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SQUALENE: A POTENTIAL ANTI-ALZHEIMER'S COMPOUND IN ALBINO RAT

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Keywords:

Alzheimer's disease (AD), Acetyl choline esterase (AChE), Acetyl choline (ACh) and squalene

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ABSTRACT: Alzheimer's disease, the most scattered neurodegenerative disease causes progressive destruction in serviceable performances and continuous reduction in cognitive activities and memory in aged people. It is estimated that people suffering from AD is about 10% between 65-70 years and 50% in people above 85years of age and this number will double every two decades. Even though extensive research has been carried out for decades, there is no specific therapeutic drug available for the permanent cure of Alzheimer`s Disease. In this study, squalene, a natural compound synthesized from marine shark liver oil is selected to evaluate its Anti-Alzheimer's properties in Experimentally AD-induced Male Albino Rats. In the present study, *in-vivo* experiments were carried out to test the Anti-Alzheimer's property of Squalene on Male Albino Rats under D-galactose-induced AD. The estimation of ACh and AChE content in the Cerebral Cortex and Hippocampus was done in different groups of rats at different time intervals. The results revealed that in experimentally AD-induced Rats, Squalene caused significant ACh content elevation while reducing AChE activity levels in both selected regions *viz*. Cerebral Cortex and Hippocampus, which is the key requirement for treating AD. From the results obtained in the present investigation, it was finally inferred that squalene has a neuroprotective role against D-Galactose-induced Alzheimer's disease. Since it resulted in the elevation of the cholinergic neurotransmitter in Hippocampus and Cerebral Cortex regions of the rat brain on both selected days of experimentation.

INTRODUCTION: Alzheimer's disease is an incurable, irreversible, progressive Neuro-degenerative brain disorder and the most common cause of Dementia. Characterized clinically by gradual damage and death of neurons, severely affecting the patient's ability to carry out routine daily activities and finally, progressive loss of behavioral and intellectual functionalities [1].

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In the present scenario, Alzheimer's disease affects nearly 46.8 million people worldwide [2] and the Epidemiological data today indicate a potentially considerable increase in the prevalence of the disease over the next two decades [3]. AD affects up to 5% of people over 65 years, rising to 20% of those over 80 years.

The cholinergic system, associated with normal age-related cognitive decline such as Alzheimer`s disease, is one of the most important hypotheses explaining the causes of Alzheimer`s disease. The basal for brain cholinergic neurons degenerate due to the oxidative stress under AD conditions affecting the cholinergic transmission. As such, one of the most promising approaches for treating this

disease is to enhance the Acetylcholine level in brain using Acetylcholinesterase inhibitors can interface with the progression of Alzheimer`s Disease(AD).

Among several Treatment strategies, only a few medications (Donepezil, Rivastigmine, and Galantamine) are currently approved by regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) to treat the cognitive manifestations of AD.

Despite extensive research investigations, there is still no permanent cure for Alzheimer`s. There is ample evidence for the efficacy of these medications in treating mild to moderate Alzheimer's disease [4] and some evidence for their use in the advanced stage too.

Only Donepezil is approved for the treatment of advanced AD dementia. Antipsychotic drugs are modestly useful in reducing aggression and psychosis in Alzheimer's patients with behavioral problems but are associated with serious adverse effects, such as cerebrovascular vents, movement difficulties, or cognitive decline, and have not been shown to associate with increased mortality [5] on long-term usage.

In this context, researchers started exploring natural bioactive compounds of Plant and Animal origin for the treatment of several neurological diseases, including AD, since they were proved to be less toxic with minimum side effects. Based on the above cited literature, in our present study, Squalene [6] a natural organic compound derived from 2 deep-sea shark species (Squalus) liver and commonly known as "Shark liver oil" was selected.

In the brain, squalene is involved in *de novo* biosynthesis of cholesterol through the Mevalonate pathway and is thus essential for normal Synaptogenesis, neurotransmitter release, axonal development, and neuro-steroid production.

Apart from this, squalene has several health benefits as Anti-tumor, Anti-microbial, anti-oxidant as it is rich in bioactive compounds that help boost the immune system, and synthesize hormones, vitamin D, and cholesterol. The other reason for selecting squalene is that it is involved in cholesterol homeostasis and inhibits regulation of Amyloid Beta production responsible in Tau hyperphosphorylation and formation of Amyloid Plaques, one of the Hall-Marks of AD.

Considering the close relationship between the most accepted cholinergic hypothesis and Alzheimer's disease, the present study focused on the investigations to identify and assess the neuroprotective role of Squalene in AD-induced Male Albino rats with reference to the cholinergic system.

