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Anubhuti Pasrija
Dabur India Ltd., Site IV, Sahibabad-Ghaziabad, Uttar Pradesh, India

Harvinder Popli
Delhi Pharmaceutical Sciences and Research University, Mehrauli-Badarpur Road, Sector 3, Pushp Vihar, New Delhi, Delhi, India

Ranjan Mitra
Dabur India Ltd., Site IV, Sahibabad-Ghaziabad, Uttar Pradesh, India

Chhavi Singla
Department of Pharmacy, School of Health Sciences, Sushant University erstwhile, Ansal University, Gurugram, Haryana, India

Corresponding Author:
Anubhuti Pasrija
Dabur India Ltd., Site IV, Sahibabad-Ghaziabad, Uttar Pradesh, India

In vitro anti-ageing activity of Brihat Panchamoola species

Anubhuti Pasrija, Harvinder Popli, Ranjan Mitra and Chhavi Singla

Abstract
Senescence is a cellular response that is characterized by a stable growth arrest that limits the proliferation of aged or damaged cells. There are several biomarkers, which are associated with the cellular senescence. B-Galactosidase is one such biomarker which is widely used to detect cellular senescence *in vitro*. This marker is present in senescent cells only and is a reliable marker for senescent cell detection. Brihat Panchamool is a part of Dashmoola herb which is a combination of ten ayurvedic herbs i.e. Bilva, Patala, Agnimantha, Shyonaka, Gambhari, Bruhati, Kantakari, Prishniparni, Shalparni, Gokshura, where part used are moola of all the drugs in a group. The first five herbs of Dashmoola are collectively called Brihat Panchamoola which is used traditionally in various ayurvedic formulations like Dashmolarishta, Dashmool ghritam, chywanprash, Mahanarayan Tail etc. The present study has been undertaken with a view to establish the *in vitro* anti-ageing activity of young roots and bark of Brihat Panchamoola species. The aqueous extracts of roots and bark showed profound anti-ageing potential.

Keywords: *Aegle marmelos* (L.) Correa, β-galactosidase, Brihatpanchmool, *Clerodendrum phlomidis* L.f., *Dashmool*, *Gmelina arborea* Roxb., *Oroxylum indicum* (L.) Benth. Ex. Kurz., *Stereospermum chelonoides* (L.f.) DC

1. Introduction
Combination drugs or Fixed Dosage Combinations (FDCs) is a concept propounded in Ayurvedic tradition of Pharmaceutics. Accordingly, a selected group of botanical species are combined in a fixed proportion (mostly in equal quantities). Brihat panchamool constitutes an important part of Dashmoola which comprises of a combination of 10 herbs. Dashamoola as the name suggests, is a fixed dose combination described in Ayurveda and contains roots of ten different plants. Of these, first five species are known as *brihad panchamoola* and the remaining as *laghoo panchamoola* [1]. These species are mentioned individually in Charakh Samhita and their grouping into Laghu and Brihat Panchamoola was done by Sushrita Samhita for the first time. It is used in the form of *kwath* or *arishta* according to Ayurveda. These formulations are used by the Ayurvedic practitioners in various conditions for headache, relief of pain and swelling related to arthritis, pyrexia, abdominal distension and costo-chondral pains. Dashmool consists of two categories as Brihat panchamool which includes *Aegle marmelos, Clerodendrum phlomidis, Oroxylum indicum, Stereospermum suveolens, Gmelia arborea* while Laghu panchmul includes; *Solanum indicum, Solanum xanthocarpum, Uraria picta, Desmodium gangeticum and Tribulus terrestris* [2].
P1ants of Brihatpanchmoo1a species are under threat due to extensive use of roots and bark in herba1 formu1ations and requires an obvious necessity to re- examine the fixed-dose-combination used in Ayurveda fo11owing suitab1e scientific approach [3]. The roots of the p1ant are co11ected by destructive method which reduces the opportunity for rejuvenation and affects p1ant demography. A number of possib1e strategies are proposed from time to time for p1ants under-threat to curtai1 overharvesting, among which the most promising is the use of renewab1e p1ant parts as a1ternative to bark, roots and rhizomes for medicina1 purposes. Dashmoola as a whole and the individual species of Brihat Panchamoola are good source of phenolic compounds and antioxidants and play a role as anti-ageing drugs, however very little scientific studies are available which substantiate their use as anti-ageing drugs. The present study therefore, proposes to screen aqueous extracts of Brihat Panchamoola species using '*in-vitro'* testing protocols. β-Galactosidase is a biomarker which is widely used to detect cellular senescence *in vitro*. This marker is present in senescent cells only and is a reliable marker for senescent cell detection [4-6].

