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## DEVELOPMENT OF STANDARDISED ANTI-AGEING HERBAL DRUG FORMULATION

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### ABSTRACT

Ageing is complex biological process, characterized by diminishing or impairment of functional capacity to respond stress. In ageing both intrinsic and extrinsic determinants lead progressively to a loss of structural integrity and physiological function. Oxidative stress play major role in ageing process, varieties of antioxidants are used to reduce the ageing. The aim of the present study is to investigate the anti-ageing activity of formulation made from appropriate standardized extracts of *Withania somnifera*, *Bacopa monniera*, *Tinospora cordifolia*, *Emblica officinalis*. The standardization of extracts were done by HPTLC method. *In-vivo* evaluation of antiageing activity was done by using D-galactose induced ageing model. *In-vitro* evaluation of antioxidant activity of extracts by using DPPH assay method was performed. The levels of malondialdehyde and lipofuscin pigments were increased in D-galactose induced ageing mice. After formulation administration the total protein content is increased in formulation treated group than ageing induced group, it suggest that formulation prevent formation of malondialdehyde and lipofuscin pigment which are indicators of ageing.

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### Key Words

Ageing, standardization,  
malondialdehyde, lipofuscin  
pigment, HPTLC

## INTRODUCTION

Ageing is universal but complex biological process characterized by impairment of various functions and decreased ability to respond stress. The population of world is ageing rapidly<sup>1</sup>. The present study is involved with developmental studies on antiageing activity of polyherbal formulation contains extracts of *Bacopa monniera*, *Withania somnifera*, *Tinospora cordifolia*, *Embllica officinalis*, which mainly act by antioxidant mechanism. *Bacopa monniera* shows antioxidant activity<sup>2</sup> and improves memory and mental function<sup>3</sup>, *Withania somnifera* shows anti stress, antioxidant activity<sup>4,5</sup>, *Tinospora cordifolia* decrease lipid peroxidation, Increase antioxidant enzymes<sup>6</sup>, *Embllica officinalis* is major ingredient of Triphala. Triphala has been reported to posses antiageing properties and improve mental faculties<sup>7</sup>, and show antioxidant activity<sup>8</sup>. Hence, in the present study these drugs were screened for antiageing activity including two parameters, i.e. *In vitro* antioxidant activity and *In vivo* antigeing activity in experimental D-galactose ageing model.

## MATERIAL AND METHODS

### Plant material

The drugs *Bacopa monniera*, roots of *Withania somnifera*, stem of *Tinospora cordifolia*, fruit of *Embllica officinalis* were procured and authenticated from Green pharmacy, Pune.

### Chemicals and reagents

Ethanol, Distilled water, Petroleum ether, Chloroform, Toluene, Acetone, Methanol, Ethyl Acetate, Formic Acid, all solvents used were of analytical grade. 1,1-diphenyl-2-picryl hydrazyl (Merck Ltd., India), Thiobarbituric acid (TBA) - Loba Chemie, Mumbai, India, Trichloroacetic acid (TCA)- E.Merck India Ltd. Mumbai, India, D-galactose- Loba Chemie, Mumbai, India, Quinine sulphate- Loba Chemie, Mumbai, India, Standard protein (bovine serum albumin fraction V) - Loba Chemie, Mumbai, India, Pune, Ascorbic acid (Merck Ltd., India), gallic acid (Fluka (India), Foiln-Ciocalteu's phenol reagent- E. Merck (I) Ltd. Mumbai, India.

### Preparation of extracts

The powdered plant material of *Bacopa monniera* was defatted with petroleum ether and then extracted with ethanol (95%) in a soxhlet apparatus. The extract was greenish black residue<sup>9</sup>. The powdered plant material of *Withania somnifera* extracted with aqueous alcohol (1:1) in soxhlet apparatus at 55<sup>0</sup>c<sup>10</sup>. The dried powder of *Tinospora cordifolia* was soaked in distilled water for 2 hours and boiled for 4 hours, decanted and concentrated<sup>11</sup>. The powdered plant material of *Embllica officinalis* was extracted with distilled water. Polyherbal formulation was prepared by mixing all plant extracts in equal proportion<sup>12</sup>.

### Standardization of the extracts

The *Embllica officinalis*, *Bacopa monniera*, *Withania somnifera* were standardized using standards gallic acid, bacoside A, withaferin A respectively by HPTLC Method. For *Embllica officinalis* standardization is done by method reported<sup>13</sup>. In brief, Toluene: ethyl acetate: methanol: formic acid (3:3:0.2:0.8) was used as mobile phase and scan at 280 nm. For *Bacopa monniera* standardization is done by method reported<sup>14</sup>. In brief, ethyl acetate: methanol: water (6:1.4:1) (v/v/v) was used as the mobile phase and scan at 615 nm after derivatization with vanillin (1 g), ethyl alcohol (95 ml) and sulphuric acid (5 ml), and heating at 110<sup>0</sup> C for 15 min. For *Withania somnifera* standardization is done by method reported<sup>15</sup>. In brief Toluene: ethyl acetate: formic acid (5:5:1) was used as mobile phase and scan at 530 nm after derivatization with vanillin-boric acid-conc. sulphuric acid-methanol (0.5 g-10g-20ml-1,000ml). Linomat IV applicator was used. Densitometry scanning was performed using TLC scanner III (Camag, Switzerland) CATS 4 (CAMAG) software was used for analysis.

