). Besides inhibiting the binding of DIAP1 with initiator and effecter caspases, the RHG proteins also greatly decrease DIAP1 abundance through enhanced ubiquitin-mediated degradation. Further, Rpr and Grim also suppress global protein translation, so that levels of free Dronc increase since Dronc has a longer half life than DIAP1 (Holley *et al.* 2002; Yoo *et al.* 2002). Present results showed that formulation feeding substantially reduced the abundance of ectopically induced Grim protein in larval eye discs of *GMR-GAL4>UAS-grim*. Although the levels of Rpr or Hid were not directly examined, it appears that levels of Rpr and Hid too get similarly reduced in formulation fed larval tissues since the three genes are often similarly affected in response to different signalling pathways that toggle the cell between survival and death (Fuchs and Steller 2011). It may be noted that over-expression of Hid caused maximum damage and the AR- or RS-mediated suppression of apoptosis was correspondingly less. This may be related to the fact that Hid's pro-apoptotic role in eye discs may involve a divergent path as well (Hays *et al.* 2002; Mallik and Lakhotia 2009). In view of the pivotal roles of RHG proteins in triggering apoptosis in *Drosophila* (Arya *et al.* 2007; Fuchs and Steller 2011; Denton *et al.* 2013), reduced induction of RHG proteins is likely to be one of the factors contributing to suppression of the induced apoptosis by AR or RS. It remains to be examined if the changes in levels of the different proteins noted in this study are due to changes at transcriptional level or altered stability/synthesis of the given protein.

DIAP1 is the most crucial inhibitor of apoptosis in *Drosophila* (Goyal *et al.* 2000; Hawkins *et al.* 2000; Muro *et al.* 2002) since it keeps initiator as well as effecter caspases, Dronc and Drice, respectively, inhibited. DIAP1 prevents accumulation of the auto-processed active form of Dronc in cell cytoplasm (Muro *et al.* 2002) and carries out non-proteolytic ubiquitination of Dronc and Drice to keep

them inactive (Ryoo *et al.* 2002; Ditzel *et al.* 2008; Fuchs and Steller 2011; Denton *et al.* 2013). DIAP1 also inhibits interaction of Dronc and DARK and thus prevents formation of apoptosome complex (Dorstyn *et al.* 2002; Igaki *et al.* 2002). DIAP1 has further been shown to inhibit apoptosis by activating the ligand-independent hedgehog signalling in *Drosophila* (Christiansen *et al.* 2013). The significantly enhanced levels of DIAP1 in wild type as well as *rpr, grim* or *hid* transgene expressing larval eye imaginal discs following AR or RS dietary supplement seem to be the other major factor contributing to the observed suppression in induced apoptosis.

DIAP2 does not appear to be a crucial inhibitor of apoptosis, although it can functionally substitute DIAP1 in inhibiting developmental or induced apoptosis (Vucic *et al.* 1997; Leulier *et al*. 2006) as it also prevents activation of Drice (Ribeiro *et al.* 2007). Therefore, the remarkably enhanced levels of DIAP2 following formulation feeding also appear to contribute to suppression of the RHG mediated apoptosis.

In agreement with the earlier report (Dwivedi *et al.* 2012), the present results showed an increase in levels of Bancal (Hrb57A) in wild type as well as in *rpr, grim* or *hid* over-expressing larval eye imaginal discs. More significant is the finding that the cytoplasmic granules of Bancal showed varying degrees of colocalization with DIAP1, which, while directly proportional to the elevation in levels of the respective proteins, was inversely related to the extent of induced apoptosis. A comparable greater association of DIAP1 and Bancal was also seen in other conditions of reduced apoptosis (Mallik and Lakhotia 2009). Enhanced levels of Bancal and other hnRNPs (Dwivedi *et al.* 2012; 2013) also thus contribute to suppression of induced apoptosis through stabilization of DIAP1 in the formulation fed larval tissues.

