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Evaluation of immunostimulatory activity of Chyawanprash using *in vitro* assays

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Chyawanprash is an ayurvedic formulation used in Indian traditional medicinal system for its beneficial effect on human health. We investigated the immunostimulatory effects of Chyawanprash (CHY) using *in vitro* assays evaluating the secretion of cytokines such as Tumor Necrosis Factor-alpha (TNF-α), Interleukin-1beta (IL-1β) and Macrophage Inflammatory Protein-1-alpha (MIP-1-α) from murine bone marrow derived Dendritic Cells (DC) which play pivotal role in immunostimulation. The effects of CHY on phagocytosis in murine macrophages (RAW264.7) and Natural Killer (NK) cell activity were also investigated. At non-cytotoxic concentrations (20–500 μg/ml), CHY enhanced the secretion of all the three cytokines from DC. CHY also stimulated both, macrophage (RAW264.7) as well as NK cell activity, *in vitro*. In conclusion, the data substantiates the immunoprotective role of CHY at cellular level mediated by immunostimulation in key immune cells viz. dendritic Cells, macrophages and NK cells.

Keywords: Dendritic cells, Cytokines, Interleukin-1beta, IL-1β, Macrophage inflammatory protein-1-alpha, MIP-1-α, NK cells, Phagocytosis, Tumor necrosis factor-alpha, TNF-α

Ayurveda is a traditional medicinal system practiced in India since ancient days. Chyawanprash (CHY), an ayurvedic formulation, considered as rasayana in Ayurveda, is characterized by benefits of maintaining youth, vigor, vitality via antioxidant and Immunostimulatory action[1-3]. Chyawanprash is a combination of more than 40 herbs in appropriate proportions prepared in a base of Indian gooseberry, *Emblica officinalis*, fruit pulp. It is used in the treatment of respiratory tract infections and tuberculosis[4], shows genoprotective activity against tobacco smoke[5], antioxidant[6], anti-aging[7] and antiamnesic[8] activity in mice. The major ingredient of CHY, *Emblica officinalis* (Amla), is known to possess immunostimulatory[9] and anti-inflammatory properties[10]. Other ingredients such as *Withania somnifera* (Ashwagandha) and *Tinospora cordifolia* (Guduchi), are also known for their immunostimulatory properties[11,12].

In the present study, we evaluated the immunostimulatory potential of CHY *in vitro* using key immune cells. Also, we have assessed the effects of CHY at non-cytotoxic concentrations on the phagocytic ability of macrophages (RAW 264.7) and NK cell activity.

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Materials and Methods

Chemicals/Reagents—FBS (Tissue Culture Biologics), Penicillin/Streptomycin (Krishgens biosystems), RPMI-1640 and DMEM medium (Lonza), rmGMCSF and ELISA kits for murine TNF-α, IL-1β, MIP-1α (Quantikine, R & D systems, Minneapolis, MN), Cytoselect™ 96-well Phagocytosis assay kit (Cellbio Labs), Lipopolysachharide (LPS, from *Escherichia coli* serotype 0127:B8), DMSO, HBSS and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) were used in the study. Chyawanprash (CHY) was obtained from Dabur India Limited, Ghaziabad, Uttar Pradesh, India. The composition details of D-CHY is given in table 1.

Animals—Specific pathogen-free male C57BL/6 mice (20-25 g, 8–10 weeks) were obtained from National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Hyderabad, India, and were kept in the in-house animal facility maintained at 22±3 °C and 55±15% relative humidity with 12 h L:D cycle. They were given autoclaved pelleted feed and filtered drinking water *ad libitum*. All experiments involving the mice were performed under the protocols approved by the Institutional Animal Ethics Committee (IAEC).

Preparation of CHY stock solutions—CHY was dissolved in sterile DMSO (200 mg/ml) and diluted in serum free medium to achieve final concentrations in

