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STUDY OF PHARMACOLOGICAL EFFECT OF ETHANOLIC EXTRACT OF *TRIKATU* (EET) ON LEARNING AND MEMORY IN WISTAR ALBINO RATS

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Background: *Trikatu* is a mixture of black pepper (*Piper nigrum Linn.*), long pepper (*Piper longum Linn.*), and ginger (*Zingiber officinale Rosc.*) in equal proportions. Piperine is the major alkaloidal constituent of *trikatu*. Piperine has been demonstrated to have potential neuroprotective properties and contribute to preventing cognitive decline due to neurodegenerative diseases. *Trikatu* stimulates the metabolic process by rapid nutrient absorption. It is a spice which can provide natural nutritional and medicinal benefit. It has analgesic, antipyretic, anti-inflammatory, antimicrobial and antineoplastic properties etc. **Objectives:** The current study was conducted to evaluate the effect of Ethanolic Extract of T*rikatu* (EET) on learning and memory in rats. The phytochemical constituents present in ethanolic extract were also evaluated. **Methodology:** Three models used were Elevated Plus maze, Y Maze and Novel Object Recognition. Dose of 500mg/kg and 1000 mg/kg respectively were selected. Piracetam was used as a standard drug for learning and memory which was administered intraperitoneally. The test doses were administered continuously for a period of seven days. The Test Groups (500mg/Kg and 1000 mg/Kg) animals were subjected to Elevated plus maze, Y maze and Novel Object Recognition test and the observations were recorded at different time intervals on the first, fourth and seventh day and the results were statistically analyzed using one way ANOVA by Dunnett's test. **Results:** In elevated plus maze EET at 500 mg/kg and 1000 mg/kg showed significantly shorter transfer latency on 8th day as compared to Control. In Y maze Both EET 500mg/kg and 1000mg/kg showed significant shorter transfer latency on Day 1, 4 and 7 compared to control. The Novel Object Recognition test at 30mins, 2hrs and 24hrs after dosing and testing showed significant increase in exploration time and discrimination index for dose of EET 1000mg/kg as compared to EET 500mg/kg. **Conclusion:** Based on the findings, it can be suggested that EET at both doses may possess learning and memory activity. Further *in vitro* analysis is required to understand exact mechanism of improved learning and memory.

Key words: learning, memory, *Trikatu,* Y Maze, Novel Recognition Test, Elevated Plus Maze

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1.0 INTRODUCTION:

Trikatu is a very well far-famed 'Rasayana' in Ayurveda and widely used as a polyherbal ayurvedic formulation in India. Trikatu means the mixture of three acrids. In Ayurveda this has been outlined in Sanskritic language as tri: three; katu: acrids.[1] It's conjointly called trishuna (Thirst) and katutrika (Harsh way). It consists of dried fruits of black pepper (*Piper* nigrum *L*. (PN), long pepper (*Piper* longum *L*. (PL) family: Piperaceae) and dried rhizomes of ginger (Zingiber officinale Rosc. (ZO) family: Zingiberaceae). The PN, PL and ZO are the main three ingredients of trikatu. Trikatu was formulated by taking weight ratio 1:1:1 (w/w/w) of the three crude drugs for therapeutic functions. The antioxidant, antitumor, antineoplastic and oth er pharmacological effects of *P.* nigrum, *P.* longum and *Z.* officinalis are principally because of pungent constituents like 6-gingerol, piperine, capsaicin, volatile oil and different bioactive molecules.[2] Trikatu has been prescribed for cough, cold, fever, asthma, metastasis issues and improvement of digestive disorders etc[2] Piperine is the major alkaloidal constituent of *trikatu*. Piperine has been demonstrated to have potential neuroprotective properties and contribute to preventing cognitive decline due to neurodegenerative diseases. The current study was conducted to evaluate the effect of Ethanolic Extract of T*rikatu* (EET) on learning and memory in rats.

2.0.0 Material and methods

2.1.0 Materials

2.1.1 Chemicals and Drugs

Dried ethanolic extract of *Trikatu* (obtained from Amsar Pharmaceuticals) , Piracetam 200 mg/kg, alcoholic alpha- naphthol solution, Concentrated sulphuric acid, Fehling's A and B solution, Benedict's reagents, Barfoed's reagent, Bial's reagent, Iodine solution, 4% Sodium hydroxide solution, 1% Copper sulphate solution, Millon's reagent, Ninhydrin solution, Lead acetate solution, Chloroform, Acetic anhydride, Picric acid, Pyridine, alkaline sodium nitroprusside solution, Glacial acetic acid, Dichloromethane, Ammonia, 5% Aqueous FeCl3, Benzene, Zinc dust and Conc. Hydrochloric acid, Dragendorff's reagent ,Mayer's reagent , Wagner's reagent , Bromine water, Potassium permanganate, dilute nitric acid ,Oral feeding syringes and other relevant glass ware.

2.1.2 Experimental Animals

Wistar albino rats of either sex, weighing about 150-200g were used in this study. The

rats were housed in polypropylene cages and were maintained under standard condition (temperature 25± 2°C, relative humidity 55±10% and 12 hours light and dark cycle) allowing food and water ad libitum. All the animals were acclimatized to laboratory condition for a week before commencement of experiment (CPCSEA guidelines). All experimental protocols were reviewed and accepted by the Institutional Animal Ethics Committee (IAEC) of Goa college of Pharmacy, Panaji prior to the commencement of the experiment- GCP/IAEC/2019/04.

