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A STUDY OF NEUROBEHAVIORAL AND BIOCHEMICAL ACTIVITIES OF *ACORUS CALAMUS* LINN. ON RESTRAINT STRESSED WISTAR RATS

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ABSTRACT: Life span of human, a gradually increase can occur with change in diet and life style which play an important role in delay or even block the progression of age related degenerative problems like dementia, alzheimer's disease. Exposure to various stress is the emerging greatest challenge to the society recent years. The ill-effect of stress is release of oxidants which is found to be always associated with cognitive decline. The natural plants like *Acorus Calamus* Linn. (ACL) has been proved to have antioxidant effect and ameliorating behavioral deficits caused due to neurodegenerative by exposure to stress. To investigate the behavioural activity and the biochemical effect of ethanolic extract of *Acorus Calamus* Linn. (EE-ACL) and the active principle α-asarone (AA) in restraint induced stressed rats. Adult male Wistar rats were divided into five groups six in each. Group I treated with 0.5% of DMSO 1 mL/kg/p.o, Group II treated with 0.5% of DMSO 1 mL/kg/p.o + restraint stress 6 h for 21 days, Group III treated with EE-ACL 100 mg/kg/p.o + restraint stress 6 h for 21 days, Group IV treated with AA 9 mg/kg/p.o + restraint stress 6 h for 21 days, Group V treated with *Tinospora cordifolia* TC 40 mg/kg/p.o + restraint stress 6 h for 21 days. The behavioural performance, biochemical analyses were done. The anxiety like behaviour was analysed by elevated plus maze (EPM) and open field test (OFT). The spatial learning and memory was assessed using Y-maze and eight arm radial maze (EARM). The Corticosterone level is estimated in all groups. Statistical analysis was done by one-way analysis of variance, followed by post-hoc Tukey's test for multiple comparison of groups. $p<0.05$ was considered statistically significant. The results suggest that EE-ACL and the active principle AA has significantly improved cognitive functions in rats subjected to chronic restraint stress. The corticosterone concentration was decreased in rats pre-treated with the plant compound. Improvement in cognition could be due to the antioxidant action of ACL. On comparing with the TC which served as a standard drug the EE-ACL treated rats showed statistically significant result. The ethanolic extract of ACL and the active principal showed neuro-cognitive effect due to the presence of phytoconstituents such as triterpenoids, flavonoid, tannins and saponins. Hence, ACL could be an adjuvant therapy as it plays a role in neuronal stress adaptation mechanism and have potential to prevent progression of neurodegenerative diseases.

INTRODUCTION: Stress is one of the most prevalent and life-threatening forms of mental illness affecting more than one fifth of the world's population.

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Stress is the common cause for dementia which is the emerging greatest challenge to the society in recent years. Dementia is always associated with cognitive decline [1]. The WHO 2012 have reported that 35.6 million people suffer from dementia worldwide. It is expected to increase by double in the year 2030 [2]. Prolonged exposure to stress is known to cause anxiety, depression, neuro psychiatric and neuro degenerative disease [3]. Stress is known to elevate circulating levels of glucocorticoids with stimulation of hippocampal

production of neurotrophies and inflammatory cytokines that bring about molecular, structural and functional changes in the brain. Animals exposed to restraint stress produces an imbalance between oxidants and antioxidants, which is the main pathogenesis of various neurodegenerative diseases. Oxidative neurodegeneration is a key factor in worsening the behavioral recoveries. Increased level of corticosterone from adrenal cortex causes damage to hippocampal neurons as restraint effectively produces endoplasmic damage which initiates hippocampal apoptosis and cognitive impairment [4]. The hippocampus is one of the central nervous system (CNS) which is extensively studied in relation to find how cognitive impairment occurs because of exposure to chronic stress. Repeated neuronal stress has been proved to cause a release of oxidants which affects the neuronal function in the hippocampus [5]. Glucocorticoids regulates learning and memory in hippocampus is one of the center for cognition mainly involved in the consolidation of memories and had more glucocorticoid receptors (GR). The behavioral reactivity of the rats is correlated with a high secretion of corticosterone in response to stress [6]. With the above facts, the stress has a role in altering the learning and memory *via* elevated the plasma corticosterone in hippocampus.