Table 1—Details of the Dabur Chyawanprash contents*

Contents	Quantity (g)
Emblica officinalis	90.0
Crystal Sugar	61.4
Clarified butter	2.08
Sesamum indicum seed oil	1.20
Pueraria tuberosa	1.195
Piper longum	1.12
Honey	1.0
Bambusa arundinecia	0.8
Asparagus racemosus, *Dioscorea bulbifera*, *Withania somnifera*	0.796
Elettaria cardamomum	0.588
Adhatoda vasica, *Aegle marmelos*, *Boerhaavia diffusa*, *Cyperus rotundus*, *Desmodium gangeticum*, *Glycyrrhiza glabra Curcuma zedoaria*, *Gmelina arborea*, *Inula recemosa*, *Leptadenia reticulata*, *Maritima annua*, *Nymphaea stellata*, *Oroxylum indicum*, *Phaseolus trilobus*, *Phyllanthus niruri*, *Pistacia integerrima*, *Premna integrifolia*, *Sida cordifolia*, *Solanum indicum*, *Solanum xanthocarpum*, *Stereospermum suaveolens*, *Teramnus labialis*, *Terminalia chebula*, *Tinospora cordifolia*, *Tribulus terrestris*, *Uraria picta*, *Vitis vinifera*	0.398
Calcined Mica	0.188
Syzygium aromaticum	0.128
Anacyclus pyrethrum	0.126
Cinnamomum tamala, *Cinnamomum zeylanicum*, *Mesua ferrea*	0.116
Dried aqueous paste of oyster shell	0.063
Crocus sativus	0.021
Santalum album oil	0.0092

*Each 100g of Dabur Chywanprash

the range 0.1-500 µg/ml. Final DMSO in cells did not exceed 0.25%.

Dendritic cell (DC) activity

Generation of DC cultures from bone marrow—DCs were generated from murine bone marrow by Lutz *et al*. Method[13] with some modifications. Femurs were excised from C57BL/6 mice and bone marrow was harvested by gently flushing the femur with HBSS using a 23-gauge needle. Cells were cultured in 90-mm culture petri dishes (2×10^6 cells/10 ml) with growth medium (RPMI 1640 medium supplemented with 10% FBS, 100U/ml penicillin, 100 µg/ml streptomycin and 20 ng/ml rmGMCSF) for 6 days at 37 °C in a humidified 5% CO_2 atmosphere. On day 3, cells were replenished with additional 10 ml of growth medium containing rmGMCSF. Semi-adherent, immature DCs were harvested on day 6 by gentle pipetting and used in experiments. Viable cells were counted using trypan blue exclusion method.

Estimation of cytokines (*TNF-α*, *IL-1-β and MIP-1-α*) *in DCs*—Effect of D-CHY on viability of DCs was assessed after 24 h of treatment. DCs (50,000 cells/well) were plated in 96-well culture plates and treated with D-CHY in the concentration range of 0.1-500 µg/ml in triplicates. The cytotoxic effect of D-CHY on DCs was determined after 24 h of incubation by MTT assay. Concentrations of D-CHY that resulted in >80% viability of DCs were selected for subsequent cytokine analysis studies.

For estimation of cytokines activity, day-6 DCs (0.16×10^6 cells/well in 24-well culture plate) were treated with D-CHY at selected non-cytotoxic concentrations, 20-500 µg/ml in triplicates. DCs treated with 0.25% DMSO were included as control cells. After 24 h of incubation, cell-free culture supernatants were analyzed for secreted levels of TNF-α, IL-1-β and MIP-1-α by ELISA. LPS (*E. coli.*) (10 ng/ml) was included as a positive control.

Phagocytosis assay in macrophages (*RAW264.7*)—Mouse macrophage cell line (RAW264.7) was procured from the National Centre For Cell Science (NCCS, Pune, India) and grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a 5% humidified incubator with a 95% air atmosphere. Effect of D-CHY on viability of RAW264.7 cells was determined after 24 h. Cells were seeded at a density of 5×10^4 cells/well in 96-well culture plates. After overnight incubation, cells were treated with D-CHY

(0.1-500 µg/ml) in triplicates for another 24 h. Any cytotoxic effect of D-CHY on mouse macrophages (RAW 264.7) was determined by MTT assay.

For phagocytosis assay, RAW 264.7 cells were seeded at a density of 5×10^4 cells/well in 96-well culture plates for 24 h. Cells were treated with D-CHY at non-cytotoxic concentrations, 20-500 µg/ml in triplicates for another 24 h. Cells treated with 0.25% DMSO were used as controls. LPS (*E. coli.*) (50 µg/ml) was used as a positive control. Phagocytosis was estimated using CytoSelect™ 96-well phagocytosis assay kit (Zymosan, Colorimetric Format).

