Evaluation of antioxidant, immunostimulatory and antifatigue properties of *Dashmularishta* using *in vitro* and *in vivo* assays

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*Dashmularishta* is an Ayurvedic formulation used widely as a health tonic. Since the mode of action of *Dashmularishta* has not been explored much in detail so far, we investigated its potential effects on antioxidant, antifatigue and immunostimulatory properties using *in vitro* as well as *in vivo* assays. The antioxidant potential was assessed by free radical scavenging (ABTS based) assay. Antifatigue potential was elucidated by DNA super coiling assay and swim endurance test model. Immunostimulatory effect on Natural Killer (NK) cells activity was evaluated using mouse splenocytes and YAC-1 based assay. *Dashmularishta* exhibited *in vitro* antioxidant and antifatigue potential and *in vivo* antifatigue properties in Swiss albino mice. Further, it also enhanced the *in vitro* NK cells activity at non-cytotoxic concentrations.

**Keywords:** Ayurvedic, Antifatigue, Free radical scavenging, Herbal, NK cells, Swim endurance, Dashmularishta, Immunomodulatory

*Asava-Aristas* are an advanced dosage form of Ayurvedic formulations. These dosage forms are considered to possess better shelf lives by virtue of microbial fermentation (*Sandhana*) that results in the transformation of several phytochemicals, thereby rendering these formulations more potent, besides aiding their rapid absorption1,2.

*Dashmularishta* (DSM) is an acclaimed polyherbal *asava-arista* formulation comprising over 50 herbs. Traditionally, DSM is indicated in gastrointestinal, respiratory, urinary, anorectal and nervous disorders3. It is also renowned for its benefits on woman health4. DSM is reported to exhibit free radical scavenging and anti-inflammatory properties5 and antibacterial6 activity against enteric pathogens. DSM comprises herbs, such as *Aegle marmelos* (L.) Correa, *Oroxylum indicum* (L.) Benth. ex Kurz, *Desmodium gangeticum* (L.) DC, and *Tinospora cordifolia* (Thunb.) Miers that are reported to possess free radical scavenging7,8, anti-inflammatory9 and immunostimulatory10 activities.

Despite its long usage, few attempts have been made to validate the efficacy of DSM. Here, we investigated antioxidant, antifatigue and immunostimulatory activities of DSM using *in vitro* as well as *in vivo* assays. Antioxidant potential was evaluated *in vitro* by free radical scavenging activity utilizing ABTS assay. The antifatigue potential was evaluated *in vitro* by assessing its ability to protect DNA cleavage (supercoiled form of plasmid DNA) under H2O2/UVB induced stress conditions; and for strength and stamina using *in vivo* swim endurance test in adult female Swiss albino mice. Immunostimulatory potential was elucidated using NK cells, at non-cytotoxic concentrations.

**Materials and Methods**

**Chemicals/Reagents**

- ABTS (Amresco), Potassium per sulphate (Sigma), Trolox (Acros Organics) Tris Buffer (Merck), Methanol (Qualigens), DNA loading dye (Promega), Agarose (Sigma), H2O2 (Rankem), Ammonium chloride (Merck), Boric acid (Merck), CFSE (BiochemiKa), EDTA (Sigma), FBS (Life Technologies), HBSS (Sigma), KHCO3, LPS, MTT, Na2EDTA (Sigma), Penicillin/Streptomycin (HiMedia), RPMI-1640 (Biosera), Sodium Bicarbonate (Merck), Sodium hydroxide (Qualigens), Triton X-100(Sigma), CD-FBS (Life Technologies), Diethanolamine (Sigma), DMEM, DMEM w/o Phenol red jKit (Cusabio), Human Hyaluronic acid ELISA kit (Cusabio), Sirius Red (Direct Red 80) (Sigma) DMSO (Cisco Research Laboratories). *Dashmularishta*11 obtained from Dabur India Limited, Ghaziabad, Uttar Pradesh, India. The composition details of D-DSM are given in Table 1.

**Animals**

For NK activity assay, 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.

