Original Article

Neuroprotective Effect of Petroleum Ether, Methanolic and Aqueous Extracts of Fruits of *Benincasa hispida* on Lipofuscinogenesis and Fluorescence Product in Brain of D-galactose Induced Aging Accelerated Mice

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ABSTRACT

Background: Aging is one of the major factors of development of neurodegenerative disorder and also involved in gradual learning and memory loss. In recent years discovery of antiaging drugs is one of the important topic of research. Many plant drugs proved to be reliable treatment for aging associated changes. **Aim:** The present investigation was aimed at determining the neuroprotective effect of petroleum ether (BHP), Methanolic (BHM) and aqueous extract (BHA) obtained from *Benincasa hispida* by the measurement of fluorescence product and biochemical parameter like lipid peroxidation, catalase activity and glutathione peroxides activity in the brains of D-galactose induced aging accelerated female albino mice. **Methods:** D-galactose administration is a well-known model to accelerate normal aging. D-galactose (0.5 ml 5%) was administered for 15 days and accumulation of fluorescence product and lipofuscin granule in cerebral cortex was evaluated. Moreover lipid peroxidation activity and the antioxidant enzymes like glutathione peroxides and catalase activity in cerebral cortex were also evaluated. Furthermore brains of mice subjected to histopathology studies for evaluation of lipofuscin granules. **Results:** Co-treatment of D-galactose with BHP, BIM and BIA (100 mg/kg, po) reduced the fluorescence product accumulated in the cerebral cortex. BIM and BHA was protective against D-galatose-induced accelerated aging by reducing lipid peroxidation and restoring the enzymatic activity of glutathione peroxidase and catalase. Moreover BIM was also showed decreased accumulation of lipofuscin granules. **Conclusion:** In conclusion, BIM and BIA were found to be an effective neuroprotective reagent which could reverse D-galactose-induced oxidative damage and acceleration of aging.

Key words: Neuroprotection, Aging accelerattion, Fluorescence product, Lipid peroxidation, Lipofuscin granules, *Benincasa hispida*.

INTRODUCTION

Aging is natural phenomenon which is always associated with diverse chronic diseases, including neurodegenerative disorders, cancer and cardiovascular diseases.[1,2] Aging also associated with gradual, incremental loss of cognitive and motor performances. Moreover normal life of patient is significantly affected by rapid cognitive decline, due to age dependent neurological disorders.[3] With the increasing elderly population in the world, aging has already become an important public issue.[4] Recent reports indicate that the chronic administration of D-galactose is responsible for aging acceleration and also influenced the age related cognitive decline in mice.[5,6] D-galactose causes the formation of advanced glycation end-products (AGEs)

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in vivo, resulting in oxidative stress which in turn leads to accumulation of Reactive Oxygen Species (ROS) and stimulation of free radical production.[7] Repeated injection of D-galactose in animals is known to cause alterations in biochemical markers, loss in propagating ability, retrograde changes in neural cells and memory impairments which are nothing but the aging like symptoms.[8,9] The D-galactose induced excessive formation of ROS followed by neuronal damage, chiefly manifested as decline in learning and memory capacity is widely cited model of neurodegeneration associated with aging acceleration.[10] The oxygen metabolism of D-galactose produces many ROS, which may result in direct or indirect impairment of learning and memory.[9] Various *in vivo* and *in vitro* studies have reported that, D-galactose is a reducing sugar that readily reacts with the free amine groups of neuronal proteins and neuropeptides to form advanced glycation end-products (AGEs). These AGEs undergo chemical oxidation and degradation via AGE-receptor binding and activation of signaling pathways to form free radicals and cause oxidative stress. In addition, the glycated protein produces 50 fold more free radicals than non glycated protein at the physiological pH, which in turn contribute to increase oxidative stress and thereby damage the micromolecules and cell organelles particularly the mitochondria.[10] These affect the mitochondrial functioning causing less production of the ATP and more production of the free radicals, leading to an extra burden on lysosomes for the degradation of autophagocytosed and damaged micromolecules and mitochondria.[11]