NK cell activity—Spleens were removed from C57BL/6 mice and a single cell suspension was prepared. Erythrocytes in the splenocytes were removed by treatment with lysis buffer (0.15 M NH_4Cl, 0.01 M $NaHCO_3$ and 0.1 mM Na_2EDTA, pH 7.4) for 5 min. Cells were washed in RPMI-1640 medium and used as NK cell population. Mouse lymphoma cell line (YAC-1) (sensitive to NK cells) was maintained in RPMI-1640 supplemented with 10% FBS, Penicillin (100 U/ml), Streptomycin (100 µg/ml) at 37 °C, 5% CO_2 and 95% humidity and used as a target cell population.

To assess the effects of D-CHY on viability, splenocytes were seeded at a density of 0.5×10^6 cells/well in 96-well culture plates and then treated with D-CHY (20-500 µg/ml) in triplicates. After 24 h of incubation, cytotoxic effect was determined by MTT assay.

For NK cell activity, splenocytes (0.5×10^6 cells/well in 96-well culture plate) were treated with D-CHY at non-cytotoxic concentrations in the range 20-500 µg/ml (in triplicates) for 24 h. YAC-1 cells (5000 cells/well) were co-incubated with NK-cells pre-treated with D-CHY (Effector: Target ratio of 100:1). NK cells alone (treated with 0.25% DMSO) (E) and YAC-1 cells alone (T) were also incubated as controls. After 4 h of incubation at 37 °C in a CO_2 incubator, NK cells mediated lysis of YAC-1 cells was determined using MTT assay. LPS (*E. Coli.*@ 100 ng/ml) was used as a positive control.

Percentage of NK cell activity was calculated as:

$$NKA\% = \frac{[1-\{O.D._{(ET)} - O.D._{(E)}\}]*100}{O.D._{(T)}}$$

Statistical analysis—Experimental values were expressed as arithmetic mean ± SEM. Statistical differences between control and treatment groups were determined using One-way ANOVA with Dunnett's multiple comparison post test. *P* values <0.01 were considered significant.

Results

Increased cytokines secretion in DCs—Dendritic cells showed ≥ 80% viability in the concentration range of 0.1-500 µg/ml for 24 h (Fig. 1A). D-CHY also induced remarkable changes in the morphology of DCs (Fig. 1B). Control DCs showed formation of numerous colonies with semi-adherent cells in the suspension. Some cells adhering to surface were also seen at the site of colony formation. At a concentration of 20-500 µg/ml D-CHY causes a dose dependent maturation in DCs. This effect was evident by increased number of adhered and branched cells in

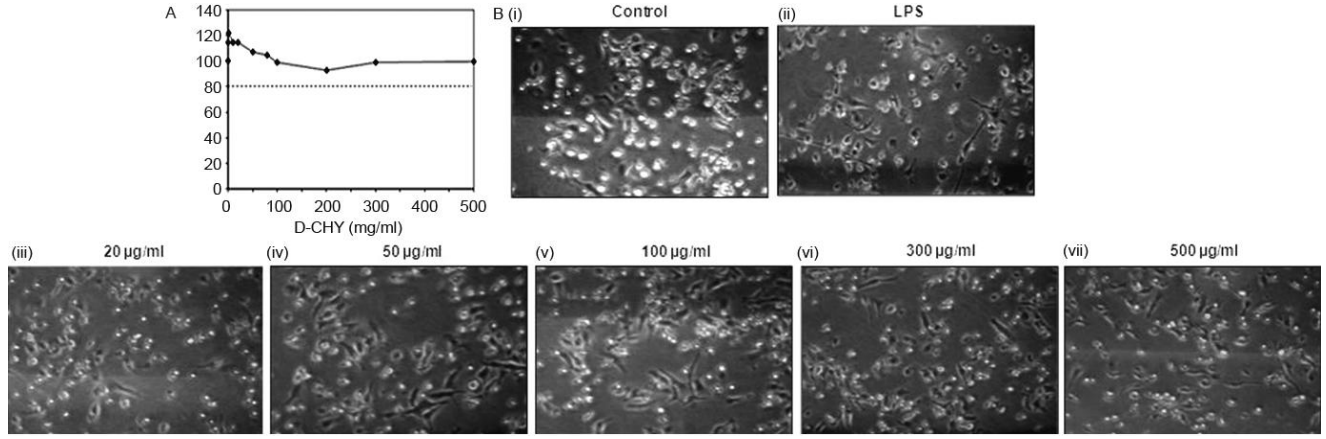

Fig. 1—Effects of D-CHY on viability and morphology of DCs. (A) Day-6 DCs (50000/well) were treated with D-CHY (0.1-500 µg/ml) for 24 h. Cells treated with 0.25% DMSO were used as control. Cell viability was determined by MTT assay as the ratio (expressed as a percentage) of absorbance of treated cells to control cells (n=3). (B) Morphology of day-6 DCs treated with D-CHY (20-500 µg/ml) (Magnification-100X).

comparison with semi-adherent cells. Maturation of DCs was also observed upon treatment with LPS (10 ng/ml), which was used as a positive control.

