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Protective effects of ethanolic extract of *Piper cubeba* L. on D-galactose induced neuronal lipofuscinogenesis in albino rats

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ABSTRACT

Lipofuscin accumulation has been implicated in post-mitotic cell aging and neurodegenerative diseases like dementia and Alzheimer's. The most characteristic feature of lipofuscin granules is auto-fluorescence. Many allopathic agents are available for treatment of dementia but having limited use. In the present study, the ethanolic extract of fruits of *Piper cubeba* L. belonging to family Piperaceae was evaluated for biochemical and histochemical changes of lipofuscin granules in the hippocampus region of albino rats against D-galactose induced neuronal lipofuscinogenesis. The results of the present study revealed that, dose 400 mg/kg, p.o. of plant extract significantly ($P < 0.001$) decreases lipofuscin fluorescence from the hippocampus region of albino rats when compared with D-galactose treated group. The histochemical observations showed increased accumulation of lipofuscin granules in D-galactose treated group animals whereas *Piper cubeba* co-treated group showed a decreased number of lipofuscin granules in hippocampus region of brain. A reference drug Piracetam 400 mg/kg, i.p. treated group animals also exhibited decreased accumulation of lipofuscin granules. Thus, protective effect of ethanolic extract of *Piper cubeba* on D-galactose induced neuronal lipofuscinogenesis may be due to presence of lignans like cubebin, hinokinin, yatein, isoyatein and their antioxidant property.

Keywords: Antioxidants; Dementia; Hippocampus; Lipofuscinogenesis; *Piper cubeba*.

1. INTRODUCTION

The most common form of dementia is Alzheimer's disease (AD) which is characterized by the loss of neurons primarily in the distinct regions of brain such as hippocampus and cerebral cortex (Prince et al., 2014). It is the fourth most common cause of death in developed nations which estimated that, by 2030 more than 65 million people of the world will be living with dementia (Tariot et al., 1995). Age is the primary risk factor for both AD and dementia. Cognitive impairment and neuropsychiatric symptoms are core syndromes of dementia (Nussbaum and Ellis, 2003). The neurons which present the tendency to loss with age invariably accumulate indigestible autofluorescent substance called lipofuscin granule. The accumulation of lipofuscin granule is associated with a progressive injurious effect on the metabolism of the nerve cells. As a result there is loss of functional ability of the neurons which may lead to age related neurodegenerative diseases like Parkinson's,

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dementia and Alzheimer's (Nakano et al., 1989). Various medicinal plants like *Celastrus paniculatus, Picrorhiza kurroa* (Russo et al., 2001), Ashwagandha (Panda and Kar, 1997), *Ginkgo biloba* (Seif et al., 1995), *Bacopa monniera* L (Deshmukh et al., 2007) containing antioxidants have been proven to possess neuroprotective effect. The medicinal plant *Piper cubeba* L. commonly known as tailed or java pepper belonging to family Piperaceae included as ingredient in various Indian and Chinese traditional remedies. The presence of lignans such as cubebin, hinokinin, yatein and isoyatein (Elfahmi and Batterman, 2007) in extract was proved to possess various pharmacological activities like anti-inflammatory and analgesic (Choi and Hwang, 2005), anti-leishmanial (Bodiwala et al., 2007), anti-proliferative (Yam et al., 2008) and anti-hepatitis C virus (Januario et al., 2002). Thus, based on literature review, the present study has been designed to elucidate the possible role of ethanolic extract of fruits of *Piper cubeba* on D-galactose induced neuronal lipofuscinogenesis.

2. MATERIALS AND METHODS

2.1 Chemicals

D-galactose (CDH, India), Quinine sulfate (Alchem, India), Zeihl Neelson Carbol Fuscin stain (Merk, Germany), Piracetam (Uni-UCB, India). All other reagents and chemicals were of analytical grade. Rotary microtome (Jainco, India), Photofluorometer (Labtronics India), Lyotrap dryer (Rolex, India), Phase contrast microscope (Praga, India).

