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STUDY OF ALZHEIMER'S DISEASE ON BARK OF *KYDIA CALYCINA* IN HYDRO-ALCOHOLIC EXTRACT AND IN-VITRO STUDY

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ABSTRACT

The present study was performed in the bark of *Kydia Calycina* for the study of Alzheimeric activity using hydro-alcoholic extraction in the ratio 1:1.It was aimed to carry out the in-vitro study of "Acetyl cholinesterase inhibition activity" by Ellman's method. The present investigation was aimed to determine Alzheimer`s disease(AD) in the bark of *Kydia Calycina*. The report of respective study shows the dose dependent increase in percentage inhibitory activity for Anti-cholinesterase activity by Ellman's method. From the results of the present study, it was concluded that, the bark of the plant exhibits Alzheimeric activity. The Alzherimeric activity produced by the hydro-alcoholic extract at 250 µg/ml concentration shows the percentage inhibition of Anti-cholinertase which is compared with that of same concentration of standard Ellman's reagent.

KEYWORDS: *Kydia Calycina*, Alzheimeric activity, hydro-alcoholic extraction, Acetyl cholinesterase inhibition activity, Ellman's method.

INTRODUCTION

Alzheimer's disease is a chronic, irreversible progressive neuro-degenerative disorder. Alois Alzheimer, who was born on 14th June 1864, Marktbreit in kingdom of Bavaria. Alzheimer is credited with identifying the first published case of "Presenile dementia", which Kraepelin would later identify as Alzheimer's Disease.[3] These abnormal protein accumulate mostly due to reduced clearance, but in some cases, due to over production, it causes neuronal damage. There is marked cholinergic deficiency in the brain, through other neurotransmitters system, especially glutamate and neuro-peptide.[2]

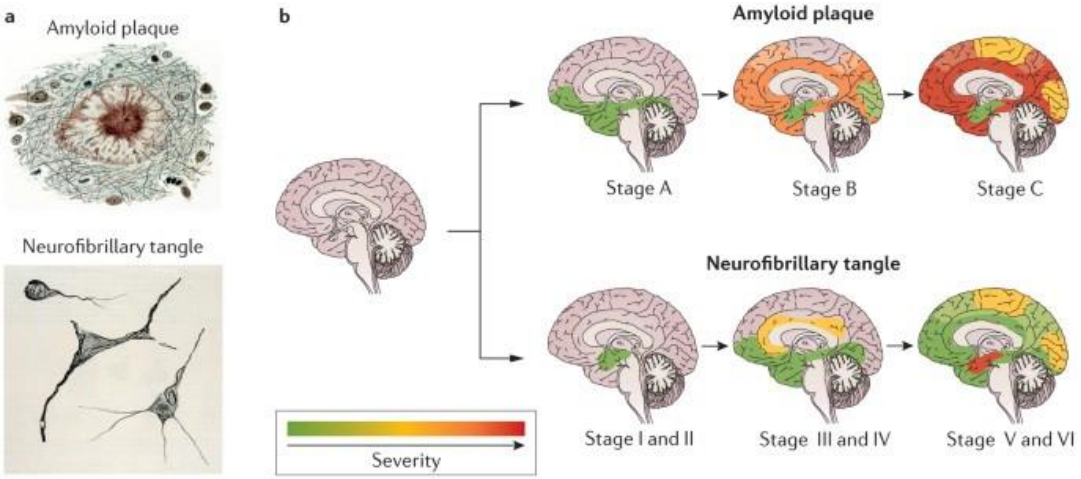
Alzheimer`s Diseases(AD) is the one of the most prevalent neuro-degenerative diseases resulting in progressive congnitive decline.[4] Alzheimer`s disease diagnosis involves a significant decline of intellectual abilities in one or more cognitive domain including learning memory language and executive function.[5] Alzheimer`s diseases is the most common cause of dementia, responsible for 60-80% of cases reported.[6] The cause of the disease is not clear, it has been reported that 35 million people worldwide have Alzheimer disease or others types of dementia and about 65 million peoples are expected to have dementia problem by 2030. Pathologically, it is caused because of intracellular neurofibrillary tangles and extracellular amyloidal protein and results in the deposition of plaques which obstruct the communication between the nerve cells resulting in this neurodegenerative disorder.[1] The common drugs used for the symptomatic management of patients with Alzheimers include cholinesterase inhibitors (Donepezil, Rivastigmine and Glantamine), neuroprospective N-methyl-D-aspartate(NMDA) receptor antagonist (Memantine)or combination therapy.[7]

The development of the disease begins years before the first symptoms. The damage most often starts in the region of the brain that controls memory. The loss of neurons spreads in a somewhat predictable pattern to other regions of the brain. By the late stage of the disease, the brain has shrunk significantly.

