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Potential Applications of Calocybe indica Ethanol Extract in Noniceptive and Neuronal Pain

Celia-Vargas De la Cruz[1], Md Shariful Islam[2],Md. Khondokar Abu Raihan[3], Teresa- Gallardo Jugo[1], César-Fuertes Ruiton[4], Mónica G-Retuerto Figueroa[5], Eva Ramos-Llica[5], Arilmi-Gorriti gutierrez[5], Sixto-Gonzalez Elera[6]

Abstract *- Calocybe indica belonging to the family Lyophyllaceae, Analgesic potential, CNS test and membrane stabilizing activity of Calocy be indicaethanolicextract (ETCL) were evaluated by some different standard methods. The results of the study showed that ETCI possess peripherally and centrally acting analgesic potential in mice model. In acetic acid induced writhing method, ETCI (100mg/kg & 150mg/kg) significantly (P<0.001, P<0.05) reduced pain sensation with 71.62% & 54.59% inhibition as compared to standard with 82.96% of inhibition respectively. In hot plate method 100mg/kg ETCI increased reaction time with 13.12%,12.90% &19.40% of latency compare to standard (14.70%, 7.24% & 2.77%) at 0min and 60 min respectively which is statistically significant (p<0.001, P<0.05). Again ETCI (150mg/kg) increased reaction time (8.19%, 15.09% and 19.79%) which is statistically significant (p<0.001, P<0.05) at 0min to 60min. In open field method ETCI showed sedative effect (100mg/kg,150mg/kg) at 0min & 60 min which is statistically significant (p<0.001, P<0.05) but not dose depending manner. In hole cross method ETCI (100mg/kg, 150mg/kg) also showed significant (p<0.001, P<0.05) sedative effect at 0min & 60 min. The ETCI were effective in membrane stabilizing activity as the extractives prevented the lyses of erythrocytes induced by hypotonic solution. During hypotonic condition the different doses of ETCI (100 &150mg/kg) showed lyses inhibition 65.27% & 10.51% respectively compared to standard with 56.65% of inhibition. The 100mg/kg dose of ETCI had showed the better result than 150mg/kg in membrane stabilizing activity. The investigations revealed that the ethanolic extracts of ETCI possess both central and peripheral analgesic potentials and also have sedative effect with membrane stabilizing activity.*

Keywords*: Calocybe Indica, Analgesic Potential, CNS Activity, Membrane Stabilizing Activity.*

INTRODUCTION

Calocybe indica belonging to the family Lyophyllaceae, also known as Milky Mushroom [1],*C. indica* was mainly used to the treatment of Diabetes. It is reduced the blood sugar level. These Mushrooms also reduced the blood pressure and It also maintains the body weight [2].The six major constituents of ethanolic extract of *C.indica* were water, proteins, carbohydrates, dietary fiber, fat, and ash [3]. These Mushrooms were considered good source of fats and minerals; fat fraction in Mushrooms has been composed of unsaturated fatty acids and Mushrooms has been got low calorie food with very little fat which is free of cholesterol and rich in linoleic acid [4-5]. High protein and fiber with low fat content has been evaluated from *C. indica* which make the mushroom as a low energy and healthy food stuff [6]. It has been determined that the type of substrates and supplements used for mushroom

[1]*Faculty of Pharmacy and Biochemistry-Centro Latinoamericano de Enzeñanza e Investigación en Bacteriología Alimentaria (CLEIBA), Research Group Biotechnology and Omics in Life Science (BIOLIFS), National University of San Marcos, Lima-Perú.*
[2]*Department of Pharmacy, Southeast University, Banani, Dhaka, Bangladesh.*
[3]*Department of Microbiology, Primeasia University, Banani, Dhaka, Bangladesh.*
[4]*Faculty of Pharmacy and Biochemistry-Laboratory of Organic Chemistry, Research Group Natural Resources Research, National University of San Marcos, Lima-Perú.*
[5]*Faculty of Pharmacy and Biochemistry-Laboratory of Pharmacognosy, Grupo de InvestigaciónFarmacognosia y MedicinaTradicional , National University of San Marcos, Lima-Perú.*
[6]*Faculty of Pharmacy and Biochemistry-Laboratory of Toxicology, National University of San Marcos, Lima-Perú.*
Corresponding author: cvargasd@unmsm.edu.pe

