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Research Article

STUDIES ON THE ROLE OF DRIED ETHANOLIC EXTRACT OF *AGARICUS BISPORUS* IN THE TREATMENT OF DEPRESSION IN MICE

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ABSTRACT

Objectives: Depression is a widespread mental illness with an estimated 322+million people worldwide and one of the most frequent reasons for medical and physical disability. However, the standard treatments available are having side effects such as addiction, physical dependence, and to overcome the lacuna researchers are looking for natural remedies Thus our study aimed to investigate the anti-depressant effect of *Agaricus bisporus* (White button mushroom) in mice.

Methods: The anti-depressant action of dried ethanolic extract of *A. bisporus* (EEAB) was evaluated in the Swiss albino mice by various models. After extraction and standardization of the white button mushroom the dose-dependent anti-depressant effect of the white button mushroom was determined. Further, with the best chosen dose, the chronic anti-depressant effect of the white button mushroom was carried out, and also to further substantiate the data brain bioamine estimation was done.

Results and Discussion: Statistical analysis with EEAB200 mg/kg and 400 mg/kg showed marked anti-depressant action in dose dependent manner. The calibration of EEAB extract by HPLC guided a determination that the presence stigmasterol. Brain estimation of the EEAB with 400mg/kg dose displayed upregulation of Serotonin in the treated mice. Since Serotonin is a major neurotransmitter which is vital role anti-depressant activity. So, the upregulationofSerotoninsuggeststhatEEABishavingprofound anti-depressant activity.

Conclusion: At 400 mg/kg, the standardized extract showed anti-depressant efficacy in an acute pharmacological trial. Furthur Agaricus treated group of animal for consecutive 14, 21, 28 days chronic study showed significant increased in brain GABA and serotonin level in comparison with the untreated group of animals analysed by HPLC and flurimetric analysis.

Keywords: Depression, High-performance liquid chromatography, Serotonin, Dopamine, Stigmasterol, White Button Mushroom.

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INTRODUCTION

According to the World Health Organization, depression is a type of mental illness and is a common mental health problem in today's society. This mental illness affects more than 322 million people worldwide. The most common symptoms of depression are persistent sadness, lack of interest, loss of appetite, sleep, fatigue, depression, and cognitive problems [1].

The interaction of psychological, social, and biological factors is a major cause of depression. People who have experienced difficult times in life, such as the loss of a loved one and long-term unemployment, are more likely to experience depression. Depression is more common in many diseases, such as heart disease and neurodegenerative diseases [1].

Current treatments for depression include behavioral therapy, counseling, and antidepressants. However, some of the strange and even serious side effects of this class of antidepressants have created a need for better treatment [2].

Mushrooms belong to the fungi kingdom and are often eaten as vegetables due to their unique nutritional value. Micronutrients are mostly found in vegetables, meats, and grains, and mushrooms are rich in them. These micronutrients include the sulfur-containing amino acid ergothioneine, fiber-related monosaccharides and polysaccharides, riboflavin, niacin, pantothenic acid, copper, phosphorus, and selenium. Plants that provide only Vitamin B12 and Vitamin D to vegetarians, produced by the conversion of ergosterol to ergocalciferol under ultraviolet (UV) radiation [3].

Today, white mushrooms (*Agaricus bisporus*) are a valuable food because they are the best food and an excellent medical tool [17-32]. Nowadays, white mushrooms are widely used as alternative medicine or health supplement. Many studies have shown that white mushrooms have neuroprotective effects related to their cholinesterase inhibitory, antioxidant, and anti-inflammatory properties [2].

METHODS

Animals

Male Swiss albino mice were kept at the animal center of the Department of Pharmacology, College of Pharmacy and Allied Health Sciences, Durgapur, Dr. M. O. Roy said. The first batches sold are from companies registered with CPCSEA in India. The mice were housed at 21°C on a 12:12 h light/dark cycle. Male rats aged 18–25 years were housed in a climate-controlled environment at 250°C with limited access to water and rat chow. The Institute of Animal Ethics (IAEC) reviewed and approved the study after ethical review in accordance with BCRCP/1348/C/10/CPCSEA/16-IAEC/2/23.

Drugs and chemicals

The laboratory at Dr. M. O. Roy School of Pharmacy and Allied Health Sciences has the following materials: Ethylenediaminetetraacetic acid (EDTA), phosphate buffer, perchloric acid, sodium citrate, citric acid, sodium citrate buffer, and ninhydrin solution.

