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Amelioration of intracerebroventricular streptozotocin induced cognitive impairment by *Evolvulus alsinoides* in rats: *In vitro* and *in vivo* evidence

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ABSTRACT

Evolvulus alsinoides, also known as Shankpushapi, is a commonly used traditional medicine for enhancing memory. We evaluated the *in vitro* free radical scavenging and enzymes [acetylcholinesterase, butyrylcholinestrase, glycogen synthase kinase-3-β (GSK-3-β), rho kinase (ROCK II), prolyl endopeptidase (PEP), catechol-*O*-methyl transferase (COMT) and lipoxygenase (LOX)] inhibitory activities of aqueous and hydro-alcoholic extracts of *E. alsinoides*. Hydro-alcoholic extract of *E. alsinoides* demonstrated more free radical scavenging activity as compared to aqueous extract. Hydro-alcoholic extract also showed higher cholinesterase, GSK-3-β, ROCK II, PEP, COMT and LOX enzyme inhibitory activities as compared to aqueous extract. Phytochemical analysis revealed more flavanoids in hydro-alcoholic extract as compared to aqueous extract but no significant difference in phenolic content of the two extracts was observed. Based on *in vitro* data, hydro-alcoholic extract (100, 300 and 500 mg/kg, p.o.) was selected for *in vivo* study in intracerebroventricularly injected streptozotocin (STZ) induced cognitive impairment in male Wistar rats. Elevated plus maze, passive avoidance and Morris water maze were used for assessment of cognitive function on 14th, 21st and 28th day after STZ injection. Oxidative stress parameters (malondialdehyde, reduced glutathione, nitric oxide levels and superoxide dismutase activity), cholinergic dysfunction and rho kinase (ROCK II) expression were studied in cerebral cortex and hippocampus of rat brain at the end of the study. Hydro-alcoholic extract of *E. alsinoides* dose dependently prevented STZ induced cognitive impairment by reducing the oxidative stress, improving cholinergic function and preventing the increase in rho kinase expression. The results suggest an anti-Alzheimer potential of hydro-alcoholic extract of *E. alsinoides*.

1. Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disorder is characterized by decline in cognitive functions including learning and memory (Monczor, 2005; Han, 2005). The pathological hallmarks of AD include the formation of beta-amyloid (A-β) plaques, neurofibrillary tangles (NFT) and degeneration of cholinergic neurons (Weiss et al., 2008; Johnson et al., 2008). Formation of A-β plaques and NFT causes cholinergic dysfunction, generation of free radicals and activation of astrocytes. The resulting oxidative stress and inflammation play an important role in the pathogenesis of AD (Stuchbury and Munch, 2005; Gary and Hsueh-Meei, 2005). Increased breakdown of acetylcholine (ACh) by cholinesterase enzyme leading to ACh deficiency in the cerebral cortex is primarily responsible for cognitive impairment in AD (Herholz, 2008). However, various other enzymes like glycogen synthase kinase-3-β (GSK-3-β), rho kinase (ROCK II), prolyl endopeptidase (PEP), catechol-*O*-methyl transferase (COMT) and lipoxygenase (LOX) also play role in the etiopathology of AD (Ellis, 2005; Takashima et al., 1998; Huang et al., 2008; Toide et al., 1995; Sweet et al., 2005; Firuzi et al., 2008). Alongside these enzymes, oxidative stress due to imbalance between oxidant and antioxidant system plays an important role in pathogenesis of AD (Gary and Hsueh-Meei, 2005; Butterfield, 2004). It is well known that brain is particularly vulnerable to oxidative damage because of high content of polyunsaturated fatty acid (Gutteridge, 1995).

In addition to currently available therapy, many herbal drugs have been used for the treatment of memory related disorders. *Evolvulus alsinoides* commonly known as Shankpushapi, is a popular herb used in the Ayurvedic system of medicine for treatment of various neurological disorders including epilepsy and aging. It is one of the common ingredients in many formulations available in Indian market which are considered as a Medhya Rasayana – meaning a drug which rejuvenates, maintains and enhances intellect and memory. Hydroxyl radical scavenging, immunomodulatory, adaptogenic, anti-amnesic and antiulcer activities of

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E. alsinoides have been reported in previous studies (Auddy et al., 2003; Ganju et al., 2003; Siripurapu et al., 2005). Recently, memory enhancing activity of ethanolic extract of *E. alsinoides* and its ethyl acetate as well as aqueous fractions has also been reported in rats (Nahata et al., 2010).

Intracerebroventricular (ICV) injection of streptozotocin (STZ) in rats has been closely linked with various pathological conditions of AD like oxidative stress, neuro-inflammation and impaired brain glucose and energy metabolism (Tota et al., 2010; Nitsch and Hoyer, 1991) and leads to progressive loss of memory (Lannert and Hoyer, 1998; Awasthi et al., 2010; Sharma and Gupta, 2003). Along with decreased choline acetyltransferase (ChAT), increased cholinesterase activity has also been observed. Therefore, this model has been described as an appropriate experimental model for AD (Lester-coll et al., 2006).

Considering traditional use of *E. alsinoides* as memory enhancer and the known role of free radicals and cholinesterase, GSK-3-β, ROCK II, PEP, COMT and LOX enzymes in Alzheimer's disease, the present study was undertaken to evaluate the *in vitro* free radical scavenging and enzyme inhibitory activity of aqueous and hydro-alcoholic whole plant extracts of *E. alsinoides*.