2.1.3 Instruments

The instruments used in the study were Elevated Plus Maze (Columbus instrument), Novel Object Recognition Apparatus, Y maze (Panlab), Water bath (Classic Scientific), Precision balance (Contech Ltd.) and Refrigerator (Samsung).

2.2.0 Methods

2.2.1 Preparation of ethanolic extract of *Trikatu*

Trikatu was prepared by mixing the three powdered fruits of black pepper (dried fruit of *Piper nigrum Linn.),* long pepper (dried fruit of *Piper longum Linn*.), and ginger (dried rhizome of *Zingiber officinale Rosc*.) in equal proportions (w/w) according to the Indian Ayurvedic formula. 100 g of *trikatu* were extracted in sterile milli-Q water using ultra sonication at room temperature for 1 h. *Trikatu* extract was then dried using a rotary evaporation under reduced pressure at a temperature of 45 °C and lyophilized. The extract final yield after lyophilization was stored at 20 °C until use. The extract was further dissolved in culture medium and filtered through a 0.22 mm filter before treatment to the cells.

2.2.2 Pharmacological Screening of Ethanolic Extract of *Trikatu* on CNS of Rats

The rats were divided into 4 groups, each group consisting of 6 rats, and were administered the following:

Group I was administered DW, Group II was administered Piracetam (200mg/kg) ,Group III was administered Ethanolic Extract of Trikatu (EET) (500mg/kg) and Group IV was administered Ethanolic Extract of Trikatu (EET) (1000mg/kg).

EET was administered to the rats by oral route using oral feeding syringes.

Piracetam was administered to the rats by intra peritoneal injection.

The following methods were used to screen EET for their effect on learning and memory

2.2.2.4.1 Elevated Plus Maze Model[4]

It consists of two open arms measuring 50x10 cm and two closed arms of 50x10x40 cm with open roof in the shape of plus and is elevated from the ground to a height of 50cm. It has a central square of 10x10 cm.

The rat was placed at the end of any open arm facing away from the central platform and the time taken by the rat to move into any of the close arm was recorded. This time was noted as the transfer latency. The rat was allowed to explore the maze further for 5 minutes. If the rat did not enter any of the arms then it was forced into any one closed arm and transfer latency was recorded. Next day i.e after 24 h again transfer latency was noted. The significant decrease in transfer latency on 8th day as compared to 7th day suggested improvement in memory. Each animal was weighed and numbered, according to their body weight dose to be administered was calculated, animals were divided in groups and corresponding doses of test and standard drugs were administered orally daily for 7 days,the rats were placed on the end of any one of the open arms facing away from the central square, on the 7th day, 90 mins after administration of the last dose the rats were exposed to the training session on the elevated plus maze where transfer latency into any of the closed arms was noted, on the 8th day (after 24 hours) transfer latency into any of closed arm was recorded and compared with transfer latency of 7th day to evaluate the retention of memory.

2.2.2.4.2 Novel Object Recognition Model[5]

Apparatus

The apparatus consisted of an open box (100×100×50 cm high) with the inside painted in black. The objects to be discriminated were of an inert material, plastic. The weight of the objects ensured that they could not be displaced by the rats. **Pre-training:** The animals were handled for 1 week and then all animals were given one habituation session in which they were allowed to explore the arena without stimuli (without objects) for 5 mins. This habituation is especially important for the animal to become familiar with the environment, increasing the interest of the animal by the objects presented in the training phase. **Training Phase:** The animals were placed into the arena facing the center of the opposite wall and exposed for a set length of time to two identical objects (Object 1 and Object 2) that are located in the corner a specified distance from each other (15 cm from each adjacent wall) and allowed to explore for 3 mins, time that the animal explored each object was measured. The rats were then returned to its home cage. **Test phase:** This phase was conducted for 30 minutes, 2h or 24 h after the administration of the drug to measure working memory, short term memory or long-term memory. In the test phase the animal was re-placed in the arena, presented with two objects in the same positions: one object (familiar object) that is used in the training phase and the other object

is a different object (novel object). The positions of the objects in the test and the objects used as novel or familiar are counterbalanced between the animals. The following parameters were analyzed: the time spent exploring each object (Object 1 and Object 2) in the training phase, the time spent exploring each objects (familiar and novel object) in the test phase and the discrimination index. Discrimination index was calculated by the formula Tn-Tf/Tn+Tf, where Tn is time spent exploring novel object and Tf is time spent exploring familiar object. The discrimination index helps in determining the preference for novel or identical object and the time taken to explore the novel object is considered as an index of memory retention.

2.2.2.4.3 Y Maze Model[6]

The Y maze apparatus consists of a stem arm of 27.5cm long connected to the other two arms of 27.5cm at 60˚ angle to form a **Y. Training phase :** A day before experiment, the rat was placed in the start arm and was allowed to explore the maze for 5 minutes. During this training period, the arms were kept empty and no feed was placed in any of the corners of the arm. An arm entry was considered when all 4 paws of the rat cross the threshold of the central zone and into the arm and the animal's snout is oriented toward the end of the arm.