Instruments and methods

All bioanalytical methods were performed in 1.5 mL closed Eppendorf

tubes (Eppendorf, Germany). Eppendorf tubes were covered with aluminum foil to protect from sunlight. Temperature can be more easily controlled by placing the entire sample in a temperature-controlled oven (Sunsim, India). Thin-layer chromatography (TLC) was performed on silica GF254 plates (Sigma-Aldrich Canada Co. or Millipore [Canada] Ltd., Germany). Use a UV chamber with a wavelength of 254 nm to view the TLC surface. Spectrophotometric measurements were carried out with a UV visible spectrophotometer model 1800 (Shimadzu, Japan) equipped with a quartz cuvette with a 1 cm optical path length [8-35].

Drug solution and dose

Dried ethanolic extract of *A. bisporus* (EEAB) (400 mg/kg, orally) was dissolved in saline.

Pharmacognostical part

Collection and authentication of the fungi

The entire *A. bisporus* fungi was collected and authenticated by Rising Fungi Mushroom Seed Supplier/Spawn Laboratory and Mushroom Cultivation Centre, Durgapur, West Bengal, 713213.

Drying and size reduction

Only the fruit of *A. bisporus* is dried in a cold place at a temperature of 25°C. The powder is then passed through a 60-mesh sieve to ensure consistency and used for processing.

Preparation of the extract

Place 25 g of rice flour in a beaker filled to the bottom. Attach the Soxhlet extractor to the beaker and perform the extraction using 95% ethanol (250 mL) at the appropriate temperature.

The extract was concentrated using a distillation machine at 65°C for about 3 h to remove the solvent and obtain the crude material. After that, the extract was evaporated to dryness to obtain a dark brown product extract used in this study.

Standardization of fungi extract

Marker analysis of fungi extract using TLC

A qualitative and quantitative method known as marker analysis finds substances or preformed markers in a mixture. Stigmaster Ol was chosen as the marker. Prepare the mobile phase in hexane: ethyl acetate: ethanol: chloroform (4:3:2:1) for two TLC plates, each containing 20 mL of solution. We then use a pencil to mark seven different places on two TLC sheets [4].

For the solubility test, equal amounts of the fungal extract were mixed with four other solvents including n-hexane, chloroform, ethyl acetate, and ethanol. Then, dissolve 3 mg of pure stigmasterol in 2 mL of chloroform. Then, draw four areas using the capillary: Two for the initial fungal infection, one for pure stigmasterol, and two for the different solvents on two separate plates. After detection, place the two TLC pieces in the TLC chamber and leave them there until the solvent flows through them. Remove the TLC plate from the outside, let it dry completely, and examine in the UV chamber.

Marker analysis by high-performance liquid chromatography (HPLC)

A simple analytical chemistry method called HPLC is used to separate the different components of a chemical mixture. The marker selected for this study, stigmasterol, was analyzed qualitatively and quantitatively using the HPLC method. For analysis, 15 mg of stigmasterol was diluted in 3 mL of chloroform. Then remove the fungal solution and dilute 0.5 mL with 4.5 mL of ethanol. Take 10 µL of the product, filter through Whatman filter paper and put into the HPLC system.

Pharmacological part

Toxicity study

Already done by previous researchers.

Fig. 1: Picture of white button mushroom (*Agaricus bisporus*)

Fig. 2: Extraction performs using the decoction method

Fig. 3: Ethanol evaporation using rotary evaporator

Acute behavioral study

Tail suspension test

This test is a way to measure the behavior of mice that appear depressed. The animals first need to get used to the laboratory. Twenty male Swiss albino mice were then collected and divided into four groups of five. The first group received 200 mg/kg EEAB, the second group received 400 mg/kg, the third group received the combination group, and the

Fig. 4: Thin-layer chromatography

Fig. 5: Picture of oral administration

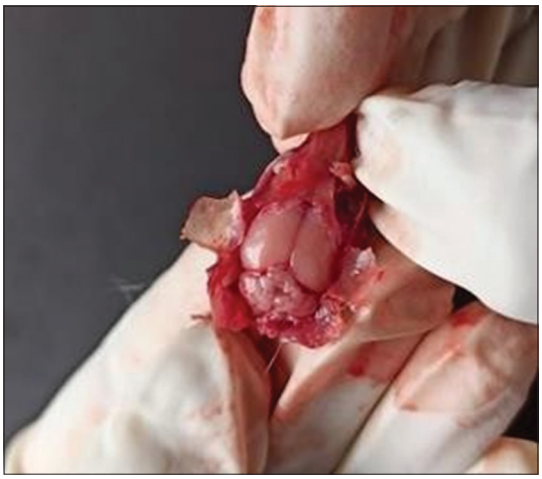

Fig. 6: Picture of internal brain tissue of mice

fourth group received the control group. Use a support-like device to immobilize the mouse's tail using nonabrasive techniques. The duration of inactivity was measured as an indicator of depression during the six-minute test. Create an experimental group for clinical evaluation and a control group for baseline comparison.

