Section 4

15

Pharmacology

Ayurvedic *Amalaki Rasayana* and *Rasa-Sindoor* Suppress Neurodegeneration in Fly Models of Huntington's and Alzheimer's Diseases

India

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Summary

With a view to understanding the basic biology underlying the traditional Ayurvedic system of healthcare, we used the *Drosophila* fly model and examined the biological effects of two Ayurvedic *Rasayana* formulations, *viz*., *Amalaki Rasayana* (AR) and *Rasa-Sindoor* (RS), at organismal, cell and molecular levels. Rearing first instar larvae on food supplemented with 0.5% AR or RS affected biological parameters including development, life-span, fecundity and stress-tolerance in a formulation-specific manner, which generally agreed with the suggested therapeutic applications in Ayurveda. Both formulations significantly increased levels of several heterogeneous nuclear ribonucleoproteins (hnRNPs) and cAMP response element binding protein (CBP) in different cell types in the fly model. We showed that dietary supplementation of either of these formulations during the larval period substantially suppressed neurodegeneration in fly models with polyglutamine neurodegenerative disorders and Alzheimer's disorder without any side-effects. Additionally, both of these dietary supplements substantially inhibited induced apoptosis (cell death), which adds to the survival of diseased neuronal cells that would otherwise have undergone apoptosis. Thus our studies suggest, for the first time, the potential of these Ayurvedic formulations in providing holistic relief from increasingly common neurodegenerative disorders.

Background and Justification

Ayurveda is the traditional Indian medicine system, widely practiced uninterruptedly at least for the past four thousand years. Classical Ayurvedic texts like the *Sushruta Samhita* divide Ayurveda into eight branches, of which the rejuvenating *Rasayana* therapy aims at promoting long life, enhancing physical and mental strength, and strengthening resistance against the infirmities and ailments of old age. An ethical lifestyle in conjunction with protocols involving diet, cleansing procedures and the intake of medicinal formulations are part of the *Rasayana* therapy. Under *Rasayana* therapy, orally-administered drugs are mostly based on plant products but may also include drugs derived from animal and mineral/metal sources. Etymologically, *Rasayana* implies the supply of the nutrient sap (*Rasa*) resulting from the digestion of food to the target (*Ayana*) body tissues. Classically, *Rasayanas* are believed to augment the transport and supply of *Rasa* to the tissues.

The available ancient Ayurvedic literature does not elaborate the mechanism/s and effect/s in terms of a contemporary understanding of biology or physiology. Although recent times have witnessed an increased interest in traditional and herbal medicine systems, most studies have used specific extracts or "active principles" derived from herbal

or other traditional drugs and formulations. However, since the Ayurvedic medicines and formulations are complex integrated derivatives involving specific preparatory steps, results of studies employing isolated active compounds may not provide full insight into the efficacy or mode of action of the traditional formulations. Therefore, it is necessary to undertake in-depth scientific investigations on the action/s of traditional Ayurvedic drugs or formulations using sound *in vivo* experimental model systems. Accordingly, we used the fruit fly, *Drosophila melanogaster*, as a model for understanding the cellular biological, biochemical and genetic bases of action for Ayurvedic formulations.

As a first approach, we used *Amalaki Rasayana*, a herbal derivative, and *Rasa-Sindoor,* an organo-metallic derivative of mercury. *Amalaki Rasayana* (AR) is a prominent drug in Ayurvedic classic texts such as the *Charak Samhita* and the *Ashtang Hridaya* and is claimed to enhance life expectancy, body strength, intellect and fertility, and to provide freedom from age-related illnesses. *Rasa-Sindoor* (RS) is indicated in a wide variety of disorders including chronic and recurrent infections (including pneumonia and bronchitis), anal fistulas, rheumatological diseases especially those of auto-immune origin, general and sexual debility as well as benign and malignant neoplasms. We found that these formulations do indeed affect some of the basic biological life parameters in the fly model, mirroring their expected usages in humans (Dwivedi *et al*., 2012).