The root cause for cognitive decline due to exposure to stress is release of oxidants. The antioxidants released by the body to counteract the oxidants is not enough to prevent cellular damage. Hence the compound from natural plant material with antioxidant effect is essential to prevent the damage [7]. One such natural plant is *Acorus calamus* Linn. (ACL) of Araceae family is a semiaquatic, perennial, aromatic herb with its rhizome grown all over India [8]. It has been used for hundreds of years to cure diseases especially the CNS abnormalities [9]. One of the active principles is α-asarone and It has been proved for its antioxidant effect, ACL possesses a beneficial memory enhancing property for memory impairment, learning performance, and behavior modification [10]. The ethanolic extract demonstrated to possess potential anti-oxidative, anti-inflammatory as well as neuroprotective actions [11]. ACL has also been reported to decrease free radical generation via enhancement of anti-oxidant mechanisms such as increase in superoxide dismutase, catalase, reduced glutathione and glutathione peroxidase levels [12]. Oral administration of alcoholic extract exhibited an antidepressant like activity which may be by modulating the central neurochemical as well as HPA axis in response to stress induced by FST [13]. *Tinospora cordifolia* (TC) has been proved to possess learning and memory enhancing [14] antioxidant, [15] 2002 and anti-stress activity [16]. Oral administration of TC in healthy volunteers and it has enhanced verbal learning and memory and logical memory of immediate and short term type [17]. The evaluation of exploratory behaviour, anxiety like behaviour was done using open field test and elevated plus maze. The spatial learning and memory and spontaneous alteration behaviour was evaluated by eight arm radial maze and Y maze.

The present study is aimed to evaluate the effects of ethanolic extract of *Acorus calamus* Linn. (EE-ACL), active principle alpha-asarone (AA) on behavioural and biochemical responses in restraint induced stress in rats.

MATERIALS AND METHODS:

Animals: A total of 30 adult Wistar albino rats (*Rattus norvegicus*), with 180 to 220 g body weight were purchased from Center for laboratory animal research, Department of Research Development, Saveetha Institute of Medical and Technical Sciences, Chennai. IAEC Reference number - (SU/CLAR/RD/037/2017) 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.

Acorus calamus was purchased from Tampcol Ltd., Chennai, India. It was identified and authenticated by The Director of Centre for Advanced Studies on Botany, University of Madras, Chennai, India. The authentication number is NIAS 1502014.

Preparation of Ethanolic Extracts: The shade dried rhizome (100 g) of ACL was ground to coarse powder, placed in a Soxhlet extractor containing 70% of ethanol and resulting extract was concentrated in a rotatory evaporator under reduced pressure. Extracts were stored in refrigerator (4 °C) until further use. The drug AA was purchased from Fluka, Sigma-Aldrich Ltd., St. Louis, MO, USA).

TC was purchased from Sanjeevani pharmacy, Chennai. Other chemicals were purchased from Southern surgical ltd, Chennai. Corticosterone assay kit was purchased from Thermo Fisher-Scientific company, USA. The suspension of EE-ACL, AA and TC for Intra oral administration using oral gavage was prepared by dissolving it in 0.5% of DMSO to the required volume.

Experimental Groups: The Wistar albino rats were divided into 5 group, each group consisting of 6 animals. The groups are given in **Table 1**.

TABLE 1: DIFFERENT GROUPS OF ANIMALS AND EXPERIMENTAL SCHEDULE

Groups	Drugs and dose injected (p.o.)	No. of days	Restraint Stress
1	0.5% of DMSO 1 mL/kg	21	No
2	0.5% of DMSO 1 mL/kg	21	Yes
3	EE-ACL 100 mg/kg	21	Yes
4	AA 9 mg/kg	21	Yes
5	TC 40 mg/kg	21	Yes

Restraint Stress Procedure: The animals subjected to restraint stress and the material used to produce restraint stress is made up of stainless steel dimensions of 15 cm × breadth 7 cm × width 9 cm. was used for the experiments for 6 h per day (9.00 am to 3.00 pm) and followed for 21 consecutive days. The restraint apparatus had multiple holes allowing the animals to stretch the legs, but will not allow the animal to move within the restraint cage [18, 19, 20].

Behavioural Assessment:

Open Field Test (OFT): The OFT apparatus was made of a large square shaped arena of 80 cm × 80 cm by 40 cm high walls. The floor was marked into 25 equal square segments and divided into outer and central region. A 40-W frosted bulb was suspended above the arena. The exploring and emotional behaviour of the animal was measured in 5-minute test time. Each rat was placed at the center of the arena and was observed for the following.

Time spent in the center and the periphery of the arena (ambulation), standing on the hind legs with or without a support of the wall, the number of fecal pellets passed, Number of rearing and grooming activity were calculated [21, 22].

