EFFECTS OF AQUEOUS EXTRACT OF *GLYCYRRHIZA GLabra* LINN. AND DIOSMETIN ON MODULATION OF SPATIAL MEMORY THROUGH ACETYLCHOLINESTERASE AND BRAIN-DERIVED NEUROTROPHIC FACTOR IN ETHANOL-INDUCED COGNITIVE IMPAIRMENT MODEL RATS

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ABSTRACT

Objective: The objective of this research was to evaluate the cognitive impairment due to excessive consumption of alcohol and memory enhancement action of *Glycyrrhiza glabra* Linn. (AEGGL) and diosmetin (Dm).

Methods: In this study, 36 adult male Wistar rats were divided into the six groups (n=6) and eight-arm radial maze, narrow beam test, and open field behavior parameters were assessed on day 1, 10, and 21. After the 21 days of experiment, animals were sacrificed, and blood samples were collected for serum acetylcholinesterase (AChE) and brain-derived neurotrophic factor (BDNF) estimation. We have also analyzed the morphology of CA3 region of the hippocampus.

Results: The results of this study suggested that AEGGL and Dm treatment could be the potential drugs for ethanol-induced cognitive impairment.

Conclusion: Ethanol-induced cognitive impairment was recovered by AEGGL and Dm treatment, we suggested that this might be due to anticholinesterase activity and increased synthesis of BDNF levels in the brain. Further, researches are warranted to understand the exact mechanism of action of drugs.

Keywords: *Glycyrrhiza glabra* Linn., Diosmetin, Ethanol, Cognitive impairment, Spatial memory.

INTRODUCTION

Alcohol is one of the most consumed products and becomes a trend among the youngsters in India. The World Health Organization has reported that Indians reached the second place in South East Asian Region in alcohol consumption [1]. Excessive exposure to alcohol leads nervous system-related diseases with or without nutritional deficiencies. Metabolism and toxic effects of ethanol vary in the brain region and depend on age, dosage, and duration of consumption. However, recent studies show that alcohol affects neuronal cells, specifically astrocytes and microglial cells are the major targets. This leads to white matter atrophy, neuroinflammation, and impaired neurogenesis [2]. Catalysis of alcohol produces reactive oxygen species ends up with oxidative stress and results in many irreversible changes in the brain [3]. Cognitive abilities are fully depend on the hippocampus structure integrity and neurotransmitter acetylcholine (ACh) levels. Acetylcholinesterase (AChE) is considered as treatment target to keep up the ACh level in some neurodegenerative disorders such as Alzheimer’s disease and dementia [4]. Accumulative evidence have demonstrated that ethanol caused cognitive impairment and cholinergic dysfunction associated with oxidative stress [5,6]. Long-term cognitive deficits, alteration in memory processing, and changes in synaptic transmission were associated with acute and chronic alcoholic models through action on glutamate, gamma-aminobutyric acid, and N-methyl-D-aspartate receptors (NMDAR) [7]. Ethanol-induced mild cognitive impairment (MCI) was produced at the dose of 1.75 mg/kg of ethanol for 21 days to identify the better cholinesterase inhibitors between donepezil and rivastigmine for treating cognitive deficits [8]. Neuroprotective effects of neurotrophins are playing key role in modulation of cognitive impairment and functional recovery after brain tissue inflammation. Especially, brain-derived neurotrophic factor (BDNF) involved with neuronal survival and plasticity which is synthesized primarily from neurons secondarily from neuroglial cells of inflamed brain tissue [9]. Elevated BDNF levels were ameliorating the cognitive impairment by supporting the injured hippocampus. Estimation of BDNF exhibits the neuronal integrity of CA3 pyramidal neurons of hippocampus [10].