MATERIALS AND METHODS:

Procurement of Chemicals: All the chemicals used in the present study were obtained from the standard chemical companies *viz*. Sisco Research Lab, Mumbai, India: Hi-Media Chemicals, Mumbai, India; Qualigen Chemicals, Mumbai, India; SD-fine Chemicals, Mumbai, India, and CDH Chemicals, New Delhi, India for estimation of selected biochemical assays by employing the suitable equipment.

Induction of Alzheimer's disease in Rats: In the present study, AD was induced by Intra Peritoneal Injection of D-Galactose, a reducing sugar as per reference [7]. Institutional Animal Ethical Committee (09/(i)/a/CPCSEA/IAEC/SVU/ZOOL/KY dt.19.04.2012).

The Effective Dose, 150 mg/kg body weight, already reported for squalene (150mg /kg body weight), which we again confirmed in our Lab, was used to evaluate its Anti-Alzheimer's potential on the cholinergic system.

Experimental Design:

Animal model	Male albino rat
Age of rat	3 months old
Body weight	180 ± 20grams
The chemical used for Induction of Alzheimer's Disease	D-Galactose (Zhang *et. al.*, 2011) (120 mg/kg body weight)
Route of Administration	Intra Peritoneal injection (IP)
Test Substance	Squalene (Subramanian *et. al.*, 2006) (150 mg/kg body weight (0.4ml for each rat)
Route of administration	Oral
Selected tissues for estimation	Cerebral Cortex (CC) & Hippocampus (HP)
Selected days of experiment	60^{th} and 90^{th} day of treatment

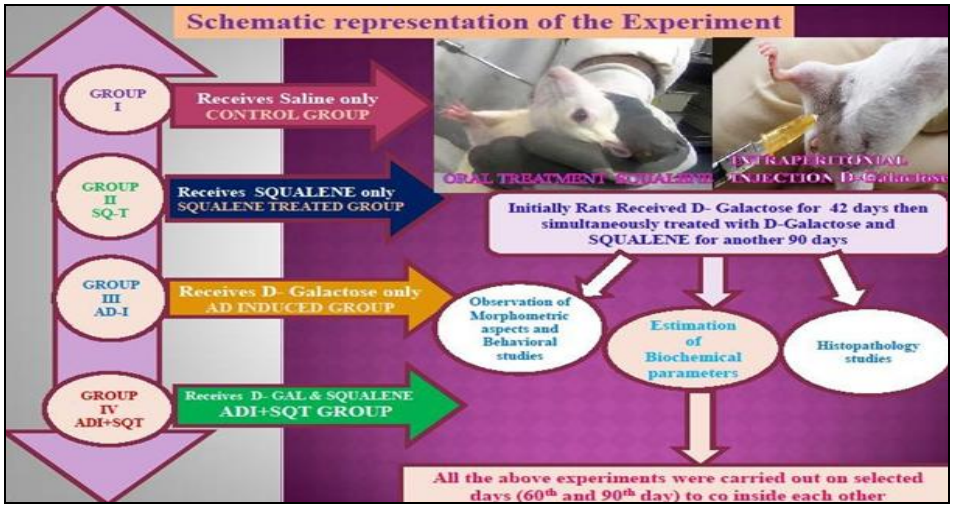

FIG. 1: SCHEMATIC REPRESENTATION OF THE EXPERIMENT

Isolation of Tissues: After induction of Alzheimer's disease, the Rats were treated with the marine bioactive compound, squalene, for 90 days. The above selected four groups of rats *viz.* Control, SQ-T, AD-I model and Protective group (ADI+SQT)) were kept under fasting for 12 hrs before scarifying them by cervical dislocation on selected days of experimentation *i.e.*, 60th and 90th day. The selected brain regions, such as Hippocampus and Cerebral Cortex, were collected from each rat quickly, washed thoroughly with ice-cold saline, and frozen in liquid nitrogen or stored in a Deep freezer -80 °C for estimation of the 2 Cholinergic constituents, ACh and AChE on 60th and 90th day of treatment with the following techniques.

Cholinergic System						
Acetylcholine content (ACh) Acetyl cholinesterase (AChE)	Metcalf	method Augustinsson (1957). Ellamasn *et. al.* (1961)	(1957)	As	Given	By

Statistical Analysis: All the experiments were carried out in triplicates, and values of the measured parameters were expressed as Mean±SEM. Two-way ANOVA was used to test the significance of difference among nine groups, followed by Dunnet's Multiple Range Test (DMRT). Statistical analysis was performed by using the Statistical Program of Social Sciences (SPSS) for windows (Version 16; SPSS Inc., Chicago, 1L, USA). The data was regarded as significantly different at $P<0.05$.