Elevated Plus Maze (EPM): The apparatus was made of two open arms and two closed arms crossed in the form of plus sign open arm was 50 × 10 × 2 cm and closed arm was 50 × 10 × 40 cm with an open roof. The device was elevated 50 cm from the floor. The animals were placed in the center facing closed arm. Time spent in open arm and closed arm, number of fecal pellets and rearing were recorded and analyzed for 5 min [22].

Y Maze Test: Spatial learning and spontaneous alteration task were analysed by Y-Maze. The apparatus consists of three arms connected into a Y shape 40 × 8 × 15 cm arms. Animal is placed in Arm A facing away from center and allowed to move through apparatus for 5 min. Scoring consists of recording each arm entry. An alteration was defined as entry into all three arms consecutively and the sequence of arm entries was manually recorded. The alteration percentage was then calculated as the ratio of actual to possible alterations. Number of triads = (total number of entries-2). Triad (set of three letters) containing all three letters is scored as alteration.

Percent alteration = [(number of alterations / total number of triads) × 100].

Rats with less than 8 times arm entries during the 5-min trial were excluded from the analysis [23, 24].

Eight Arm Radial Maze (EARM): Each Radial arm was equally spaced and contains food cups at the end. Animals were allowed to explore all eight arms for first two days of the training period. The four arms (arm number 2, 4, 6 and 8) were provided with food for each training trial and the other four arms (arm number 1, 3, 5 and 7) were kept empty. The training period continued until food was consumed or 5 min had lapsed. Number of working memory errors indicated the re-entry of animals into already visited arm- with food pellet, Working memory error (WME) is re-entry of animal into already visited arm with-no food pellet, and reference memory errors is re-entry of animal into the arm without food and with food were calculated. In addition to that latency is the time taken to visit all four arms is also calculated [25, 26].

Estimation of Corticosterone Level: The blood samples were centrifuged at (6000 rpm at 4 ºC for 15 min), and the plasma was stored at -80 ºC until

used for the corticosterone assay. Plasma corticosterone levels were measured by using ELISA.

Statistical Analysis: The data were expressed as mean ± SE and analysed using one way analysis of variance followed by post-hoc Tukey's test for multiple comparison of groups. All statistical analysis and graph plotting was carried out using Sigma Plot 13.0 (Sys stat software, USA).

RESULTS:

Open Field Test: In OFT, the time spent in the centre is significantly decreased in stress group than control group Time spent in centre of the arena is significantly increased in EE- ACL group than stress group with (F = 4.072, p<0.011).

AA group did not show significant difference in time spent in the center of the field when compared to stress. Over all the time spent in the center in drug treated groups are increased towards control whereas the time spent in the periphery is decreased when compared towards control are shown in **Fig. 1** and **2**.

The Rearing and grooming activities was significantly decreased in stress group when compared to control. EE-ACL and AA treated rats showed increased in rearing and grooming activities on comparing with stress group. Overall the rearing and grooming in different groups were not statistical significant shown in **Fig. 3** and **4** the (F = 2.293, p<0.088), (F = 2.661, p< 0.056).