Stimulatory effects of D-CHY on DCs activity were determined by estimating the levels of cytokines secreted in culture supernatants using ELISA. D-CHY @ 20-500 µg/ml for 24 h resulted in increased secretion of TNF-α, IL-1-β and MIP-1-α (Fig. 2 A-C) as compared to control DCs. TNF-α recorded a 5.2 fold significant increase (P <0.01) at 500 µg/ml followed by 2.2 fold increase of IL-1β. Levels of MIP-1α secreted into extracellular medium also showed 2.9 and 3.3 fold increase at 300 µg/ml and 500 µg/ml, respectively (P <0.01). With the positive control LPS (10 ng/ml) also, DCs showed significantly (P <0.01) increased secretion of cytokines, TNF-α, IL-1β and MIP-1α.

Enhanced phagocytosis in macrophages—The mouse macrophages (RAW 264.7) did not show any significant loss of cellular viability with D-CHY @ 0.1- 500 µg/ml after a time period of 24 h (Fig. 3A).

The stimulatory effect of D-CHY on phagocytosis was measured after 24 h of treatment by the extent of internalization of pre-labeled zymosan particles using a colorimetric kit based method (Cytoselect, Cellbio Labs). Phagocytosis of macrophages showed significant increase upon treatment with D-CHY in the concentration range of 50-500 µg/ml (P <0.01) (Fig. 3B). Within this concentration range, D-CHY stimulated the extent of zymosan-particles internalization by 28.9-65.2% as compared to control RAW 264.7 cells. LPS (50 µg/ml) also significantly (P <0.01) enhanced phagocytosis of macrophages, when used as a positive control.

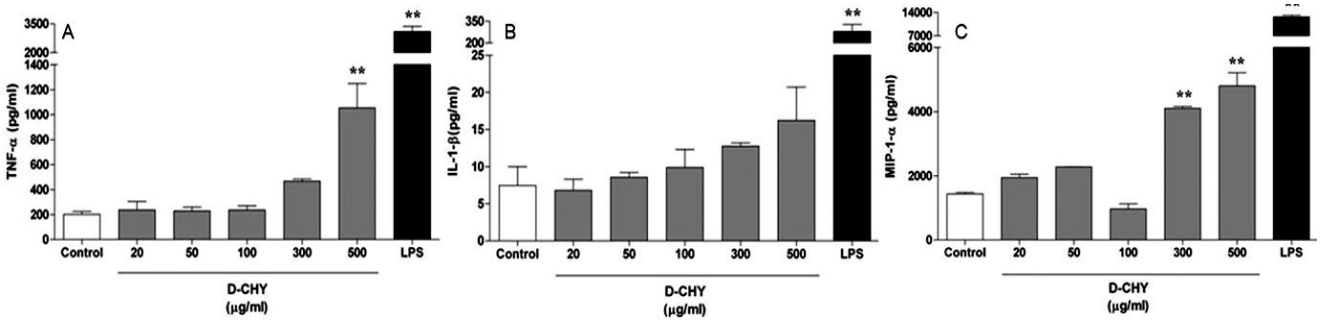

Fig. 2—Effects of D-CHY on secretion of cytokines from DCs. Day-6 DCs (0.16×10^6/well) were treated with D-CHY (20-00 µg/ml) for 24 h. DCs treated with 0.25% DMSO were used as control. LPS (10 ng/ml) was used as a positive control. Levels of (A) TNF-α, (B) IL-1β and (C) MIP-1α production in culture supernatants of DCs were determined by ELISA (n=3). *P <0.01, significantly different from the control DCs (Analyzed using One-way Anova with Dunnett's multiple comparison post test).