Though there is continuous advancement in current pharmacotherapy for the neurodegenerative disorders still there is lack of effective and widely applicable pharmacological treatments which may explain a growing interest in the traditional medicines.[12] The use of medicines from plant sources has increased globally due to their lower adverse effects, price and good efficacy in the majority of human illnesses.[13,14] *Benincasa hispida* (BH) is used as food in different part of India and other tropical countries and belongs to the family Cucurbitaceae.[15] In Ayurveda, BH is recommended for treatment of peptic ulcer, hemorrhages from internal organs, epilepsy and other nervous disorders.[16,17] BH was also reported for its histamine release inhibition activity in the rat exudates cell, where histamine release was induced by antigen-antibody reaction.[18] Alcoholic and petroleum ether extract of BH exhibited antiulcerogenic effect. BH probably has a CNS component in prevention of stress induced ulceration.[19] Aqueous extract of seeds of BH also showed immunopotentiator activity. The juice of BH was found to be effective against morphine withdrawal symptoms.[20] Researcher also reported anxiolytic activity of BH.[21] In our earlier reports BH showed inhibition of locomotor activity, nootropic, anxiolytic and analgesic activity. Moreover BH also showed potentiation of haloperidol induced catalepsy.[22]

The present investigation was aimed at determining the neuroprotective activity of petroleum ether (BHP), Methanolic (BHM) and aqueous extract (BHA) obtained from *Benincasa hispida* by the measurement of fluorescence product and biochemical parameter like lipid peroxidation, catalase activity and glutathione peroxides activity in the brains of D-galactose induced aging accelerated female albino mice.

MATERIALS AND METHODS

Plant material

The plant material (fruits of *Benincasa hispida*) was collected from Pune region of Maharashtra, India and was authenticated by botanical survey of India.

Preparation and standardization of Extract

Petroleum ether extract (BHP), methanolic extract (BHM) and aqueous extracts (BHA) were prepared by succsessive extraction method. The fruit pulp of BH were dried in shade and coarsely powdered. The powder was successively extracted with petroleum ether followed by methanol in a soxhlet apparatus. Powder remaining after methanolic extraction was subjected to aqueous extraction.[23] The aqueous extract was prepared by maceration with distilled water for 24 hr. The extracts were concentrated under reduced pressure and were stored at 8–10°C throughout the study. The yield of BHP, BHM and BHA were 4.2% w/w, 3.2% w/w and 5.1% w/w respectively.

Preliminary thin layer chromatography study was carried out for checking the presence of phyto-constituents such as beta-sitosterol, lupeol, isovitexin, isomultiflorinol, cucurbitacin and other amino acid etc. which have been reported to present in BH. Further quantitative estimation of phyto-constituents using high pressure thin layer chromatography (HPTLC) fingerprinting was also carried out using RP-18 silica as follows.[24]

Betasitosterol in BHP, Petroleum ether: Acetonitrile: Methanol (1:2:2)

Lupeol in BHP, Benzene: Ethyl acetate (9.5: 0.5)

Isovitexin in BHM, Ethyl acetate: n-butanol: Water (2:1:3)

Chemicals and drugs

D-galactose, 5, 5-dithiobisnitrobenzoic acid (Loba chemicals, Mumbai, India), thiobarbituric acid,

trichloroacetic acid, ascorbate (SD Fine Chemical, Mumbai, India) and glutathione (Ozone, Mumbai, India) were used.

Animals

Six months old female Swiss albino mice (18-22g) certified as healthy by a veterinary physician were used. The female mice were reported to be ideal for D-galactose model and are more susceptible towards D-galactose induced changes in brain as compared to male mice.[10] These mice were maintained at 25°C ± 2°C and 45-55% RH and under standard environmental conditions (12:12 h L:D cycle). These mice had free access to food and water. Institutional Animal Ethics Committee (IAEC) of Marathwada Mitra Mandals College of pharmacy has approved the protocol (CPCSEA/IAEC/PC-10/12) and entire study has carried out as per standard guideline of IAEC.

Acute toxicity test

Mice were subjected to acute oral toxicity study as per guidelines suggested by the Organization for Economic Co-operation and Development.[25] The mice were observed continuously for 2 hr for behavioral and autonomic profiles and for any sign of toxicity or mortality up to 7 days.