An earlier study from our laboratory (Mallik and Lakhotia 2009) showed that down-regulation of the non-coding hsrω transcripts inhibits induced apoptosis, with one of the contributing factors being stabilization of DIAP1 by Bancal. This raised the possibility that formulation feeding may down-regulate hsrω transcripts, which results in increased binding of Bancal with DIAP1. However, we found (data not presented) that the levels of hsrω transcripts are not altered by the formulation feeding, neither in wild type nor in RHG over-expressing genotypes.

Down-regulation of JNK signalling is reported to suppress induced apoptosis in *Drosophila* (Yang *et al.* 1997; Mallik and Lakhotia 2009). Rpr induced degradation of DIAP1 stabilizes DTRAF1 (Drosophila tumour-necrosis factor receptor-associated factor 1) and activates the JNK-mediated cell death (Holley *et al.* 2002). It would be interesting in future studies to see if the JNK signalling is also modulated by these Ayurvedic formulations.

Members of the cell death machinery have roles, other than in developmental or induced apoptosis, in several non-apoptotic events like the border cell migration during oogenesis, sperm individualization, shaping of aristae, dendrite pruning, development of sensory organ precursor, etc. (Kuranaga and Miura 2007; Koto *et al.* 2009; Fabian and Brill 2012). In view of such multiple and significant roles, the enhanced levels of DIAPs and reduced levels of pro-apoptotic proteins and the caspases in formulation-fed wild type larvae may be expected to adversely affect development. However, our results clearly show that while induced apoptosis is largely, although not completely, suppressed, the developmental apoptosis is not at all affected by these formulations since the characteristic histolysis of larval salivary glands and other tissues progressed as in regular-food-reared organisms. Further, strong support for the developmental apoptosis remaining unaffected by AR or RS is provided by the fact that none of the AR- or RS- fed wild type larvae suffer any developmental defects (Dwivedi *et al.* 2012), which inevitably would have occured if developmental apoptosis was to be compromised in any way (Abrams *et al.* 1993; Fuchs and Steller 2011). The flies reared since larval period on AR- or RS-supplemented food were actually better in many biological parameters like life span, development time, fecundity, stress tolerance, etc. (Dwivedi *et al.* 2012, 2013). Increase in levels of histone acetyl transferase like CBP300, different hnRNPs and stress tolerance that follows these rejuvenating dietary supplements appear to positively modulate multiple cellular pathways and thus buffer the cells so that while non-apoptotic cells have reduced levels, those destined to die during development have the required levels of apoptotic machinery components.

It is interesting that the two Ayurvedic formulations used in this study have comparable effects in spite of their very different constituents. The major constituent of AR is obtained from extracts of fruits of 'Amla' or *Emblica officinalis*, while RS is a sublimate product of mercury and sulphur (Dwivedi *et al.* 2012). We are currently examining global transcriptome changes effected by each of the two dietary supplements to understand the basis of their comparable effects on cell physiology. Present studies further emphasize that, contrary to the common perception of heavy metal toxicity of Ayurvedic formulations like RS, a properly processed RS, whose high mercury content is nearly in the nano-particle range (Singh *et al.* 2009), is not at all toxic. It is actually beneficial for the fly's life (Dwivedi *et al.* 2012, 2013), as also claimed for human applications (Sharma 1979). Heavy metals like mercury and arsenic are also widely used in the traditional Chinese medical systems as well (Zhang *et al.* 2012).

Unlike the stringently regulated developmental apoptosis, cell death induced in response to unfavourable external or internal signals may often be an 'over-reaction'. The different components of cell stress response delicately balance the survival and death pathways (Arya *et al.* 2007). Therefore, even small disturbances can sometimes tilt the balance so that the death response gets triggered, especially in cells with compromised homeostasis. DNA damage and cell stress, which strongly affect apoptotic signalling (Roos and Kaina 2006; Arya *et al.* 2007; Moon *et al.* 2008; Fuchs and Steller 2011; Denton *et al.* 2013), are omnipresent. Therefore, average cells remain prone to death signals. AR has been shown to suppress age-related DNA damage (Swain *et al.* 2011) and to enhance tolerance to oxidative stress (Dwivedi *et al.* 2012), while AR and RS both confer better tolerance to thermal and starvation stresses (Dwivedi *et al.* 2012). Such findings suggest that, as claimed in traditional literature (Sharma 1979; Singh 2009), AR and RS formulations help in better maintenance of cellular homeostasis. Elevated levels of DIAPs and reduced levels of RHG proteins and caspases, as seen in normally developing non-apoptotic cells of wild type larvae reared on AR or RS supplemented food, apparently reflect a more robust physiological state of these cells so that they can tolerate greater insults without triggering the death response.