Alzheimer's disease is a complex, multifactorial, neurodegenerative disease, resulting from complicated interactions of one's genetic makeup, education, age, and environment. Many hypotheses have laid the foundation

to gain understanding of the etiology of the disease, with one of the oldest being the cholinergic hypothesis. This hypothesis is based upon the fact that AD patients show reduction in activity of choline acetyltransferase and acetylcholinesterase in the cerebral cortex compared with the normal brain. Postmortem brain tissue from patients with AD confirmed the reduced neurotransmitter pathway activity, revealing that degeneration of cholinergic neurons and loss of cholinergic neurotransmission significantly contributes to the cognitive impairment seen in those with AD. The Tau hypothesis has also been proposed, considering AD histopathology reveals intraneuronal neurofibrillary lesions made up of tau proteins. Tau proteins are mainly found in neurons and are involved in the assembly and stabilization of the neuronal microtubule network. Tau protein becomes pathological when the phosphorylation regulation becomes unchecked and hyperphosphorylated tau proteins polymerize into filaments and become neurofibrillary tangles. This leads to malfunction of the structural and regulatory actions of the cytoskeleton and then leads to abnormal morphology, axonal transport, and synaptic function of neurons, thus leading to neurodegeneration.

These prior theories paved the way to the widely accepted hypothesis for the pathogenesis of AD: the amyloid cascade hypothesis. This theory attributes clinical sequelae of the disease to the overproduction or decreased clearance of amyloid beta (Ab) peptides, which then leads to increased deposition of Ab, furthermore, leading to neuronal damage (**Figure 1**).



The length of Ab varies depending on the posttranslational cleavage pattern of the transmembrane amyloid precursor protein (APP). Ab is generated by cleavage of APP via either b- or g-secretases, resulting in the infamous insoluble Ab fibrils. Two main types of Ab polymers play a direct role in the pathology of AD: Ab40 and Ab42. Ab40/Ab42 then oligomerizes, travels to synaptic clefts, and interferes with synaptic signaling. These eventually further polymerize into insoluble amyloid fibrils that aggregate into amyloid plaques. Within the plaques, Ab peptides in b-sheet conformation polymerize into structurally distinct forms, including fibrillar, protofibers and polymorphic oligomers. It is the deposition of these plaques diffusely throughout the brain that lead to microglial activation, cytokine release, reactive astrocytosis, and an overall inflammatory response. These structural changes lead to synaptic and neuronal loss and eventual gross cerebral atrophy. On the other hand, should APP be processed by a-secretase in the healthy adult, soluble b-amyloid is produced, which has been linked to play a role in neuronal plasticity/survival, is protective against excitotoxicity, is important for early CNS development, and has been shown to be important for promoting synapse formation.

MATERIALS AND METHODS

A. PLANT MATERIAL

The Present Study as per the literature review uses the plant *Kydia Calycina*, Roxb (Family: Malvaceae).

1. Collection and Authentification of Plant

The fresh bark were collected, identified and authenticated by the "kottakkal Aryavydhyashala, Kottakkal, Malappuram, Kerala."

2. Drying And Powdering

The fresh barks was shade dried and then crushed to a coarse powder by a mechanical grinding.

3. Preparation of the Extract

The hydro-alcoholic extract was prepared by mixing ethanol and distilled water in the ratio of (1:1). After 18 hours of the extraction period, it was removed and the crude mixture solution was evaporated and concentrated for further in vitro studies.

B. PRELIMINARY PHYTO-CHEMICAL TEST

Qualitative analysis for various phytoconstituents in the dried powder and extract of *Kydia calycina*. Chemical test were carried out to know the nature of compounds

present in the hydro-alcoholic extract and to identify the presence of various phyto-constituents present in it.

1. Test for Alkaloids

The extract was stirred with few ml of dilute hydrochloric acid and filtered. The filtrate was tested with various alkaloid reagents.