Increasing life span combined with life-style changes in recent decades has been associated with a significantly elevated incidence of a variety of neurodegenerative disorders. These include several polyglutamine (polyQ) expansion neurodegenerative disorders such as Huntington's disease (HD) and diverse spinocerebellar ataxias (SCA). Alzheimer's disease (AD), the other common form of senile dementia in humans, is associated with truncated Aβ peptides produced by aberrant proteolytic cleavage of the transmembrane receptor amyloid precursor protein (APP). A characteristic feature of these neurodegenerative diseases is the accumulation of polyQ inclusion bodies (IB) or the formation of amyloid plaques, respectively, by the repeat expanded or truncated protein. These inclusions and aggregates disrupt cellular homeostasis by sequestering a range of critical cellular proteins including molecular chaperones, transcription factors, proteasome subunits and cytoskeletal components resulting in cellular damage and consequent death of the affected neuronal cells. With a view to understanding the molecular and cellular pathophysiology of neurodegeneration and to discover potential drug targets for therapeutic applications, several human neurodegenerative diseases like HD, different SCAs and AD, among others, have been modelled in diverse organisms including *Drosophila*.

Several traditional Ayurvedic formulations are claimed to facilitate "healthy aging" (Singh, 2003) and thus have the potential to mitigate the suffering from neurodegenerative diseases (Lakhotia, 2013). We (Dwivedi *et al*., 2012) found that feeding *Drosophila* larvae and adult flies on food supplemented with 0.5% (weight/volume) of AR or RS significantly improved their tolerance to thermal or starvation stresses and enhanced cellular levels of various heterogeneous RNA-binding proteins (hnRNPs) and CAMP response element binding protein/p300 histone-acetyl-transferase in wild-type larval tissues. These proteins have key roles in gene expression and RNA processing and transport. Several earlier studies in different model systems (Sofola *et al*., 2007; Mallik and Lakhotia, 2010; Caccamo *et al*., 2010; Berson *et al*., 2012) have also shown that elevated levels of hnRNPs, CBP and better tolerance to thermal and/or oxidative stress suppress neurodegeneration. Therefore, we examined if dietary supplementation with AR or RS affects neurodegeneration in fly models of polyQ disorders or Alzheimer's disease (AD). Interestingly, both the formulations notably suppressed pathogenesis in the fly models of human neurodegenerative disorders (Dwivedi *et al*., 2013).

Description

Pilot experiments with food supplemented with 0.125%, 0.25%, 0.5%, 2%, 4% or 6% of the formulations were carried out and, based on the results, in subsequent experiments either of the two formulations were used at 0.5% (i.e., 500mg/100ml food) concentration for feeding *Drosophila* larvae and/or adult flies (Dwivedi *et al*., 2012). Rearing on AR or RS supplemented diet resulted in substantial suppression of the different polyQ-dependent neurodegenerative phenotypes, *viz*., eye morphology, differentiation and organization of rhabdomeres, apoptosis in developing eye discs, accumulation of inclusion bodies, induction of the chaperone-like Hsp70 and Hsp60, etc, clearly showing that these two formulations effectively suppress neurodegeneration in fly models of polyQ toxicity (Dwivedi *et al*., 2013).

PolyQ transgene expression leads to an accumulation of polyQ inclusion bodies posterior to the morphogenetic furrow in differentiating late third instar larval eye discs (Fig. 1a,e). The polyQ inclusion bodies were significantly reduced in the eye discs of larvae reared on AR or RS supplemented food (Fig. 1b-d, f-h). Along with the reduction in polyQ inclusion bodies levels in eye discs in larvae reared on food supplemented with AR or RS, the differentiating ommatidial units posterior to the morphogenetic furrow also showed a remarkably improved organization (Fig. 1i-o). Measurement of the polyQ immunofluorescence intensity in eye discs confirmed that the accumulation of inclusion bodies in AR- and RS-fed larvae was significantly reduced when compared to those

reared on regular food (Fig. 1d, h). Since the levels of polyQ transcripts in formulation-fed larvae were similar to those observed in normally fed larvae (inset in Fig. 1c), the reduced accumulation of inclusion bodies may be the result of post-transcriptional events.