Our present findings have clinical implications since the more robust physiological state following AR or RS supplements may prevent unwarranted cell death associated with several diseases and ageing (Higami and Shimokawa 2000; Muradian and Schachtschabel 2001; Lu *et al.* 2012) and thus contribute to 'healthy ageing', one of their suggested effects in traditional Ayurvedic practices (Sharma 1979; Singh 2009).

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References

Abrams JM, White K, Fessler LI and Steller H 1993 Programmed cell death during *Drosophila* embryogenesis. *Development* **117** 29–43

Aplin AC and Kaufman TC 1997 Homeotic transformation of legs to mouthparts by proboscipedia expression in *Drosophila* imaginal discs. *Mech. Dev.* **62** 51–60

Arya R and Lakhotia S 2008 Hsp60D is essential for caspase-mediated induced apoptosis in *Drosophila melanogaster*. *Cell Stress Chaperones* **13** 509–526

Arya R and Lakhotia SC 2006 A simple nail polish imprint technique for examination of external morphology of *Drosophila* eyes. *Curr. Sci.* **90** 1179–1180

Arya R, Mallik M and Lakhotia SC 2007 Heat shock genes - integrating cell survival and death. *J. Biosci.* **32** 595–610

Baehrecke EH 2002 How death shapes life during development. *Nat. Rev. Mol. Cell Biol.* **3** 779–787

Berry DL and Baehrecke EH 2007 Growth arrest and autophagy are required for salivary gland cell degradation in *Drosophila*. *Cell* **131** 1137–1148

Cakouros D, Daish TJ and Kumar S 2004 Ecdysone receptor directly binds the promoter of the *Drosophila* caspase dronc, regulating its expression in specific tissues. *J. Cell Biol.* **165** 631–640

Chih HW, Chiu HF, Tang KS, Chang FR and Wu YC 2001 Bullatacin, a potent antitumor annonaceous acetogenin, inhibits proliferation of human hepatocarcinoma cell line 2.2.15 by apoptosis induction. *Life Sci.* **69** 1321–1331

Christiansen AE, Ding T, Fan Y, Graves HK, Herz HM, Lindblad JL and Bergmann A 2013 Non-cell autonomous control of apoptosis by ligand-independent Hedgehog signaling in *Drosophila*. *Cell Death Differ.* **20** 302–311

Claveria C, Caminero E, Martinez AC, Campuzano S and Torres M 2002 GH3, a novel proapoptotic domain in *Drosophila* Grim, promotes a mitochondrial death pathway. *EMBO J.* **21** 3327–3336

Daish TJ, Mills K and Kumar S 2004 *Drosophila* caspase DRONC is required for specific developmental cell death pathways and stress-induced apoptosis. *Dev. Cell* **7** 909–915

Denton D, Aung-Htut MT and Kumar S 2013 Developmentally programmed cell death in *Drosophila*. *Biochim. Biophys. Acta* **1833** 3499–3506

Ditzel M, Broemer M, Tenev T, Bolduc C, Lee TV, Rigbolt KT, Elliott R, Zvelebil M, *et al*. 2008 Inactivation of effecter caspases through nondegradative polyubiquitylation. *Mol. Cell* **32** 540–553

Dorstyn L, Colussi PA, Quinn LM, Richardson H and Kumar S 1999 DRONC, an ecdysone-inducible *Drosophila* caspase. *Proc. Natl. Acad. Sci. U S A* **96** 4307–4312