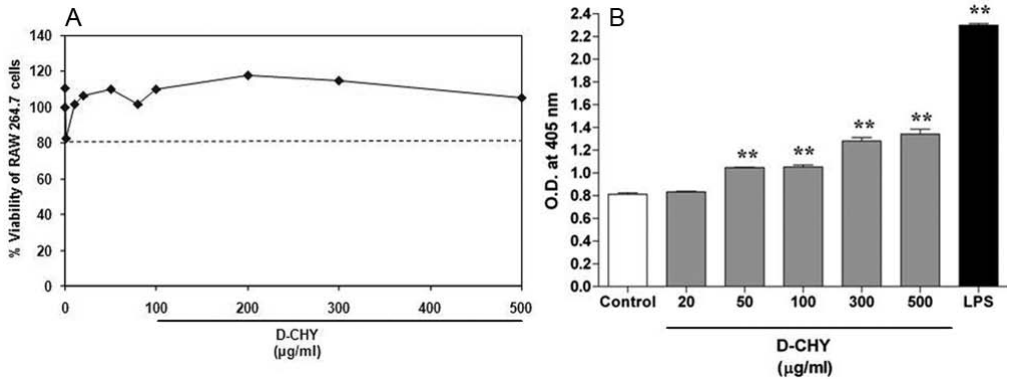

Fig. 3—Effects of D-CHY on viability and phagocytic activity of mouse macrophages (RAW 264.7). (A) RAW264.7 cells (50000/well) were treated with D-CHY (0.1-500 µg/ml) for 24 h. Cells treated with 0.25% DMSO were used as control. Cell viability was determined by MTT assay as the ratio (expressed as a percentage) of absorbance of treated cells to control cells. (B) RAW264.7 cells (50000/well) were treated with D-CHY (20-500 µg/ml) for 24 h. Phagocytic ability of macrophages was estimated by engulfment of pre-labeled zymosan particles. Unbound particles were blocked and internalized zymosan particles were quantified using colorimetric kit based method. LPS (50 µg/ml) was used as a positive control (n=3). *P <0.01 significantly different from the control cells (Analyzed using One-way Anova with Dunnett's multiple comparison post test).

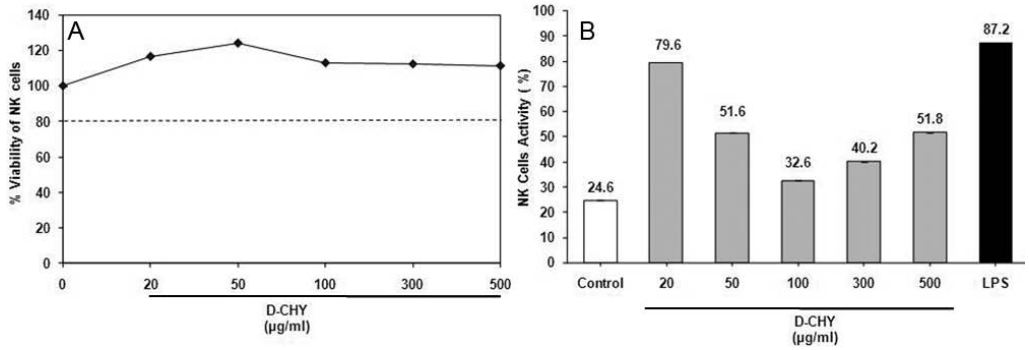

Fig. 4—Effects of D-CHY on NK cells viability and activity. (A) 0.5×10^6 NK cells were treated with D-CHY (20-500 µg/ml) for 24 h. Cells treated with 0.25 % DMSO were used as control. Cell viability was determined by MTT assay as the ratio (expressed as a percentage) of absorbance of treated cells to control cells; (B) 0.5×10^6 NK cells (E) pre-treated with D-CHY (20-500 µg/ml) for 24 h were co-incubated with YAC-1 cells (5000 cells/well) (T). NK cells mediated lysis of Yac-1 cells was measured by MTT assay (n=3). LPS (100 ng/ml) was used as a positive control.

Stimulation of NK cell activity—NK cells on treatment with D-CHY (20-500 µg/ml) for 24 h did not show any significant loss of cell viability (Fig. 4A). To further evaluate the immunostimulatory activity, NK cells were first treated with D-CHY for 24 h and then co-cultured with YAC-1 cells at E:T ratio of 100:1. D-CHY, at the concentration range of 20-500 µg/ml, considerably stimulated NK cell activity (Fig. 4B). A basal level of 24.6% NK cell activity was observed in control cells, which increased up to a maximum of 79.6% (by 3.2 fold) at 20 µg/ml of D-CHY. Positive control LPS (100 ng/ml) also enhanced NK cell activity to 87.2% (by 3.5 fold).