RESULTS: From the results, it was noticed that, in general, squalene had caused significant alterations in both Acetylcholine (ACh) content and the Acetylcholinesterase (AChE) activity levels in selected brain regions *viz.*, Cerebral Cortex (CC) and Hippocampus (HP) of control and different groups of experimental rats on selected days of time intervals.

Acetylcholine (ACh) Content: (Graph.1)

Effect on 60th Day: Analysis of the data on control groups of rats revealed that the Acetylcholine content was highest in the Hippocampus (195.21 µ moles of ACh/gm) than in the Cerebral Cortex (172.20 µ moles of ACh/gm). Squalene alone treated group rats showed increased ACh content in the Cerebral Cortex and Hippocampus by 30.79% and 15.36%, respectively. In the AD-induced group, both Cerebral Cortex and Hippocampus recorded significantly declined ACh content (12.16% and 16.30%). Interestingly, simultaneous treatment of AD-Induced rats with squalene caused a significant elevation in ACh content in the Cerebral Cortex (42.42%) as well as the Hippocampus (25.51%).

Even though a similar trend was observed in all rats on 90th day of the experiment, the impact of squalene, either in terms of elevation or suppression, was more pronounced compared to that of 60th day. For example, the control group rats showed significant improvement in ACh content in both the Hippocampus (215.89 µ moles of ACh/gm) and Cerebral Cortex (148.20 21 µ moles of ACh/gm), while squalene treated group

exhibited more ACh in Cerebral Cortex (41.85%) than the hippocampus (18.20%). There was a gradual increase in the percentage change, with maximum percent change noticed in Hippocampus (32.72%) followed by Cerebral Cortex (15.98%). As in the case of 60th day, the Protective group of rats showed a much more elevation in ACh content in Cerebral Cortex (72.21%) followed by Hippocampus (22.79%). Further, it was also noticed that the recovery effect of Squalene on AD-induced changes were seem to be dependent on the duration of treatment *i.e.*, longer the duration more recovery.

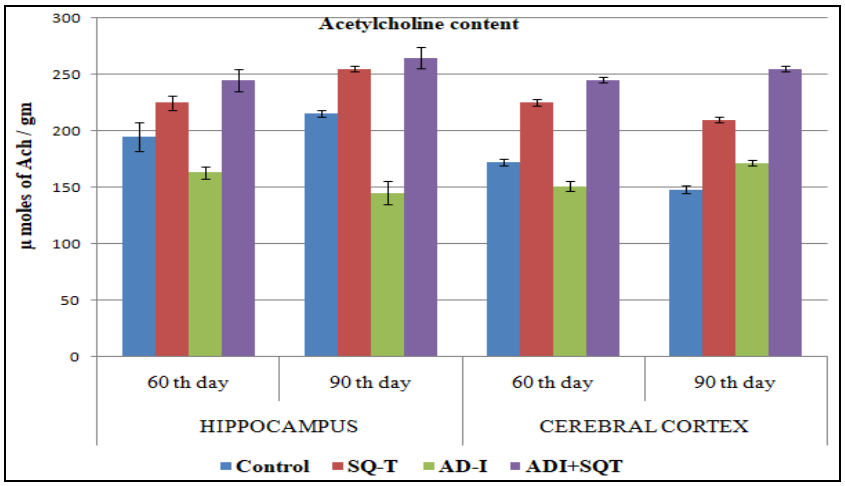

FIG. 2: CHANGES IN ACETYLCHOLINE CONTENT IN HIPPOCAMPUS AND CEREBRAL CORTEX REGIONS OF CONTROL AND EXPERIMENTAL GROUPS OF RAT BRAIN ON SELECTED DAYS. VALUES ARE MEAN ± SEM (N=6). *P<0.05 *VS*. CONTROL; #P<0.01 *VS*. AD MODEL

Acetyl Cholinesterase (AChE) Activity: (Graph 2):

Effect on 60th Day: The activity levels of AChE in both brain regions were quite contrasting to the ACh content. The activity level of AChE in control groups of rats recorded was highest in the Cerebral Cortex (8.00µ moles of ACh hydrolyzed/mg protein/h) followed by Hippocampus (7.68 µ moles of ACh hydrolyzed/mg protein/h). Administration of Squalene caused significant decrease in AChE levels in Hippocampus (29.42%) and Cerebral Cortex (15.00%), AD induction elevated AChE in both regions. However, in a protective group of rats, the AChE levels showed a recovery tendency.