Fig. 7: Picture of hominization process of brain tissue of mice

Fig. 8: Ion exchange column chromatography

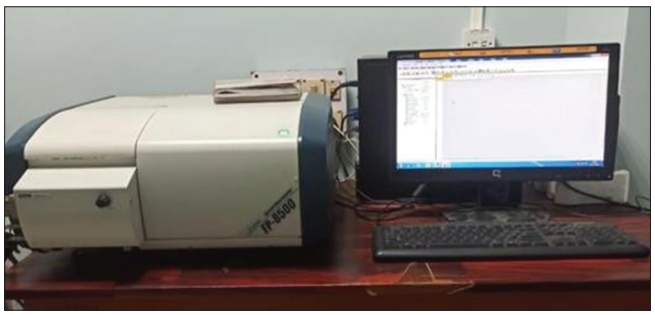

Fig. 9: Picture of spectrofluorometer

Open field test (locomotor activity): (400mg/kg)

The test was performed in an open area with a white plastic floor and black rubber sheeting if necessary. The animals were placed in a corner of the arena and monitored for 5 min. The total distance the mouse traveled was recorded as a function of the mouse.

Chronic study: (400mg/kg)

Animal use ethanol *A. bisporus* extract (400 mg/kg): The experimental animals were studied for 14, 21, and 28 days. Two groups of five animals were formed from the subjects: A test group and a control group. An additional group was formed for the chemical additive piperine. After

the injection period, all animals were sacrificed and the brain was removed for further analysis.

Determination of brain bioamine levels

Extraction of bioamine from brain tissue: For GABA: Animals were killed by cervical dislocation. After removing the skull, the entire brain was collected and stored in cold storage. According to research, an average mouse brain weighs 400 mg. Then add 0.1 mL of 5% sodium metabisulfite ($Na_2S_2O_5$), 0.2 mL of 10% EDTA, 0.1 mL of 1 m citric acid, and 10 mL of 0.4 m perchloric acid. Homogenize the tissue in very cold. The homogenate was centrifuged at 14,000 g for 10 min at 0°C.

For serotonin: The brain tissue was cooled and 0.2 mL of 10% EDTA, 5% $Na_2S_2O_5$, and 10 mL of 0.4N perchloric acid were added. The tissue was homogenized and frozen. Centrifuge the homogenate at 14,000 rpm for 10 min. After collecting the supernatant, lower the pH to 2 using 5 N potassium carbonate (K_2CO_3).

Column preparation: After mixing them with DOWEX 50W in phosphate buffer, a glass tube of approximately 5 cm in length and 4.0 mm in diameter was used to construct the column. After washing as indicated, flush 20 mL of 2 N sodium hydroxide (NaOH) in 1% EDTA through the column. Then wash the column with a glass distilled water until neutral.

After which, 20 mL of 2 N hydrogen chloride (HCL) was passed through the column. Then washing was done with glass distilled water until neutral.

Elution: Elution for GABA: Samples passed through the line. Use 8 mL of clean water for pre-cleaning of the lines. After passing 8 mL of 0.025 m sodium citrate (pH 4.5) through the column, 4 mL of 0.05 m sodium citrate buffer (pH 5.3) was added to balance the mixture. After using the first 0.5 mL, 3.5 mL was collected for the study. Wash the column with glass distilled water, NaOH, and HCL.

Elution for serotonin: The sample is washed with distilled water and passed through the chromatography tube. Then wash with 15 mL of distilled water. The column is washed with 15 mL of sodium phosphate buffer (pH = 6.5) and then washed with 15 mL of distilled water. The first 3.5 mL are discarded and the remaining 7 mL are used to study norepinephrine and epinephrine. Then wash the column with 4.7 mL of methanol (50%) HCL and collect the last 3.5 mL to capture serotonin and dopamine. Elution is carried out in the dark and at room temperature, approximately 25–26°.

Detection: Detection of GABA: Ninhydrin reaction: Add 50 µL of 2.5 m K_2CO_3 to the eluate to make it alkaline. Then, 0.4 mL of ninhydrin solution was added to 0.8 mL of eluate. After 30 min incubation at 60°C, store in a refrigerator at room temperature for 30 min. Fluorescence was read at 380 nm excitation and 450 nm emission.

Detection of serotonin: For the detection of serotonin the reagents that were added to the standard, control eluate and the sample eluate have been tabulated in Table 1.

Estimation: Estimation of serotonin using fluorimetry method: Fluorometry is a method of measuring fluorescence intensity at specific wavelengths using a spectrofluorometer or filter fluorometer. Following preparation for serotonin analysis, brain samples were analyzed by fluorometric analysis. Serotonin samples were prepared using analytical procedures described previously. Fluorescence was measured with 355 nm emission and 310 nm excitation.