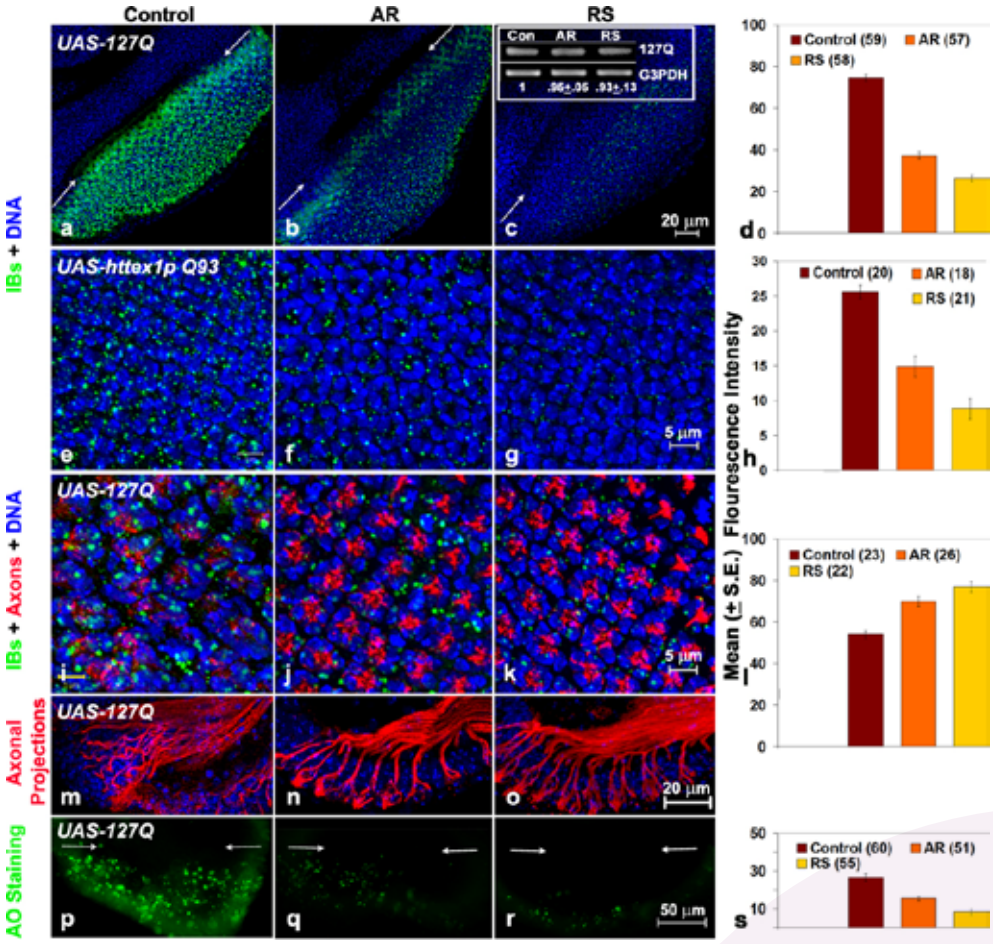

Figure 1: Eye discs expressing *GMR-GAL4>UAS-127Q or GMR-GAL4>UAS-htt1 p Q93* (indicated in left column of each row) from *Drosophila* larvae fed with Ayurvedic formulations. Left column (a,e,i,m): untreated control; Centre column (b,f,j,n), reared on *Amalaki Rasayana* (AR); Right column (c,g,k,o), reared on *Rasa-Sindoor* (RS).

(a-k) show reduced the accumulation of polyQ inclusion bodies (green); (i-k) show improved rhabdomere arrays; (m-o) show reduced damage to axonal projections in the optic stalk (red); and (p-r) show reduced cell death (green).

White arrows in (a-c and p-r) indicate position of the morphogenetic furrow in eye discs. The scale bars apply to all panels in the same row.

The inset in (c) shows the 127Q (upper row) and G3PDH (lower row) amplicons generated by semi-quantitative RT-PCR with total RNA from larval eye discs from *GMR-GAL4>127Q* larvae reared on control (Con), AR and RS supplemented food (indicated on top of the columns). The values below each lane indicate the mean (±SE, n = 3) levels of polyQ transcripts relative to that in the control sample, which was taken as 1.0.

Histograms (d,h,l,s) show the mean (±SE) fluorescence intensities (measured in arbitrary fluorescence units) of polyQ inclusion bodies (d,h), mab22C10 (l) and AO (s) staining, respectively, in *GMR-GAL4>UAS-127Q* expressing eye imaginal discs of late third instar larvae reared on different feeding regimes. Numbers in parentheses after the bar legends indicate the number of eye discs examined for each data point (Figure reproduced from Dwivedi *et al*., 2013).