D-galactose treatment

60 female albino mice were divided in to five groups of 12 each. First group served as control and received 0.5 ml saline per day for 15 days. Second group mice were injected with 0.5 ml 5% D-galactose (sc) per day for 15 days as served as aging accelerated group. The mice of third, fourth and fifth group were injected (sc) with a daily doses of 0.5 ml 5% D-galactose plus BHP, BHM and BHA 100mg/kg (po). The dose was selected from our previous neurobehavioral study of BH.[22] On 15th day, 60 min after the doses of BIP, BIM and BIA and D-galactose mice were sacrificed. From each group six mice were subjected for biochemical estimation of lipofuscin content, lipid peroxidation, glutathione peroxides and catalase activity. Remaining six mice were subjected to histopathological examination.[5,6,10]

Measurement of fluorescence

Lipofuscin contents from whole cerebral cortex were extracted in chloroform: methanol mixture (2:1 v/v) and fluorescence was measured on photofluorometer using 1µg of quinine sulfate/ml of 0.1N sulfuric acid as standard and 0.1N sulfuric acid as blank.[5,6,10]

Antioxidant activity

As a measure of lipid peroxidation, malonaldialdehyde (MDA) levels was estimated according to method of Kakkar *et al.*[26] by measuring thiobarbituric acid reactive substances (TBARS). Initially the color developed was read at 532 nm using spectrophotometer and expressed as nM/mg of protein.

Activities of glutathione peroxidase (GPx) was estimated;[27-29] the color developed was read at 420 nm using spectrophotometer and expressed as U/mg of protein.

The activity of catalase (CAT) was measured according to the method of Claiborne (1991)[30] and the protein content of brain tissue was estimated by following the method of Lowry *et al.*[31]

Histopathological examination

The mice were sacrificed by decapitation and brains were taken out. Individual entire brain was fixed in neutral buffered 10% formalin for 24 hr. Paraffin blocks were prepared by microtechnique and saggital sections of 5µm forebrain were prepared using rotary microtome (INCO, Ambala, India). This section then stained with ZeilNeelson Carbolfuscin method and microscopical examination was carried out under 40x lens.[32]

Statistical analysis

The data were expressed as mean ± SE. One way ANOVA followed by Tukey's *post hoc* test was performed by using GraphPadInStat version 3.01,32, GraphPad Software, San Diego California, USA.

RESULTS

Phytochemical Analysis

Presence of beta-sitosterol and lupeol was found in thin layer chromatographic study of BHP while presence of isovitexin was found in BHM.

The 6.18 % w/w of beta-sitosterol and 2.23 % of lupeol was found in HPTLC study of BHP. The level of isovitexin in BHM was found to be 0.78 %.

Acute toxicity test

BHP, BHM and BHA was found safe up to the dose of 2 gm/kg. All three extract reported non-significant sedation above the dose of 500 mg/kg.

Fluorescence content

The biochemical extraction of fluorescence product in vehicle treated control mice and 15 days D galactose

treated (ageing accelerated) mice was 0.45±0.008 and 1.29±0.022 ug/mg of protein respectively and there by showed significant (P< 0.001) increase in the fluorescence product in D galactose treated mice. While in BHP, BHM and BHA co-treated ageing accelerated mice it were 0.992 ± 0.029, 0.88±0.01 and 0.56±0.03ug/mg of protein (Table 1).

Assay of lipid peroxidation

The MDA levels in cerebral cortex of vehicle treated control mice and 15 days D galactose treated (ageing accelerated) mice was 1.49 ±0.02nM/mg of protein and 2.19±0.04 nM/mg of protein respectively and there by showed significant (P<0.001) increase in MDA levels of ageing accelerated mice. In BHP, BHM and BHA co-treated mice the MDA levels was significantly (P<0.01) decreased to 1.876±0.078, 1.59.±0.05 and 1.8197±0.03nM/mg of protein as compare to ageing accelerated mice (Table 1).

Glutathione peroxidase activity

The GPx activity levels in cerebral cortex of control mice were found to be 31.83±1.47 mU/mg of protein. In ageing accelerated mice GPx levels was significantly (P<0.001) decreased to 25.35±0.92 mU/mg of protein. While significant (P<0.01) attenuation in GPx levels was found in BHP, BHM and BHA co-treated mice as compare to ageing accelerated mice. In BHP, BHM and BHA the GPx activity levels was 27.71 ±0.36, 28.59 ±0.49and 25.18±1.20mU/mg of protein (Table 1).