Estimation of serotonin using HPLC: After the separation process of brain biogenic amine extraction, the separated eluates were obtained and used as injection samples in HPLC for biogenic amine measurement. Before injection, the cell was treated with a 50:50 methanol: water mixture and filtered through a membrane filter. The flow rate was controlled at 0.4 mL/min. Inject 20 µL of the sample using a Rheodyne syringe. Serotonin levels in brain samples isolated from treated and untreated animals were compared.

The fluorescence emission for different samples was read at the following wavelength for different catecholamines after UV irradiation for 5 min – Serotonin was read at 310/355 nm.

RESULTS

Result of marker analysis fungi extract by using TLC

From the above figures (Figs. 10 and 11), it was confirmed that there is presence of marker that is stigmasterol in EEAB and when the mother extract is mix with chloroform the presence of stigmasterol more than other solvent (n-hexane, ethanol, and ethyl acetate) solution.

Marker analysis by HPLC of EEAB and stigmasterol

Stigmasterol Pure (1 mg/mL)

From the above Fig. 12, two peaks are prominently visible. By observing the sharpness of the peak, it can be concluded that the peak at 14.718 min (Table 2) is the peak for pure Stigmasterol.

A. bisporus extract (1:10)

By observing (Fig. 13), it can be clearly state that the peak at 14.214 min can be the presence of Stigmasterol. As earlier discussed in (Fig. 12), the peak of pure stigmasterol was at 14.718 min and the one peak has observed at 14.214 min both peaks are almost close enough to conclude the presence of stigmasterol. Hence, concluded that there was a presence of stigmasterol in the extract.

Acute behavioral study: (Dose-dependent study)

Chronic study: (14, 21 and 28 days study)

Estimation of serotonin level for 14 days study by using HPLC

Serotonin (control group) 400 mg/kg in 14 days

There are two major peaks at 5.273 min, 7.016 min, and

Table 1: Concentration of reagents that were added for the fluorometric determination of serotonin

Reagent	Standard (mL)	Reagent Blank (mL)	Sample (mL)	Control (mL)
H_2O	--	0.05	0.05	0.05
4% EDTA	0.05	0.05	0.05	0.05
0.25% K3(Fe (CN) 6)	--	--	--	0.05
3.225 NaOH in 0.125M $Na_2S_2O_3$	--	--	--	0.4
Wait for more than 1 min				
Glacial acetic acid	--	--	--	0.2
Eluting solution	1.0	1.0	--	--
Eluate	--	--	1.0	1.0
Standard drug solution (1 µg/mL)	0.05	--	--	--
0.25% K3(Fe (CN) 6)	0.05	0.05	--	--
Wait for 3–6 min				
3.225 NaOH in 0.125M $Na_2S_2O_3$	0.4	0.4	0.4	--
Glacial acetic acid	0.2	0.2	0.2	

H_2O: Water, EDTA: Ethylenediaminetetraacetic acid, K3(Fe (CN) 6): Potassium ferricyanide, NAOH: Sodium hydroxide, $Na_2S_2O_3$: Sodium thiosulfate

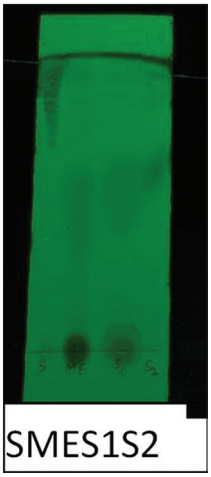

Fig. 10: Thin-layer chromatography of ethanolic extract of *Agaricus bisporus* with the respect of stigmasterol, ethanol, and ethyl acetate

Fig. 11: Thin-layer chromatography of ethanolic extract of *Agaricus bisporus* with the respect of chloroform and n-hexane

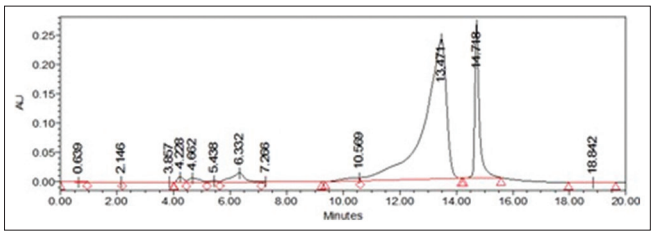

Fig. 12: High-performance liquid chromatography peaks of pure stigmasterol

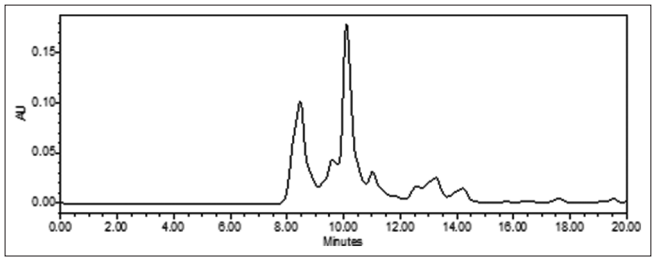

Fig. 13: Resulted peaks from high-performance liquid chromatography of ethanolic extract of *Agaricus bisporus*