Depending on the *in vitro* results, hydro-alcoholic extract of *E. alsinoides* was selected for *in vivo* evaluation against intracerebroventricular streptozotocin induced cognitive impairment, oxidative stress, cholinergic dysfunction and rho kinase (ROCK II) expression in cerebral cortex and hippocampus of rat brain.

2. Materials and methods

2.1. In vitro study

Whole plant of *E. alsinoides* was collected from Chennai, Tamil Nadu, India. Plant material was authenticated in the Department of Botany, Natural Remedies Pvt. Ltd., Bangalore, Karnataka, India (voucher specimen No. NPL-867).

2.1.1. Preparation of extracts

Dried plant material (whole) was coarsely powdered and subjected to extraction using distilled water (aqueous extract) and 50% methanol (hydro-alcoholic extract) as solvent. Hot extraction was performed for 3 h and the filtrate was collected. The process was repeated twice using dried residue of the same plant material. The filtrates obtained from 1st, 2nd and 3rd extractions were pooled and concentrated under vacuum at 40 °C by rota-vapor. Semi-solid form of the extract was dried in vacuum tray drier to get the powder form. Percentage yield of aqueous and hydro-alcoholic extracts was 13.5% and 13.0%, respectively. By HPLC analysis, aqueous and hydro-alcoholic extracts contain caffeic acid (0.0018% and 0.0049%) and umbelliferone (0.0089% and 0.0116%), respectively.

2.1.2. Phytochemical analysis

The total phenolic content of the aqueous and hydro-alcoholic extracts was measured using Folin–Ciocalteu assay with slight modification (Singleton and Rossi, 1965). Gallic acid was taken as standard and the total phenolic content of extracts is expressed as micrograms gallic acid equivalents per gram of extract.

The total flavonoid content of the extracts was determined as described previously with slight modifications (Zhishen et al., 1999). Rutin was taken as standard. The values are expressed as microMolar of rutin equivalent per gram of extract.

2.1.3. Free radical scavenging activity

2.1.3.1. Oxygen radical absorbance capacity (ORAC) assay. The ORAC value of the extracts was determined as described earlier (Davalos et al., 2004). The net area under curve (AUC) corresponding to the extracts was calculated by subtracting the AUC corresponding to the control. Standard graph was plotted with various concentrations of Trolox. The ORAC values are expressed as Trolox equivalents for each extract.

2.1.3.2. 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging assay. ABTS radical scavenging assay was performed as described by Auddy et al. (2003). Percentage inhibition was calculated as ABTS radical scavenging activity (%) = $[(Abs_{control}-Abs_{sample})/Abs_{control}] \times 100$ where $Abs_{control}$ is absorbance of ABTS radical; Abs_{sample} is absorbance of ABTS radical along with extracts/positive control. The IC_{50} of the extracts was calculated.

2.1.3.3. Peroxynitrite radical absorbance capacity (NORAC) assay. The assay was performed as described by Kooy et al. (1994). The NORAC values are expressed as Trolox equivalents for each extract.

2.1.4. Enzyme inhibitory activity

2.1.4.1. Acetylcholinesterase and butyrylcholinesterase inhibitory activity. The cholinergic markers, acetylcholinesterase (AChE) and pseudocholinesterase (BuChE) were estimated according to the method of Ellman et al. (1961). Percentage inhibition was then calculated.

2.1.4.2. Glycogen synthase kinase-3-β (GSK-3-β) inhibitory activity. GSK-3-β causes the senile plaque and neuro-fibrillary tangle formation, microglia-mediated inflammation, pathological hallmarks of AD which leads to neuronal death (Lucas et al., 2001; Hooper et al., 2008). In previous studies, it has been reported that GSK-3-β inhibitors prevented the neuro-fibrillary tangle formation in animal models (Takashima et al., 1998).

Therefore, GSK-3-β inhibitory activity was determined using a homogeneous time resolved fluorescence (HTRF) assay kit based on proximity of a europium cryptate donor label and streptavidin-XL665 acceptor label. The IC_{50} values were calculated.

2.1.4.3. Rho kinase (ROCK II) inhibitory activity. The previous findings indicate that rho kinase activity is increased under various pathological conditions including stroke and AD; therefore drugs targeting Rho-ROCK pathway is under development for treating central nervous system disorders. Huang et al. (2008) has reported that hydroxyfasudil, a rho kinase inhibitor ameliorated the memory deficit caused by permanent bilateral carotid artery ligation by decreasing the neuronal loss and oxidative stress.

In the present study, rho kinase (ROCK II) inhibitory activity was determined using a homogeneous time resolved fluorescence (HTRF) assay kit. The IC_{50} values were calculated.

2.1.4.4. Prolyl endopeptidase (PEP) inhibitory activity. Portevin et al. (1996) has shown that PEP activity has increased significantly in AD patients as compared to control subjects suggesting its potential in the treatment of AD. In previous studies, it has also been reported that PEP inhibitor improves learning and memory in scopolamine and dorsal hippocampal-lesioned induced amnesia in rats (Toide et al., 1995) and also prevented the amyloid like deposition in senescence accelerated mouse (Kato et al., 1997).

Thus, PEP inhibitory activity of plant extracts was determined as per the method of Kobayashi et al. (2002). The IC_{50} values were calculated.

2.1.4.5. Catechol-O-methyl transferase (COMT) inhibitory activity. COMT degrades catecholamines such as dopamine, epinephrine and norepinephrine (Sweet et al., 2005). A functional valine to methionine polymorphism in COMT gene has been associated