Currently, very few approved drugs are available for cognitive impairment and neurodegenerative disorders caused by alcoholism because of less bioavailability and adverse side effects [11]. Therapeutic benefits of natural products were considered in drug discovery field to the find better molecule for neurological disorders. *Glycyrrhiza glabra* Linn. (GGL) is a medicinal plant commonly known as licorice (Fabaceae family) and athiramburum in Tamil which has many therapeutic potential to treat as hepatoprotective, antiulcerative, and antimicrobial agents [12]. Enhancement action of GGL (AEGGL) is one of the herbal extracts, commonly used Indian system of medicine for enhancing learning and memory [13]. AEGGL has lethal dose as higher that can be used for chronic consumption [1,4]. Dm is known antioxidant molecule which is responsible for the protective effect against neurotoxicity [15]. Dm produced improvement of spatial learning, working memory and role in modulation of cognitive impairment and functional recovery after brain tissue inflammation. Especially, brain-derived neurotrophic factor (BDNF) involved with neuronal survival and plasticity which is synthesized primarily from neurons secondarily from neuroglial cells of inflamed brain tissue [9]. Elevated BDNF levels were ameliorating the cognitive impairment by supporting the injured hippocampus. Estimation of BDNF exhibits the neuronal integrity of CA3 pyramidal neurons of hippocampus [10].

The results of this study suggested that AEGGL and Dm treatment could be the potential drugs for ethanol-induced cognitive impairment.
Thus, this study was aimed to investigate the effects of AEGGL and Dm on spatial memory, cholinergic function, and motor activities of Wistar rats with ethanol-induced cognitive impairment.

**METHODS**

**Experimental animals**

A total of 36 adult male Wistar albino rats (*Rattus norvegicus*), with 180–280 g body weight were purchased from the Center for Laboratory Animal Research, Department of Research Development, Saveetha Institute of Medical and Technical Sciences, Chennai. This study was approved by IAEC Reference number - (SU/CLAR/RD/038/2017) dated 25/08/17.

The rats were housed in polypropylene cages with paddy husk bedding, standard food pellets, and drinking water ad libitum, and 12 h light and 12 h dark schedule in 23°C ± 2°C were provided.

**Chemicals and drugs**

Ethanol (95%, Nanda, Inc.), normal saline, dioxetin (DM) (Sigma, Inc.), AChE (Sigma, Inc.) and BDNF assay kit (Bosterbio, USA) dimethyl sulfoxide (DMSO), and other laboratory chemicals (Labthi, Inc.) were purchased for this experiment.

**Plant material and extract preparation**

The roots of GGL were purchased from local herbal market in Chennai and got authenticated from medicinal botanist, Department of Medicinal Botany, National Institute of Siddha, Tamil Nadu, India. The specimen was deposited for future reference, and the authentication reference number is NIAS 1502015.

The roots were dried and powdered to mix with distilled water in Soxhlet apparatus for aqueous extract preparation. The filtrate was collected and evaporated using reduced pressure evaporator, and the extract yield was collected for the study [17].

**Experimental groups**

- Control group received DMSO for 21 days intragastrically (i.g).
- ETOH group received ETOH (1.75 mg/kg/d) for 21 days (i.g).
- AEGGL group received AEGGL (150 mg/kg/d) along with DMSO for 21 days (i.g).
- Dm group received Dm (4 mg/kg/d) along with DMSO (1 mg/kg/d) for 21 days (i.g).
- AEGGL+EIOH group received AEGGL (150 mg/kg/d) along with ethanol (1 mg/kg/d) for 21 days (i.g).
- Dm+EIOH group received Dm (4 mg/kg/d) along with ethanol (1.75 mg/kg/d) for 21 days (i.g).

**Standardization of behavioral tests**

All animals were transferred to experimental room from quarantine room, after acclimatization behavioral training was given for 1 week. In this period, animals were familiarized with apparatus and test procedures. During behavioral training period standardize the experimental procedure was done. Behavioral assessments were studied using open field test (OFT), eight-arm radial maze (EARM), and narrow beam test. Behavioral studies were conducted on the 1st, 10th, and 21st day of the experiment for six groups after 4 h of drug administration.

**EARM**

The radial arm maze consists of eight arms, numbered from 1 to 8 arms of 48 cm × 3 cm × 12 cm, extending radially from a central platform (40 cm in diameter), with a 5 cm edge around the apparatus. Each radial arm is equally spaced and contains food cups at the end. Removable blocks of 9 cm × 3 cm × 13 cm were used to block the selected arm of the maze. The maze was elevated 40 cm from the ground or floor. The following variables were scored for 5 min and recorded: Number of reference memory errors (entry of animal into the non-baited arm, number of working memory errors (reentry of animal into already visited baited arm), and time taken to visit all four baited arm latency [18].