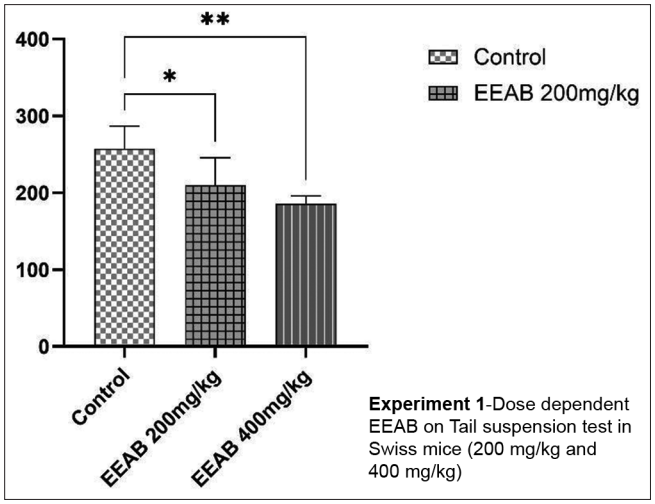

Fig. 14: Effect of ethanolic extract of *Agaricus bisporus* on tail suspension test in Swiss albino mice. Results are expressed as mean ± standard error of the mean for five animals. Statistical comparison was performed using one-way analysis of variance followed by Dunnett's test; *p<0.01, **p<0.001 when compared to the control group

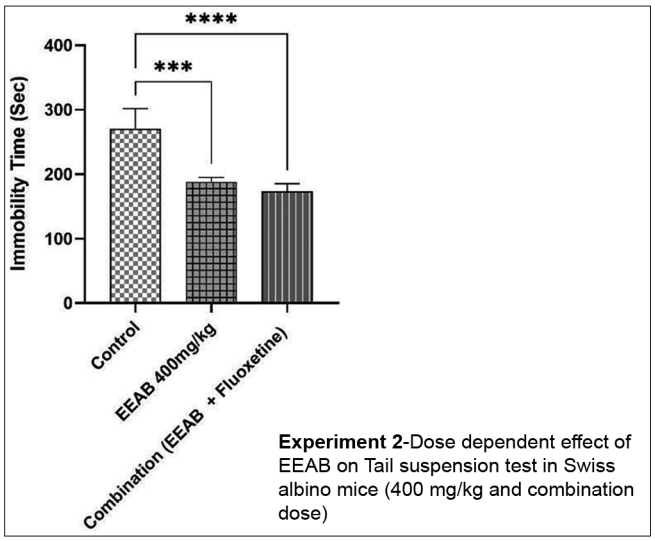

Fig. 15: Effect of ethanolic extract of *Agaricus bisporus* on tail suspension test in Swiss albino mice. Results are expressed as mean ± standard error of the mean for five animals. Statistical comparison was performed using one-way analysis of variance followed by Dunnett's test; ***p<0.0001, ****p<0.00001 when compared to the control group

Table 2: HPLC Datatable for pure stigmasterol

S. No.	Retention time	Area	Percentage of Area
1.	13.471	12,862,611	75.88
2.	14.718	2,834,259	16.72

HPLC: High-performance liquid chromatography

8.617 min in which one of them should be of serotonin. For our quantification, we took the additive sum of two peaks containing area 452,445.

There are three major peaks at 4.777 min, 6.374 min, and 8.089 min which peaks are closed to the control group's peaks. By comparing

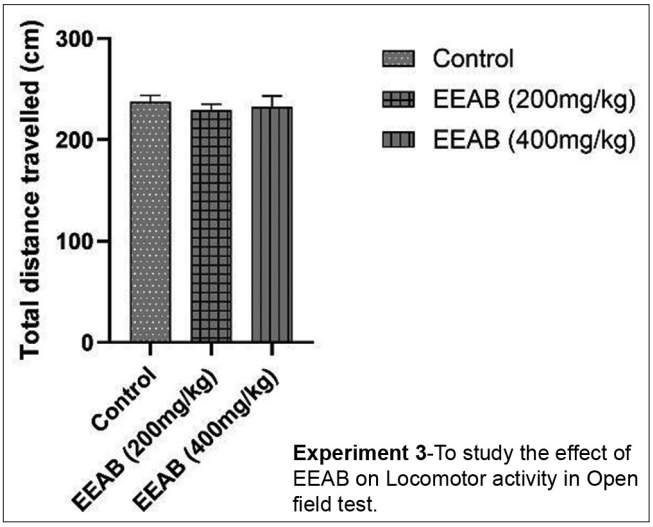

Fig. 16: Effect of ethanolic extract of *Agaricus bisporus* on locomotor activity in open field test. Each bar represents the mean ± standard error of the mean of five animals. Effect of EEAB does not show any changes in locomotor activity in different doses of EEAB. Thus, none of the doses showed any effect on locomotor activity