We examined the cellular levels of two hnRNPs, *viz*. Hrb87F (hnRNP-A homologue) and Bancal (hnRNP K homologue) and CBP in *GMR-GAL4>UAS-127Q*-expressing eye discs in larvae reared on normal food and those reared on AR- or RS-supplemented food. Immunostaining with appropriate antibody and confocal microscopy showed that compared to normally-fed larvae (Fig. 2a,d,e and h), dietary supplement of either of the formulations resulted in significant increase in cellular levels of Hrb87F (Fig. 2b-d) and Bancal (Fig. 2f-h). The increase was more apparent in RS-fed larval eye discs.

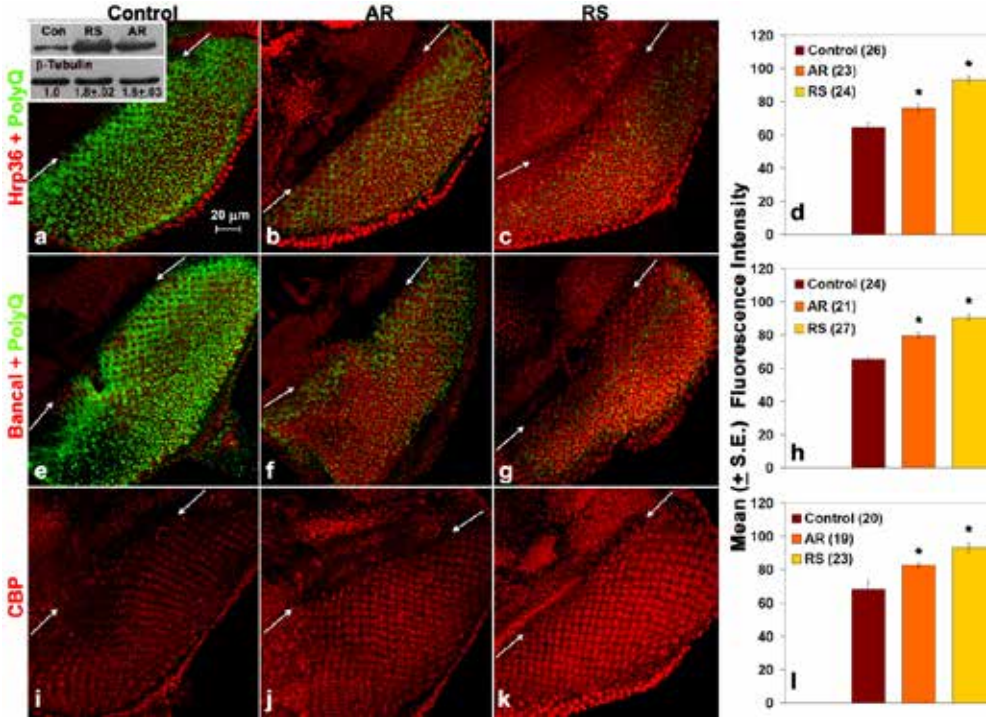

Figure 2: *Drosphila* larval eye discs expressing *GMR-GAL4>127Q* fed with Ayurvedic formulations. Left column (a,e,i): untreated control; Centre column (b,f,j), reared on *Amalaki Rasayana* (AR); Right column (c,g,k), reared on *Rasa-Sindoor* (RS).

(a-d) show elevated cellular levels of Hrb87F (red); (e-h) show elevated levels of Bancal (red); and (i-l) show elevated levels of CBP (red). Also, (a-c and e-g) show the reduction in accumulation of polyQ inclusion bodies (green).

White arrows indicate position of the morphogenetic furrow in the eye discs. The scale bar in (a) corresponds to 20 µm and applies to all the panels.

The inset in (a) is a western blot of total protein from eye discs of 127Q-expressing larvae reared on normal (Con), RS- or AR-supplemented food to show the relative levels of Hrb87F (upper row); β-tubulin (lower row) was used as a loading control. The values below each column indicate the mean (±SE, n = 3) levels of Hrb87F relative to that in control sample, which was taken as 1.0.

Histograms (d,h,l) represent the mean (±SE) fluorescence intensities (in arbitrary fluorescence units) of Hrb87F (d), Bancal (h) and CBP (l), respectively, in *127Q*-expressing larval eye imaginal discs following different feeding regimes while the numbers in parentheses after the bar legend indicate the number of eye discs examined (Figure reproduced from Dwivedi *et al*., 2013).