Testing phase :Each animal was weighed and numbered, the dose to be administered was calculated according to their body weight, animals were divided in groups and corresponding doses of test drugs were administered orally daily for 7 days, on the 1, 4 and 7 day, the experimentation was carried out where the rat was placed in the stem arm, one of the two arms was baited with the feed and one was kept empty, the trial was carried out at the time intervals of 2, 4 and 8 hours after the drug administration for 5 minutes for each subject, on 8th Day, transfer latency of the subject into the baited arm and the time spent in the baited arm was recorded, the apparatus was cleaned after each trial.

Method Used for Statistical Analysis: Graphpad instat -One-way Anova followed by Dunnet's test

3.0 Results

3.1 Qualitative Phytochemical Screening

Preliminary phytochemical screening of *Trikatu* extract revealed the presence of carbohydrates, proteins, flavonoids, cardiac glycosides, anthraquinone alkaloids and tannins.

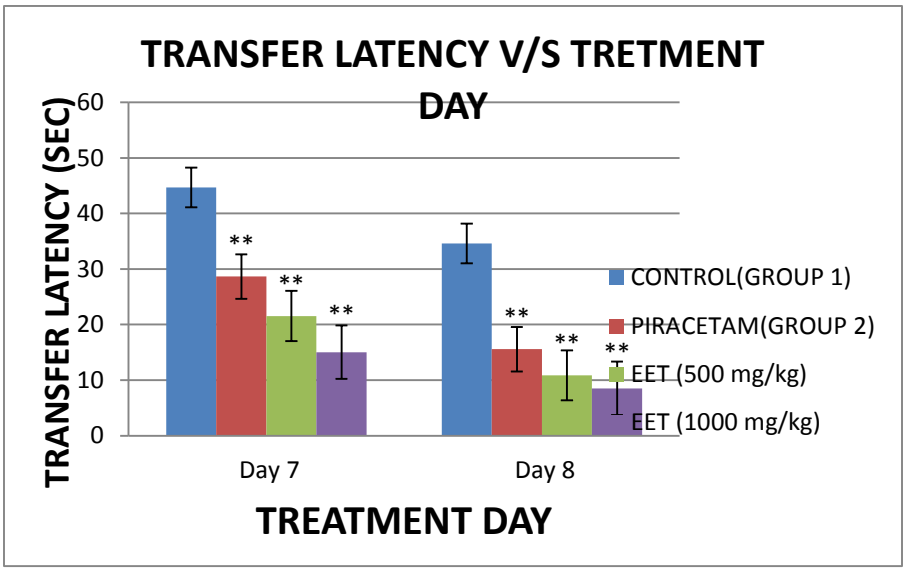
3.2 Screening for Learning and Memory Activity

3.2.1 Elevated Plus Maze

Table No. 1 Effect of EET on transfer latency in elevated plus maze test

REATMENT	Transfer Latency on Day 7	Transfer Latency on Day 8
CONTROL	44.693 ± 0.028	34.613 ± 0.036
PIRACETAM	28.662 ± 0.021**	15.570 ± 0.109**
EET (500 mg/kg)	21.553 ± 0.371**	10.876 ± 0.223**
EET (1000 mg/kg)	15.041 ± 0.251**	8.521 ± 0.390**

Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 v/s Control



Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 v/s Control

Based on the above findings it can be suggested that EET at both doses may possess learning and memory activity.

3.2.2 Novel Object Recognition Model

Table No. 3: Effect of Piracetam, EET 500 mg/kg and EET 1000 mg/kg on Novel object recognition at 30 minutes time interval. (Values compared against control)

Treatment		Control (Group I)	Piracetam (Group II)	EET 500 mg/kg (Group III)	EET 1000 mg/kg (Group IV)
TRAINING DAY					
Exploration Time (sec)	Object A	4.699± 1.221	8.587 ± 2.368	26.333 ± 1.647	26.833 ± 2.088
	Object B	5.595 ± 1.041	7.518 ± 1.829	26.833 ± 7.085	29.667 ± 2.266
	TEST DAY				
	Familiar Object	4.650 ±1.511	1.611± 0.1905	11.333 ± 2.275*	23.500 ± 2.940**
	Novel Object	6.597± 2.268	19.498± 2.829*	54.333 ± 4.135**	32.167 ± 3.304**

Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 vs Control

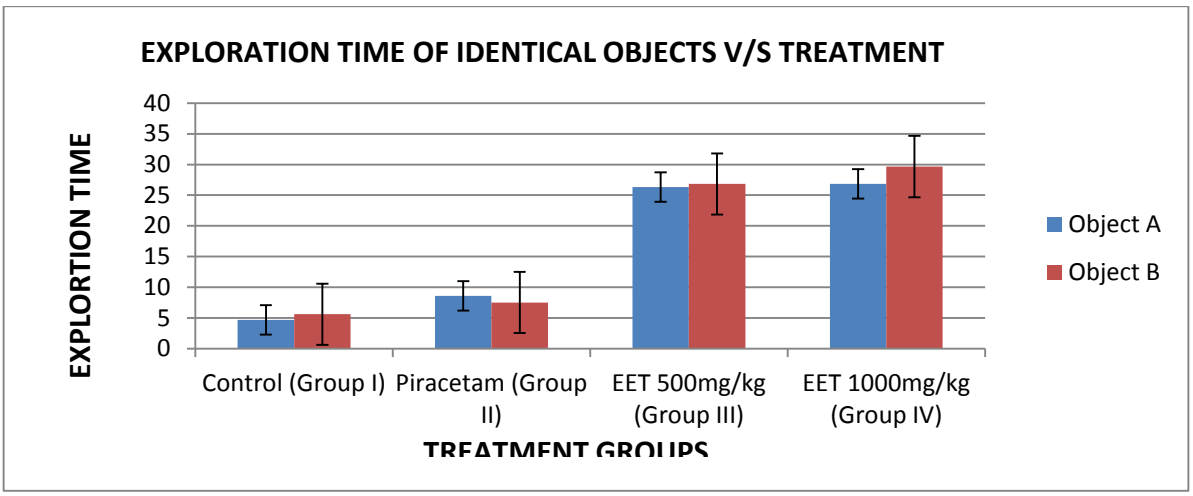


Figure No. 2: Exploration time for identical objects during training phase at 30 minutes time interval for Group I, Group II, Group III and Group IV.

Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 vs Control

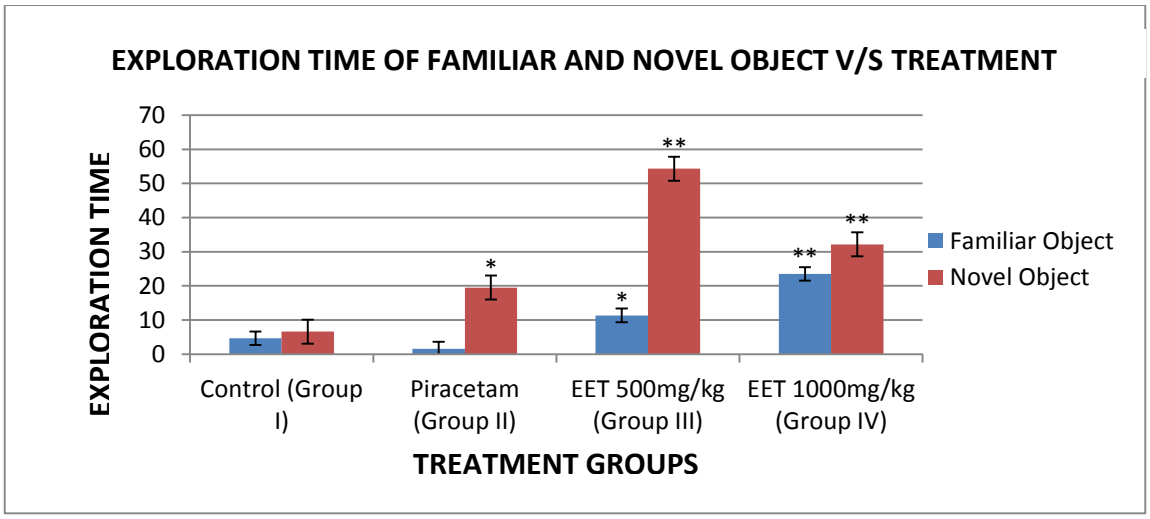


Figure No. 3: Exploration time for familiar and novel object during test phase at 30 minutes time interval for Group I, Group II, Group III and Group IV

Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 vs Control

Table No. 4: Effect of Piracetam, EET 500 mg/kg and EET 1000 mg/kg on Novel object recognition at 2hrs time interval. (Values compared against control)

Treatment		Control (Group I)	Piracetam (Group II)	EET 500 mg/kg (Group III)	EET 1000 mg/kg (Group IV)
TRAINING DAY					
Exploration Time (sec)	Object A	4.572± 1.221	6.494 ± 2.368	28.500 ± 1.647	27.333 ± 2.088
	Object B	3.492 ±	6.410 ±	20.167 ± 7.085	28.500 ± 2.266

	1.041	1.829		
TEST DAY				
Familiar Object	4.648 ±1.511	1.457± 0.1905	12.833 ± 2.275**	13.667 ± 2.940**
Novel Object	2.590± 2.268	10.424± 2.829	54.333 ± 4.135**	49.000 ± 3.304**

Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 vs Control

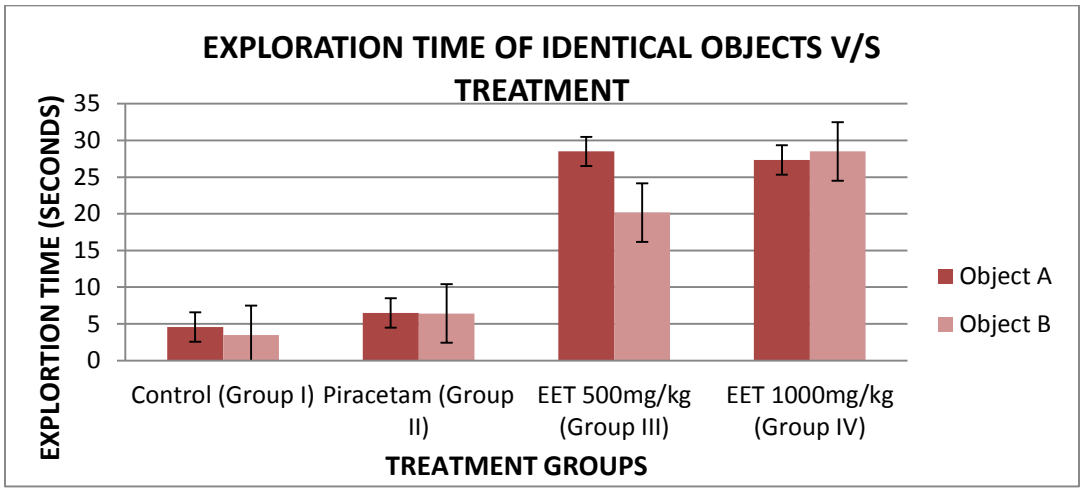


Figure No. 4: Exploration time for identical objects during training phase at 2hrs time interval for Group I, Group II, Group III and Group IV

Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 vs Control

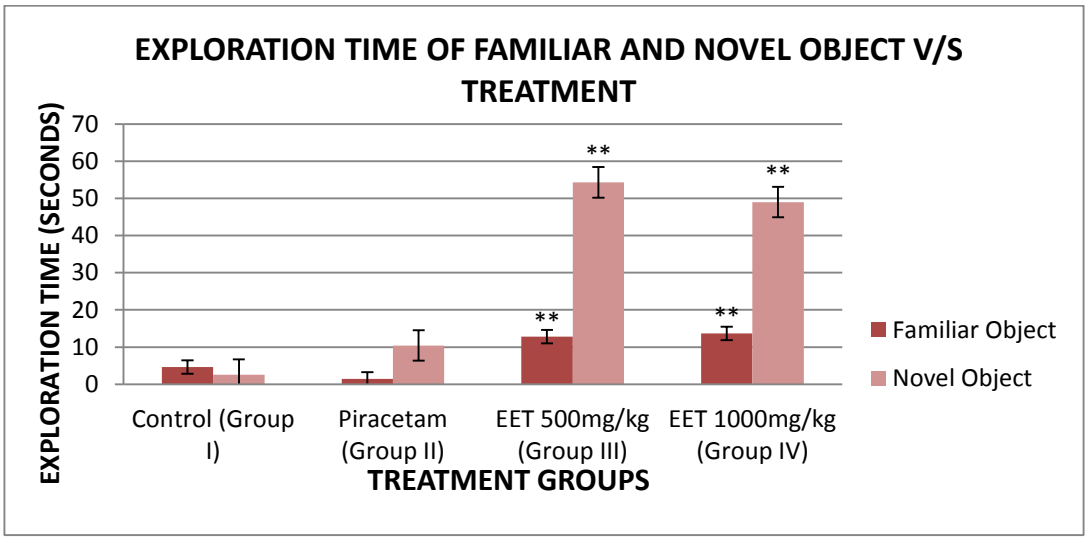


Figure No. 5: Exploration time for familiar and novel object during test phase at 2hrs time interval for Group I, Group II, Group III and Group IV.

Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 vs Control

Table No. 5: Effect of Piracetam, EET 500 mg/kg and EET 1000 mg/kg on Novel object recognition at 24hrs time interval. (Values compared against control)

Treatment		Control (Group I)	Piracetam (Group II)	EET 500 mg/kg (Group III)	EET 1000 mg/kg (Group IV)
TRAINING DAY					
Exploration Time (sec)	Object A	4.491± 1.221	3.369 ± 2.368	24.833 ± 1.647	23.833 ± 2.088
	Object B	3.682 ± 1.041	3.358 ± 1.829	19.000 ± 7.085	27.000 ± 2.266
	TEST DAY				
	Familiar Object	4.517 ±1.511	2.529± 0.1905	23.500 ± 2.275**	19.833 ± 2.940**
	Novel Object	4.572± 2.268	9.481± 2.829	32.167 ± 4.135**	45.167 ± 3.304**

Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 vs Control

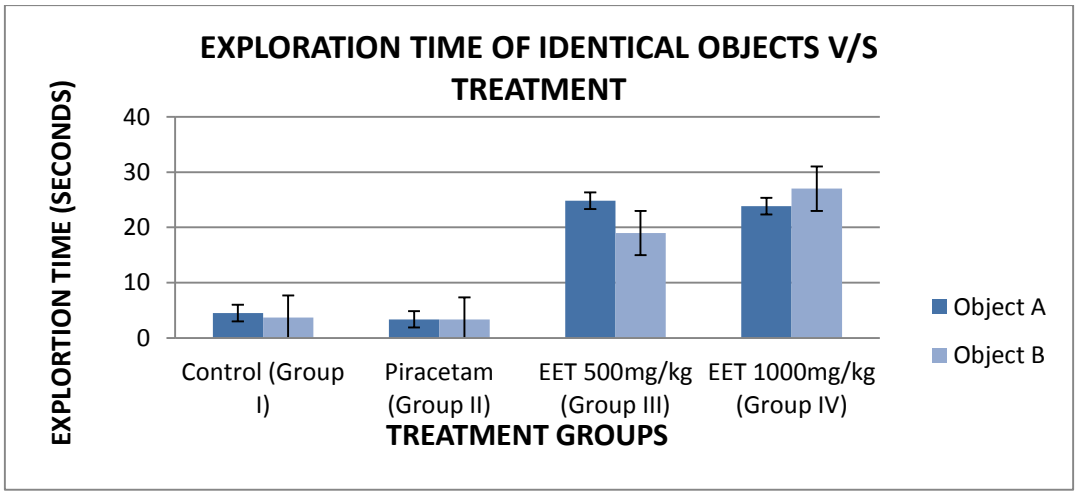


Figure No. 6: Exploration time for identical objects during training phase at 24hrs time interval for Group I, Group II, Group III and Group IV

Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 vs Control

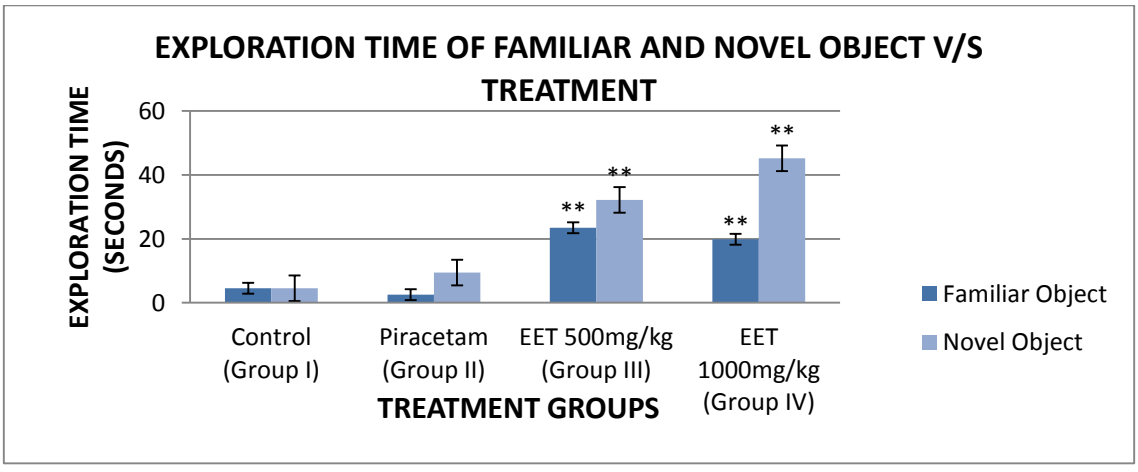


Figure No. 7: Exploration time for familiar and novel object during test

phase at 24hrs time interval for Group I, Group II, Group III and Group IV Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 vs Control

Table No. 6: Effect of Piracetam, Ethanolic extract of *trikatu* (EET) on discrimination index at 30 minutes, 2 hours and 24 hours time interval. (Values compared against control)

DISCRIMINATION INDEX				
TREATMENT	**CONTROL (GROUP I)**	**PIRACETAM (GROUP II)**	**EET 500mg/kg (GROUP III)**	**EET 1000mg/kg (GROUP IV)**
30 MINUTES	0.1590 ± 0.0246	0.8483 ± 0.0239**	0.654± 0.0369**	0.155± 0.1476
2 HOURS	0.0621± 0.0395	0.7371± 0.0570**	0.617± 0.1441**	0.563± 0.1242**
24 HOURS	0.0630± 0.0825	0.6345± 0.0649**	0.160± 0.1128**	0.389± 0.0525**

Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 vs Control

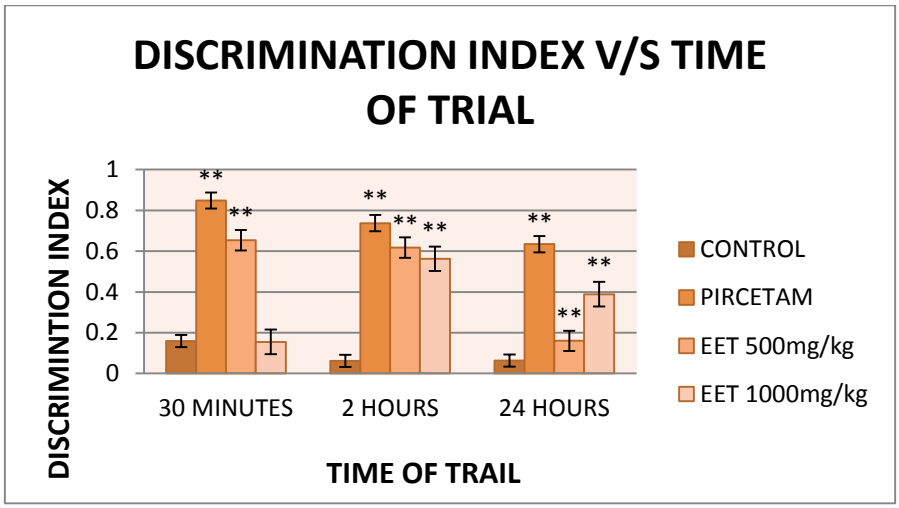


Figure No. 8: Discrimination index at 30 minutes, 2 hours and 24 hours time interval for Control, Piracetam, EET 500mg/kg and EET 1000mg/kg.

Values are expressed as mean ± SEM (n=6) *P< 0.05, **P<0.01 vs Control

3.2.3 Y Maze Model

The effect of EOCC on learning and memory of rats were evaluated using Y maze. The transfer latency on day 1, 4 and 7 was compared.