2. Material and Methods

2.1 Collection and Identification of raw materials

Bark and young root samples of Brihat Panchamoola species (*Aegle marmelos* (L.) Correa, *Clerodendrum phlomidis* L.f., *Oroxylum indicum* (L.) Benth. Ex. Kurz., *Stereospermum chelonoides* (L.f.) DC and *Gmelina arborea* Roxb.) were received from the Bioresource Development Group, Dabur Research & Development Centre against Voucher Nos. DRDC-1258-BRD/AM, DRDC-1259-BRD/CP, DRDC-1260-BRD/SS, DRDC-1261-BRD/GA and DRDC-1262-BRD/OI). The plant materials were identified by Dr. G.P. Kimothi & Dr. C.S. Rana, Taxonomist, Dabur Research & Development Centre, Sahibabad, Ghaziabad. A voucher specimen has been retained in the department for future reference.

2.2 Chemicals and Reagents

The list of chemicals, reagents and the details of media used in the study are mentioned in Table 1.

Table 1: List of Chemicals and Reagents

Name of Chemical/ Kits/Consumables	Manufacturer	Catalog No./ Product No.	Batch/Lot No.
Antibiotics solution (Penicillin-Streptomycin)	Himedia	A001	0000370232
DMSO	Fischer Scientific	23125	2327521017
DMEM	Himedia	AL007A	0000403969
EDTA	Sigma	E5134	SLBF7702V
FBS	Gibco	16000-044	2101161
Cellular Senescence Kit	Enzo Lifescience	ENZ-KIT 129-0210	01161902
MTT	Sigma	M2128	MKBZ4723V

2.3 Experimental procedure

2.3.1 Culture and Maintenance of Cell Lines

HFF-1 cell line was maintained under suitable conditions. Cells were sub-cultured by trypsinisation, followed by splitting the cell suspension into fresh flasks and supplementing with fresh cell growth medium.

2.3.2 Formulation preparation of positive control

Resveratrol was reconstituted in DMSO to achieve stock concentration of 50mM.

The above stock solution was further diluted in serum free DMEM to achieve concentrations ranging from 1µM-100µM.

2.3.3 Preparation of extracts

Stock solutions of aqueous extracts of samples were prepared in DMEM at concentration of 100 mg/ml. The above stock solution for each TIs was further diluted in serum free DMEM to obtain concentrations ranging from 0.01µg/ml-250µg/ml

2.3.4 Determination of non-cytotoxic concentrations of extracts

Cells were trypsinized and a single cell suspension of HFF-1 cells was prepared. Cells were counted on a hemocytometer.

Cells were seeded at a density of 10,000 cells/well/180µl in DMEM + 15% FBS in 96-well plates and incubated in CO_2 incubator at 37 °C, 5% CO_2 and 95% humidity for 24 h. After 24 h, following cells were treated as follows:

For extract group, 180 µl of DMEM was added to wells. 20µl of Test item was added from the respective 10X stock solutions to achieve final concentrations ranging from 0.01µg-250µg. For Positive Control group, 180 µl of DMEM was added to wells. 20 µl of Resveratrol was added from the respective 10X stock solutions.

For Control group, 200µl of DMEM was added to wells. After incubation for 48h, the effect of extracts on cell viability was assessed by 3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

20 µL of 5 mg/ml of MTT was added to all the wells and incubated at 37 °C for 3h.

The supernatant was aspirated and 150 µL of DMSO was added to all wells to dissolve formazan crystals. The O.D. of each well was read at 540 nm using Biotek Reader.

Effect of extracts on viability of RAW264.7 cells was determined as

% Cell viability = 100-% Cytotoxicity

Where

% Cytotoxicity = (A-B/A) * 100

A = O.D. of Control cells, B = O.D. of cells treated with TI

Concentrations resulting in ≥70% cell viability were selected as safe/non-cytotoxic for further marker assays.