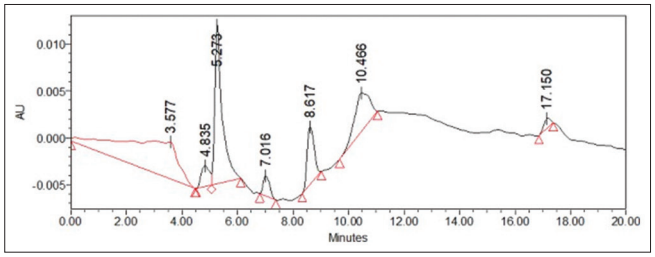

Fig. 17: Resulted peaks of high-performance liquid chromatography of control *Agaricus bisporus* serotonin level for 14 days chronic study

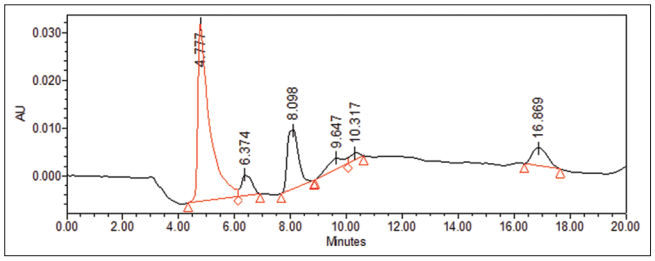

Fig. 18: Resulted peaks of high-performance liquid chromatography of test *Agaricus bisporus* serotonin level for 14 days chronic study

from the control group data, it is assured that the peak area has been increased by 1,529,790. Hence, the level of serotonin has increased as compared to the control group.

Estimation of serotonin and GABA in the EEAB by fluorimetry of 14 days chronic study

Analysis of serotonin level (14 days) by fluorimetry

From the above fluorimetry results, it can be clearly displayed that the *A. bisporus* test serotonin level obtained 4458.9199 fluorescence units/g and *A. bisporus* control serotonin level obtained 3758.3495 fluorescence units/gm. Hence, is clearly visible from the (Fig. 19) that the level of *A. bisporus* test has increased when compared to *A. bisporus* control.

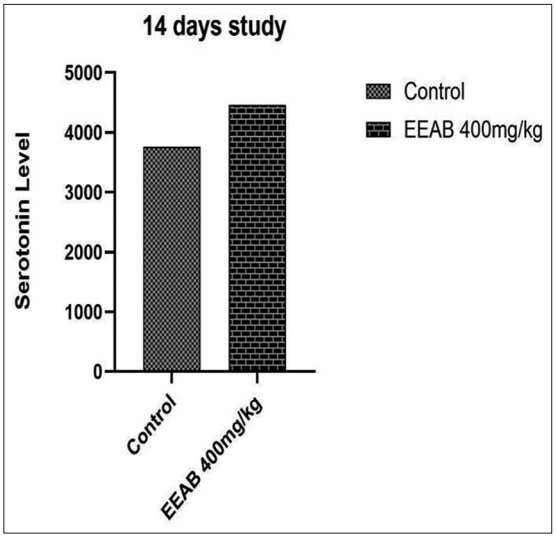

Fig. 19: Comparison of serotonin level in mice by fluorimetry (14 days)

Analysis of GABA level (14 days) by fluorimetry

From fluorometric analysis, it was revealed that the *A. bisporus* test GABA level displayed 160.2159 fluorescence units/g of dissected mice brain. Hence, from the bar chart (Fig. 20), it might be suggested that there is a decrease in compared to the control group.

Estimation of serotonin and GABA in the EEAB by fluorimetry of 21 days chronic study

Analysis of serotonin level (21 days) by fluorimetry

The above fluorometric data in (Fig. 21) revealed that there was a significant decrease in the level of serotonin in the test group as compared to the control group.

Another group of animals treated with combined dose of 400 mg/kg of extract and 70 mg of Piperine (Bioenhancer). This study also shows a deduction in the level of serotonin in the mice brain after treatment with bioenhancer as compared to the control group of animals.

Analysis of GABA level (21 days) by fluorimetry

From the above fluorometric analysis, it was revealed that in *A. bisporus* test group, GABA level displayed 1021.4017 fluorescence units/g of dissected mice brain. Hence, from the bar chart (Fig. 22), it might be suggested that there is an increase in compared to the control group.

Another group of animals treated with the combination of 400 mg/kg of extract and 70 mg of Piperine (bioenhancer). This treatment shows a significant increase in comparison to the control group when treated for 7 days just to observe its effectiveness in animal model.