Dorstyn L, Read S, Cakouros D, Huh JR, Hay BA and Kumar S 2002 The role of cytochrome c in caspase activation in *Drosophila melanogaster* cells. *J. Cell Biol.* **156** 1089–1098

Doumanis J, Quinn L, Richardson H and Kumar S 2001 STRICA, a novel *Drosophila melanogaster* caspase with an unusual serine/threonine-rich prodomain, interacts with DIAP1 and DIAP2. *Cell Death Differ.* **8** 387–394

Dwivedi V, Anandan EM, Mony RS, Muraleedharan TS, Valiathan MS, Mutsuddi M and Lakhotia SC 2012 In vivo effects of

traditional ayurvedic formulations in *Drosophila melanogaster* model relate with therapeutic applications. *PLoS ONE* **7** e37113

Dwivedi V, Tripathi BK, Mutsuddi M and Lakhotia SC 2013 Ayurvedic Amalaki Rasayana and Rasa-Sindoor suppress neurodegeneration in fly models of Huntington's and Alzheimer's diseases. *Curr. Sci.* **105** 1711–1723

Edinger AL and Thompson CB 2004 Death by design: apoptosis, necrosis and autophagy. *Curr. Opin. Cell Biol.* **16** 663–669

Fabian L and Brill JA 2012 *Drosophila* spermiogenesis: big things come from little packages. *Spermatogenesis* **2** 197–212

Fan Y and Bergmann A 2010 The cleaved-caspase-3 antibody is a marker of caspase-9-like DRONC activity in *Drosophila*. *Cell Death Differ.* **17** 534–539

Farkas R and Mechler BM 2000 The timing of *Drosophila* salivary gland apoptosis displays an l(2)gl-dose response. *Cell Death Differ.* **7** 89–101

Freeman M 1996 Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. *Cell* **87** 651–660

Fuchs Y and Steller H 2011 Programmed cell death in animal development and disease. *Cell* **147** 742–758

Goyal L, McCall K, Agapite J, Hartwieg E and Steller H 2000 Induction of apoptosis by *Drosophila* reaper, hid and grim through inhibition of IAP function. *EMBO J.* **19** 589–597

Harvey NL, Daish T, Mills K, Dorstyn L, Quinn LM, Read SH, Richardson H and Kumar S 2001 Characterization of the *Drosophila* caspase, DAMM. *J. Biol. Chem.* **276** 25342–25350

Hawkins CJ, Yoo SJ, Peterson EP, Wang SL, Vernooy SY and Hay BA 2000 The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. *J. Biol. Chem.* **275** 27084–27093

Hay BA and Guo M 2006 Caspase-dependent cell death in *Drosophila. Annu. Rev. Cell Dev. Biol.* **22** 623–650

Hays R, Wickline L and Cagan R 2002 Morgue mediates apoptosis in the *Drosophila melanogaster* retina by promoting degradation of DIAP1. *Nat. Cell Biol*. **4** 425–431

Holley CL, Olson MR, Colon-Ramos DA and Kornbluth S 2002 Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. *Nat. Cell Biol.* **4** 439–444

Higami Y and Shimokawa I 2000 Apoptosis in the aging process. *Cell Tissue Res.* **1** 125–132

Hongmei, Z. (2012). Extrinsic and intrinsic apoptosis signal pathway review. Apoptosis and Medicine, InTech *http://dx.doi.org/10.5772/50129*.

Igaki T, Yamamoto-Goto Y, Tokushige N, Kanda H and Miura M 2002 Down-regulation of DIAP1 triggers a novel *Drosophila* cell death pathway mediated by dark and DRONC. *J. Biol. Chem.* **277** 23103–23106

Kornbluth S and White K 2005 Apoptosis in *Drosophila*: neither fish nor fowl (nor man, nor worm). *J. Cell Sci.* **118** 1779–1787

Koto A, Kuranaga E and Miura M 2009 Temporal regulation of *Drosophila* IAP1 determines caspase functions in sensory organ development. *J. Cell Biol.* **187** 219–231