Catalase activity

A significant (P<0.001) decline in the catalase activity levels in cerebral cortex of ageing accelerated mice was found. In aging accelerated group catalase activity level was 0.51±0.02 umol/min/mg of protein as compare to control mice 0.99±0.06 umol/min/mg of protein. Where as in BHP, BHM and BHA co-treated mice the catalase activity levels was significantly (P<0.01) increased to 0.56±0.01, 0.693±0.02 and0.67±0.01 umol/min/mg of protein as compare to ageing accelerated mice (Table 1).

Histopathology

From histopathology studies it was revealed that, there is increased accumulation of lipofuscin granules in the brain of D-galactose treated aging accelerated group as compared to control (Figure 1 and 2). BHA co-treated group showed decreased accumulation of lipofuscin granules as compared to aging accelerated group (Figure 3). No decline in lipofuscin granules was found in BHP and BHM co-treated group as compared to aging accelerated group (Figure 4 and 5).

DISCUSSION

Neurodegeneration is often age-associated and generally a result of process of aging. Aging is due to decline in

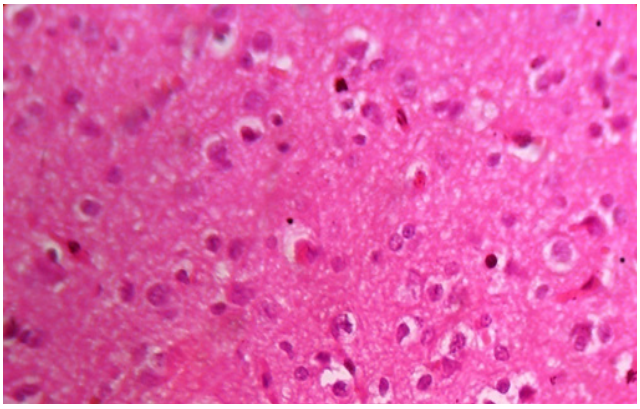

Figure 1: Saggital section of cerebral cortex of control mice demonstrating normal distribution of lipofuscin granules.

Table 1: Effect of BHP, BHM and BHA on fluorescence product, lipid peroxidation, glutathione peroxidase and catalase activity.

SL No	Group	Fluorescence product (ug/mg of protein)	TBARS (nM/mg of protein)	GPx (U/mg of protein)	CAT (µmol/min/mg of protein)
1	Control	0.456 ± 0.008	1.49±0.02	31.83±1.47	0.99±0.06
2	D galactose treated Ageing acc. Group	1.29 ± 0.022***	2.19±0.04*	25.35±0.92*	0.51±0.02*
3	BIP + D-galactose treated (Gr. III)	0.992 ± 0.029	1.876±0.078	27.71±0.36	0.56±0.01
4	BIM + D-galactose treated (Gr. IV)	0.88±0.01#	1.59.±0.05##	28.59±0.49###	0.693±0.02###
5	BIA + D-galactose treated (Gr. V)	0.56 ±0.03##	1.8197±0.03##	25.18±1.20	0.67±0.01###

Results are expressed as mean ± SEM. (n = 6). Data was analysed by one way analysis of variance (ANOVA) followed by Tukey test. *,#P<0.05. **,##P<0.01, ***,###P<0.001. * - Ageing accelerated group against control, #- BH co-treated group against ageing accelerated group.

various biochemical and physiologic functions in most organs which leads to increased susceptibility to age-associated neurodegeneration.[33]

The process of aging is accelerated by two important parameters i.e oxidative stress and reactive oxygen species (ROS). This two parameters are main cause for development of many age linked neurodegenerative diseases such as Alzheimer's and Parkinson's diseases.[34] Many studies reported that, injecting the D-galactose in brains of laboratory animals showed increase in formation of ROS, neuronal damage and a decline in learning and memory capacity.[7,9] Moreover it has been reported that free radicals were increased in D-galactose treated animals.[35,36]

Generation of oxidative stress is main mechanism by which D-Galactose can induce aging. The oxygen metabolism of D-galactose produces many ROS. This ROS then directly or indirectly causes impairment of learning and memory.[9] Advanced glycation end-products (AGEs) are formed due to administration of D- galactose. Since D-galctose is a reducing sugar that reacts readily with the free amine groups of amino acids in proteins and peptides both *in vitro* and *in vivo*. This reaction is mechanism of formation of advanced glycation end-products (AGEs). The chemical oxidation and degradation of AEGs, via AGE-receptor binding and activation of signaling pathways generates free radicals which causes the oxidative stress. Furthermore glycated protein produces fifty fold more free radicals than non glycated protein at the physiological pH. This results in an increased oxidative stress and thereby damages to the micromolecules and cell organelles particularly the mitochondria, which is one of the sites of ROS formation. These affect the mitochondrial functioning causing less production of the ATP and still more production of the free radicals. Which in turn leads to an extra burden on lysosomes for the degradation of autophagocytosed and damaged micromolecules and mitochondria.[5,6,10]