Discussion

The immune system is a complicated network of cells, tissues and organs, functioning in a coherent manner. Generation of an immune response against infectious pathogens such as virus, bacteria, fungus, toxins, carcinogens, etc. depends on various protective mechanisms mediated by immune cells in the body[14]. Dendritic cells act as a link between innate and adaptive immune arms by capturing and processing pathogenic microbes and then presenting the antigens to naïve T cells for mounting an efficient immune response against infection[15,16]. Activated DCs secrete immunostimulatory cytokines such as TNF-α, IL-1β and MIP-1α, which act as effective messenger molecules in an immune response[17]. Under normal conditions, a balanced secretion of these cytokines ensures proper functioning of the immune system. Although, an overproduction of these cytokines above the threshold levels is deleterious causing several chronic inflammatory disorders and cancer, proper regulation of DC's function by immunomodulating agents could be helpful in protection of the host from various invading pathogenic and cancerous attacks. For instance, in immuno-compromised patients with suppressed immunity levels, the functional ability and maturation state of DCs is considerably reduced. Activation of DCs and increased production of immunostimulatory cytokines is helpful in such conditions. Therefore, an agent which stimulates the level of immunostimulatory cytokines resulting in DCs activation and subsequently enables efficient functioning of T-cells is said to possess strong immunostimulatory activity.

In addition, macrophages are important regulatory cells, which recognize invading foreign bodies and are central to cell-mediated and humoral immunity[18]. Phagocytosis (internalization of pathogens by macrophages) is the first step of defense against infection. Activation of phagocytosis results in enhancement of the innate immune response[19,20]. Also, NK cells function as essential elements against pathogens and tumor cells by modulating both innate and adaptive immune responses. These cells mediate the spontaneous killing of certain virus-infected and tumor cells in a major histocompatibility complex (MHC)-unrestricted manner[21]. Activated NK cells play a crucial role in generation of protective immune response[22].

Considering the crucial role of these 3 important immune cell populations in immunostimulation, we used *in vitro* models to assess cytokine secretion in murine DCs, phagocytic capacity in macrophages and NK cell activity in splenocytes to demonstrate the immunostimulatory potential of Chyawanprash. The D-CHY altered the morphology of DCs by inducing cellular maturation. This change in DCs morphology by D-CHY reflects its stimulatory effects on the immune

system. Substantially increased secretion of key cytokines *viz.*, TNF-α, IL-1β and MIP-1α from murine bone-marrow derived DCs after 24 h of treatment with D-CHY indicates the immunostimulatory potential of D-CHY via enhanced DC's activity.

Also the increased phagocytic activity of mouse macrophages (RAW 264.7) after 24 h of treatment with Chyawanprash as shown by the increased engulfment of pre-labeled zymosan particles suggests enhanced clearance of pathogens in body by D-CHY and its potential to fight against common infections. Furthermore, enhanced NK cell activity by specific lysis of target murine lymphoma, YAC-1 cells as observed upon 24 h treatment with D-CHY supports its immunostimulatory potential.

The present study helps in elucidation of mode of action of Chyawanprash as an immunostimulatory agent. The D-CHY resulted in activation of 3 different arms of immune response based on key effector cells via an overall increase in the activity and maturation of DCs, phagocytosis by macrophages and NK cell activity. These findings are in-accordance with the immunostimulatory action of Chyawanprash, where activation of a suppressed immune system is required. At cellular level, D-CHY helps in maturation and activation of DCs by inducing changes in cellular morphology and stimulating the secretion of immunostimulatory cytokines. Increase in the extent of phagocytosis by D-CHY may be beneficial in effective clearance of infection-causing microbes and improved immunity levels. D-CHY also induced enhancement of NK cell activity, which strengthens its immunostimulatory potential and infection fighting properties.

This is noteworthy that immune-potentiating effects of D-CHY in various immune cell populations were observed at non-cytotoxic concentrations, ignoring any possible interference of loss of viability with functional activity. In fact, at certain concentrations, treatment with D-CHY resulted in proliferation of cells, corresponding to >100% viability (where OD of treated cells was higher than the OD of control untreated cells). This implies that cells have increased in number and there is no loss of viability due to D-CHY induced cytotoxicity.

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