Kumar S and Doumanis J 2000 The fly caspases. *Cell Death Differ.* **7** 1039–1044

Kuranaga E and Miura M 2007 Nonapoptotic functions of caspases: caspases as regulatory molecules for immunity and cell-fate determination. *Trends Cell Biol.* **17** 135–144

Lakhotia SC 2013 In-depth basic science studies essential for revival of Ayurveda. *AAM* **2** 58–60

Leulier F, Ribeiro PS, Palmer E, Tenev T, Takahashi K, Robertson D, Zachariou A, Pichaud F, *et al*. 2006 Systematic in vivo RNAi analysis of putative components of the *Drosophila* cell death machinery. *Cell Death Differ.* **13** 1663–1674

Lisi S, Mazzon I and White K 2000 Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in *Drosophila. Genetics* **154** 669–678

Lu B, Chen H-D and Lu H-G 2012 The relationship between apoptosis and aging. *Adv. Biosci. Biotechnol.* **3** 705–711

Mallik M and Lakhotia SC 2009 The developmentally active and stress-inducible noncoding hsromega gene is a novel regulator of apoptosis in *Drosophila*. *Genetics* **183** 831–852

Meier P, Silke J, Leevers SJ and Evan GI 2000 The *Drosophila* caspase DRONC is regulated by DIAP1. *EMBO J.* **19** 598–611

Moon N-S, Di Stefano L, Morris EJ, Patel R, White K, Dyson NJ and Perrimon N 2008 E2F and p53 Induce apoptosis independently during *Drosophila* development but intersect in the context of DNA damage. *PLoS Genet.* **4** e1000153

Muradian K and Schachtschabel DO 2001 The role of apoptosis in aging and age-related disease: update. *Z. Gerontol. Geriatr.* **6** 441–446

Muro I, Hay BA and Clem RJ 2002 The *Drosophila* DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. *J. Biol. Chem.* **277** 49644–49650

Murthy KRS 2000 Ashtanga Hridaya (Sanskrit with English Translation) (Varanasi: Krishnadas Academy)

Oshima K, Takeda M, Kuranaga E, Ueda R, Aigaki T, Miura M and Hayashi S 2006 IKK epsilon regulates F actin assembly and interacts with *Drosophila* IAP1 in cellular morphogenesis. *Curr. Biol.* **16** 1531–1537

Ouyang L, Shi Z, Zhao S, Wang FT, Zhou TT, Liu B and Bao JK 2012 Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Prolif.* **45** 487–498

Patwardhan B 2014 Ayurveda and systems biology. *AAM.* **3** 5–7

Prasanth KV, Rajendra TK, Lal AK and Lakhotia SC 2000 Omega speckles - a novel class of nuclear speckles containing hnRNPs associated with noncoding hsr-omega RNA in *Drosophila*. *J. Cell Sci.* **113** 3485–3497

Quinn LM, Dorstyn L, Mills K, Colussi PA, Chen P, Coombe M, Abrams J, Kumar S, *et al*. 2000 An essential role for the caspase dronc in developmentally programmed cell death in *Drosophila*. *J. Biol. Chem.* **275** 40416–40424

Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y and Jacobson MD 1993 Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* **262** 695–700

Rajeshkumar NV, Pillai MR and Kuttan R 2003 Induction of apoptosis in mouse and human carcinoma cell lines by Emblica officinalis polyphenols and its effect on chemical carcinogenesis. *J. Exp. Clin. Cancer Res.* **22** 201–212

Ribeiro PS, Kuranaga E, Tenev T, Leulier F, Miura M and Meier P 2007 DIAP2 functions as a mechanism-based regulator of drICE that contributes to the caspase activity threshold in living cells. *J. Cell Biol.* **179** 1467–1480

Roos WP and Kaina B 2006 DNA damage-induced cell death by apoptosis. *Trends Mol. Med.* **12** 440–450

Ryoo HD, Bergmann A, Gonen H, Ciechanover A and Steller H 2002 Regulation of *Drosophila* IAP1 degradation and apoptosis by reaper and ubcD1. *Nat. Cell Biol.* **4** 432–438