Estimation of serotonin and GABA in the EEAB by fluorimetry of 28 days chronic study

Analysis of serotonin level (28 days) by fluorimetry

The above fluorometric data in (Fig. 23) revealed that there was a significant increase in the level of serotonin in the test group as compared to the control group.

Another group of animals treated with combined dose of 400 mg/kg of extract and 100 mg of Piperine (Bioenhancer). This study also showed an increase in the level of serotonin in the mice brain after treatment with combined bioenhancer as compared to the control group of animals.

Analysis of GABA level (28 days) by fluorimetry

From the above fluorometric analysis, it was revealed that in *A. bisporus* test group, GABA level displayed 308.5315 fluorescence units/gm of

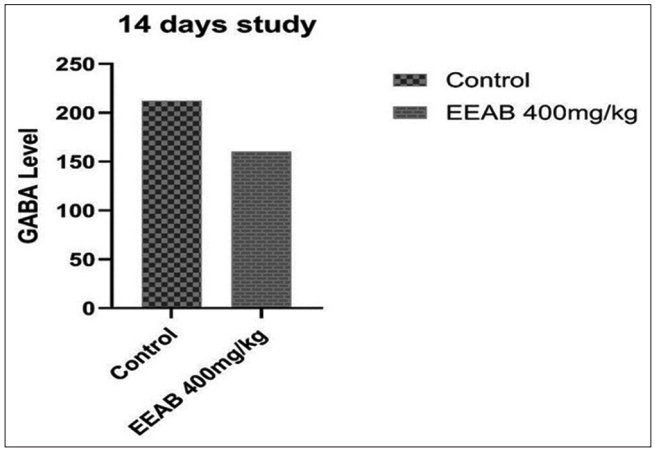

Fig. 20: Comparison of GABA level in mice by fluorimetry (14 days)

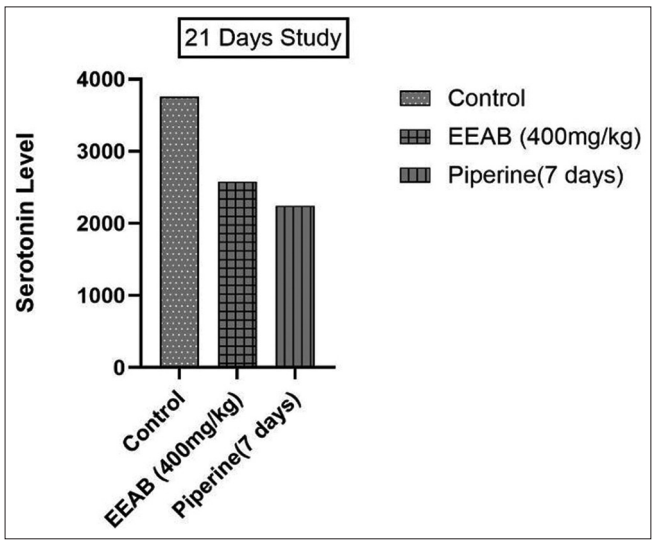

Fig. 21: Comparison of serotonin level in mice by fluorimetry (21 days)

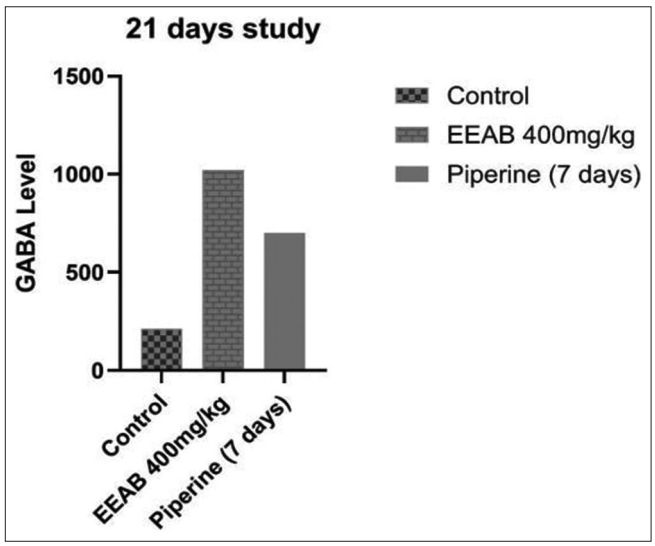

Fig. 22: Comparison of GABA level in mice by fluorimetry (21days)