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cultivation had generally influenced the proximate composition including antioxidants [7]. These Mushrooms were good sources of minerals (Ca, K, Mg, Na, and P), trace elements (Cu, Fe, Mn, and Zn) and sometimes, toxic heavy metals (Cd and Pb) as compared to vegetables [8]. It has contained polysaccharides glycogen and chitin, occurring in animals, not starch and cellulose as plants. Chitin was a water- insoluble structural N- containing polysaccharide accounting for up to 80-90 % of dry matter in mushroom cell walls [9]. *C. indica* has been possessed enrich proteins and medicinal properties including antibacterial, antiviral, and anti-AIDS. Terpenoids has been isolated from this mushroom which help to kill bacteria and viruses and exert anti- inflammatory effects [10]. These Mushrooms were an excellent source of iron, selenium, potassium, phosphorus, riboflavin, panthothenic acid, copper and zinc in addition to providing antioxidant value; these nutrients play a role in enhancing immunity and preventing diseases[11] and polysaccharides were being investigated for their immune modulatory as well as anticancer activities were showed by *C. indica*[12]. Antioxidants were chemical compounds that protect cells from the damage caused by unstable molecules known as free radicals. They were capable of damaging all components of the body viz. lipids, proteins, DNA, sugars and are involved in mutations and cancers [13]*C. indica* consists of non-enzymatic antioxidant include Vitamin A, vitamin C, vitamin E and reduced glutathione which scavenge a wide variety of free radicals[14]. The antitumor components of Mushrooms vary in their chemical nature and include polysaccharides such as hetero-β-glucan, heteroglycane, β-glucan-protein and hetero glycan protein complexes. Many species of Mushrooms have been found to be highly potent immune enhancers against cancer [15].

METHODS AND MATERIALS

Extraction of ETCL

Powder of ETCI extracted with ethanol at room temperature of their weight in a round bottom flask container with 1:2 sample and solvent ratio through occasional shaking and stirring for 7 -15 days. After 7-15 days, the whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through filter paper. This extract needs to dried until gel form. Then the crude extract was ready for assaying of analgesic, CNS &membrane stabilizing activity.

Analgesic activity studies

Acetic acid induced writhing method

The acetic acid-induced writhing test in mice was investigated the methods of [16-18]. Three groups containing four mice each, got normal saline solution (10 mL kg−1) (i.e., control), indomethacin (10 mg kg−1), plant extract (100 and 150 mg kg−1) orally. Therefore, thirty minutes later, acetic acid (10 mL kg−1) solution was given intraperitioneally to all animals in different groups. The number of writhing occurring (abdominal constrictions) of 5 to 15 minutes afterwards acetic acid injection was calculated. As a consequence, in each treated group was likened to that of a control group while indomethacin as a reference standard (positive control). The percentage inhibition of analgesic activity of writhing was counted using the below formula;

$$\text{Percent Inhibition}=\{(A-B)/A\} \times 100$$

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There, A = Average number of writhing of the control group; B = Average number of writhing of the test group.

Eddy's hot plate method

Analgesic activity was assessed by the hot plate method, this is another method for testing the analgesic activity had been performed the method of [16] with slightly modification. Twelve experimental animals were randomly selected and divided into three groups denoted. The test samples were administered at the doses of 100mg and 150mg kg−1 body weight in animal; at the dose of 10mg kg−1 body was administered in the positive control of mice, and 1% Tween 80 solution in distilled water was treated vehicle control group at the dose of 10 mL kg−1 orally in mice. The experimental animal was placed on the Eddy's hot plate at a temperature of 55±0.5°C. The opening of the experiment earlier on 30 minutes the extract samples and the standard drug were administered. When animals licked the hind paws or jumped at 1, 2, 3, 4, and 5 hours was noted as the reaction time, reaction time was calculated; a lop-off period of 20 seconds was accomplished to get off paw damage. PAS (Percent Analgesic Score) was counted up as:

$$PAS=(Ta\ Tb\ /\ Ta\times100$$

There: Tb= Before drug administration reaction time (sec); Ta= After drug administration reaction time.