2.2 Plant material and preparation of extract

The dry fruits of *Piper cubeba* were acquired from local market (Dhorle & Sons Ayurveda, Kolhapur, India) and herbarium was authenticated by Department of Biodiversity and Palaeobiology, Agharkar Research Institute Pune, India (Auth.15-118*)*. The dried fruits were grinded to fine powder (sieve no: 44). The 500 g powder was subjected to extraction with the help of continuous Soxhlet's apparatus using 700 mL ethanol for 48 h. The excess solvent was distilled out and the residue was concentrated by using Lyotrap dryer. The dry extract was stored in air tight container for further use.

2.3 Animals

Female Wistar albino rats of six month old with body weight 180-220 g were used in the present investigation under standard husbandry conditions at an ambient temperature of 25 ± 1°C and 45-55% relative humidity, with 12 h light/dark cycle. The animals had free access to standard pellet (Hindustan Lever Ltd., India) and water *ad libitum*. The study protocol was approved by departmental animal house (MCPL/IAEC/15-16/04).

2.4 Experimental design

Based on previously reported articles, the dose 400 mg/kg, p.o. (post oral) of plant extract (Shakil and Mohammed, 2016), 0.5 ml of 5% aqeous D-galactose (Deshmukh et al., 2006; Song et al., 1999) and Piracetam 400 mg/kg, i.p. (intraperitonial) (Milind and Bansal, 2010) was selected in the present investigation.

Animals were divided into four groups, each containing six animals.

Group-I: Control - injected with 0.5 ml saline per day for 15 days subcutaneously

Group-II: D-galactose treated - injected 0.5 ml of 5% aqeous D-galactose per day for 15 days subcutaneously

Group-III: Piracetam treated - 400 mg/kg, i.p. co-treated with D-galactose for 15 days

Group-IV: Extract treated - 400 mg/kg, p.o. co-treated with D-galactose for 15 days

After the 24 h of last dose administration, animals were sacrificed by cervical dislocation for biochemical and histochemical study of lipofuscinogenesis (Deshmukh et al., 2007).

2.5 Biochemical estimation of lipofuscin granules

The brain was isolated and hippocampus region was homogenized (10% w/v) in a reaction mixture containing 75 mM potassium phosphate buffer (pH 7.04), 1 mM ascorbic acid, 1 mM ferric chloride and 0.001 ml of chlorotetracycline. The lipofuscin granules were extracted according to method (Dillard and Tappel, 1971). Briefly, was added 1 ml of reaction mixture to 6 ml of chloroform: methanol (2:1 v/v) organic solvent mixture. The fluorescence was measured with photofluorometer by using 1 µg quinine sulfate/mL of 0.1N sulfuric acid as a standard and 0.1N sulphuric acid was used as a blank. The fluorescence intensity of the mixture was measured with the excitation and emission wave lengths set at 365 and 455 nm respectively and the fluorescence product was expressed in µg/mg of protein.

2.6 Histochemical study of lipofuscin granules

The brain was excised and longitudinally cut into two equal halves and fixed in 10% neutral buffered formalin for 24 hours at 4°C and then washed under running tap water for 24 hours, dehydrated through alcohol grades, cleared with xyline and embedded to paraffin blocks. Sections of 5µM thickness from hippocampus region were taken with rotary microtome and lipofuscin granules were demonstrated histochemically by Zeihl Neelson Carbol Fuscin method (Troyer, 1980). The histochemical changes were observed by using phase contrast microscope (40X).

2.7 Statistical analysis

Values are expressed as mean ± SD. Data were analyzed statistically using one way ANOVA followed by Turkey's *post hoc* test at $\alpha = 0.01$.

3. RESULTS

3.1 Biochemical estimation of lipofuscin granules

The effect of ethanol extract of *Piper cubeba* on autofluorescent product (lipofuscin) in neurons of hippocampus region of various groups of rat brain was illustrated in Table 1. The research showed a significant accumulation of fluorescent product in D-galactose administered group animals as compared to control group. The ethanolic extract of *Piper cubeba* co-treated group showed significant ($P < 0.001$) decrease in hippocampus neuronal autofluorescent product as compared to D-galactose administered group. Piracetam, a reference drug also significantly ($P < 0.001$) reversed the effect of D-galactose in hippocampal neurons.