**RESULTS**

OFT

OFT is a measure of emotional behavior in animal. It is a systematic assessment of new environment exploration, locomotor activity, and anxiety-related behaviors in rats. The OFT apparatus is made of a large square-shaped arena of 80 cm × 80 cm with 40 cm high walls. The floor is marked into 25 equal square segments to allow quantification of locomotor activity. Each rat was placed at the center of the arena and was observed the time spent in the periphery of the arena, time spent in the center of the arena, number of squares (NOS) crossed (NSC) by the animals, and number of fecal pellets passed [19].

**Narrow beam test**

The rats were trained to walk on a 10–20 mm wide wooden beam elevated 100 cm above the floor. After training, traversing time through beam to reach safe platform (100 mm distance) and NOS was quantified [20].

**Blood sample collection and tissue preparation**

After behavioral assessment, animals were anesthetized with isoflurane and blood was collected for serum BDNF and AChE estimation. Estimation of acetylcholinesterase and BDNF level in plasma was done as per manufacturer instruction [21,22]. The animals were sacrificed and brain tissue was harvested and placed forma buffered solution for 48 h for dehydration. The dehydrated brain was washed with cold saline and embedded with paraflin wax. The blocks were sectioned as 3 μm thickness by microtome at levels of hippocampus in the rat brain. The sections were mounted on glass slides and left for dry in hot plate (40°C). The slides were dewaxed using xylene followed by hematoxylin and eosin staining, and sections were used to count the number of neuron by trinocular microscope (Olympus-BX40) and image J2 software [23].

**Statistical analysis**

The data were expressed as mean ± SEM and analyzed using one-way analysis of variance followed by post hoc Tukey’s test for multiple comparisons of groups. All statistical analyses and graph plotting were carried out using SPSS software 22.0 (Chicago, USA). p<0.05 is considered as statistically significant.

**RESULTS**

**Effect of AEGGL and Dm on spatial memory**

Figs. 1-3 showed that progressive reduction of spatial memory as decreased latency period and increased working methanol extract (WME) and reference methanolic extract (RME) in EARM test in ETOH group in time-dependent manner. AEGGL+EIOH group and Dm+ETOH group have shown significant improvement in spatial memory compared with ETOH group as the effect of drugs given for 21 days. Comparing the results in between groups by Tukey’s post hoc analysis on the 21st day, WME (F=20.429, p<0.001), RME (F=20.078, p<0.001), and latency period (F=27.889, p<0.001) were statistically significant. Drug only treated groups such as AEGGL and Dm group was shown less WME, RME, and increased the latency period than control group after 21 days (F=2.543, p<0.05). Specifically, AEGGL group has shown significant effects on ETOH-induced MCI rats than Dm group after the 10th and 21st day.

**Effect of AEGGL and Dm on open field exploration**

The anxious behavior of animals in OFT such as time spent in center of arena, near peripheral walls, and fecal pellet was shown in Figs. 4-7. ETOH group spent more time in periphery as day progressively than control group, AEGGL and Dm group after the 10th (F=12.184, p<0.001) and 21st day (F=24.291, p<0.001). AEGGL+EIOH group and Dm+ETOH spent more time in center of arena than ETOH group. Ethanol reduces the NOS crossing in OFT in a day-dependent manner that AEGGL and Dm groups showed significant ambulation than control group on the 10th day (F=24.943, p<0.001) and 21st day (F=32.089, p<0.001). AEGGL+EIOH and Dm+EIOH group exhibit the significant difference in NOS than the ETOH group that drugs improve the ambulation.
Effect of AEGGL and Dm on ambulation
Figs. 8 and 9 demonstrated that the effects of AEGGL and Dm on latency to cross the narrow beam and NOS during the traverse. The results showed that the motor activity is affected in ETOH group than AEGGL+ETOH and Dm+ETOH groups throughout the days statistically (F=2.018, p<0.04). NOS were significant in control, AEGGL and Dm treated groups than ETOH group after 21 days of experiment. NOS were prevented in AEGGL+ETOH and Dm+ETOH group (F=2.265, p<0.05) than ETOH group as showed the effect of drugs on motor activities in time-dependent manner.

Effect of AEGGL and Dm on AChE activity and BDNF levels
Since cholinergic neurons played a key role on spatial memory, we also investigated the effect of AEGGL and Dm on AChE activity in the plasma, and the results were shown in Table 1. The activity of AChE
The significant reduction of AchE activity was observed in AEGGL+EtOH and Dm+EtOH treated animals which maintain the cholinergic neurons after 21 days of experiment. Interestingly, AEGGL and Dm only treated groups were shown less AchE activity (p=0.001) compared with control rats which mean they could act as cholinesterase inhibitors. Overall, the results suggest that the AchE level was increased by ethanol induction (F=14.208, p<0.001), and the drug components were used tried to bring back the AchE level to normal by decreasing the AchE esterase level.