- **Mayer's test**

To a few ml filtrate, 2 drops of Mayer's reagent (potassium mercuric iodide solution) was added along the sides of the test tube. A cream color precipitate indicates the presence of alkaloids.

- **Dragendorff's test**

To a few ml of the filtrate, 2 drops of Dragendorff's reagent (potassium bismuth iodide solution) was added along the sides of the test tube. Areddish brown precipitate indicates the presence of alkaloids.

- **Wagner's test**

To a few ml of filtrate, 2drops of Wagner's reagent (Iodine potassium iodide solution) was added along the sides of the test tube. A reddish brown precipitate indicates the present of alkaloids.

- **Hager's test**

To a few ml of the filtrate, 2-3 drops of Hager's reagent (saturated solution of picric acid) along the sides of the test tube. Yellow precipitate indicate the presence of alkaloids.

2. Test for carbohydrate and their glycoside

- **Molish's test**

To 2 ml of sample, few drops of 5%, alcoholic Napthol weresolution added, mix well and cool. To this mixture 1ml of concentrate sulfuric acid was added along the sides of the tube, while cooling the tube in ice water violet colour develops at the junction of the test tube indicates the presence of carbohydrate/glycoside.

- **Barfoed's test**

To 1ml of test solution was heated with 1ml of Barfoed's reagent on water bath, if red cupric acid is formed, monosaccharide is present. Disaccharide on prolonged heating (about 10 min) may also cause reduction, owing to partial hydrolysis to monosaccharide.

3. Test for Saponins

- **Foam test**

Few ml of water as added to extract and shake vigorously and kept aside. The froth (foam) produced was observed after 15 min. Formulation of persistent foam indicate the presence of saponins.

4. Test for flavanoids

- **Shinoda test**

A small quality of the extract was taken in a test tube and dissolved in 1ml of methanol. A pinch of magnesium powder was added followed by con, HCL. Appearance of pink scarlet or crimson red colour indicates the presence of flavonoids, bioflavonoids and their glycosides.

5. Test for Tannin

- **Ferric chloride test**

A small quantity of extract dissolved in methanol and few drops of neutral ferric chloride was added, if the phenol and enol are present it gives a violet color shows the presence of tannins.

6. Test for steroid/triterpenoids and their glycosides

- **Liebermann-Burchard Reaction**

Few mg extract dissolved in chloroform to this1 of chilled acetic anhydride was added and mixed well. Then 2 drops of chilled conc.H_2SO_4 was added along with sides of test tube. The color developed at the junction of the two liquid was observed. Steroids/terpenoids gives a characterized red color.

ELLMAN'S METHOD[31]

This method was first introduced and described by Ellman and his colleagues and is based on thiocholine-derivative (acetyl- or butyryl) hydrolysis by ChEs (true or pseudo) and reaction of resulting thiocholine with thiol reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and formation of 5-thio-2-nitrobenzoic acid (TNB) anion. Measurement of TNB ion absorption at 410 nm for pseudo-ChE and 440 nm for reticulocytic acetyl-ChE indirectly determines ChE activity. Some authors proposed that indoxylacetate is better substrate than Acetylthiocholine (ATCH) as it does not react with oxime antidotes and thiol used for Ellman's method.

PHYSIOCHEMICAL STANDARDISATION

1. Determination of ash value(Indian pharmacopoeia, 1966, 1996)

Ash value are helpful in determining the quality and purity of the drug in powder form. Ash values were determine according to Indian pharmacopoeia, such as total ash, acid insoluble ash, water soluble ash and sulphated ash, for determination of different ash value.

A. Total Ash

About 3 gms of the powdered drug was accurately weighed and are taken in a silica crucible which was previously ignited and weighted, scattered the ground drug in a fine even layer on the bottom of the crucible. The crucible was incinerated gradually by increasing the temperature to make it dull red hot until free from carbon. The crucible was cooled and weighted. The procedure was repeated to constant weight. The percentage of total ash was calculated with reference to air dried drug.

B. Acid insoluble ash

The ash obtained as described in the above method was boiled with 25 ml of 2N HCL for 5min. The insoluble ash was collected in ash less filter paper and washed with hot water. The insoluble ash was transferred into silica

crucible, was ignited and weighted. The procedure was repeated to get the constant weight.

