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Original Article

Formulation and Evaluation of Anxiolytic Activity of Nanogel of CSN1S2 Protein from Goat Milk

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ABSTRACT

Objectives: This study is targeted at investigating the nanogel formulation of a protein from the milk of goat; CSN1S2 for anxiolytic activity in mice. **Materials and Methods:** After isolating CSNIS2, its nanogel was formulated by emulsification diffusion method. The nanogels' physical appearance, particle size, viscosity, spreadability, Zeta potential, particle size, Zeta potential, and *in vitro* diffusion profile were observed. Fourier transform infrared diffraction was used to characterize the structure of casein nanogels. The pharmacological screening of the formulation at 500 and 750 mg/kg was performed using Elevated Plus Maze test (EPM), Open Field Test (OFT) and Light and Dark Test (LDT) in mice. In addition, attempts have been conducted to explore the level of brain monoamine neurotransmitters like GABA and serotonin using flame fluorimeter. **Results:** The results from the models followed in the present study were interpreted and observed that the nanogels of the goat milk CSN1S2 protein significantly (p<0.001) exhibited anxiolytic activity in a dose dependent manner which was found to be well comparable the standard treatments; diazepam. An increase in brain GABA and decrease in brain serotonin concentration supported the anxiolytic activity of CSN1S2 protein. **Conclusion:** The findings of the current study indicate that goat milk CSN1S2 protein may possess anxiolytic activity by the elevated brain GABA and demoted brain serotonin concentration which provides scientific proof for its traditional argument.

Keywords: Anxiolytic, Nanogel, Goat Milk, CSN1S2, GABA, Serotonin.

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INTRODUCTION

In the present modernized lifestyle, anxiety is one of the most prevailing psychiatric disorders which affect people of different age groups worldwide. Anxiety is an unpleasant feeling of stress, discomfort, tension, insomnia, worried thoughts, and physical changes like elevated blood pressure, sweating, difficulty in breathing and increased heart rate along with educational and professional impairment with adverse quality of life.[1,2] It is evident that psychotropic drugs are one type of pharmacotherapy for anxiety disorders, but their effectiveness is constrained by their adverse effects, dietary restrictions, and drug interactions like the frequent benzodiazepines causes cognitive impairments, addictions, psychomotor impairments, anterograde amnesia, confusion, aggressive behaviour, physical depletion.[3] These are some of the aspects that have prompted numerous researchers to investigate novel compounds derived from nature in the hope of discovering new anxiolytic treatments with no or fewer undesirable side effects. In our previous study and other scientific investigations, it was evident that goat milk was found to reduce the risks of gastrointestinal disorders, diabetes and many other chronic diseases.[4-7] Literature shows that goat milk can reduce the anxiety-like behaviour and also helps in brain development along with its traditional use to manage anxiety.[8,9] Additionally, goat milk has a high concentration of bioactive peptides derived from the proteins α-CN-S1, α-CN-S2 (CSN1S2), β-casein (CSN2) and κ-casein (CSN3)[10] and casein account for most of the protein in milk approximately 80% of the total protein content that may have multiple functions.[10] In the present study the most prominent protein fraction of goat milk CSN1S2 has been considered to explore its antianxiety potentiality. As it is well known that anxiety is mediated by imbalance of brain neurotransmitters mainly GABA and serotonin which remain the main targets for most of the anxiety agents to modulate and give relief from anxiety.[11] It was found that goat milk has high levels of GABA (gamma-aminobutyric acid) and CSN1S2 is a protein which may be a source for its synthesis.[12] Revolutionized research on nanogels proved them with high drug loading capacity, biocompatibility along with high permeability with

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their intranasal application for neurological therapy.[13,14] Casein nanoformulations are also scientifically proven to cross the blood-brain barrier with potent neurotherapy.[15] Considering the medicinal importance of goat milk and nanogels in neurotherapy, the present research is attempted to screen the anxiolytic activity of CSN1S2 nanogels along with the estimation of its effect on the brain bioamine concentrations mainly GABA and serotonin.

MATERIALS AND METHODS

Isolation of CSN1S2

From 250 mL goat milk purchased from a local breeder of Greater Noida, CSN1S2 protein was isolated. To 40°C, the temperature of milk was raised, and then glacial acetic acid was added drop wise (5 mL) while stirring until precipitates are formed. The caprine CSN1S2 protein was purified by standard method. The protein precipitation was then separated by filtration through a nylon mesh. The obtained protein was subjected to chemical tests for its chemical nature and then stored at -20°C till further chemical test to confirm its pharmacological studies.[16]

Preparation of Nanogel of CSN1S2

The casein nanogel was prepared by following emulsion-solvent diffusion method. As per the considered dose from previous research[16] (500 and 750 mg/Kg), CSN1S2 was weighed accurately and so ethyl cellulose was dissolved in dichloromethane. Whereas Carbopol 934 was used to prepare the aqueous phase by stirring and heating continuously with addition of tween 80. Further, the phase with drug was added into the aqueous phase with homogenization for emulsification. The nanogel was obtained by adding triethanolamine to the gel to maintain the pH.[17-19]