2.4 Estimation of B-Galactosidase activity

HFF-1 cells were counted using hemocytometer and plated in 12 well plates at the density of 50,000 cells/well in DMEM supplemented with 15% FBS. Cells were incubated in CO_2 incubator at 37 °C, 5% CO_2 and 95% humidity for 24 h. Cells were treated with Test Items at non-cytotoxic concentrations and incubated in CO_2 incubator for 24 h. After 24 h, treated cells were exposed to 400µM of H_2O_2 (oxidative damage) for 24h.

After 24h, Cell lysates were prepared and stored at -80 ˚C.

Levels of SA-β-Gal in supernatants were estimated at non-cytotoxic concentrations ranging from 0.01µg/ml-250µg/ml using ELISA as per manufacturer's instructions.

3. Results and Discussion

3.1 Non-cytotoxic concentration of test items

HFF-1 cells were treated with 13 extract samples in the concentration range of 0.01µg/ml-250µg/ml. The effect of extracts on cellular viability was determined after 48 h of treatment. Table-2 demonstrates the effect of TIs on viability of HFF-1 cells.

TIs (1.5 yr Mix, Bael 1yr, Padam Stem Bark, Agnimantha 1.5yr, Sona Chal 1yr, Bael Stem Bark, Gambhar Stem, Gambhar 6M, Sona Chal Mature, Padal 1.5yr) in the concentration range of 0.01µg/ml-250µg/ml demonstrated >70% cell viability as compared to Control cells. Hence this concentration range was considered non-cytotoxic and selected for further assay.

TIs (Mature Mix, Agnimantha Mature, Bael 2yr) in the concentration range of 0.01µg/ml-50µg/ml demonstrated >60% cell viability as compared to Control cells. Hence this concentration range was considered non-cytotoxic and selected for further assay.

Positive Control (Resveratrol) in the concentration range of 1µM-100µM demonstrated >70% cell viability as compared to control.

Table 2: Effect of TIs on viability of HFF-1 cells

Sample	Conc.	% Cytotoxicity	%Viability	Sample	Conc.	% Cytotoxicity	%Viability
Untreated		0	100	Untreated		0	100
DRDC-07 (Sona Chal 1yr) (µg/mL)	0.01	22.0	78.0	Resveratrol(PC) (µM)	1	14.6	85.4
	0.1	17.7	82.3		10	13.7	86.3
	10	25.7	74.3		25	19.3	80.7
	100	24.4	75.6		50	28.6	71.4
	250	29.5	70.5		100	26.0	74.0
DRDC-08 (Bael Stem Bark) (µg/mL)	0.01	9.1	90.9	DRDC-01 (Mature Mix) (µg/mL)	0.01	14.9	85.1
	0.1	16.4	83.6		1	10.1	89.9
	10	23.3	76.7		50	15.8	84.2
	100	20.1	79.9		100	46.7	53.3
	250	27.8	72.2		250	48.1	51.9
DRDC-09 (Bael 2yr) (µg/mL)	0.01	12.2	87.8	DRDC-02 (1.5yr Mix) (µg/mL)	0.01	7.4	92.6
	0.1	13.3	86.7		0.1	7.0	93.0
	50	26.8	73.2		10	14.6	85.4
	100	48.6	51.4		100	29.7	70.3
	250	57.9	42.1		250	25.8	74.2
DRDC-10 (Gambhar Stem Bark) (µg/mL)	0.01	14.4	85.6	DRDC-03 (Bael 1yr) (µg/mL)	0.01	13.8	86.2
	0.1	9.4	90.6		0.1	11.8	88.2
	10	13.2	86.8		10	12.4	87.6
	100	17.9	82.1		100	15.5	84.5
	250	19.1	80.9		250	14.6	85.4
DRDC-11 (Gambhar 6M) (µg/mL)	0.01	4.8	95.2	DRDC-04 (Padam stem Bark) (µg/mL)	0.01	18.2	81.8
	0.1	-0.2	100.2		0.1	25.7	74.3
	10	-0.4	100.4		10	26.2	73.8
	100	9.7	90.3		100	27.4	72.6
	250	8.6	91.4		250	24.6	75.4
DRDC-12 (Sona Chal Mature) (µg/mL)	0.01	16.5	83.5	DRDC-05 (Agnimantha 1.5yr) (µg/mL)	0.01	19.9	80.1
	0.1	24.3	75.7		0.1	19.1	80.9
	10	8.5	91.5		10	23.3	76.7
	100	27.6	72.4		100	21.9	78.1
	250	26.6	73.4		250	20.2	79.8
DRDC-13 (Padal 1.5 yr) (µg/mL)	0.01	17.9	82.1	DRDC-06 (Agnimantha Mature) (µg/mL)	0.01	22.8	77.2
	0.1	17.3	82.7		0.1	16.9	83.1
	10	14.1	85.9		10	34.7	65.3
	100	14.7	85.3		100	60.4	39.6
	250	14.0	86.0		250	65.3	34.7