Co-immunostaining with antibodies against polyQ also revealed that the increase in the cellular level of these hnRNPs following formulation feeding is associated with a reduction in the accumulation of inclusion bodies (Fig. 2a-g). An increase in levels of Hrb87F was also confirmed by western-blotting which also showed that RS-fed larval samples displayed a greater increase (inset in Fig. 2a). Immunostaining for CBP/p300 in *GMR-GAL4>UAS-127Q* expressing discs (Fig. 2i-l) showed that AR-or RS-feeding significantly enhanced the levels of CBP, more so in RS-fed samples (Fig. 2k,l). AR- or RS-feeding significantly improved proteasomal activity in formulation-fed larval tissues. The ubiquitin-proteasomal activity (UPS), involved in the degradation and clearance of unwanted proteins in cells, is compromised in the affected neuronal cells in polyQ/HD and AD, leading to enhanced accumulation of pathogenic proteins. Therefore, we examined the UPS activity in *GMR-GAL4>UAS-127Q*-expressing eye discs using the *UAS-Ub^{G76V}-GFP* transgenic line (Dantuma *et al*., 2010) in which the green fluorescent protein (GFP) is tagged with ubiquitin so that under conditions of compromised UPS activity, GFP fluorescence persists. As expected, because of the compromised UPS activity, eye discs of normally-fed *GMR-GAL4>UAS-127Q*-expressing larvae showed high levels of GFP fluorescence (Fig. 3a). However, in AR- or RS-fed larval eye discs, the GFP fluorescence was significantly reduced, especially in RS-fed samples (Fig. 3b, c and d).

In order to further assess whether the improved UPS is indeed playing a role in reducing the accumulation of inclusion bodies and disappearance of *UAS-Ub^{G76V}-GFP* fluorescence, *GMR-GAL4>UAS-127Q*-expressing eye discs from differently fed late third instar larvae were incubated *in vitro* for two hours in medium containing 1μM clastolactacystin β-lactone, a proteasome inhibitor, prior to immunostaining for polyQ inclusion bodies. As expected, the accumulation of inclusion bodies was much higher in discs from normally fed larval eye discs in which the proteasomal activity was inhibited for two hours (Fig. 3i, compared with Fig. 3e). Interestingly, however, the accumulation of inclusion bodies even in the presence of proteasome inhibitor was much less in discs from AR- (Fig. 3j and l) or RS- (Fig. 3k and l) fed larvae, although they were slightly more abundant than in discs which were not exposed to the proteasomal inhibitor (Fig. 3f and g). Taken together, these results confirm that AR- or RS-feeding indeed improves proteasomal activity.