Analysis of Nanogels

Physical Appearance: For the appearance, presence of aggregates, colour, settling period and homogeneity of the nanogel formulation were detected. They were also subjected to examine any sign of aggregation and settling period in a container.[17-20]

pH assessment: The pH of the CSN1S2 nanogel was checked with Digital pH meter (Syntronics, India) by immersing the glass electrode inside the aqueous nanogel. It was carefully monitored to prevent any irritation to the nasal mucosa.[17-20]

Spreadability: To know the extent of surface area the CNN1S2 nanogel spreads on its application to the skin, between two horizontal planes the gel is placed and a 200 g (M) standard weight is applied to the top plate. After 1 min (T) the spreading diameter (L) was measured.[20] The spreadability (S) was measured following the formula:

$$S=M*L/T$$

Viscosity: The viscosity of the nanogel formulation was measured using Brookfield type rotating viscometer. The cone from the holding rod was land in the middle of the nanogels.

Particle size: By following proton correlation spectroscopy, the particle size of the nanogel was recorded using a Zeta sizer (Malvern aster sizer 2000 MS). In distilled water, 1 mL nanogel was diluted and still get a clear solution. The zeta potential and droplet size were determined to get idea about its absorption rate.[17-20]

Experimental animals

In the present study, swiss albino mice of weight 25-30 g and of either sex was preferred for the pharmacological screening procedures. The animals were availed from Noida Institute of Engineering and Technology (Pharmacy Institute), Greater Noida after approval from the Institutional Animals Ethics Committee (IAEC/NIET/2020/01/08) of the Institute; Registration No: 1845/Re/S/16/CPCSEA. They were kept in polypropylene cages under standard animal house conditions like temperature (27±1°C), humidity (55-65%) with adequate amount of palate diet and drinking water.

Gross behavioural study

The gross behavioural alteration in mice on CSN1S2 nanogel treatment was assessed to estimate its impact on Central Nervous System (CNS) of the animals. The whole behavioural studies are done after appropriate training for estimated duration and to avoid any external factors to cause anxiety or antianxiety effect constant noise free and standard animal housing and careful handling. The parameters under consideration were reflexes like righting, pinna, corneal reflexes. Additionally, traction tests, muscle tone, awareness and stereotyped behaviour were monitored to obtain extensive insight of behavioural changes in mice by the formulation at different intervals.[21,22]

Assessment of anxiolytic activity

Elevated Plus Maze (EPM) apparatus, Open Field Test (OFT) and Light-Dark Tests (LDT) were employed for examining anxiolytic activity of CSN1S2 nanogels. The mice were segregated into four groups, six in each. Groups 1, 2, 3 and 4 were employed with normal saline (0.9%; intranasal route), diazepam (1 mg/kg; intraperitoneal), CSN1S2 nanogels 500 and 750 mg/kg intranasal respectively.

Elevated Plus Maze (EPM) test

For anxiolytic property of the formulation, standard elevated plus maze apparatus was utilized. The animals were administered with saline, diazepam (1 mg/kg) and CSN1S2 protein (500 and 750 mg/kg) once daily for 7 days before the experiment. In this test, the mice were placed gently in the central square facing one of the open arms. The number of entries and average time spent in both arms was recorded for duration of 5 min.[22]

Open Field Test (OFT)

A standard open field test apparatus was employed for this study. Following the administration of diazepam, saline, CSN1S2 nanogels at the same sequence, route and dose once daily for 7 days, the mice were kept gently on one of the squares at the corner facing towards the open side. The parameters observed for 5 min were the number of rearing, time spent in the central square and the number of squares crossed.[23]

Light and Dark Test (LDT)

The light and dark test apparatus comprised of a wooden box, open from the top. Two separate chambers were utilized: a white and a black. After 7 days of routine treatment, the mice were independently positioned in the centre of the light box, and their time spent in the light area, number of admissions into the bright chamber, and instances of rearing in both the bright and dark chambers were recorded during a duration of 5 min.[24]

Estimation of Neurotransmitters in mice brain

Estimation of GABA

After development of anxiety by EPM, 20 mice were utilised to evaluate the effect of CSN1S2 nanogels on the GABA level in the brain. Tissue specimens of brain were collected and homogenized in 0.01 M HCl. Glutamic acid (0.05 M) in sodium sulphate (0.2 M) buffer at pH 6.4, ninhydrin (14 mM) in sodium bicarbonate (0.05 M) buffer at pH 9.9-10 and copper tartare reagent. All dissolved together in 1 L of distilled water. 0.25 mL of homogenate was diluted with hydrochloric acid (0.01 M) and trichloroacetic acid (10%). The proteins were collected from the final homogenate. After next homogenisation, 100 µL supernatant was combined with glutamic acid and ninhydrin solution. Final mixture was incubated at 60°C for 30 min and cooled before adding copper tartrate reagent. By using fluorometric technique, the GABA concentration was quantified at wavelength 380-460 nm.[25]