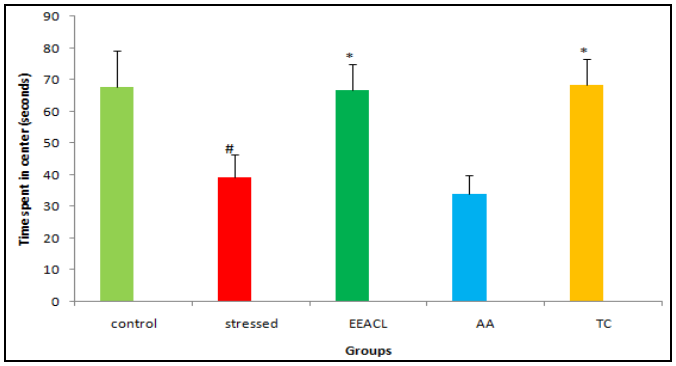

FIG. 1: OFT- TIME SPENT IN CENTER

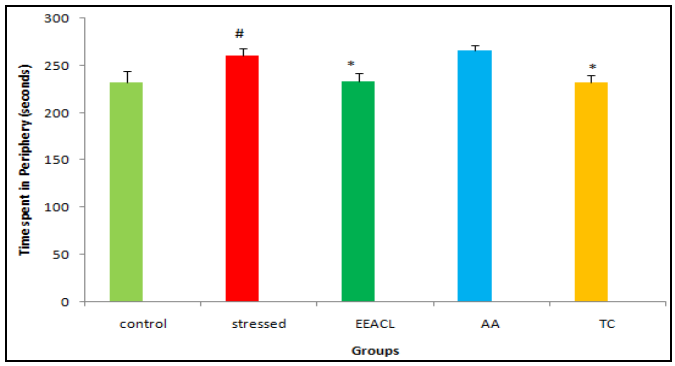

FIG. 2: OFT - TIME SPENT IN PERIPHERY

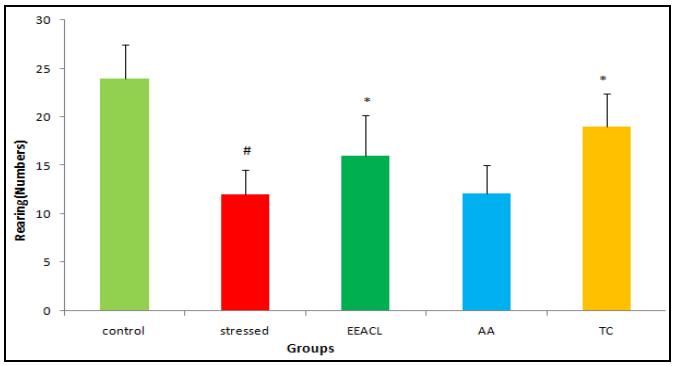

FIG. 3: OFT- REARING

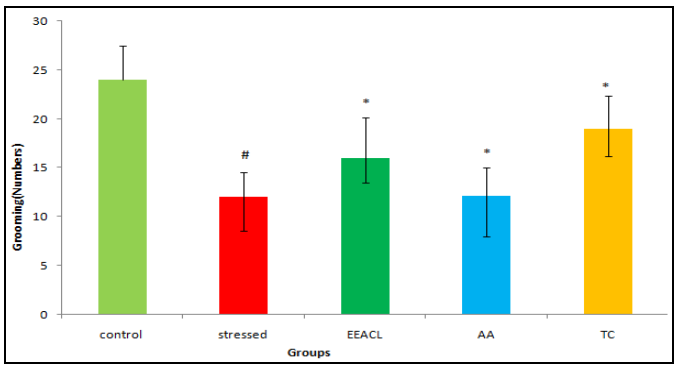

FIG. 4: OFT- GROOMING

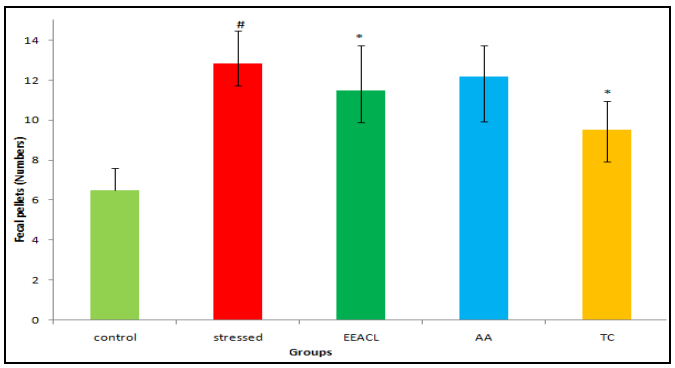

FIG. 5: OFT- FECAL PELLETS

Data were expressed as mean ± SEM and analysed by One way analysis of variance followed by post-hoc Tukey's test for multiple comparison of groups. # is significant from control and * is significant from stressed.

The fecal pellets of stressed groups increased compared with control significantly but not significant on comparing with drug treated group with F = 2.433, p<0.076. The **Fig. 5** shows the number of fecal pellets passed in different groups. On comparing with the EE-ACL, AA treated group TC showed significant increased behavioural performance when compared with the other groups. TC groups served as the standard drug group. In all behavioural parameters TC treated groups shows similar to that of EE-ACL and AA treated group.

Elevated Plus Maze Test: Time spent in open arm and closed arm in the EPM test of various groups were shown in **Fig. 6** and **7**. Time spent in the open arm is decreased in stressed group when compared to control group. EE-ACL treated group were found to be significantly increased when compared to stressed groups. Time spent in the closed arm in EE- ACL group shows similar results with standard drug TC with F = 4.501, p<0.007. Rearing activities and number of fecal pellets during EPM were analyzed and shown in **Fig. 8** and **9**. Stress group showed reduced rearing activities than control group. EE-ACL showed more rearing activities than stress group. These values were shown as statistically significant (F = 4.351, p<0.008). Number of fecal pellets passed by stress group was increased more than the control group. EE-ACL groups were passed less number of fecal pellets comparatively other groups and like control groups. Statistically significance was noticed (F = 3.417, p<0.023) among the all groups. Over all the EE- ACL group rats showed similar performance with TC treated standard drug group animals.