CNS activity studies

Open field test: Described by the method of [19] was followed to carry out open field test was slightly modified and used for screening action of the test drugs on CNS in mice. Above all, the animal divided into four groups where four mice were in group; mice were divided into control group and experimental group. The experimental group got ethanolic extracts of *C. indica* at the doses of 100 and 150 mg/kg were administered orally, indeed the control group got vehicle 1% Tween 80 in water. The number of squares which was visited by the animals was recorded for 3 minutes on 0, 30, and 60 minutes. The test samples (200 mg/kg and 400 mg/kg b.w.), normal saline (10ml/kg), and diazepam as reference drug (1mg/kg).

Hole cross test

The hole cross test was investigated by [20], a separation was permanent in the central of a cage having size 30×20×14 cm was measured. In the center of the cage with a 3 cm diameter was made at a height of 7.5 cm. The animals were divided and treated with control, standard, and test group, and were placed in one side of the cage. In other words, the number of the chamber of an animal through the hole from one chamber to other was recorded for a period of 3minutes on 0, 30, 60, 90 and 120 minutes later the administration of the test drug and reference drug. % movements inhibition was determined by following equation.

$$(Cm\ –Tm/Cm)\ \times100$$

Cm= Movement of control mean; Tm= Movement of test mean.

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Membrane stabilizing activity

The method as described by [21] was adopted with some modifications. Blood was collected into sterile plane bottles and animals not given any NSAIDS for two weeks before to the experiment were dedicated by cervical dislocation. The blood samples were compounded with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0,5 % citric acid and 0.42 % NaCl) and centrifuge at 3,000 rpm. The packed cells were washed with isosaline and then 10% suspension was made. To each extract's concertation, 1 ml of phosphoric buffer, 2 ml hyposaline and 0.5 ml of RBC suspension was appended thereafter incubated at 37^0C for 30 minutes and centrifuged at 3,000 rpm for 20 minutes. The reactions were performed in triplicates in 96-well micro plate reader Spectra Max 384 plus (Molecular Devices, USA) and hemoglobin content of the supernatant solution was estimated spectrophotometrically at 540 nm. Indomethacin (100µg/ml) was used as reference standard and a control was prepared by omitting the extracts. % of RBC membrane stabilization was calculated using the following equation.

% Protection = 100 – [(Optical density of Test sample / Optical density of Control) X 100]

Result

Acetic acid induced writhing test (peripheral pain).

In this test, the antinociceptive effects of ETCI was investigated by administering 100 mg/kg and 150 mg/kg dose to mice. By applying this test, it was seen that there was significant effect of extract compare to standard drug (Indomethacin). Among the ETCI extract 100mg/kg showed better results compare to the standard which was not ETCI 150mg/kg in dose depending manner.

Table-1: Evaluation of Analgesic activity of ETCI by Acetic acid induced writhing method.

Treatment	Number of writhing	% of inhibition
Standard	4.87±1.1149**	82.96%
ETCI(100mg)	8.125±0.9537**	71.62%
ETCI(150mg)	13±3.6265**	54.59%

Values are reported as mean ± S.E.M. for group of four animals (n = 4). Values are analyzed as compared to control using one-way ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control, * indicates P < .05, ** indicates P <.001.