### Determination of total phenolic content

The content of the total phenolic in plant extract was determined by Folin Ciocalteu method spectrometrically<sup>16</sup>. To 2 ml of Folin-Ciocalteu's reagent was added to the samples (200,400,600 µg/ml) and mixed thoroughly. To the mixture distilled water were added and it was made upto 25 mL by adding sodium carbonate (29%) solution. These solutions were kept in dark for 30 min for colour development. After

incubation, the absorbance of this solution was measured at 760nm using UV Spectrophotometer. Gallic acid was used as a standard. Total polyphenolic content of the extract as Gallic acid equivalent (GAE) was calculated.

### Evaluation of Antiageing activity

#### *In-vitro* Antioxidant Activity

The scavenging activity of the extracts on DPPH radicals was assayed according to the method described<sup>16,17</sup>. One millilitre of aqueous solution containing various concentrations of the extract (100–500 µg/ml) were mixed with 5ml of 0.2mM DPPH in methanol. Absorbance at 517 nm was determined every 30 s for 6 min after adding the extract. Ascorbic acid was used as the standard. The DPPH radical scavenging activity of the test substance was calculated.

#### *In-vivo* Antiageing activity

**Animal Experimentation:** Male Swiss albino mice (40 g) were used. Animals were housed 6 per cage; they were allowed free access to water and food ad libitum, and maintained at a constant temperature (23±1 °C) as well as humidity (60±10%), and under a 12 h light/dark cycle. The protocol for experimentation was approved by Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Pune, India (Approval no.CPCSEA/28/2010) Constituted under Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), India (CPCSEA/100/1999). The mice were divided into following eight groups of six animal each

Control GroupI: Animal were injected subcutaneously with 0.5ml sterile water/day/animal for 20 days. Standard GroupII: The mice were injected (s.c) with 5% D-galactose, and vitamin C (500mg/kg) (p.o) for 20 days. Ageing induced GroupIII: The mice were injected (s.c) with 5% D-galactose, 0.5ml/day/animal for 20 days. Polyherbal Formulation (PHF) co-treated GroupIV: The mice were injected (s.c) with 5% D-galactose, along with formulation (500 mg/kg), orally for 20 days. PHF co-treated Group V: The mice were injected (s.c) with 5% D-galactose, along with formulation (750 mg/kg), orally for 20 days. Natural recovery GroupVI: This group

injected of 5% D-galactose for 20 days and then they were kept without any dose of formulation for next 20 days. PHF recovery GroupVII: The mice were injected with 5% D-galactose for 20 days and then they were injected with formulation (500mg/kg) for next 20 days. PHF recovery GroupVIII: The mice were injected with 5% D-galactose for 20 days and then they were injected with formulation (750mg/kg) for next 20 days. The change in body weight was recorded daily. All the animals were sacrificed on the next day of last treatment by cervical dislocation. Different organs like prostate and liver were dissected out and weighed. The prostate and liver tissue was homogenized by using mixture containing 75 mM phosphate buffer (PH 7.04), 1 mM ascorbic acid, 1mM ferric chloride and used for biochemical investigation<sup>18</sup>.

i. Lipid peroxidation was studied by Will's method<sup>19</sup> in which the thiobarbituric acid reactive substance (TBARS) i.e. malondialdehyde (MDA) is measured in the formed of red coloured malondialdehyde-TBA complex colorimetrically at 532 nm. Lipid peroxidation was measured in the form of n mol MDA/mg wet of tissue and The percentage of anti-lipid peroxidation effect (% ALP) was calculated.

ii. Lipofuscinogenesis was studied by method of Dillard and Tappel<sup>20</sup>. The prostate and liver tissues was homogenized by using mixture prepared earlier for lipid peroxidation. The extraction is carried out by addition of chloroform:methanol (2:1 v/v) to 0.5ml tissue homogenized sample. The fluorescence was measured on photoflurometer calibrated with quinine sulphate. 1µg of quinine sulphate/ml of 0.1 N H<sub>2</sub>SO<sub>4</sub> was used as standard and 0.1N H<sub>2</sub>SO<sub>4</sub> was used as blank.

iii. The total protein was estimated by Lowry's method<sup>21</sup>. Homogenized sample of liver and prostate was used. To 0.2 ml of sample or standard 1ml of freshly prepared complex reagent was added, stand at room temperature for 10 min. Then 0.1 ml of diluted Folin reagent was added, using a vortex mixer and solution was allowed to stand at room temperature for 30-60 min. Different concentrations of standard protein (Bovine serum albumin) were taken. Color developed was read at 750 nm.