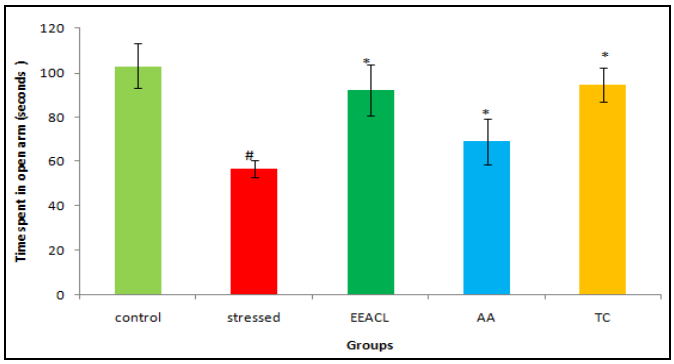

FIG. 6: EPM- TIME SPENT IN OPEN ARM

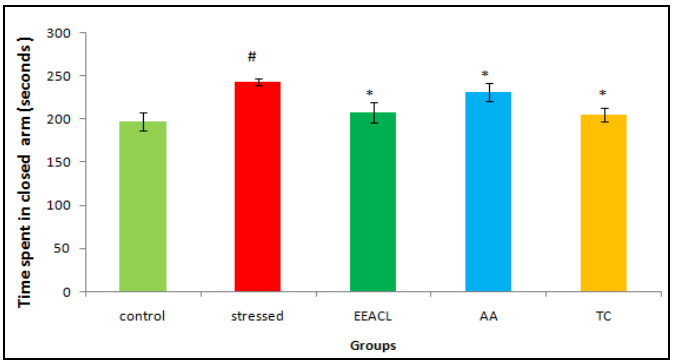

FIG. 7: EPM- TIME SPENT IN CLOSED ARM

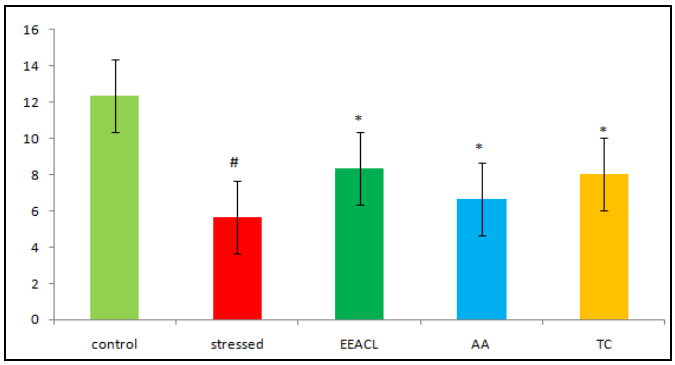

FIG. 8: EPM- REARING

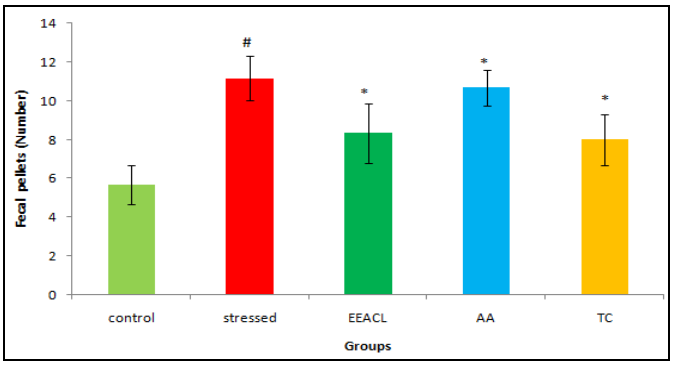

FIG. 9: EPM- FECAL PELLETS

Data were expressed as mean ± SEM and analysed by One way analysis of variance followed by post-hoc Tukey's test for multiple comparison of groups. # is significant from control and * is significant from stressed.

Eight Arm Radial Maze Test (EARM): The number of WME-baited arm was high in restraint stress group compared with control group. EE-ACL and AA treated groups showed less WME-baited arm error compared with stress group. Statistical significant was seen (F = 3.152. p<0.032) in between the groups shown in **Fig. 10**.