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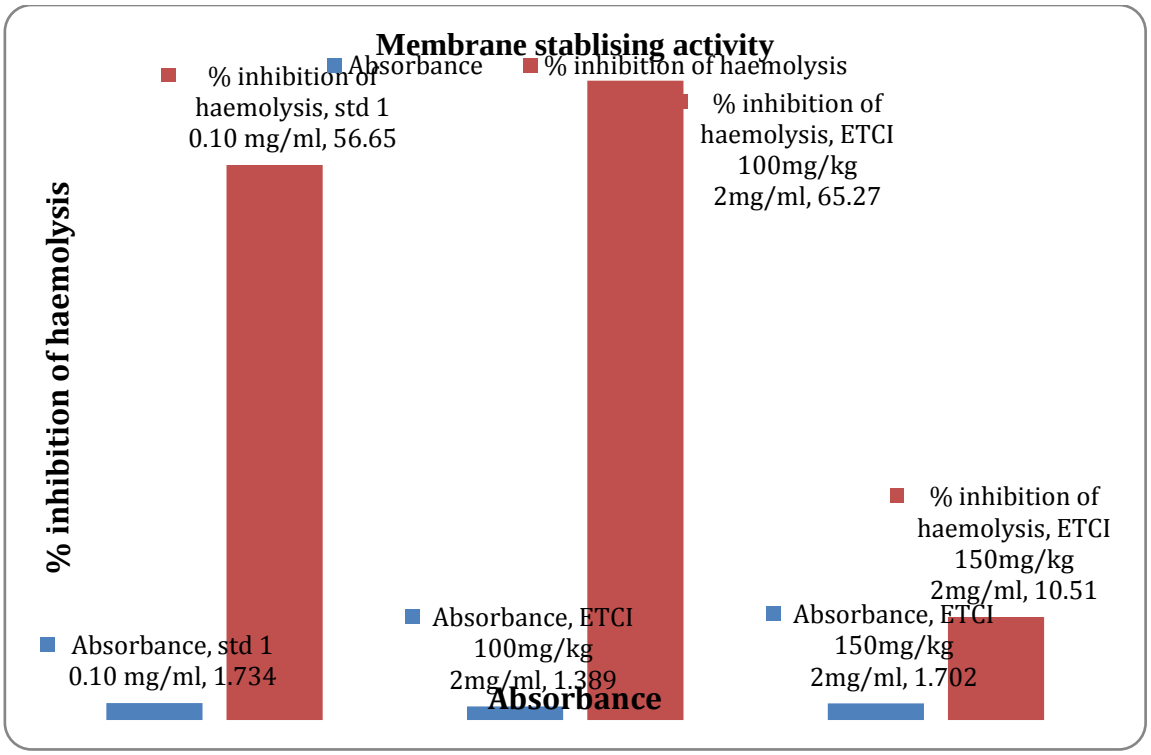

Figure 1: Antinociceptive effect of *C. indica*by acetic acid induced writhing method.

Eddy's Hot Plate method

The analgesic activity of ETCI was assessed using hot plate method of Eddy. The temperature was maintained at55±0.5°C.Animals licked their limbs and jumped as an indication of pain. These rats were treated with suspensions as follows: control group received normal water. The test groups received 100mg/kg and 150mg/kg of ETCI. The standard group received Indomethacin 10mg/kg by the oral route. One hour after dosing group specific drugs, rats were placed on the hot plate and the time until eitherlickingand jumping occurs was recorded by a stop watch. The latency period was recorded before and after 0min, 30min and 60min following oral administration of group specific drugs. The cut off time of 18 sec was employed for hot plate test.

Table-2: Evaluation of Analgesic activity by Eddy's Hot plate method of ETCI

Treatment	Reaction Time					
	0 min	% of Latency	30 min	% of Latency	60 min	% of Latency
Standard	17±1.15*	14.7	17.25±0.866	7.24	18±0**	2.77
ETCI(100mg)	15.08±0.481	13.12	15.5±1.374	12.9	16.44±0.601**	19.4
ETCI(150mg)	14.16±1.737	8.19	15.9±0.949	15.09	16.52±0.732**	19.79

Values are reported as mean ± S.E.M. for group of four animals (n= 4). Values are analyzed as compared to control using one-way ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control, * indicates P < .05, ** indicates P <0.001.