In the present investigation, the administration of D-galactose in mice for 15 days, significantly ($P<0.001$) increased the fluorescence product in cerebral cortex. There was a significant decrease in fluorescence product

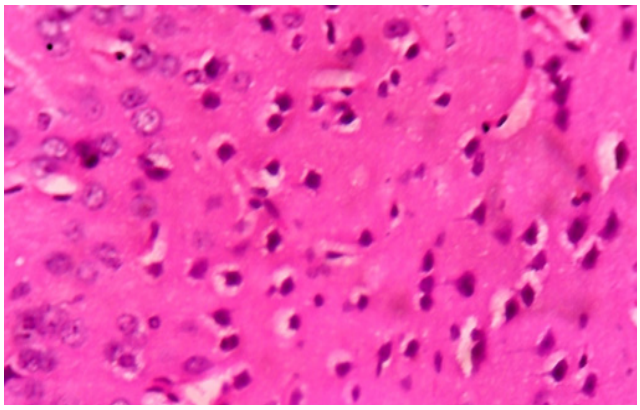

Figure 2: Saggital section of cerebral cortex of age accelerated mice demonstrating increased accumulation of lipofuscin granules.

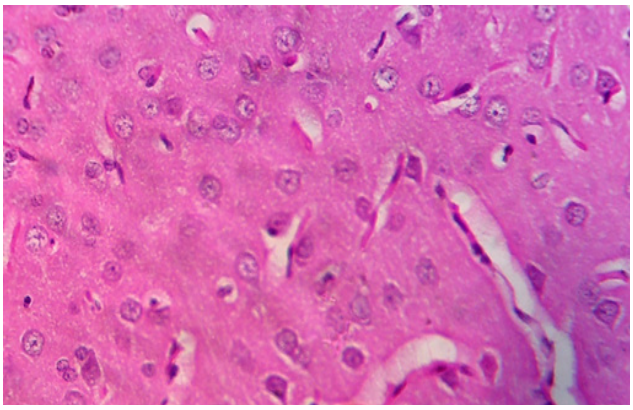

Figure 4: Saggital section of cerebral cortex of BHM cotreated mice.

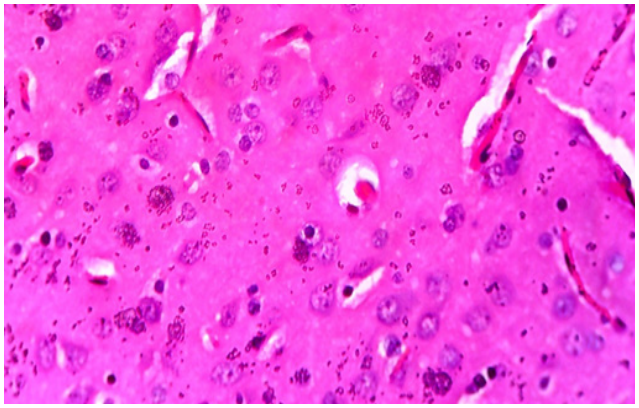

Figure 3: Saggital section of cerebral cortex of BHA cotreated mice demonstrating decreased of lipofuscin granules.

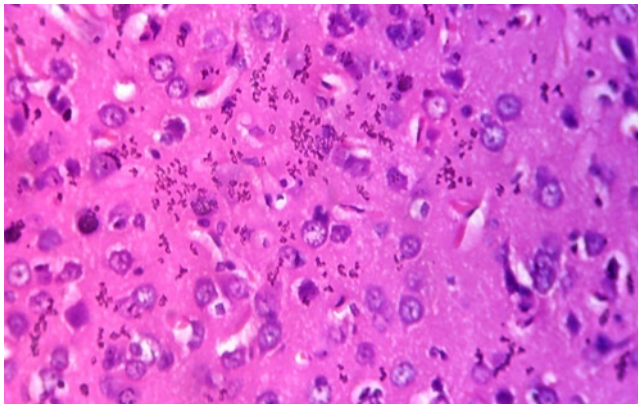

Figure 5: Saggital section of cerebral cortex of BHP cotreated mice.

in cerebral cortex after cotreatment of BHA and BHM. Furthermore, the increased accumulation of lipofuscin granules were observed in brains of D-galactose treated aging accelerated group as compared to control. On contrary in BHM co-treated group, there were decrease accumulations of lipofuscin granules as compared to aging accelerated group.