For swim endurance model, thirty-two 5–8 weeks old female Swiss albino mice (Mus musculus) weighing 23.67–38.91 g, procured from Althea Lifesciences Ltd. were used. They were kept in the in-house animal facility maintained at 22.2–24.2°C temperature, 55–63% humidity, 12 h L:D cycles ×7 days before study initiation. They were given conventional feed and filtered drinking water ad libitum. The studies were conducted at Althea Lifesciences Ltd. New Delhi. All animal experiments were performed under the protocols approved by the Institutional Animal Ethics Committee (IAEC approval no. IAEC/21/193; Date of Approval: 15/MAY/2012).

Antioxidant assay

Antioxidant assay was conducted as per the method described by Roberta Re et al. DSM was serially diluted in 80% methanol to obtain concentrations ranging from 0.001-100% (v/v). Trolox (positive control) was dissolved in 80% methanol to obtain concentrations ranging from 0.0025-0.25% (v/v). ABTS radical cation (ABTS+) was produced by mixing 7 mM ABTS with 2.45 mM potassium persulfate at a ratio of 1:0.5. Twenty μL of DSM and trolox at concentrations ranging from 0.001-100% and 0.0025-0.25% (v/v), respectively were added into 24-well assay plates. About 1980 μL of ABTS reagent with reference to Trolox in terms of TEAC (Trolox Equivalent Antioxidant Capacity):
where, $Ac = \text{Absorbance of Blank}$; and $As = \text{Absorbance of Sample}$.

TEAC value was calculated as: $\text{TEAC} = \% \text{ Inhibition of Sample}/\% \text{ Inhibition of Trolox}$

(at the same concentration of sample and Trolox i.e. value of Y when intercept equals to zero).

**Immunomodulatory activity**

**NK cell activity**

*Preparation of splenocytes* — Spleens were removed from C57BL/6 mice and a single cell suspension was prepared. Cells were pelleted at 1200 rpm for 8 min and erythrocytes in the splenocytes were removed by treatment with lysis buffer (0.15 M NaH4Cl, 0.01 M NaHCO3 and 0.1 mM Na2EDTA, pH 7.4) for 5 min. Cells were washed in RPMI-1640 medium and used as NK cell population.

*Cell viability* — DSM was diluted with DMSO to obtain the stock solution of 90% (%v/v) and diluted further in serum free RPMI-1640 to achieve final dilutions for treatment of cells of concentrations 0.1, 0.1, 0.25, 0.5 & 0.9% (v/v) (0.01-0.9% v/v). Non-cytotoxic concentrations of DSM (≥80% viability) were determined using MTT assay. The 0.2×10^6 splenocytes (NK cells)/well were treated with DSM (0.01-0.9%) (v/v) in triplicates for 24 h. About 20 μL of MTT (0.5 mg/mL) was added to each well and incubated at 37°C. After 3 h, cells were centrifuged at 2000 rpm for 8 min. Supernatants were removed and cell pellets were resuspended in 150 μL of DMSO. Absorbance of the samples was measured at 540 nm. Viability was calculated as:

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\text{Viability} = 100 - \% \text{ Cytotoxicity}
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where \( \% \text{ Cytotoxicity} = \frac{(Y-X)/Y}{100} \), in which \( X = \text{Absorbance of NK cells treated with DSM at 540 nm} \) and \( Y = \text{Absorbance of control NK cells treated with 0.1% DMSO at 540 nm} \).

**NK activity** — For NK cells activity, NK Cells (0.5×10^6 cells/180μL/well in 96-well plate) were treated with non-cytotoxic concentrations of DSM (0.01-0.9%) (v/v) in triplicates and incubated at 37°C, 5% CO2 and 95% humidity for 24 h. NK cells treated with 0.1% DMSO were used as controls. LPS (E. coli) was used as a positive control at the concentration of 10 ng/mL. YAC-1 cells were resuspended in 1 mL of carboxyfluorescein diacetate succinimidyl ester (CFSE) (10 μM) and incubated at 37°C for 1 h. After washing, CFSE-stained YAC-1 cells were then co-incubated with NK cells (0.5×10^6/well) pretreated with DSM (Effector:Target ratio of 12.5:1) in a total volume of 200 μL in each well (ET) in the 96-well cell culture plate. Unstained NK cells alone (0.5×10^6 cells/well/200 μL) (E), CFSE stained YAC-1 cells alone (40000 cells/well/200 μL) (T) were also incubated in different wells as controls. After 24 h of incubation at 37°C in a CO2 incubator, NK cells mediated lysis of YAC-1 cells was determined using fluorescent concentration release method. Plate was centrifuged at 700 g for 5 min, supernatant was removed and 100 μL of 1% Triton X100 in 0.5 M borate buffer (pH 9.0) was added to each well. The plate was then kept for 24 h at 4°C and the fluorescent release was measured at excitation wavelength 485/20 and emission wavelength 528/20. The percentage of specific lysis/NK cells activity was calculated as:

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\text{NK cells activity} (\%) = \frac{[(F_{\text{med}} - F_{\text{exp}})/F_{\text{med}}] \times 100}{\text{(RT)}. After 24 h of incubation at 37°C in a CO2 incubator, NK cells were treated with DSM or 0.1 % DMSO (control).

**Antifatigue activity**

*In vitro model* — The antifatigue assay was performed as described by Tharakan et al. DSM was dissolved in 50% methanol to obtain a stock solution of 50% (v/v). This stock solution was subsequently diluted in water to achieve final concentrations in the range of 0.006–25%. Trolox (positive control) was dissolved in 80% methanol to obtain the stock solution of 1M, which was used for the preparation of subsequent dilutions in water to achieve a final concentration of 500 μM. About 1 μg of pcDNA 3.1 was incubated with different concentrations of DSM (0.006-25 %) (v/v). Similarly, 1 μL of 30% H2O2 was added to each reaction and immediately irradiated with UVB at a dose of 230 mJ/cm² to induce pcDNA damage for 5 min at room temperature of 23°C (RT). After irradiation, each sample was mixed with 3 μL of 6X DNA loading dye and run on 1% agarose gel electrophoresis at 50 V for 3 h. pcDNA treated with H2O2 and irradiated with UVB alone was included as control. Segregated form of pcDNA bands both in the presence and absence of D-DSM was analyzed and captured using BIORAD Gel documentation system.

*In vivo model* — The swim endurance test was employed to evaluate antifatigue activity of DSM in female mice.
Acclimatized animals were randomized based on Swim Endurance (SE) time on day 0 (baseline) into four groups (G1-G4) comprising eight animals each. The in vivo efficacy study of DSM was evaluated in mice at 500, 1000 & 2000 mg/kg dose levels in groups G2 - G4, respectively. Test item was prepared in distilled water and fed orally at dose volume of 10 mL/Kg, QID from Day 1-28 consecutively. Group (G1) served as control receiving only distilled water at similar dose volume. Human dose of DSM was considered to calculate the animal (Mice) dose as: Human dose = Rat dose *(Mice Km/Human Km), where Mice Km = 3 & Human Km = 37.

The swimming exercise of mice was measured with a plastic round tub (63x49x35cm) filled with water maintained at 34±1ºC. All the animals were allowed to swim till exhaustion. End point of SE test was considered when the mouse drowned more than thrice. Swim Endurance (SE) test was performed on Day 0, 14 and 28; per cent anti-fatigue activity was calculated using the formula:

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\% \text{ Anti-fatigue Activity} = \left( \frac{\text{Test SE Time} - \text{Control SE Time}}{\text{Control SE Time}} \right) \times 100
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Statistical analysis
Experimental values were expressed as Arithmetic Mean±SD/SEM. Statistical differences between control and treatment groups were determined using One-way ANOVA with Dunnett’s multiple comparison posttest. P values <0.05 were considered significant.

Results and Discussion
This study investigated the antioxidant, immunomodulatory and antifatigue potential of Dashmularishta (DSM) utilizing in vitro and in vivo models.

Antioxidant potential
The antioxidant potential was evaluated in vitro using the free radical scavenging activity utilizing ABTS assay which is the most widely reported method for the estimation of antioxidant activity of test compounds/plan. The reduction of the radical cation was measured at 734 nm. D-DSM in the concentration range of 0.05-100% (v/v) resulted in decrease in absorbance and exhibited a dose-dependent increase in antioxidant potential by 1.2-91.7%. TEAC value of D-DSM was found to be 0.628275 (Fig. 1 A and B) indicating DSM might have potential in protection against various diseases mediated by oxidative stress. Trolox (0.0025-0.25%) used as a positive control demonstrated an increase in antioxidant potential by 2.5-93%.