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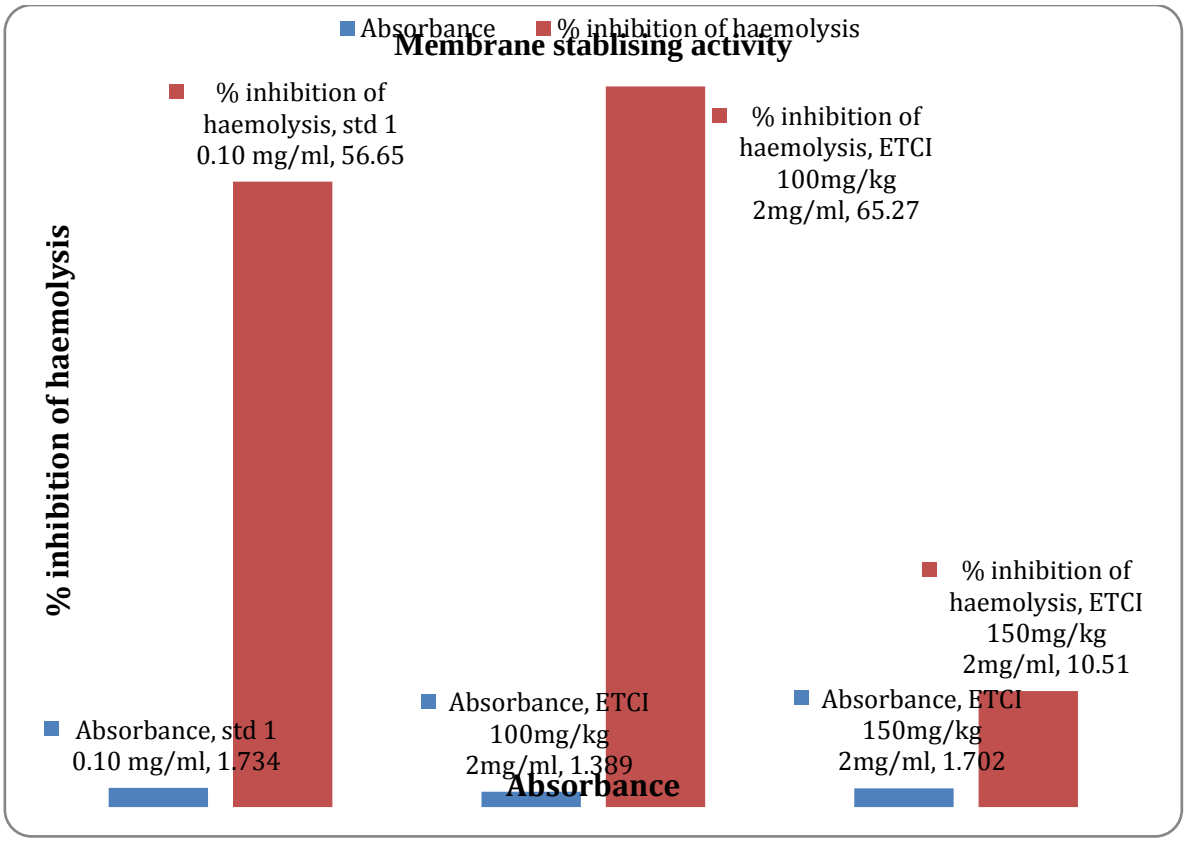

Figure 2: Effect of ethanolic extract of C. India on latency to hot plate test (Eddy's Hot Plate).

Open field test method

At 100mg/kg and 150 mg/kg dose, experimental ETCI extracts were administered to mice. As a result, the movements of mice were reduced but not dose depending manner. Also, it was comparable with diazepam (standard). This movement lowering effect of extract on mice was observed at 30 min interval from zero minute up to 90 minutes. The extracts caused reduction in movement and this may be connected to CNS depression, as reduction or depression of movement is common to most antipsychotics.

Table-3: Evaluation of CNS activity test of ETCI by Open field method

Treatment	0min	%of movement inhibition	30min	% of movement inhibition	60min	% of movement inhibition
Standard	26.2±3.27**	83.14	30±6.41**	68.66	37±9.85**	57
ETCI100mg	84±4.02**	65.32	68±4.02	28.98	51.25±5.32**	40
ETCI150mg	94±**	37.07	82.5±	13.83	56.5±5.21**	34