Incresed level of Malondialdehyde is an indication of increased lipid peroxidation in D galactose treated mice that results due to increased oxidative stress. In BH co-treated mice, the MDA levels was significantly (P<0.01) decreased as compare to ageing accelerated mice and these decreased may be due to inhibition of D galactose induced oxidative damage by BH. The ROS can be scavenged by endogenous antioxidants including GPx and catalase.[37] In this study, the activities of catalase and GPx in the cerebral cortex showed a statistically significant (P<0.001) decline in model group mice compared to control group mice. Treatment with BH for two weeks significantly (P<0.05) improve the activities of GPx and catalase.

Mitochondria causes more and more damage and cross linking of macromolecules by continuous production of free radicals. This includes enzymes and membrane components of lysosomes. Furthermore this process finally result in to an indigestible autofluorescent material commonly known as lipofuscin granules.[11] In present investigation the administration of D galactose in mice for 15 days significantly (P< 0.001) increased the fluorescence product in cerebral cortex. Whereas in BH co-treated animals, significant decrease in fluorescence product in cerebral cortex was found which indicate its effectiveness against aging process.

CONCLUSION

BHM and BHA demonstrated significant neuroprotective activity by decreasing oxidative stress induced by D-galactose administration. The neuroprotective activity is though activation of catalase and glutathione peroxidase and consequently reducing lipid peroxide damage.

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CONFLICT OF INTEREST

The authors declare no conflict of interests. The authors alone are responsible for the writing and contents of the paper.

ABBREVIATIONS

BHP: Petroleum ether extract *Benincasa hispida*; **BHM:** Methanolic extract *Benincasa hispida*; **BHA:** Aqueous extract of *Benincasa hispida*; **ROS:** Reactive Oxygen Species; **AGE:** Advanced glycation end-products; **ATP:** Adenosine triphosphate; **BH:** *Benincasa hispida*; **HPTLC:** High pressure thin layer chromatography; **IAEC:** Institutional Animal Ethics Committee; **MDA:** Malonaldialdehyde; **TBRAS:** Thiobarbituric acid reactive substances; **GPx:** Glutathione peroxidase; **CAT:** Catalase.

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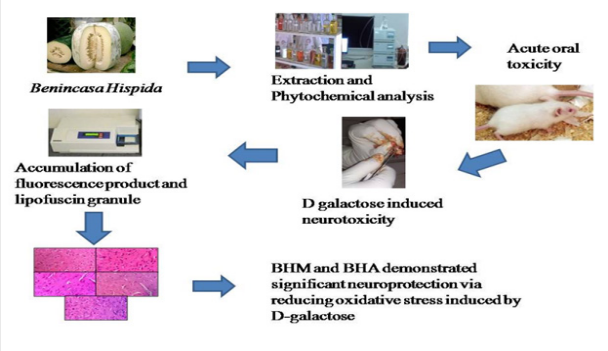
PICTORIAL ABSTRACT



SUMMARY

Aging is a major cause for development of neurodegenerative disorders and loss of memory. Degenerative changes occurring in brain are irreversible. So neuroprotective drugs are ideal treatment for aging associated disorder. Due to unavailability of synthetic neuroprotective drug, research on plant drug is getting the popularity. D galactose induced age acceleration is well studied and validated model in laboratory animals. In our study we found that *Benincasa hispida* showed significant neuroprotective activity against D galactose induced aging acceleration.

About Authors

Dr. Digambar Balkrishna Ambikar is having 13 years of experience of teaching to students of Pharmacy, Medicine, Nursing and Public health. He has 22 publication and 29 presentations to his credit. He received Young talent award by APP, 2019 and Gold medal-2008 to his PG research. He is known for his Pharmacology digital content for MBBS students preparing for various entrance and eligibility examinations.

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