Values represent mean of triplicates. Values highlighted in red indicate cytotoxic doses.

3.2 Effect of test items on B-Galactosidase activity

The β-galactosidase activity/anti-aging activity of 13 TIs in the non-cytotoxic concentration range was assessed in HFF-1 cells. HFF-1 cells were treated with TIs for 24h. After 24 h of incubation with TIs, cells were given oxidative damage (H_2O_2) with 400µM concentration for 20 h. β-Galactosidase Activity was investigated using kit-based assay.

Table 3 and Figure 1 demonstrate the effect of aqueous extracts on anti-aging/β-galactosidase activity.

Mature Mix (Aqueous extracts of bark mixed in 1:1 ratio) (0.01µg/ml-50µg/ml) demonstrated inhibition in β-galactosidase activity by 6.6%-58.9% as compared to damage control cells. 1.5 yr Mix (0.01µg/ml-250µg/ml) demonstrated inhibition in β-galactosidase activity by 42.6%-61.5% as compared to damage control cells. Beal 1 yr Extract (0.01µg/ml-250µg/ml) demonstrated inhibition in β-galactosidase activity by 13.9%-66.6% as compared to damage control cells. Padal Bark Stem (0.01µg/ml-250µg/ml) demonstrated inhibition in β-galactosidase activity by 27.4%-62.0% as compared to damage control cells.

Agni Mantha (1.5 yr) (0.01µg/ml-250µg/ml) demonstrated inhibition in β-galactosidase activity by 3.5%-49.5% as compared to damage control cells. Agni Mantha bark (0.01µg/ml-10µg/ml) demonstrated inhibition in β-galactosidase activity by 12.4%-41.0% as compared to damage control cells. Sona Chal (1 yr) (0.01µg/ml-250µg/ml) demonstrated inhibition in β-galactosidase activity by 1.9%-34.4% as compared to damage control cells. Bael Stem Bark (0.01µg/ml-250µg/ml) demonstrated inhibition in β-galactosidase activity by 30.3%-62.9% as compared to damage control cells. Bael 2yrs root (0.01µg/ml-50µg/ml) demonstrated inhibition in β-galactosidase activity by 10.6%-63.2% as compared to damage control cells.

Gambhar Chal Stem Bark (0.01µg/ml-250µg/ml) demonstrated inhibition in β-galactosidase activity by 26.9%-54.5% as compared to damage control cells. Gambhar 6 Month root (0.01µg/ml-250µg/ml) demonstrated inhibition in β-galactosidase activity by 42.0%-55.0% as compared to damage control cells. Sona Chal Bark (0.01µg/ml-250µg/ml) demonstrated inhibition in β-galactosidase activity by 2.2%-51.8% as compared to damage control cells.

Padal 1.5 yr (0.01µg/ml-250µg/ml) demonstrated inhibition in β-galactosidase activity by 10.8%-45.9% as compared to damage control cells.

Resveratrol (PC) (10µg/ml-100µg/ml) demonstrated inhibition in β-galactosidase activity by 54.6%-60.6% as compared to damage control cells.

As per the data obtained, we can infer that young roots can be equally effective to bark samples of Brihat Panchamoola. Further, young roots can be made available through tissue culture technique and it will be very useful for timely availability of raw materials. The findings of the work can be further explored on a commercial scale.