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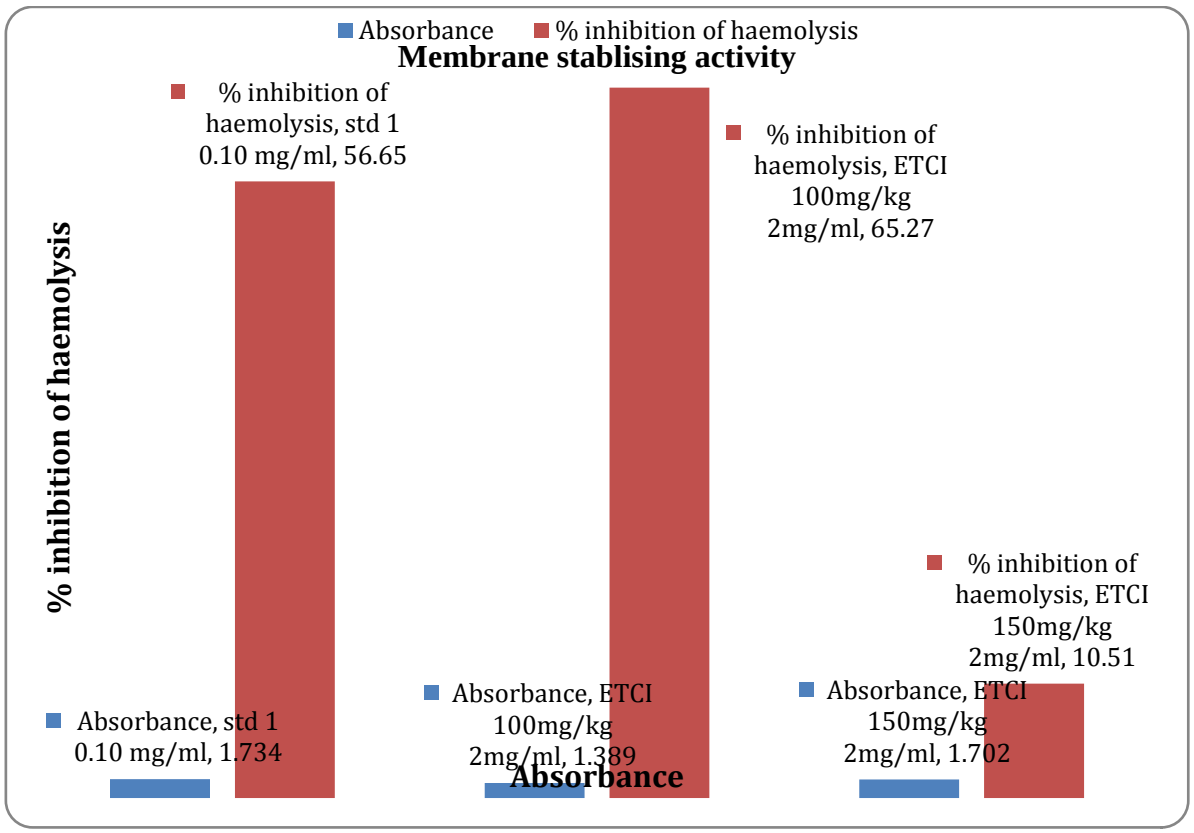

Figure-3: Effect of ethanolic extract of *C. indica* on open field test in mice.

Hole Cross method

In this technique, the *C, indica* extract at 100 mg/kg and 150 mg/kg dose showed irregular decrease of movement in the experimental animals from the second observation to last observation. Here, the result was dose depending.

Table-4: Evaluation of CNS activity of ETCI by Hole Cross method.

Treatment	0min	% of movement inhibition	30min	% of movement inhibition	60min	% of movement inhibition	
Standard	10±0.33**	23.07	7±0.81	48.14	4±0.47**	69.81	
ETCI100mg	11±2.62	65.89	10±1.24	68.10	5.7±0.55**	78.54	4±0.47** 69.81
ETCI150mg	11.2±1.72	65.11	10±1.65	67.30	5±0.47**	81.30	