Table 1 demonstrated that the effects of AEGGL and Dm on biochemical parameters. We have investigated the plasma BDNF level that was high in drug only treated groups compared with control group. Plasma BDNF level is less in ETOH group than AEGGL+EtOH and Dm+EtOH group significantly (F=39.503, p<0.001) as shown as the beneficial effect of drugs. It shows that AEGGL increases the BDNF level than Dm that can be potential drug to manage the neuronal damage and increases the cognitive abilities after 21 days of ethanol induction.

**Effect of AEGGL and Dm on hippocampal pyramidal neurons**

Histological sections divulged the hippocampal pyramidal neurons in the CA3 region in each group (Fig. 4a-g). The average number of neurons in the CA3 region decreased and scattered layer was noticed in ETOH group (Fig. 4b) than control group. Ethanol ensues the decreased number of neurons in the AEGGL+ETOH (Fig. 4e and g) group and Dm+ETOH group (Fig. 4f) than control group (Fig. 4a); irregularities of neuronal layer were reduced from ETOH group. Normal pyramidal neurons were increased in AEGGL (Fig. 4c and g) and Dm group (Fig. 4d and g) than the control group. These findings suggest that AEGGL and Dm can be the potent drugs for ethanol-induced MCI (Fig. 10).

**DISCUSSION**

Many people have MCI by various causes, but identification and diagnostic aspects of cognitive dysfunction are difficult part, especially alcohol consumers. Awareness about alcohol-induced MCI and appropriate treatment strategies is required without adverse effects. We have observed the results clearly that the rats with ethanol-induced MCI advocate impaired spatial memory, locomotion, elevated AchE activity, and decreased plasma BDNF level. AEGGL and Dm protect the cognitive abilities from 21 days of ethanol neurotoxicity. In this study, dosage of ethanol may cause motor activity did not much affect after 21 days ethanol induction. However, the cognitive skills were enhanced by AEGGL and Dm, but motor activity did not much affect after 21 days ethanol induction. In brief, AEGGL and Dm might be act on receptor and ion channels at molecular level to protect from chronic ethanolic effects. The lethal dose 50 (LD50) of AEGGL was observed at the dose of 833.3 mg/kg, however, LD50 of Dm was not observed as showed in the LD50 of Dm, LD50 of AEGGL, and LD50 of drug only treated groups compared with control group. Plasma BDNF level is less in ETOH group than AEGGL+EtOH and Dm+EtOH group significantly (F=39.503, p<0.001) as shown as the beneficial effect of drugs. It shows that AEGGL increases the BDNF level than Dm that can be potential drug to manage the neuronal damage and increases the cognitive abilities after 21 days of ethanol induction.

Ethanol ensues the decreased number of neurons in the CA3 region decreased and scattered layer was noticed in ETOH group (Fig. 4b) than control group. Ethanol ensues the decreased number of neurons in the AEGGL+ETOH (Fig. 4e and g) group and Dm+ETOH group (Fig. 4f) than control group (Fig. 4a); irregularities of neuronal layer were reduced from ETOH group. Normal pyramidal neurons were increased in AEGGL (Fig. 4c and g) and Dm group (Fig. 4d and g) than the control group. These findings suggest that AEGGL and Dm can be the potent drugs for ethanol-induced MCI (Fig. 10).
the learning, spatial, and reference memory is related with the enhanced level of AChE and decreased BDNF in ETOH group rats. Overall, the biochemical results suggest that the AChE level was increased by ethanol induction as the result of it the Ach level might have decreased. The drug components used are tried to bring back the Ach level to normal by decreasing the ACh esterase level. AEGGL and Dm have the beneficial effects on behavioral and biochemical parameters to enhance the cognitive abilities. Scanty studies are available about potential of Dm on lipophilicity and blood–brain barrier (BBB) permeability. Dm molecule has the high probability to cross BBB and high lipophilicity among the flavonoids and dock with P38 MAPK, c-JNK ERK1/2 signaling pathways in brain [33]. Some in vivo studies exhibit the neurocognitive effects and protective effects of Dm on hippocampal integrity cognitive dysfunctions [34]. Plasma AChE is a marker for Alzheimer’s disease.

