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AN IN-VITRO STUDY TO SCREEN DOOSHIVISHARI AGADA FOR ITS GSK3 INHIBITION IN ALZHEIMER'S DISEASE

Ayurveda

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ABSTRACT

Recent understanding of Alzheimer's disease reveals the role of toxins as potential etiological factors, where the person is exposed to many toxins on daily basis like metals environmental pollutants, excessive use of fertilizers, cigarette smoking, Genetic and immunological factors leads to Alzheimer's disease[1,2,3,4]. A similar kind of concept is also mentioned in Ayurveda under the concept of Dooshivisha &Garavisha, where small doses of visha which are accumulated in the body on daily basis lead to some dreadful disease after years of exposure. Literature review suggests etiological factors & signs and symptoms of Alzheimer's disease can be co-related to Dhatugata manifestation of Dooshivisha[5]. In modern science Alzheimer's disease is treated with anticholinesterase & GSK3 inhibition, which may lack in preventing disease progression, Dooshivishari agada is described for the treatment of Dooshivisha. It is found that all most all of the ingredients of Dooshivishari agada are proven to possess activities like anti-stress activity, preventing loss of memory, and preventing hyperactive deep tendon reflexes[6]. In the present in-vitro study aqueous and methanol extracts of Dooshivishari Agada showed GSK3 activity in Cell line study[7,8].

KEYWORDS

Alzheimer's disease, Anticholinesterase, Dooshivisha, Dooshivishari Agada, Gara visha, GSK3 Inhibition, Visha

INTRODUCTION

Alzheimer's disease is the most common cause of degenerative dementias and accounts for 50%-60% of all cases of dementia. It is estimated that by the year 2020, approximately 70% of the world's population aged 60 and above will be living in developing countries, with 14.2% in India, In southern India prevalence of dementia including Alzheimer's Disease is about 4.86%.[9]

In modern science, Alzheimer's disease is treated with cholinergic & GSK 3 inhibitors, among cholinesterase inhibitors-Acetyl cholinesterase, is used, which may lack in beneficial effect in preventing disease progression based on clinical long term experience, as they increase tau phosphorylation. So there is a need for some modification in treating Alzheimer's disease, particularly, cognitive and memory dysfunctions. These inhibitors also result in side effects like falls syncope, elevated hepatic enzyme concentration nausea, dizziness, headache, gastro-intestine symptoms, and rashes., Dooshivishari agada is described for the treatment of dooshivisha. It is found that all most all of the ingredients of Dooshivishari agada are proven to possess activities like anti-stress activity, preventing loss of memory, and preventing hyperactive deep tendon reflexes[6].

Dooshivishari Agada is described by vagbhata for the treatment of Dooshivisha. It is one of the formulations mentioned for the management of Dooshi visha. After reviewing the experimental study of herbs in Dooshivishari Agada it is found that all these herbs are useful in treating Alzheimer's disease

Materials and methods

Ingredients of Dooshivishari Agada:-

Pippali(~ Piper longum linn), *Dhyamaka (~Cymbopogon martini* wats), *Jatamansi (~Nardostachys jatamansi* DC), *Lodhra(~ Symplocos racemose* Roxb), *Ela (~Elettariacardamomum* Maton), *Suvarchika (~Tribulus terrestris* Linn), *kutannata (~Oroxylum indicum* Linn), *Nata(Tagara) (~ Valeriana wallichii* DC), *Kushta (~Saussurea lappa* CB Clarke), *Yashtimadhu (~Glycirhiza glabra* Linn), *Chandana (~Santalum album* Linn) Gairika(~ Red ochre) are having Neuroprotective activity

All the 12 drugs of Dooshivishari Agada are collected from the local market, taken in equal parts (10grams each) made into fine powder & forming a homogeneous mixture.

Extraction –

It is the first step to separate the desired natural products from the raw materials. Extraction methods include solvent extraction, distillation method, pressing, and sublimation according to the extraction principle. Solvent extraction is the most widely used method. The extraction of natural products progresses through the following stages: the solvent penetrates the solid matrix; the solute dissolves in the solvents; the solute is diffused out of the solid matrix; the extracted solutes are collected. Any factor enhancing the diffusivity and solubility in the above steps will facilitate the extraction. The properties of the extraction solvent, the particle size of the raw materials, the solvent-to-solid ratio, the extraction temperature, and the extraction duration will affect the extraction efficiency.