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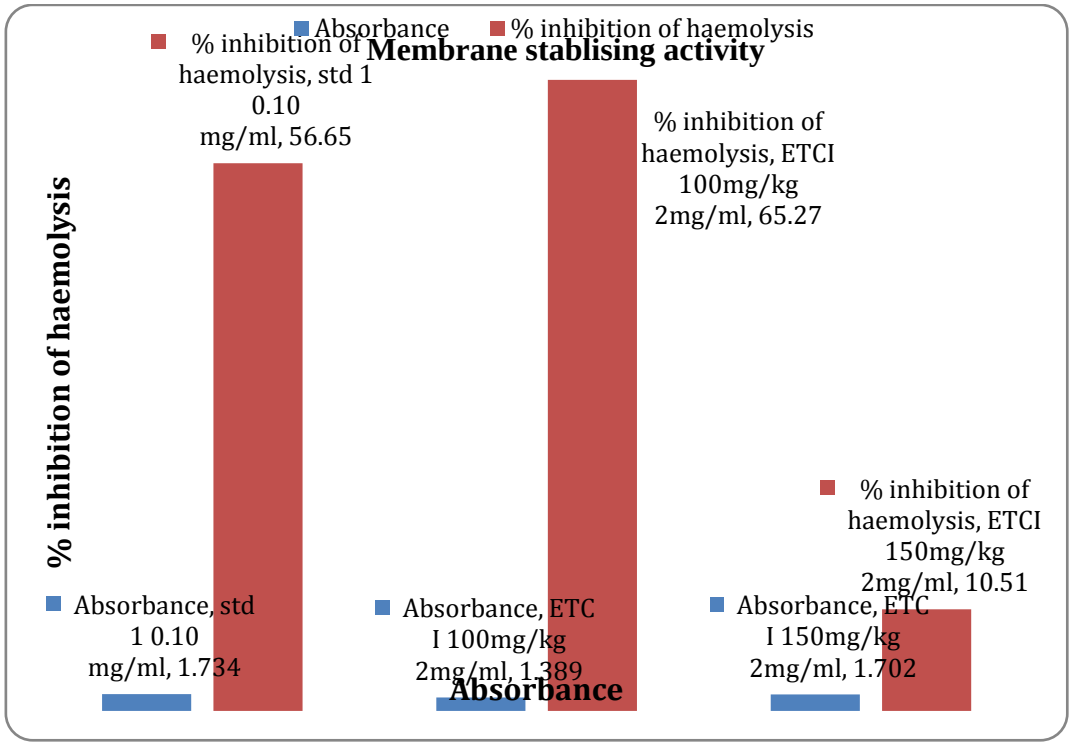

Figure-4:Effect of ethanolic extract of *C. indica* on hole cross test in mice.

Membrane stabilizing activity

Table 5 reveals the protective effects of the ethanol extracts of *C. indica* on membrane of red blood cells. It can be observed that the extracts of *C. indica* demonstrated a significant membrane stabilizing activity. The ethanolic extracts showed a concentration dependent membrane stabilization activity.

Table-5: Membrane stabilization activity of the ethanolic extracts of *C. indica.*

Treatment	Concentration	Absorbance	% inhibition of haemolysis
Standard	0.10 mg/ml	1.734	56.65
ETCI 100mg/kg	2mg/ml	1.389	65.27
ETCI 150mg/kg	2mg/ml	1.702	10.51