Table 1: Effects of AEGGL and Dm on biochemical parameters

<table>
<thead>
<tr>
<th>Groups</th>
<th>AChE (µ mol/min/mL)</th>
<th>BDNF (pg/mL)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.53±0.03</td>
<td>44.3±2.1</td>
</tr>
<tr>
<td>ETOH</td>
<td>0.92±0.04#</td>
<td>17.0±2.4#</td>
</tr>
<tr>
<td>AEGGL</td>
<td>0.52±0.03*</td>
<td>49.7±1.3*</td>
</tr>
<tr>
<td>Dm</td>
<td>0.52±0.04*</td>
<td>50.0±2.2*</td>
</tr>
<tr>
<td>AEGGL+ETOH</td>
<td>0.65±0.03#*</td>
<td>31.8±1.9#*</td>
</tr>
<tr>
<td>Dm+ETOH</td>
<td>0.68±0.06#*</td>
<td>30.8±2.3#*</td>
</tr>
<tr>
<td>Significance</td>
<td>F=14.208; P&lt;0.001</td>
<td>F=39.503; P&lt;0.001</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± SEM and analyzed by one-way analysis of variance followed by post hoc Tukey’s test for multiple comparison of groups (n=6). #is significant from control and *is significant from ETOH. SEM: Standard error mean, AEGGL: Enhancement action of Glycyrrhiza glabra Linn., Dm: Diosmetin, AChE: Acetylcholinesterase

Fig. 10: Effect of enhancement action of Glycyrrhiza glabra Linn. (AEGGL) and diosmetin (Dm) on hippocampal morphology of CA3 pyramidal neurons of rats with ethanol-induced mild cognitive impairment. (a) Control, (b) ETOH, (c) AEGGL, (d) Dm, (e) AEGGL+ETOH, (f) Dm+ETOH, and (g) total number of neurons in the CA3 region; morphological changes in different experimental groups with hematoxylin-eosin staining data expressed as mean±SEM (n=6 and magnification ×40). *Statistically significant #no significance.
and associated with cognitive decline in aging [35]. Our experiment biochemical outcome suggests that depressed AChE levels are necessary in retrieval of spatial memory. It is also been inferred that experimentally trained memory is stored in the neocortex and recalled by glutamatergic signals from the prefrontal cortex [36] because of an intercommunication between prefrontal cortex and hippocampus in memory retrieval [37]. Biochemical and pathological studies were conducted on the hippocampal parenchyma to identify hippocampal neurotoxicity in Wistar rats that results correlated with the current study [38]. Another study reported that Hypericum perforatum alleviates the neurotoxicity and AD. Histopathological studies were shown as appeared as shrunken and decreased number of the pyramidal cells [39]. These results inferred with our study and suggest that the herbal plants could be a valuable source of drug against NDD which will prove through high-throughput screening for further drug discovery efforts [40]. Our results exhibit that elevated BDNF level in drug-treated group may be implied with prevention of cognitive impairment. Decreased BDNF in ETOH group showed that progressive MCI for 21 days. BDNF is responsible for neural integrity, synaptic plasticity, and neurogenesis this might be inferred with our AEGGL group rats that they performed well in locomotion and spatial memory [41] even in AEGGL+EtOH group. We could enlighten the BDNF is a potential biomarker which is playing a key role in neuronal survival and differentiation of neuronal population of adulthood [42,43] in ETOH-induced cognitive impairment.

CONCLUSION

We have observed that AEGGL and Dm prevent the ETOH-induced cognitive impairment in Wistar rats. This might be due to anticholinesterase activity and increased synthesis of BDNF levels in brain. AEGGL and Dm could be used as precursor material to derive potential drug molecule for treating ethanol-induced MCI. Molecular level studies are required to enlighten the exact mechanism of being neuroprotective agent against alcohol-related disorders.

AUTHORS’ CONTRIBUTION

Sasikumar A - conducts the research work, acquisition of data, and interpretation of the work. Suba Malani S - drafting and revising the intellectual work of this study. Manikandan S - conception, design of work, final approval, and communication. Ramaswamy C - interpretation of data and summarize the work.

CONFLICTS OF INTEREST

Nil.

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