Effect on 90th Day: Even though a similar trend in AChE levels was noticed, the percent changes were more predominant in control and all experimental rats. An interesting observation was that the percentage of recovery was more pronounced on the 90th day when compared to that on 60th day.

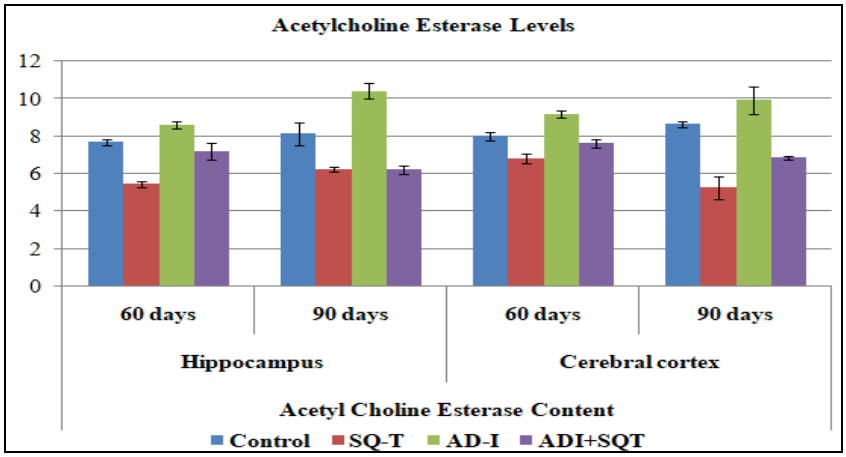

FIG. 3: CHANGES IN ACETYLCHOLINESTERASE LEVELS IN HIPPOCAMPUS AND CEREBRALCORTEX REGIONS OF CONTROL AND EXPERIMENTAL GROUPS OF RATS ON SELECTED DAYS. VALUES ARE MEAN ± SEM (N=6). *P<0.05 *VS*. CONTROL; #P<0.01 *VS*. AD MODEL

DISCUSSION: The observations in the present study demonstrated the occurrence of severe perturbations in the cholinergic system in selected brain regions of rat brains under various experimental conditions, as described in the results section. Overall, the results indicated that chronic administration of D-Galactose caused a significant reduction in ACh levels in both brain regions, such as Cerebral Cortex and Hippocampus. From the comparative studies conducted on the two constituents of the cholinergic system in all experimental groups of rats, it was evident that chronic administration of squalene for 90 days showed significant improvement and recovery in both ACh content and AChE activity levels in AD-induced rats, which was approximately near to the control levels. Further, it was also noticed that the extent of revival was directly related to the duration of AD induction and the period of treatment with squalene.

It is already well established that during the process of neurotransmission, Acetyl Choline (ACh), found at the synaptic region, will be released from the nerve into the synaptic cleft and fixes to ACh receptors on the post-synaptic membrane, relaying the signal from the nerve. The Acetylcholine Esterase, also located on the post-synaptic membrane, terminates the signal transmission by hydrolysing ACh into its two parts *i.e.* Acetic acid and Choline, wherein the liberated choline is taken up again by the pre-synaptic nerve and again ACh is synthesized by combining with acetyl-CoA through the action of choline Acetyltransferase [11].

The occurrence of ACh in various regions of the Central Nervous System, Peripheral Nervous System and Autonomic Nervous System and its importance concerning several neuronal diseases is well explained as follows: Acetylcholine is present throughout the nervous system, being essential for cerebral cortical development and activity of sleep-wake cycle, control of cerebral blood flow [12] and attention, motivation and memory [13] besides acting as a neuromodulator. Since, its identification several decades ago, it has been well established that the cholinergic neurons in the basal forebrain innervate the Cortex and Hippocampus; as such their functions may be closely related to cognitive function and memory. Degeneration of neuronal cells in these brain regions is responsible for several types of dementia including Alzheimer's disease. Several earlier research findings have reiterated, beyond a doubt, that the first and the major neurotransmitter causing Alzheimer's disease is deficiency of Acetylcholine, which is responsible for initiating short-term Memory [14]. Cholinesterases are a family of enzymes that catalyse the hydrolysis of acetylcholine into choline and acetic acid, an important process allowing restoration of the cholinergic neuronal functions. The enzymes, *viz.* Choline Acetyltransferase and Acetylcholine Esterase, the major markers for cholinergic neurons involved in the synthesis and degradation of ACh, respectively, are decreased in the Cerebral Cortex and Hippocampus areas of the brain responsible for memory and cognition. It has been generally agreed that Acetylcholine Esterase is the only cholinesterase playing a functional role in the nervous tissue.