C. Water soluble ash

The ash obtained describe in the determination of total ash was boiled for 5min with 25 ml of water. The insoluble matter was collected on an ash less filter paper and washed with hot water. The insoluble ash was transferred into silica crucible and ignited for 15min and weighted. The procedure was repeated to get the constant weight. The weight of the insoluble matter was subtracted from the weight of the total ash. The difference of weight was considered as water soluble ash.

2. Determination of extractive value

- ❖ Alcohol-soluble extractive value
- ❖ Water-soluble extractive value

A) Alcohol-soluble extractivevalue

5 gms of shade dried, coarse powder of bark of *Kydia Calycina*was macerated with 100 ml of 90% alcohol (ethanol) in a closed flask for 24 hrs shaking frequently during 1st 6 hours and allowing to stand for 18 hour, filtered rapidly, taking precautions against loss of alcohol, evaporated 25 ml of the filtrate to dryness in a tarred flat bottomed shallow dish, dried at 105° C and weighed. Calculate the percentage of the alcohol soluble extract with reference to the shade dried drug.

B) Water soluble extractive value

The determination of alcohol soluble extractive using chloroform water instead of alcohol. Calculate the percentage of water-soluble extractive with reference to the shade-dried drug.

3. Determination of moisture content(loss on drying) (The Ayurvedic pharmacopoeia, 2006, WHO, 1998)

Moisture content can be determined by heating the drug at 105 ° C to constant weight and calculating the loss of weight as per the Ayurvedic pharmacopoeia, 2006.

Procedure set forth here determine the amount of volatile matter, for substances appearing to contain water as the only volatile constituent, the procedure given below, is appropriately used. Place about 10g of drug(without preliminary drying) after accurately weighing (accurately weighed to within 0.01g) it in a tarred evaporating dish.

After placing the above set said amount of the drug in the tarred evaporating dish dried at 105^0 C for 5 hours and weighed. Continued the drying and weighing at one hour interval until difference between two successive corresponds to not more than 0.25 per cent. Constant weight is reached when two consecutive weighing after drying for 30 min and cooling for 30 min in a desiccator, shown not more than 0.01 gm. difference.

RESULT AND DISCUSSION

PRELIMINARY PHYTOCHEMICAL TESTS

Identification of active principles presentin the hydro-alcoholic extract of ***Kydia Calycina. Roxb.***

S.no	CHEMICAL TEST	KCHAE
1. 1. a	**Test for alkaloids** **Mayer' s test** T. S +Mayer's reagent	-ve
1.b	**Drangendroff's test** T. S+ Drangendroff's reagent	-ve
1.c	**Wagner's test** T.S + Wagner's test reagent	-ve
1.d	**Hager's test** T.S + Hager's reagent	-ve
2. 2.a	**Test for carbohydrates/glycosides** **Molisch test** T.S + Molisch reagent	+ve
3.	**Test for saponins** T.S + Water (shake vigorous) foam produced	-ve
4.	**Test for flavonoids** **Shinoda test** T. S + magnesium powder + conc. Hcl	+ve
5.	**Test for tannins** T.S+ $Fecl_3$ solution T. S + $Fecl_3$ solution	+ve
6.	**Test for steroids:** T. S + CHCl3 + Acetic anhydride + 2-3 drops of H_2SO_4(colour at junction of two liquids)	+ve

+ ve - presence
-ve - Absence
KCHAE -***Kydia calycina*** hydro – alcoholic extract
T. S - Test solution

PHYSIOCHEMICAL ANALYSIS OF BARK POWDER

S.NO	PARAMETER	BARK
1	WATER SOLUBLE ASH	4.35
2	TOTAL ASH	7.66
3	ACID INSOLUBLE ASH	3.51
4	ALCOHOL EXTRACT	1.57
5	LOSS ON DRYING	1.20

Chemical Reagent and glass wares used for test in our laboratory

Phyto-chemical test performed in our lab

CONCLUSION

The present investigation was aimed to determine Alzheimer`s disease(AD) in the bark of *Kydia Calycina*. The report of respective study shows the dose dependent increase in percentage inhibitory activity for Anti-cholinesterase activity by Ellman's method. It is evident that hydro-alcoholic extract of *Kydia Calycina* shows Alzheimeric activity which were compared with that of standard Ellaman's reagent(DTNB). TheAlzheimeric activity obtained weremay be due to the presence of phenolic compounds and flavanoids.

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