Saumweber H, Symmons P, Kabisch R, Will H and Bonhoeffer F 1980 Monoclonal antibodies against chromosomal proteins of *Drosophila melanogaster*: establishment of antibody producing cell lines and partial characterization of corresponding antigens. *Chromosoma* **80** 253–275

Sharma PV 1994 *Charaka Samhita* (Sanskrit with English Translation) (Chaukhambha Orientalia: Varanasi)

Sharma S 1979 *Rastarangini* (Sanskrit with Hindi translation) (Varanasi: Motilal Benarasidas)

Singh RH 2009 *Body, mind, spirit- Integrative medicine in Ayurveda Nature and Yoga* (Varanasi, India: Chaukhamba Sanskrit Pratishthan)

Singh SK, Chaudhary A, Rai D and Rai S 2009 Preparation and characterization of a mercury based Indian traditional drug Ras-Sindoor. *Indian J. Tradit. Knowl.* **8** 346–351

Sitaram B 2006 *Bhavprakash of Bhavmisra* (Sanskrit with English Translation and Notes) (Varanasi: Chaukhamba Orientalia)

Spreij TE 1971 Cell death during the development of the imaginal discs of *Calliphora erythrocephala. Netherlands J. Zool.* **21** 221–264

Steller H 2008 Regulation of apoptosis in *Drosophila*. *Cell Death Differ.* **15** 1132–1138

Swain U, Sindhu KK, Boda U, Pothani S, Giridharan NV, Raghunath M and Rao KS 2011 Studies on the molecular correlates of genomic stability in rat brain cells following Amalakirasayana therapy. *Mech. Ageing Dev.* **133** 112–117

Tapadia M and Lakhotia SC 1997 Specific induction of the hsr omega locus of *Drosophila melanogaster* by amides. *Chromosom. Res.* **5** 359–362

Thirunavukkarasu SV, Jayanthi M, Raja S and Venkataraman S 2013 Effect of Manasamitra Vatakam against aluminium induced learning and memory impairment of apoptosis in rat's hippocampus and cortex. *J. Drug Metab. Toxicol.* **4** doi:10.4172/2157-7609.1000154

Valiathan S 2006 Ayurveda – putting the house in order. *Curr. Sci.* **90** 5–6

Vucic D, Kaiser WJ, Harvey AJ and Miller LK 1997 Inhibition of Reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). *Proc. Natl. Acad. Sci. U S A* **94** 10183–10188

Wing JP, Zhou L, Schwartz LM and Nambu JR 1998 Distinct cell killing properties of the *Drosophila* reaper, head involution defective, and grim genes. *Cell Death Differ.* **5** 930–939

Wyllie AH, Kerr JF and Currie AR 1980 Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68** 251–306

Xiao D, Zeng Y, Prakash L, Badmaev V, Majeed M and Singh SV 2011 Reactive oxygen species-dependent apoptosis by gugulipid extract of ayurvedic medicine plant commiphora mukul in human prostate cancer cells is regulated by c-Jun N-terminal kinase. *Mol. Pharmacol.* **79** 499–507

Yang XL, Khosravi-Far R, Chang HY and Baltimore D 1997 Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell* **89** 1067–1076

Yin VP and Thummel CS 2004 A balance between the diap1 death inhibitor and reaper and hid death inducers controls steroid-triggered cell death in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* **101** 8022–8027

Yin VP and Thummel CS 2006 Mechanisms of steroid-triggered programmed cell death in *Drosophila. Semin. Cell Dev. Biol.* **16** 237–243

Yoo SJ, Huh JR, Muro I, Yu H, Wang LJ, Wang SL, Feldman RMR, Clem RJ, *et al*. 2002 Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms. *Nat. Cell Biol.* **4** 416–424

Zhang F, Lu Y, Wu Q, Yan J, Shi J and Liu J 2012 Role of cinnabar and realgar of WSHFD in protecting against LPS-induced neurotoxicity. *J. Ethnopharmacol.* **139** 822–828

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