The research findings on several animal models have demonstrated that loss of cholinergic function in these areas is associated with declined learning and memory. Further, the Neuropath logical demonstration that cholinergic markersin the Cerebral Cortex and hippocampus are changed in AD condition [15-16] which have been linked to its pathology and degree of cognitive impairment similar to cholinergic and memory deficits observed in normal aging populations, but for the severity of cognitive dysfunctions.

Normal aging is an advanced process related to the accumulation of oxidative damage wherein the brain undergoes morphological and functional changes resulting in the behavioral retrogression of cognition [17]. All these deteriorated changes, finally culminate in the development of several neurodegenerative diseases including the Alzheimer's disease which is accompanied by a series of abnormal alterations in neurophysiology, neuropathology and behaviour. The other neuropathological hallmarks of AD, namely neuritic plaques and Neuro Fibrillary Tangles are characterized neuro-chemically by a consistent deficit in cholinergic neurotransmission, particularly affecting cholinergic neurons in the basal forebrain [18]. In Alzheimer`s patients, dysfunction of presynaptic system is one of the causes of cognitive deficit in which decreased activity of the enzyme, Choline Acetyltransferase,

responsible for acetylcholine synthesis was observed [19]. Several hypotheses have been presented to explain the aging process, in which oxidative stress may play a key role in Alzheimer`s disease development. Several recent *in-vitro* and *in-vivo* studies have consistently demonstrated a link between cholinergic activation and APP metabolism [20].

All the above evidence lend strong support to our my present observations, wherein squalene also exhibited inhibitory activity on AChE levels along with a rise in ACh content in AD-induced rats. Enhancing several cognitive abilities by squalene treatment might provide substantial evidence that it can reverse the damage caused by AD induction, and improve brain function especially by suppressing the AChE activity levels and inhibiting neuronal apoptosis. Our findings' neuroprotective effect of squalene could be equated to several compounds extracted from marine organisms.

Several earlier researchers have evaluated the anti-Cholin Esterase effect of 134 extracts from 45 species of marine sponges and two of them *viz*. the ethyl acetate extracts of *Pericharaxheteroraphils* and *Amphimedon navalis*, where pulizer-Finali showed strong AChE inhibition [21]. To quote a few; The AChE inhibition promoted by the stigmastane type sterioidal alkaloid 4–Acetoxy-plakinamine-B suggested a mixed competitive mode of inhibition [22]. The pre-fractionated extracts from marine invertebrates and cyanobacteria against BACE1 activity, resulted in 7% of the extracts with outstanding inhibition and 11% with activity between 70% and 89% [23]. Another marine compound, Fucoidan, isolated from *Fucus vesiculosus* was able to protect rat cholinergic neuronal death induced by $A\beta_{1-42}$ [24].

Approximately, eight phlorotannis and two sterols were isolated from *E. stolonifera*, among which the compounds *viz.* Dieckol, Eckol, 2 phloroeckol and 7- phloroeckol demonstrated selective dose-dependent inhibitory activities toward AChE, where as eckstolonol and phlorofucofuroeckol-A exhibited inhibitory activities toward both Acetyl cholinesterase (AChE) and Buteryl Cholinesterase (BChE). Moreover, as per the Amyloid Hypothesis it was also been shown that the neurotoxicity of Aβ peptide aggregation depend on the amount of AChE bound to the complexes suggesting that AChE played a key role in neurodegeneration observed in AD patient's brain. Thus, inhibition of AChE could influence the APP processing and Aβ deposition leading to the reduction of one of the pathological expressions of the disease, thus suggesting that both the Cholinergic and Amyloid hypotheses are dependent. It is also important to note that changes in the central cholinergic system in AD may also contribute to various adverse behavioral symptoms, including cognitive deficits, such as depression, aggressive behaviour, psychosis, and overactivity [25-26]. other cholinergic effects have also been proposed, for example, large-scale aggregation of amyloid [27] leading to generalized neuroinflammation [28].

CONCLUSION: From the results obtained in the present investigation, it was finally inferred that squalene has a neuroprotective role against D-Galactose induced Alzheimer's disease by restoring the Cholinergic constituents to approximate normal levels.

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CONFLICTS OF INTEREST: The authors declared that there is no conflict of interest. Bhosale MD and Kulkarni KS: Bilayer Tablet- A Comprehensive Review. European J of Pharmaceutical and Medical Research 2017; 4(9): 241-251.

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