WME-unbaited arm values were showed in **Fig. 11**. Number of WME-unbaited arm was increased in stress group compared with control group. EE-ACL and AA treated rats displayed a decreased error than stress group. Number of WME-unbaited arms was observed as statistically significant (F = 4.306, p< 0.009).

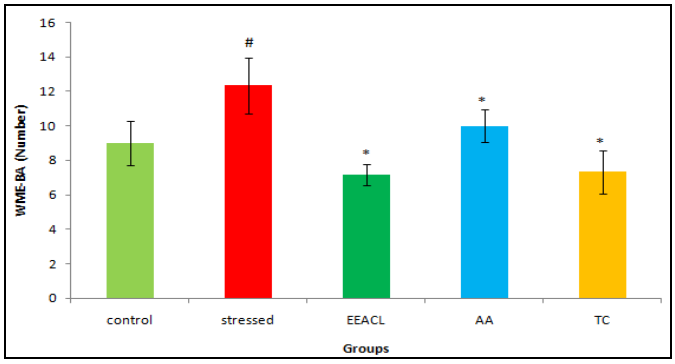

FIG. 10: EARM- WORKING MEMORY ERROR BAITED

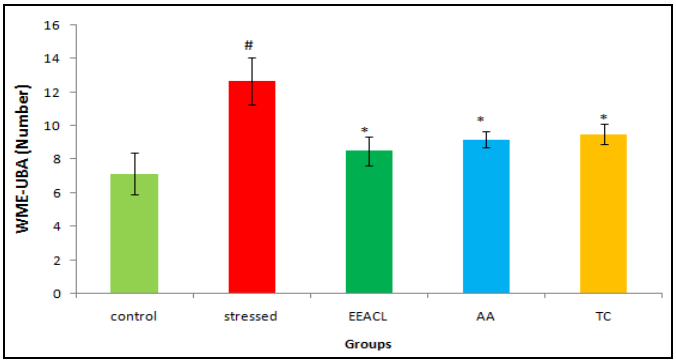

FIG. 11: EARM- WORKING MEMORY ERROR UN- BAITED

Data were expressed as mean ± SEM and analysed by One way analysis of variance followed by post-hoc Tukey's test for multiple comparison of groups. # is significant from control and * is significant from stressed.

RME in EE-ACL groups were significantly less when compared with stress group shown in **Fig. 12**. AA group was exhibited a decreased RME compared with stress group. These results were showed statistically different (F = 4.647, p<0.006) among the all groups. Latency or time taken to complete the task of EARM was shown in **Fig. 13** for all groups in this experiment. Stress group took more time to complete the task in EARM comparatively control group. AA group took more time to complete the task and but not like stress group. But EE-ACL and TC group were taken less time to complete the task with F = 6.144, p<0.001.

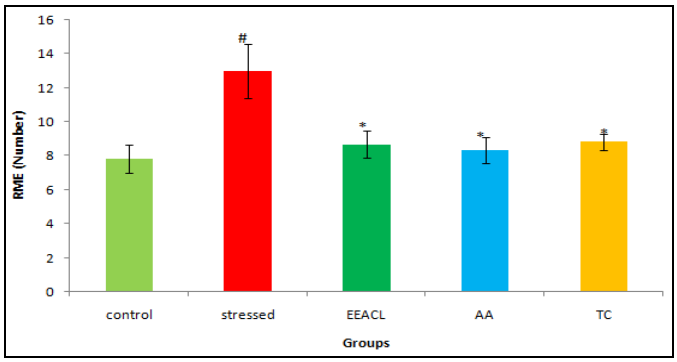

FIG. 12: EARM- REFERENCE MEMORY ERROR

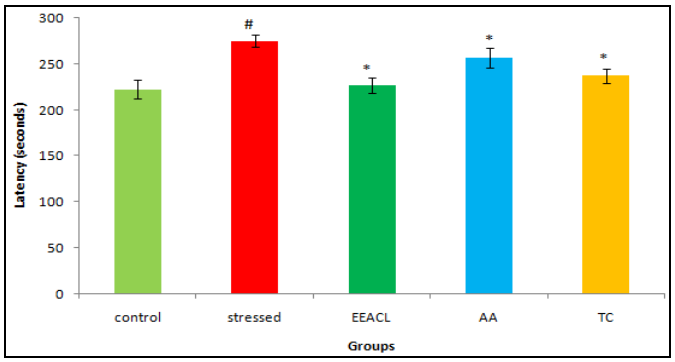

FIG. 13: EARM-LATENCY

Data were expressed as mean ± SEM and analysed by One way analysis of variance followed by post-hoc Tukey's test for multiple comparison of groups. # is significant from control and * is significant from stressed.