3.2 Histochemical changes of lipofuscin granules

The histochemical observations of lipofuscin granules in hippocampus region were demonstrated by using phase contrast microscopic and SAGLO image projector. The control group animal showed presence of negligible number of lipofuscin granules (Figure 1) and D-galactose treated animal showed an increased accumulation of lipofuscin granules (Figure 2). The ethanolic extract of *Piper cubeba* co-treated group animal showed normal distribution of lipofuscin granules in hippocampal region of brain (Figure 3). The reference drug piracetam treated animals also restores normal distribution of lipofuscin granules in the hippocampus region of brain (Figure 4).

4. DISCUSSION

Advanced glycation end products (AGEs) are produced from interaction of D-galactose with free amino groups of amino acids and proteins which are further bound to receptors for advanced glycation end products (RAGE) (Mullarkey et al., 1990). This activates intracellular signal transduction and helps to increase production of malonaldehyde which evokes formation of free radicals (Deshmukh et al., 2006) that increase oxidative stress which is one of the causal factors in the accumulation of lipofuscin granules. On the other hand, D-galactose induced oxidative stress brings insufficiency of lysosomal enzymes by cross linking

membrane proteins with lipids. Further it was suggested that, AGEs are resistant to proteolytic digestion (Zs-Nagy, 1978). This tends to neuronal damage and ageing which mimics some characters of cognitive dysfunction. Therefore, drugs and bioactive substances that prevent the D-galactose induced neuronal lipofuscinogenesis thought to be beneficial in the treatment of aging induced neuronal dysfunction and neurodenerative diseases such as AD and dementia.

In the present study, medicinal plant *Piper cubeba* prevents formation and accumulation lipofuscin granules induced by D-galactose in hippocampus region of rats. Previously published articles reported that, ethanolic extract of fruits of *Piper cubeba* contains high content of phenolics and flavonoids. The plant contains lignans such as cubebin, hinokinin, yatein and isoyatein as a chief constituents have proved for their antioxidant activity (Karthikeyan and Rani, 2003; Muchandi and Dhawale, 2017). Thus, protective effects of ethanolic extract of *Piper cubeba* on D-galactose induced neuronal lipofuscinogenesis in albino rats may be due to its antioxidant property.

Table 1 Effect of *Piper cubeba* extract on fluorescence product (µg/mg of protein) on hippocampus regions of brain in aging accelerated albino rats

Treatment group	**Fluorescence product (µg/mg of protein)**
Group-I: Control	0.29 ± 0.002
Group-II: D-galactose	1.18 ± 0.112$^{\#}$
Group-III: Piracetam co-treated	0.31 ± 0.007***
Group-IV: *Piper cubeba* extract co-treated	0.35 ± 0.008***

Values are mean ± SD. One way ANOVA followed by Turkey's post hoc test. $^{\#}$ indicates $p<0.001$ when group II compared with group I. *** indicates $p<0.001$ = highly significant when group III & group IV compared with group II.

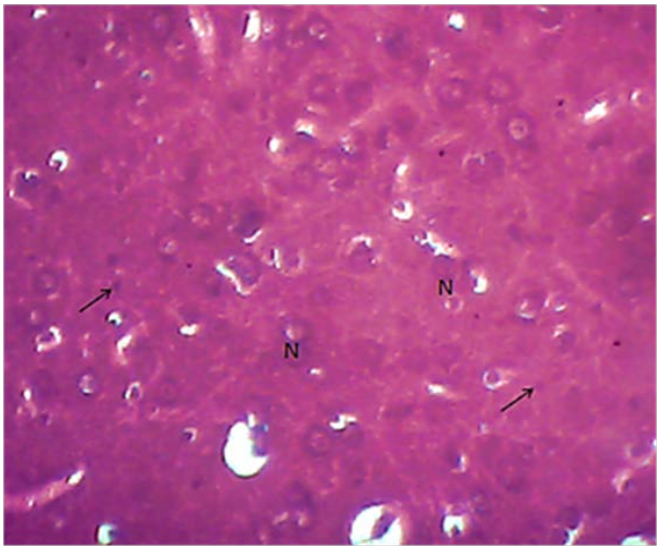

Figure 1 Saggital section of hippocampus of control group rat showing negligible distribution of lipofuscin granules (N=nucleus and black arrow indicates lipofuscin granules)