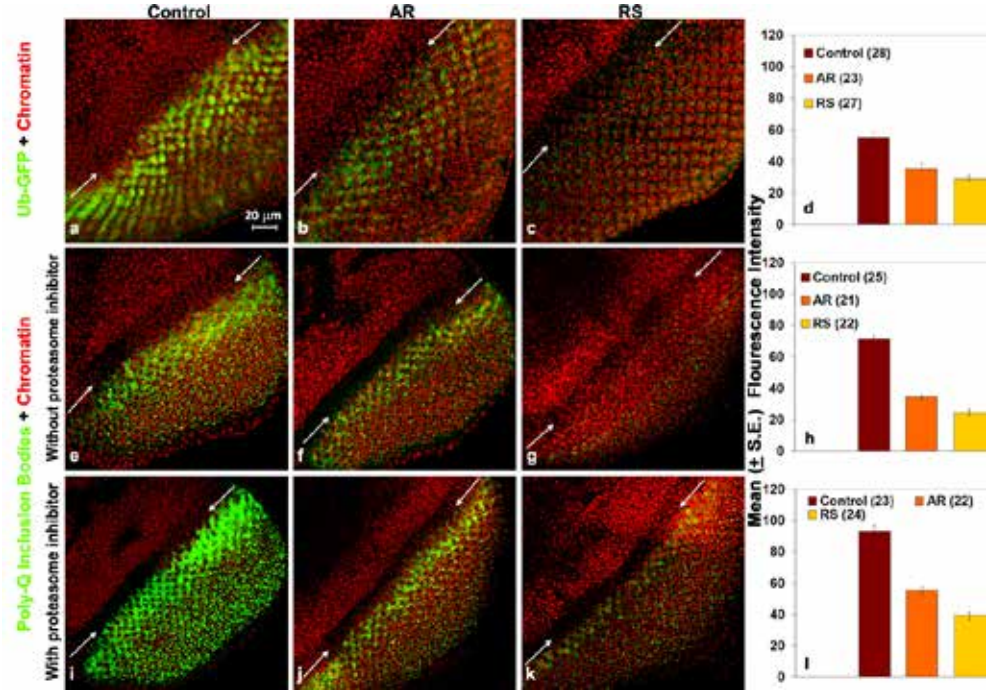

Figure 3: *Drosphila* larval eye discs expressing *GMR-GAL4>127Q* fed with Ayurvedic formulations. Left column (a,e,i): untreated control; Centre column (b,f,j), reared on *Amalaki Rasayana* (AR); Right column (c,g,k), reared on *Rasa-Sindoor* (RS).

(a-c) showing improved ubiquitin-proteasomal activity (UPS) activity through UB-green fluorescent protein (GFP) staining (green); showing polyQ inclusion bodies (green) and (a-k) and DAPI (4',6-diamidino-2-phenylindole) stained nuclei (red).

(e,f,g) are images of polyQ inclusion bodies in *127Q*-expressing larval eye discs without the 2-hour *in vitro* exposure to proteasome inhibitors while (I,j,k) are images of polyQ inclusion bodies in *127Q*-expressing larval eye discs exposed to the proteasome inhibitors.

White arrows indicate the position of the morphogenetic furrow in eye discs. The scale bar in (a) corresponds to 20 µm and applies to all image panels.

Histograms (d,h,l) represent the mean (±SE) fluorescence intensities (in arbitrary fluorescence units) of Ub-GFP, polyQ inclusion bodies without (d, h) and after (I\) treatment with a proteasome inhibitor, in *127Q*-expressing larval eye imaginal discs following different feeding regimes; numbers in parentheses after the bar legends indicate the number of eye discs examined for each data point. (Figure reproduced from Dwivedi *et al*., 2013).

Thus the enhanced levels of hnRNPs and CBP together with improved proteasomal activity following formulation feeding seem to be some of the factors that suppress the polyQ-toxicity. The inhibition of induced apoptosis or cell death (Dwivedi *et al*., 2015) by AR- or RS-feeding further adds to the suppression of neurodegeneration. A complete absence of AR- or RS-mediated suppression of neurodegeneration caused by *127Q* or Htt-ex1P Q93 toxic proteins in larvae that have reduced or complete absence of Hrb87F (data not shown) further confirmed that this hnRNP plays a pivotal role in bringing about the beneficial effects of both these formulations.

Impact

Our studies have established the fly model for understanding the "Science of Ayurveda". In addition, they have indicated, for the first time, the potential of Ayurvedic formulations such as *Amalaki Rasayana* and *Rasa-Sindoor* to provide holistic relief from the increasing burden of neurodegenerative disorders in human populations.

Publication of our papers received wide coverage in popular media and *Nature* (India). See for example:

- www.telegraphindia.com/1131227/jsp/nation/story_17724324.jsp#.Urzk-dIW04k
- www.telegraphindia.com/1120518/jsp/nation/story_15501860.jsp
- www.nature.com/nindia/2012/120515/full/nindia.2012.73.html
- www.nature.com/nindia/2013/131230/full/nindia.2013.172.html

The community of Ayurvedic practitioners also appreciated these studies and the principal investigator of the project was invited to write guest editorials in Ayurvedic journals.

These studies show, contrary to unfounded but common misapprehensions, that the traditional mercury-containing formulation does not exhibit any toxicity when prepared following traditional practices.

Based on the outcome of this project, the principle investigator has (S.C. Lakhotia) been granted a new research project for extensive genomic and proteomic studies following administration of these *Rasayanas* to *Drosophila*. In addition, several other basic science investigators in India have initiated in-depth experimental studies on the biology of Ayurveda with a view to understanding the basic science underlying this traditional healthcare system which continues to be popular in India and many other countries.

Future Plans

Our ongoing research project aims to examine in detail the transcriptomic and proteomic changes brought about by these formulations in various cell types in different genotypes, including in fly and mammalian models of neurodegenerative disorders.

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