Immunostimulatory potential (NK activity)

Cell viability
Effect of DSM on viability of NK Cells was determined after 24 h of treatment (Fig. 2A). There was no adverse effect of D-DSM (0.01%-0.9% v/v) on viability of NK cells. At all the concentrations tested, viability of cells was >80%. Hence, these concentrations were considered safe for evaluation of NK cells activity.

NK activity
The immunostimulatory potential of a compound is evaluated by assessing its effect on enhanced NK-cell mediated lyses of target tumor cell population. Stimulatory effect on NK cells activity was determined by pretreatment of splenocytes (NK cells) with DSM for 24 h. Subsequently, NK cells mediated lysis of fluorescently stained YAC-1 cells was measured. D-DSM in the concentration range of 0.1-0.9% (v/v) stimulated NK cells activity (Fig. 2B). A basal level of 13.5% NK cells activity was observed.
after 24 h incubation in the control group. This basal level of NK cells activity was considerably stimulated from 29.8-37.1% (2.2–2.8 fold increase as compared to basal levels), when D-DSM was tested in the concentration range of 0.1-0.9% (v/v). Treatment of NK cells with positive control; LPS (10 ng/mL) resulted in stimulation of NK cells activity, from basal level of 13.5-23.1% (1.7-fold increase) indicating its immunostimulatory activity and infection fighting properties.

In vitro antifatigue activity

The in vitro antifatigue potential of DSM was investigated by DNA cleavage assay. It is widely reported that reactive oxygen species (ROS) generation leads to mitochondrial DNA damage, which is directly correlated with muscle fatigue in humans. The antifatigue potential of DSM was evaluated by assessing its ability to protect DNA cleavage (supercoiled form of plasmid DNA) under H$_2$O$_2$/UVB induced stress conditions. The super coiled form was the predominant band when the normal plasmid DNA (pcDNA) was run on an agarose gel. Exposure of the plasmid DNA to H$_2$O$_2$ along with UVB resulted in DNA cleavage evident from the loss of the super coiled form of the DNA in the control sample. D-DSM (0.097-25 %) inhibited H$_2$O$_2$/UVB induced DNA cleavage in a dose dependent manner by protecting the supercoiled form of plasmid DNA (Fig. 3A). Trolox used as positive control also demonstrated protection of supercoiled form of pcDNA.

In vivo antifatigue potential

Results of swim endurance test were analyzed in two parts— (i) effect on SE time; and (ii) effect on antifatigue activity. At baseline randomization, no statistical difference in SE time was obtained among the study groups. In D-DSM treated groups, an increase in mean SE time on day 14 and 28 was observed in animals treated at all the three tested doses (500, 1000 and 2000 mg/kg) in comparison to vehicle control. Significant increase ($P <0.05$) in mean SE time was observed at test dose of 2000 mg/kg on day 28 (Fig. 3B). Antifatigue activity of D-DSM on Day 28 was found to be elevated at all the tested doses in comparison to vehicle control (Fig. 3C). Results concluded D-DSM could effectively increase the swim endurance time and postpone the appearance of fatigue at all the three doses contributing to antifatigue potential. These results were, however, not dose dependent probably because DSM being a polyherbal formulation, a
precise dose response like that of modern drugs may not be possible to obtain in biological assays for polyherbal formulations.

Conclusion

The current studies elucidated the mode of action of Dashmularishta as an antioxidant, antifatigue and immunostimulatory agent utilizing in vitro cell-based assays and in vivo models. Dashmularishta demonstrated strong anti-oxidant potential in ABTS assays. It also prevented oxidative stress induced DNA damage and exhibited prolongation of swim endurance time against forced swim concluding anti-fatigue potential. Dashmularishta was also able to stimulate NK cells mediated lyses/killing of co-cultured target tumor cells (YAC-1). It is noteworthy that the immune-potentiating effects of Dashmularishta in immune cell population were observed at non-cytotoxic concentrations, ignoring any possible interference of loss of viability with functional activity. Results of both the in vitro and in vivo studies on antifatigue potential suggested that Dashmularishta in daily usage may increase the strength and stamina. The molecular mechanism behind these observations can be elucidated.

References