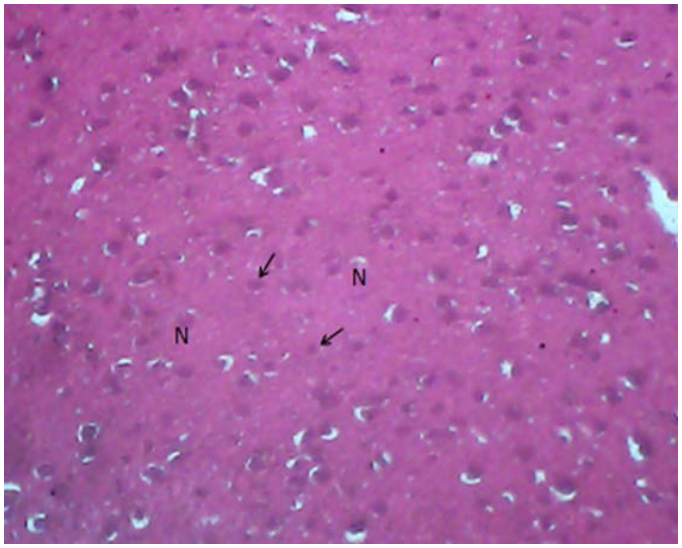

Figure 2 Saggital section of hippocampus region of D-galactose treated rat showing increased accumulation of lipofuscin granules

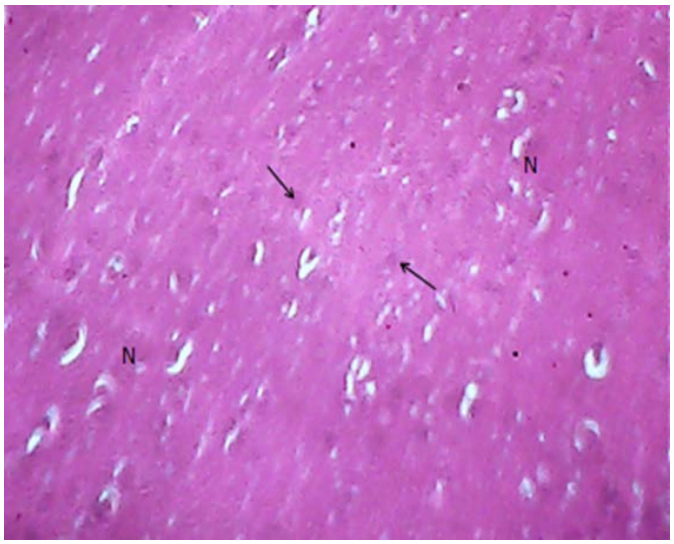

Figure 3 Saggital section of hippocampus region of rat brain treated with ethanolic extract of *Piper cubeba* showing decreased accumulation of lipofuscin granules

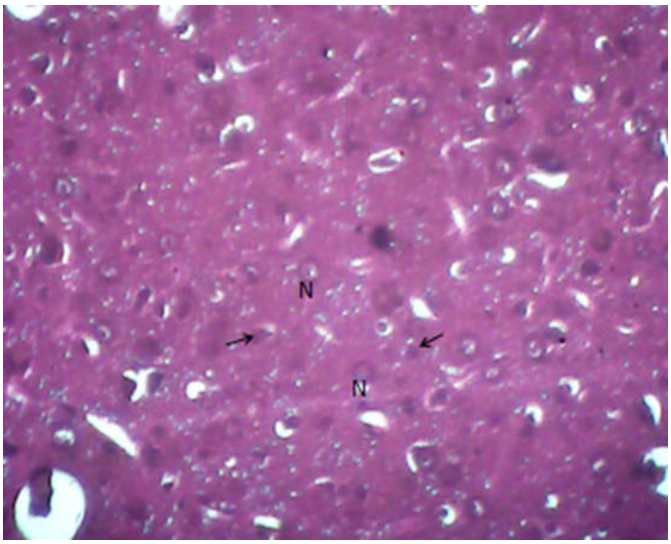

Figure 4 Saggital section of hippocampus region of piracetam treated rat showing normal restoring the lipofuscin granules

5. CONCLUSION

The ethanolic extract of *Piper cubeba* has protected the neurons from accumulation of D-galactose induced lipofuscin granules and thus may be useful in aging induced neurodegenerative diseases. However, detail cognitive functions should be evaluated to know underlying mechanism.

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