- **Preparation of Extract:**
- Weighed 20g of dried Sample powder and dissolved in 100ml of Methanol / Water in a 100ml beaker with aluminum foil covered on it.
- Then the beaker was kept on hot water bath at 50º C for 4 hours.
- After the incubation period the extract was filtered with Whatmann filter paper and the filtrate was collected in a 250ml beaker. Residue present over the filter paper was discarded and the filtrate was taken for further use.
- Then the filtrate was kept at 50ºC for a few hours until the extract got completely dried and turned into semisolid form.
- This semi-solid sample was weighed and the yield was noted.

Table 1: Yield summary after crude extraction

sample	Sample taken for Extraction	Solubility	Yield
Dooshivishari Agada	20g	Methanol	1967.5mg
	20g	Aqueous	1870.2mg

Cell line:-

SHSY5Y - *Homo sapiens*, neuroblastoma

Reagents used:

a RNase free environment, after cleaning pipettes
b. TRIzol (15596026 and 15596018)
c. DEPC
d. chloroform (67-66-3)
e. isopropanol(67-63-0)
f. 70% ethanol
g. DEPC treated water
h. 1.5 and 2ml eppendrof tube, 15ml falcon tube –DEPC treated autoclaved and oven-dried

Treatments:-

Cell line	Treatment
SHSY5Y	Control
	Dooshivishari agada - 80µg/ml
	Dooshivishari agada - 160µg/ml

Sample Preparation and RNA Isolation[10]:

Total RNA from SHSY5Ycells was extracted using TRizol Reagent (Invitrogen,) according to the manufacturer's instruction. SHSY5Ycells were washed twice with PBS and centrifuged at 2000rpm for 5min. To the cell pellet, 1ml of TRIzol (per p35 dish) was added in a 1.5ml eppendorf tube and vortexed. Samples were allowed to stand for 5 minutes at room temperature. To the reaction mixture, 0.2ml of chloroform is added and vigorously mixed for 15 seconds. The tube was allowed to stand at room temperature for 5 minutes, centrifuged the resulting mixture at 10,000rpm for 15min at 4^{0}c. The upper aqueous phase is transferred to a new clean eppendorf tube and treated with 0.5ml of isopropanol. The resultant mixture is mixed gently by inverting the sample 5 times and incubating at room temperature for 5 minutes. Samples were centrifuged at 10,000 rpm for 10 minutes at 4^{0}c. The supernatant was discarded and the RNA pellet was washed by adding 1ml of 70% ethanol. Mix the sample gently by inverting it a few times. Centrifuged for 5min at 14,000 rpm at 4^{0}c. The supernatant was discarded by inverting the tube on clean tissue paper. Later, the pellet was dried by incubating in a dry bath for 5min at 55^{0}c. The pellet was then resuspended in 25 µl of DEPC treated water.

RT-PCR[11]:

A semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using the Techno Prime system to determine the levels of GSK-3 and β-Actin mRNA expressions. The cDNA was synthesized from 2µg of RNA using the Verso cDNA synthesis kit (Thermo Fischer Scientific) with an oligo dT primer according to the manufacturer's instructions. The reaction volume was set to 20µl and cDNA synthesis was performed at 42°C for 60 min, followed by RT inactivation at 85°C for 5 min.

Primers:

Table2 Primer details

Gene	Primer pair	Sequence	Tm	Product size (bp)
β – Actin	FP	TCCTCCTGAGCGCAAGTACTCT	62.1	153
	RP	GCTCAGTAACAGTCCGCCTAGAA	62.4	
GSK	FP	GACACCTGAGCTGCCCTTGG	51.88	167
	RP	GAGGAAGTCCAGTGTGCAGC	51.83	

PCR[12]:

The PCR mixture (final volume of 20 µL) contained 1 µL of cDNA, 10 µL of Red Taq Master Mix 2x (Amplicon), and 1µM of each complementary primer specific for GSK-3 and β-Actin (internal control) sequence. The samples were denatured at 94°C for 5 minutes and amplified using 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute for GSK-3 renaturation was set to 47C and for β-Actin, the renaturation was set to 55°C for 30 seconds followed by a final elongation at 72°C for 10 minutes. The optimal numbers of cycles have been selected for amplification of these genes experimentally so that amplifications were in the exponential range and have not reached a plateau. Ten microliters of the final amplification product were run on a 2% ethidium-stained agarose gel and photographed. Quantification of the results was accomplished by measuring the optical density of the bands, using the computerized imaging program Image J. The values were normalized to β-Actin intensity levels.