Y Maze Test: The spontaneous alteration behaviour (SAB) of the different experimental groups were shown in **Fig. 14**. The rats treated with EE-ACL and AA increased spontaneous alteration when compared with stress group with the (F = 11.919, p<0.001).

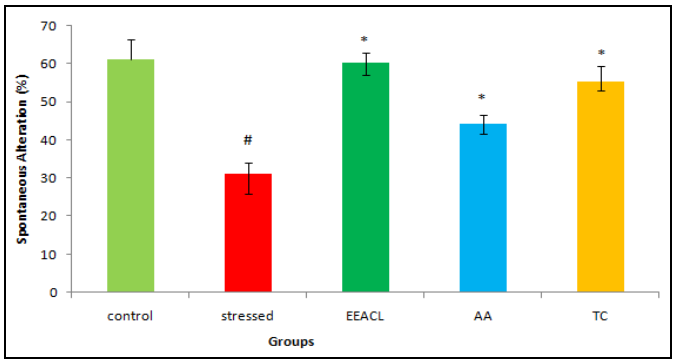

FIG. 14: Y- MAZE

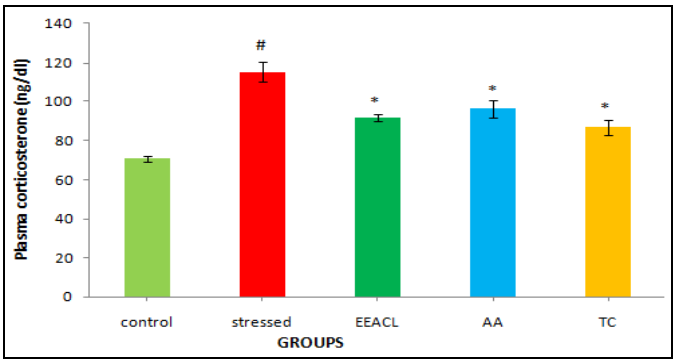

FIG. 15: PLASMA CORTICOSTERONE

Data were expressed as mean ± SEM and analysed by One way analysis of variance followed by post-hoc Tukey's test for multiple comparison of groups. # is significant from control and * is significant from stressed.

Plasma Corticosterone Level: Plasma corticosterone level in all groups was displayed in **Fig. 15**. In stress group the corticosterone was significantly higher than control group. The drug treated groups showed significantly less corticosterone level than stress but higher when compared with control with F value = 19.809 and p<0.001.

DISCUSSION: In the present study, the rats treated with the EE-ACL of dosage 100 mg/kg/po before restraint stress showed better performance in all behavioural test. The plasma corticosterone level in both EE-ACL and AA of dose 9 mg/kg/po groups showed significant decreased level when compared to stressed group. Overall the EE- ACL showed significantly increased result than AA but equal to TC of dose 40 mg/kg/po treated group.

In the present study administration of EE-ACL has produced a significant increase in grooming time, rearing time and an increase in time spent at the center of the field. Grooming behavior following exposure to stress and the increase in time spent at the center of the field clearly indicate that the compounds have anxiolytic activity [27]. Increase duration of behaviour like time spent in the central squares, rearing indicates good exploratory behaviour and less anxiety. Choleris *et al*., in 2001 has put forward that there will be increased defecation in the new environment due to activation of an autonomic nervous system after exposure to stress [28]. Similar to our study, the rats exposed to chronic restraint stress has passed more fecal pellets when compared with the rats treated with EE-ACL.

The anxiolytic behaviour of the animal is checked using the elevated plus maze in which rats avoid exposed open areas of the maze, which are assumed to be the most aversive, and a preference to be enclosed by protective walls [29]. In our study the rats treated with EE-ACL demonstrated increased activity in the open arms of EPM, the time spent in the open arms are increased. Overall, the time spent in enclosed arms were reduced in the groups treated with EE-ACL when compared to stress group animals but not much in AA treated group. Like our study, Bhutada *et al.,* in 2010 proved that the flavonoid in various natural plants provides a new potential option for prevention of cognitive dysfunction in diabetic rats in EPM [30].