Table 3: Effect of test items on β-Galactosidase activity in HFF-1 cells

Samples	Concentration	Mean RFU	% Inhibition (wrt H2O2)
Untreated		582	
Damage H_2O_2 (µM)	400 µM	1273	0.0
DRDC-07 (Sona Chal 1yr) (µg/mL)	0.01	1248	1.9
	0.1	835	34.4
	10	926	27.2
	100	875	31.2
	250	941	26.1
DRDC-08 (Bael Stem Bark) (µg/mL)	0.01	887	30.3
	0.1	650	48.9
	10	472	62.9
	100	501	60.6
	250	661	48.1
DRDC-09 (Bael 2yrs root) (µg/mL)	0.01	468	63.2
	0.1	514	59.6
	1	692	45.7
	10	994	21.9
	50	1138	10.6
DRDC-10 (Gambhar Chal Stem Bark) (µg/mL)	0.01	914	28.2
	0.1	931	26.9
	10	715	43.8
	100	724	43.1
	250	579	54.5
DRDC-11 (Gambhar 6M Root) (µg/mL)	0.01	698	45.2
	0.1	696	45.3
	10	639	49.8
	100	573	55.0
	250	738	42.0
DRDC-12 (Sona Chal Mature) (µg/mL)	0.01	613	51.8
	0.1	776	39.1
	10	775	39.1
	100	927	27.2
	250	1244	2.2
DRDC-13 (Padal 1.5 yr) (µg/mL)	0.01	1136	10.8
	0.1	1067	16.2
	10	841	33.9
	100	707	44.4
	250	688	45.9

Samples	Concentration	Mean RFU	% Inhibition (wrt H2O2)
Untreated		582	
Damage H_2O_2 (µM)	400 µM	1273	0.0
Resvaratrol (PC)(µM)	10	563	55.8
	50	578	54.6
	100	502	60.6
DRDC-01 (Mature Mix) (µg/mL)	0.01	682	46.4
	0.1	621	51.2
	1	524	58.9
	10	1142	10.3
	50	1189	6.6
DRDC-02 (1.5yr Mix) (µg/mL)	0.01	730	42.6
	0.1	490	61.5
	10	513	59.7
	100	537	57.8
	250	553	56.5
DRDC-03 (Bael 1yr extract) (µg/mL)	0.01	1095	13.9
	0.1	713	44.0
	10	479	62.4
	100	426	66.6
	250	581	54.3
DRDC-04 (Padal Bark Stem) (µg/mL)	0.01	634	50.2
	0.1	633	50.3
	10	483	62.0
	100	651	48.8
	250	924	27.4
DRDC-05 (Agnimantha 1.5yr) (µg/mL)	0.01	643	49.5
	0.1	801	37.1
	10	887	30.3
	100	1029	19.2
	250	1228	3.5
DRC-06 (Agnimantha Mature roots) (µg/mL)	0.01	751	41.0
	0.1	889	30.1
	1	1115	12.4
	10	1100	13.6
	50	1285	-0.9

Values represent mean of duplicates

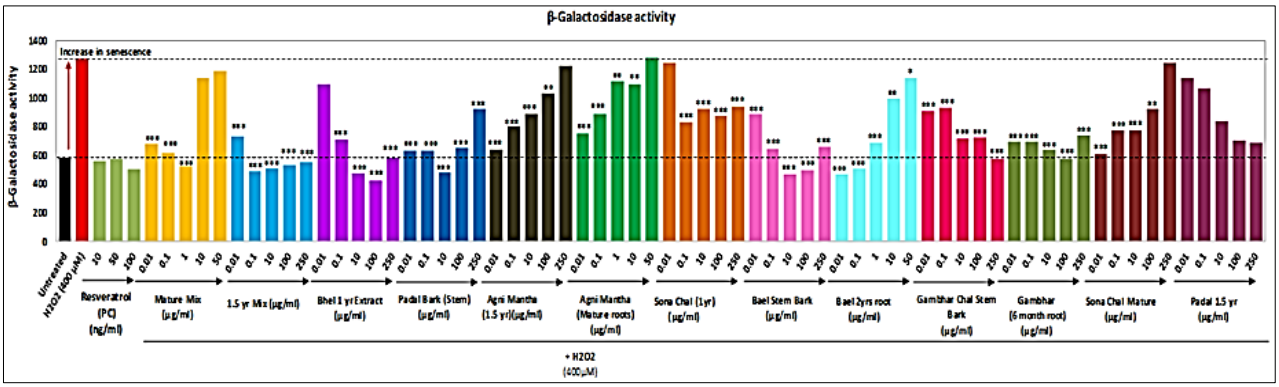

Fig 1: Percentage inhibition in SA- β-Galactosidase activity by test items in skin fibroblast

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