Table no. 7: Transfer Latency for group I, Group II, Group III and Group IV using Y maze model

TREATMENT	**TIME (HOURS)**	**TRANSFER LATENCY (SECS)**		
		DAY 1	**DAY 4**	**DAY 7**
CONTROL	2	45.132 ± 3.668	42.642 ± 2.543	35.595 ± 8.257

(GROUP I)	4	37.616 ± 5.777	25.573 ± 2.496	28.252 ± 6.555
	8	25.824 ± 7.694	31.480 ± 4.050	22.537 ± 0.668
PIRACETAM	2	39.677 ± 6.735**	40.601 ± 9.354*	43.336 ± 6.343**
	4	32.478 ± 3.006**	27.889 ± 5.289*	25.600 ± 1.842**
	8	19.315 ± 0.321**	18.593 ± 2.054**	27.803± 2.575**
EET (500 mg/kg) (GROUP III)	2	28.266 ± 9.077**	25.176 ± 1.259**	29.647 ± 4.006**
	4	28.996 ± 9.450**	21.672 ± 3.495**	29.115 ± 9.162
	8	16.764 ± 8.796**	21.295 ± 5.762**	20.791 ± 3.204*
EET (1000 mg/kg) (GROUP IV)	2	22.044 ± 3.279**	17.222 ± 2.857**	11.031 ± 3.865**
	4	15.508± 2.043**	11.059 ± 1.222**	9.930 ± 3.325**
	8	8.024± 5.396**	9.983 ± 0.973**	10.200± 5.390**

Values are expressed as mean ± SEM (n=6) *P<0.05, **P<0.01, *** P<0.001 vs. control

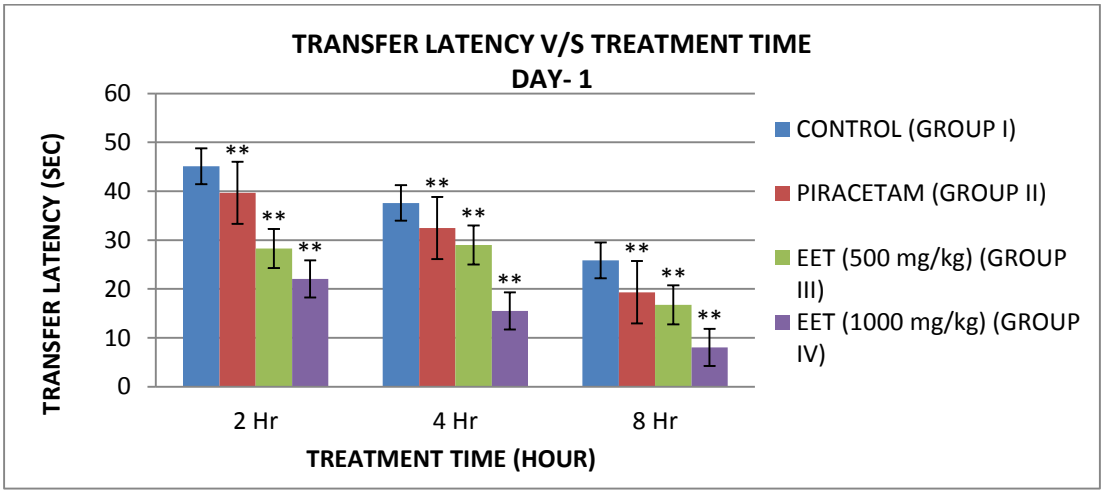


Figure no. 9 : Transfer Latency of Group I, Group II, Group III and Group IV on Y maze Model on Day 1

Values are expressed as mean ± SEM (n=6) *P<0.05, **P<0.01, *** P<0.001 vs. control

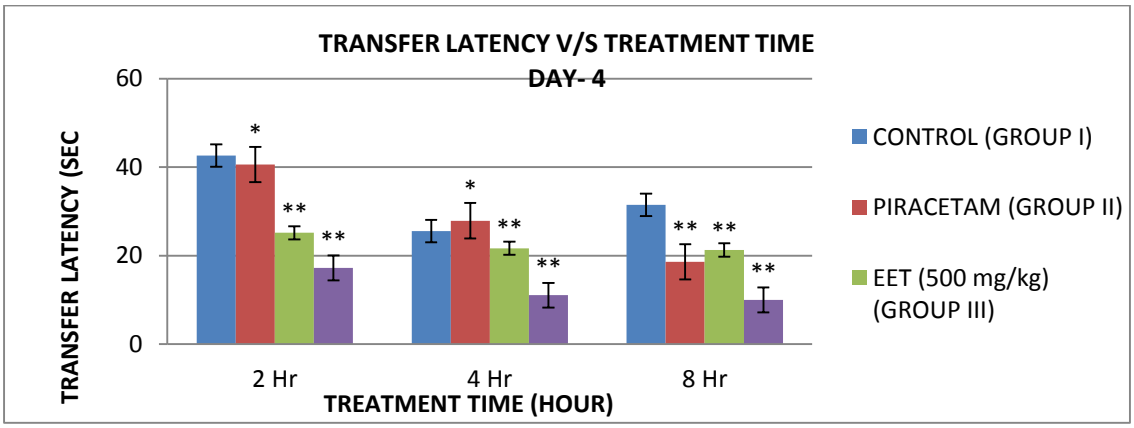


Figure no. 10: Transfer Latency of Group I, Group II, Group III and Group IV on Y maze Model on Day 4

Values are expressed as mean ± SEM (n=6) *P<0.05, **P<0.01, *** P<0.001 vs. control