**Statistical analysis**

The results are expressed as mean ± SEM. Comparisons between the groups were performed by one way analysis of variance (ANOVA) followed by Post hoc Tukey’s test. In all tests the criteria for statistical

significance was  $p < 0.05$ , very significance  $p < 0.01$ ,  $p < 0.001$

**RESULTS**

**Standardization**

**Table 1:** It shows HPTLC profile of standard and sample of plant extracts

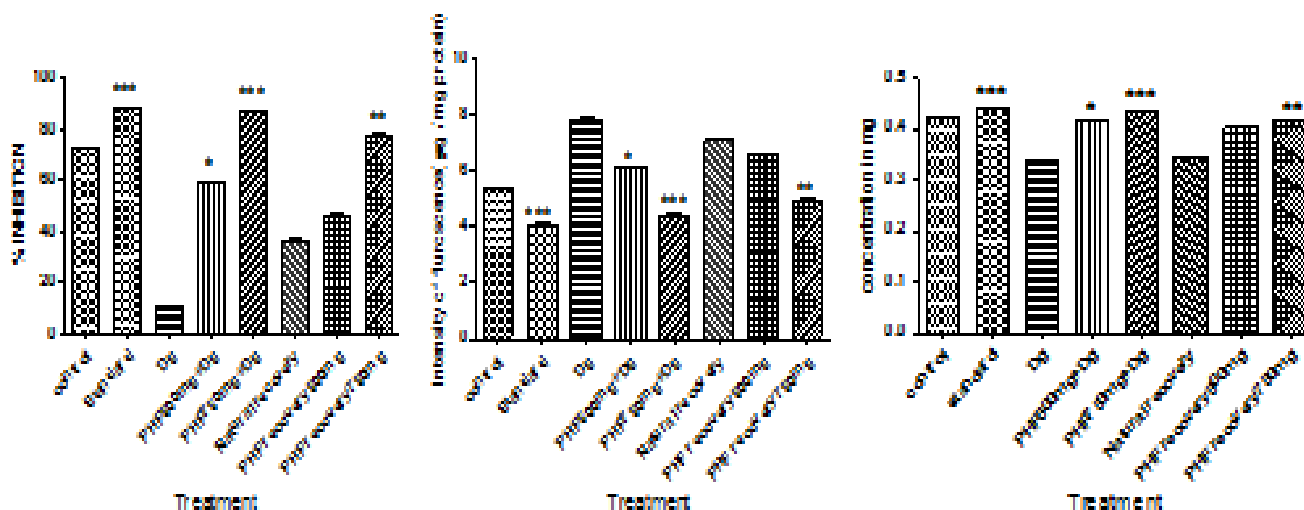
Plant extracts	UV absorbance	Rf value	
		Standard	Sample
<i>Embllica officinalis</i>	280	0.56	0.55
<i>Bacopa monniera</i>	615	0.53	0.52
<i>Withania somnifera</i>	530	0.33	0.22

**Biochemical estimation in mice liver and prostate tissues**

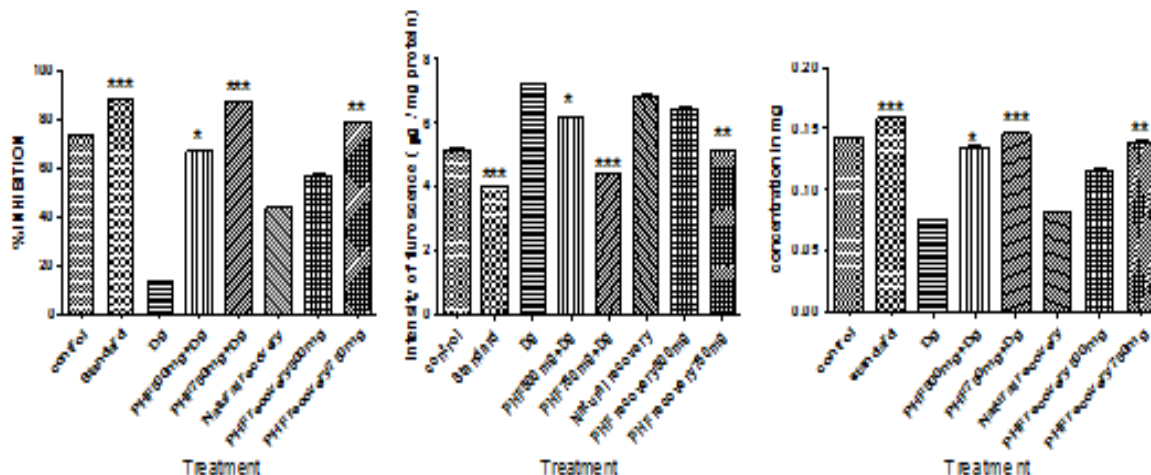
The results of biochemical estimation in liver and prostate tissues of animals exhibited by the test groups are shown in Fig.1&2 Lipid peroxidation ,which indicates endogenous MDA levels, was found to be increased in the of D-galactose treated mice as compared to standard, on the other hand it is decreased in PHF treated group as compared to D-galactose induced mice. In natural recovery group MDA concentration increased as compared to the PHF