The animals treated with *Alafia multiflora* showed less anxiety and more exploration activity when placed in EPM as the plant was rich in flavonoid [31]. The effect of *Celastrus paniculatus* on stressed rats was studied by Bhagya *et al.,* in 2016 and the animals spent more time in open arm in the EPM [22]. The possible mechanism by which the ACL produce anxiolytic behaviour may be due to ability of the drug to penetrate blood brain barrier and stimulating the target area hippocampus for improving cognition as the plant ACL is rich in flavonoids [32].

In EARM the groups treated with EE-ACL and AA showed less error in both working and reference memory. The administration of flavonoid rutin have shown improvement in spatial memory impairment induced by cerebral ischemia and the result also suggest that EARM is an ideal tool to confirm impaired cognitive function after any type of stress and also effective in the evaluation of drug effect in a reversal of impairment [33]. Increased in the errors in EARM was observed in noise stressed exposed rats group but in rats which received AA and alcoholic extracts 30 min before exposure to stress showed statistically significant improvement in retaining both working and reference memory which is like the current study [34]. In the present study the EE-ACL treated group showed better performance when compared with AA treated group. The possible mechanism of the improvement or retrieval of the memory is due to the antioxidant effect of ACL [35]. The rats treated with the whole extract of TC in a study showed significantly less latency (time to complete the task) in EARM [36]. On comparing with the standard drug TC used as memory enhancer the EE-ACL showed similar effect in behavioural performance.

The present study data showed that restraint stress impairs performance on spatial memory task in Y maze test. When spatial memory was assessed by spontaneous alteration behaviour (SAB) in Y maze test the percentage of SAB was greatly reduced in the rats subjected to restraint stress when compared to control group. The possible reason which has impaired the performance of restraint rats is likely to be due to memory deficit because of retraction of neuronal circuits in different regions of hippocampus [37].

The performance of groups treated with EE-ACL showed significant spontaneous alteration behaviour producing more triads in spatial memory task. The rats treated with vitamin C before subjecting restraint showed more triads in spatial memory task which is like the current study [20]. On comparing with EA-ACL and AA with TC group which served as a standard drug the performance were statistically significant. TC has showed neuro-protective activity in rats which are subjected to lipopolysaccharide induces bacterial endotoxin [38].

Among different animal models of stress like physical inactivity, insomnia, cold immersion psychosocial stress, the immobilization stress model has significantly increased the plasma corticosterone levels. Even 1 h acute immo-bilization stress has reported a significant increase in blood corticosterone levels [39]. In the present study, the corticosterone hormone level was significantly increased by restraint induced stress in rats. As hippocampus has glucocorticoid receptors (GR), increased in corticosterone level would have damaged the hippocampal neurons. The hippo-campus has an inhibitory effect on regulation of stress *via* hypothalamo-pitutary adrenal axis (HPA) pathway [40]. A measurement of corticosterone levels immediately after psychosocial stress episode in rats showed significantly increased corticosterone levels [41] which are relevant to the present study. One explanation for impaired learning and memory is due to glucocorticoid and mineralocorticoid receptors are not expressed, or limited expression, during elevation of corticosterone [42]. Study reports suggest that chronic stress impairs behavioural performance through changes in the HPA axis and that increases a corticosterone level which mainly impairs the learning and memory [43].

Overall improvement in behavioural performance is due to different phytoconstituents such as triterpenoids, flavonoid, tannins and saponins present in the plant ACL may be the reason for the rats to perform better [44, 45]. A few scientific reports indicates that triterpenoids produces central nervous system relaxant effect [46]. The polyphenol enhanced locomotor activity in the open field and the spatial memory in EARM as resveratrol rich in flavonoid has antidepressant properties which regulate stress induced changes [47]. Their general bioavailability and particularly their ability of the phenolic compounds to cross the blood brain barrier could have played an important role in the expression of their effects in central nervous system and thereby reducing the corticosterone level after exposure to stress [48].

CONCLUSION: The plant ACL have the potential to protect the cognitive functions in rats exposed to restraint stress through its antioxidant or anti-depressant effects equal with TC which is the plant compound used for memory enhancement. In the behavioural test OFT and EPM the EE-ACL treated rats showed significant performance which was equal to the standard drug TC. On assessing the result of the EARM and Y-maze which is a tool assess the cognition, the animals treated with EE-ACL and AA both showed statistically significant improvement in their performance. The plant ACL also has a significant role in reducing the corticosterone level to enhance the behavioural performance and can be a good drug for cognitive decline after exposure to restraint stress. The plant ACL overall can act as a nootropic drug to enhance memory or other cognitive functions.

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