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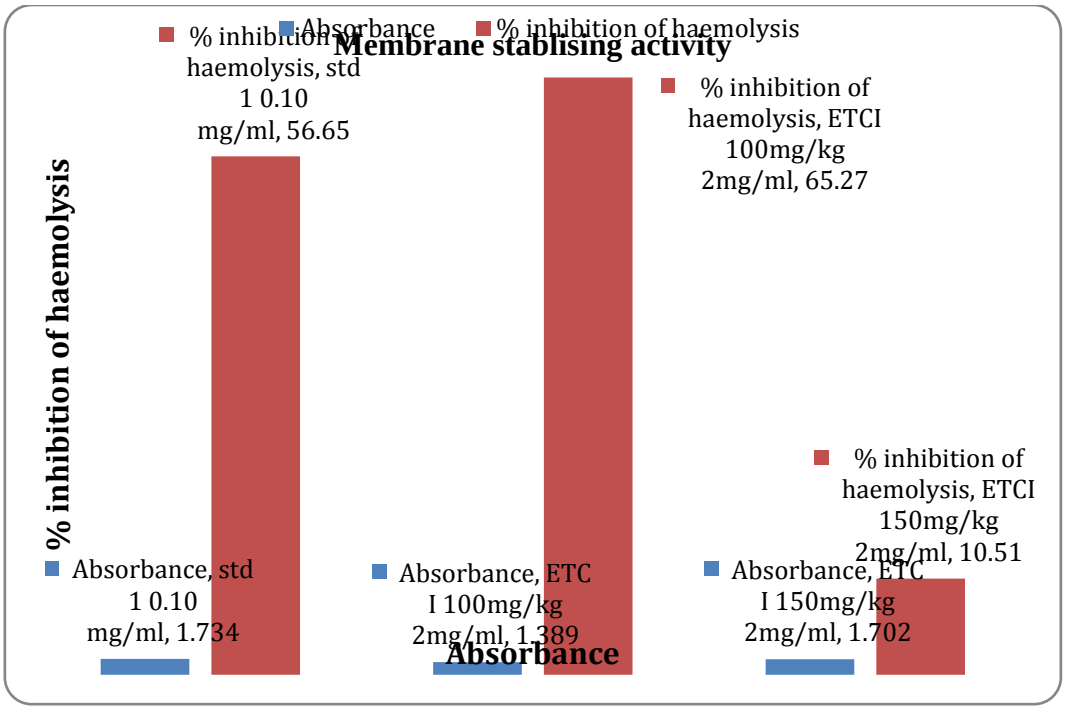

Figure-5:Effect of different doses of ETCI on hypotonic solution-induced haemolysis of erythrocyte membrane.

Discussion

According to our study it is clear that ETCI extract shows the effect compare to standard Indomethacin.100mg/kg is more effective then 150mg/dose in acetic acid induced method and 100mg/kg is also 150 mg/kg in hot plate method.Acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics and represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from the tissue phospholipid response is thought to be mediated by peritoneal mast cells acid sensing ion channels and prostaglandin pathway. The organic acid has also been postulated to act indirectly by inducing the release of endogenous mediators, which stimulate the nociceptive neurons. Hot plate method is safer than acetic acid induced method. Therefore, it is likely that ethanolic extract of ETCI might have exerted its peripheral antinociceptive action by interfering with the local reaction caused by the irritant or by inhibiting the synthesis, release or antagonizing the action of pain mediators at the largest sites. Alertness is the result of increased locomotor activity and sedative effect is the result of decrease in locomotor activity. In this investigation, locomotor activity was observed by two methods: open field and hole cross tests. The standard (diazepam) was used in this study to induce sleep as well as believed that it acts on the binding site of GABA receptors. It has been reported that flavanoids and other steroids act as ligands for GABA receptor in CNS. So, there is a chance that presence of flavanoid in the extract may be responsible for its CNS depressant activity.The ETCI were effective in membrane stabilizing activity as the extractives prevented the lyses of erythrocytes induced by hypotonic solution. During hypotonic condition the different doses of ETCI (100 mg/kg,150mg/kg) showed inhibition 65.27%,10.51% respectively. During the hypotonic condition the percent inhibition of standard was about 56.65%. The 100mg/kg dose had showed the better result than 150mg/kg. The different ethanolic extract of ETCI at

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concentration 2.0mg/ml significantly protected the lyses of human erythrocyte membrane as compared to the standard acetyl salicylic acid.

Conclusion

The popular and most versatile commercially cultivated and consumer preferred milky mushroom (*Calocybe indica)* was selected for the study. Chemical and nutritional compositionof the fresh and processed milky mushrooms was assessed with regard to macro nutrients, vitamins, minerals and other chemical constituents. Eighteen amino acidsincluding eight essential amino acids were analyzed, which yielded more precise data on the quality aspects of mushroom protein. Milky mushroom contains all the essential amino acids and the content of isoleucin, valine, threonine was exceptionally high when compared to reference protein.Thesemilky mushroom (ETCI) would be a great source of chemicals that can be synthetically manufacture as medicine in future.

Consent

It is not applicable.

Competing interests

Authors have declared that no competing interests exist.

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