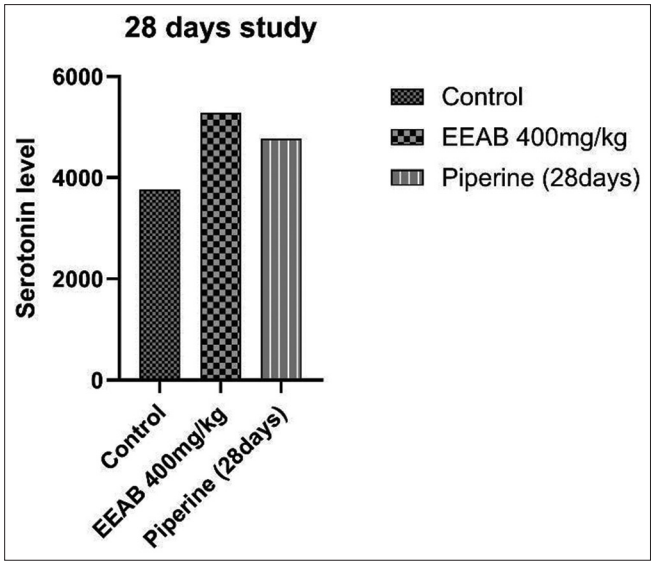

Fig. 23: Comparison of serotonin level in mice by fluorimetry (28 days)

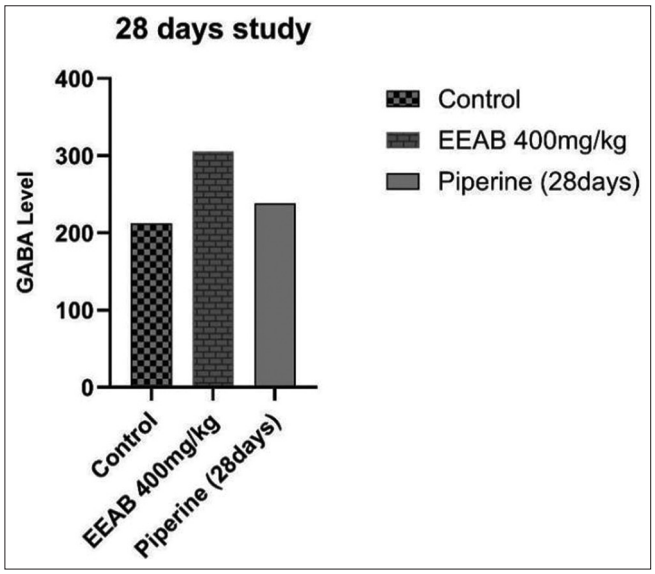

Fig. 24: Comparison of GABA level in mice by fluorimetry (28 days)

Table 3: HPLC data of the EEAB

S. No.	Retention time	Area	Percentage of Area
1.	14.214	580,734	4.46

HPLC: High-performance liquid chromatography, EEAB: Ethanolic extract of *Agaricus bisporus*

dissected mice brain. Hence, from the bar chart (Fig. 24), it might be suggested that there is much increase in compared to the control group.

Another group of animals treated with the combination of 400 mg/kg of extract and 100 mg of Piperine (bioenhancer). This treatment shows a significant increase in comparison to the control group when treated for 28 days just to observe its effectiveness in animal model.

Table 4: HPLC data of test group of EEAB

S. No.	Retention time	Area	Percentage of Area
1.	4.777	1,075,245	59.65
2.	6.374	113,253	6.28
3.	8.089	341,292	18.93

HPLC: High-performance liquid chromatography, EEAB: Ethanolic extract of *Agaricus bisporus*

Table 5: HPLC data of the control group of EEAB

S. No.	Retention time	Area	Percentage of Area
1.	5.273	311,256	26.19
2.	7.016	34,978	2.94
3.	8.617	106,211	8.94

HPLC: High-performance liquid chromatography, EEAB: Ethanolic extract of *Agaricus bisporus*

CONCLUSION

In clinical studies, a dose of 400 mg/kg has been shown to have an anti-anxiety effect. The study found that serotonin and GABA were significantly increased in the brains of treated mice compared to untreated mice. Serotonin is an inhibitory neurotransmitter, so the anti-anxiety effect of the extract may be attributed to its increase. The upregulation of GABA suggests that this neurotransmitter may play a role in the ability of *Agaricus blazei* extract to improve sleep.

Studies show that EEAB has significant effects after 14 and 28 days, but its effects wane after 21 days. On the other hand, piperine as a bioenhancer can increase the amount of biogenic amines in the brain. Higher doses of piperine caused an increase in brain biogenic amine concentrations even after 28 days. Therefore, it can be said that piperine and standardized ethanolic extracts of *A. bisporus* work well together.

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AUTHORS' CONTRIBUTIONS

Experimental design, guidance, supervision, and review work for the research were done by Ms. Sushruta Chakraborty and Dr. Souvik Basak. Experimental work, development, and optimization of the formulations, interpretation of result, and writing of this manuscript were done by Dr. Abhik Si and Mr. Sandipan Sahoo. All contributors read and approve the final manuscript.

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