recovery group. Intensity of fluorescence indicated that lipofuscinogenesis was increased in aging induced mice than the standard,where as it was reduced due to PHF treatment. The decrease in lipofuscins in PHF recovery group was higher than the natural recovery group. Also the total protein content is increased PHF treated group.The PHF recovery group show good result than the natural recovery group. Present PHF was compared with standard and formulation at 750 mg/kg was found to be more effective.

**Fig 1.** It shows effect of polyherbal formulation on lipid peroxidation, lipofuscinogenesis and total protein content in liver



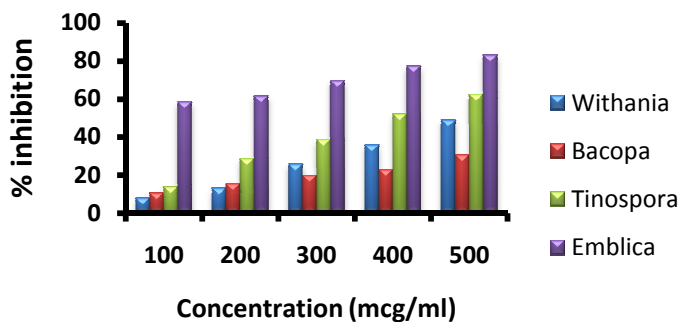
**Fig 2.** It shows effect of polyherbal formulation on lipid peroxidation, lipofuscinogenesis and total protein content in prostate



**In-vitro evaluation of Antioxidant activity**

The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The significant DPPH scavenging activity of *Emblica officinalis*, *Bacopa monniera*, *Withania somnifera* was shown in fig 3. at 500 µg/ml when compared to standard drug Ascorbic acid (79.32%), which is expressed as percentage of scavenging effect, it plays very important role in prevention of oxidation of lipids.

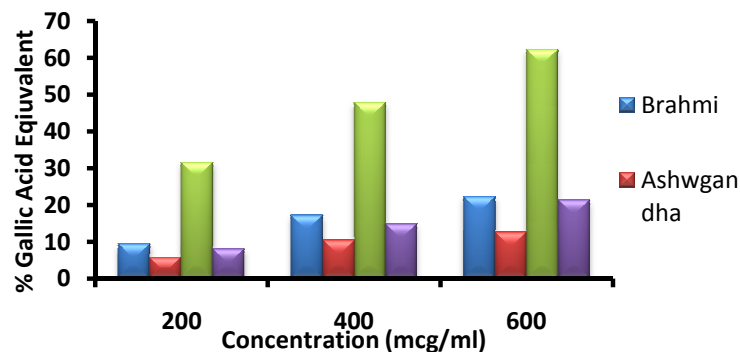
**Fig 3.** It shows DPPH scavenging activity



**Determination of Total phenolic content**

The total polyphenolic content of *B. monniera*, *W. somnifera*, *T. cordifolia* and *E. officinalis* was found to be 22.36%,12.59%,21.41% and 62.27 % respectively( Fig.4)

**Fig 4.**It shows total Phenolic Determination



**DISCUSSION**

Traditionally, Rasayana drugs are used against a diverse disorders. ‘Rasayana’ drugs exert their therapeutic actions by their ability to scavenge free radicals or by their antioxidant potential .In the present study four herbal plants included in rasayana namely, *Withania somnifera*, *Bacopa monniera*, *Tinospora cordifolia* and *Emblica officinalis* extracts show potent activity against ageing<sup>22</sup>. The lipofuscin (age pigment) accumulates slowly, universally & specifically in lysosomes. The composition of lipofuscin is nearly half protein, one-third carbohydrate & rest of lipid indicates that it is primarily composed of advanced glycation end product (AGE) rather than lipid peroxidation product <sup>23</sup>. Oxidative stress has been shown to promote lipofuscin formation whereas antioxidant reduced it.

**ACKNOWLEDGMENT**

The author sincerely acknowledge Bharati Vidyapeeth Deemed University, Poona College of Pharmacy, Pune, India, for infrastructural facilities.

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