RESULTS:

Analysis of Amplicons:

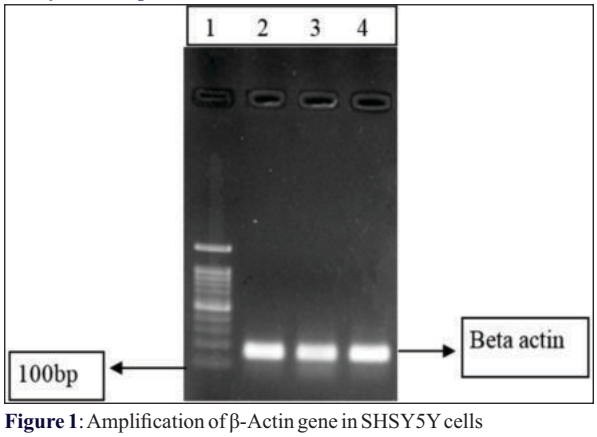

Figure 1: Amplification of β-Actin gene in SHSY5Y cells

Lane 1- Ladder; Lane 2-control: Lane 3-80µg/ml; Lane 4-160 µg/ml;

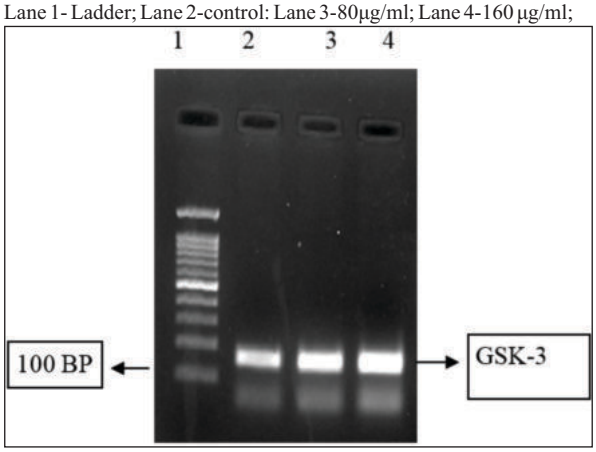

Figure 2: Amplification of GSK-3 gene in SHSY5Y cells

Lane 1- Ladder; Lane 2 control: Lane 3-80µg/ml; Lane 4-160 µg/ml;

Table 3: Relative expression GSK-3 gene in SHSY5Y cells

Samples	Band Intensity Of PCR Amplicon Of Genes		Normalized	Relative Gene Expression
	β-Actin	GSK-3		
Control	18650.418	18745.56	1.01	1.00
80µg/ml	19397.489	22710.024	1.17	1.16
160µg/ml	21727.312	28155.501	1.30	1.29

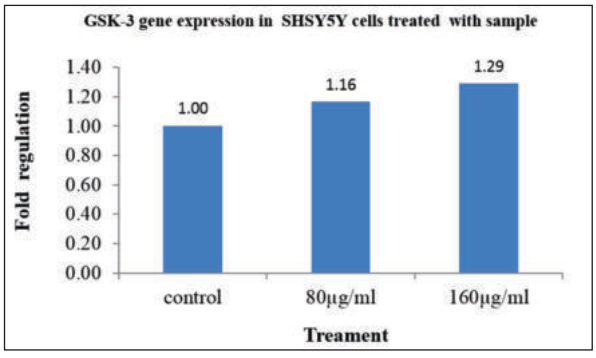

Fig 2: Relative expression GSK-3 gene in SHSY5Y

DISCUSSION

The cause of Alzheimer's disease is not known, however, several factors are thought to be included in this disease-Neurochemical factors like Acetylcholine, norepinephrine,-Environmental factors like metals, environmental pollution, and excessive use of fertilizers and hazardous toxic chemicals during the production of food materials cigarette smoking[13]. Hence Alzheimer's disease can be included under Dooshivisha. Dooshivishari Agada helps in the management of Dooshivisha & also has an immune modulatory effect. Absorption of drugs occurs quickly in a detoxified body so the use of Dooshivishari Agada can do their work effectively.

CONCLUSION

The effect of the compound on GSK-3 gene expression was studied in SHSY5Y by semi-quantitative- PCR was analyzed. The internal control β-Actin was used to normalize the gene expression. The study revealed the expression of GSK-3 was upregulated as the treatment concentrations increased. The results show that the expression levels of GSK-3 at 80µg/ml tested have shown 1.16 and 160µg/ml test has shown 1.29 fold expression compared to control. Hence Alzheimer's disease may be considered to treat with Dooshivishari Agada.

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