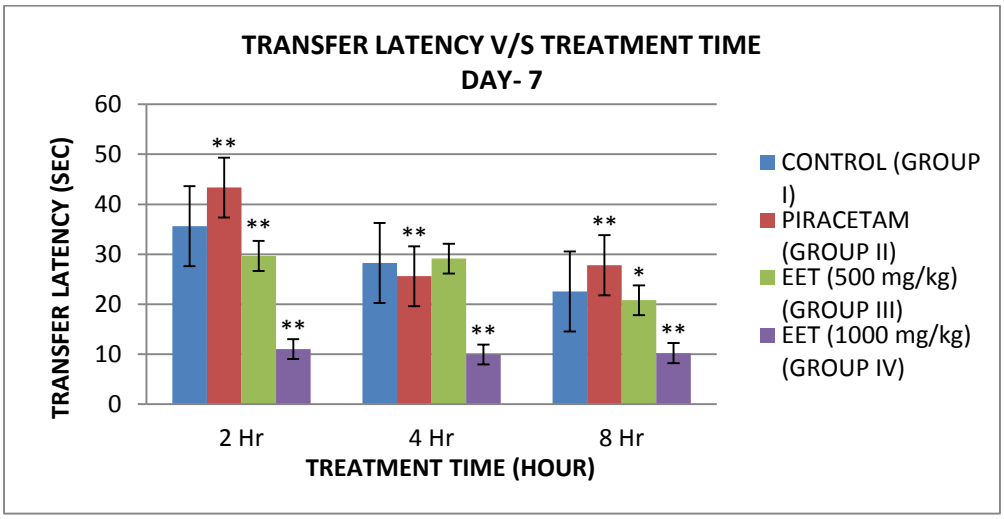


Figure no. 11: Transfer Latency of Group I, Group II, Group III and Group IV on Y maze Model on Day 7

Values are expressed as mean ± SEM (n=6) *P<0.05, **P<0.01, *** P<0.001 vs. control

4.0 DISCUSSION

Trikatu is a very well far-famed 'Rasayana' in Ayurveda and widely used as a polyherbal ayurvedic formulation in India. Trikatu means the mixture of three acrids. In Ayurveda this has been outlined in Sanskritic language as tri: three; katu: acrids. It's conjointly called trishuna and katutrika [1]. It consists of dried fruits of black pepper (*Piper* nigrum *L*. (PN), long pepper (*Piper* longum *L*. (PL) family: Piperaceae) and dried rhizomes of ginger (Zingiber officinale Rosc. (ZO) family: Zingiberaceae). The PN, PL and ZO are the main three ingredients of trikatu. Trikatu was formulated by taking weight ratio 1:1:1 (w/w/w) of the three crude drugs for therapeutic functions.

The antioxidant, antitumor, antineoplastic and other pharmacological effects of *P.* nigrum, *P.* longum and *Z.* officinalis are principally because of pungent constituents like 6-gingerol, piperine, capsaicin, volatile oil and different bioactive molecules.[7] Trikatu has been prescribed for cough, cold, fever, asthma, metastasis issues and improvement of digestive disorders[8]

The present study was conducted in order to screen the ethanolic extract of *Trikatu* (EET) for its phytochemical constituents and for its learning and memory enhancing action on the central nervous system of rats, using various pharmacological animal models.

The activity of EET was evaluated using the *in vivo* behavioral animal models. The behavioral models used to evaluate were elevated plus Maze, Novel object recognition model and the Y-Maze.

The behavioral study was carried out and the results obtained were statistically analyzed

using one way ANOVA by Dunnet's test and compared with the standard.

In Elevated Plus Maze, the corresponding doses of test and standard drugs were administered orally daily for 7 days. On the 7th day, after administration of the last dose, the rats were exposed to the training session on the elevated plus maze where transfer latency into any of the closed arms was noted. On the 8th day (after 24 hours) transfer latency into any of closed arm was recorded and compared with transfer latency of 7th day to evaluate the retention of memory. Both, EET 500 mg/kg and EET 1000 mg/kg showed significantly shorter transfer latency on 8th day on elevated plus maze compared to Control. Based on the findings it can be suggested that EET at both doses may possess learning and memory activity.

In Y Maze, the corresponding doses of test and standard drugs were administered orally daily for 7 days. On the 1st, 4th and 7th day, the experimentation was carried out where the rat was placed in the stem arm. One of the two arms was baited with the feed and one was kept empty. The trial was carried out at the time intervals of 2, 4 and 6 hours after the drug administration for 5 minutes for each subject. The transfer latency of the subject into the baited arm was recorded to evaluate the retention of memory. Both EET 500mg/kg and 1000mg/kg showed significant shorter transfer latency on Day 1, 4 and 7 compared to control. Based on the findings, it can be suggested that EET at both doses may possess learning and memory activity.

In Novel object recognition test, day 1 was considered as habituation session wherein rats were allowed to explore the area for five minutes without any objects, habituation day was followed by training on day 2 where rats are exposed to two identical objects for 3 minutes and the exploration time for object A and object B were noted during the training phase. On the test day, one object was replaced by novel object and the exploration time and discrimination index were evaluated after 30 minutes, 2 hours and 24 hours of treatment to assess working memory, short term memory and long-term memory respectively. The NOR model at 30mins, 2hrs and 24hrs after dosing and testing showed significant increase in exploration time and discrimination index for dose of EET 1000mg/kg as compared to EET 500mg/kg.

5.0 CONCLUSION

Based on the findings of study, it can be concluded that ethanolic extract of *trikatu* both at 500mg/kg as well as 1000mg/kg has significant effect on learning and memory. This may be attributed to the presence of various phytochemical constituents like carbohydrates, proteins, flavonoids, cardiac glycosides, anthraquinone alkaloids and

tannins. The concept of learning and memory is a complex process where in further studies are required to elucidate which phytochemical constituent is responsible and the exact mechanism underlying the probable enhancement in learning and memory showed by Ethanolic extract of *Trikatu* in the present behavioural study.

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