Estimation of Serotonin

As in the previous GABA estimation the mice were administered with respective standards and CSN1S2 nanogel and anxiety was introduced by EPM. Brian of the mice were dissected in a similar procedure. The tissues of brain were homogenized with HCl (0.1N) at 0°C and then incubated for 10 min. It was centrifuged at 14000 rpm for 15 min at 0°C. Into the transparent supernatant solution, zinc sulphate (10%) and sodium hydroxide (1N) were incorporated. The tubes were again agitated for 5 min and then centrifuged for 20 min at 2500 rpm. The serotonin content in supernatant with 2N HCl was estimated from degree of fluorescence by a fluorometer at an instrument sensitivity of 0.3 at the wavelength [excitation (nm) / fluorescence (nm)] 290/550, with slit openings of 5/3 (Excitation/Emission).[26]

Analysis of obtained data

All the results were presented as mean±SEM and the data were analyzed using One-Way Analysis of Variance (ANOVA) accompanied by Dunnett's "t" test. A p value of $p<0.05$ was considered as the level of significance. Whereas $p<0.001$ was considered as highest significance.

RESULTS

Physical Appearance: The formulated nanogels were observed to be clear, and transparent with good consistency. It showed a with the polymer for proper application.

pH: the pH of the nanogels was observed to be between 5.5 to 7.0 which is perfect for their intranasal administration as its measured pH is near to the pH of the nasal mucosa. The formulated nanogels will be non-irritant to the nasal mucosa with easy application.

Viscosity: The nanogel formulation was introduced into the Brookfield viscometer and the viscocity was observed to be within the standard limit. The observed viscosity of the formulated nanogel was 4458±31.7 cp.

Spreadability: The spreadability of the nanogels was found to be in a range of 8.5 gcm/s to 10.3 g cm/s which was ideal for their best apply intranasally. The observed spreadability of the nanogel was 8.96±0.09 g cm/s.

Gross behavioral study

In this test, the CSN1S2 nanogels exhibited less effect on reflexes of mice as they were seen to display alertness. CSN1S2 nanogels had comparative lesser, but impact on catalepsy and muscle relaxant properties in mice, indicating their anxiolytic activity when compared to the conventional medication diazepam. The CSN1S2 nanogels were observed to enhance stereotypical behaviors, including a rise in the rearing, sniffing, and grooming actions. The CSN1S2 protein, as demonstrated in the current behavioral investigation, is clearly helpful in reducing anxiety.

Effect of CSN1S2 nanogels on EPM

The mice of normal control group with saline exhibited more time in the closed arm and showed less entries into the open arm of the maze during a duration of 5 min. Whereas, mice treated with diazepam performed a significant ($p<0.001$) rise in both the time spent and entry frequency in the open arm. Significant rise ($p<0.05$ and $p<0.001$) in duration and frequency of entries in the open arm were noticed by CSN1S2 nanogels (500 and 750 mg/kg respectively); however, the 750 mg/kg dose demonstrated superior anxiolytic efficacy compared to the 500 mg/kg dose (Table 1).

Effect of CSN1S2 nanogels on Open Field Test (OFT)

The animals of control group were noticed to remain in the central arm for maximum time during this test. Whereas mice in standard group and CSN1S2 nanogels (500 and 750 mg/kg) treated mice exhibited a noticeable rise in the number of squares crossed, frequency of rearing and duration of stay in the middle square in 5-min periods when compared to the saline-administered control group. On the other hand, the test CSN1S2 nanogels showed more significant ($p<0.001$) results at 750 mg/kg than that of the animals treated with 500 mg/kg of CSN1S2 nanogel ($p<0.05$) (Table 2).

Effect of CSN1S2 nanogels on light and dark test

The control group animals were found to remain in the light chamber maximum time to avoid anxiety in this test. On the other hand, case of the animals treated with CSN1S2 nanogels (500 and 750 mg/kg) increased the frequency of entries into the dark chamber with more time spent in the dark chamber and rearing (Table 3) reflecting its dose-dependent inhibition of anxiety behaviour which was well compared to that of the standard drug diazepam. However, the anxiolytic activity of the nanogels at higher dose (750 mg/kg) was observed to be more significant ($p<0.001$) on comparison of the activity of mice to the control group (18.833±0.30).

Effect of CSN1S2 on GABA levels

In this study, biochemical tests inferred the reduced brain GABA level in control group animals than that of the normal GABA level due to anxiety. In the nanogel administered groups, a significant ($p<0.001$) raised GABA level in the brain tissues of the animals demonstrated, which was found to be approaching normal brain levels. The CSN1S2 nanogel at a dosage of 750 mg/kg demonstrated a significant rise in GABA concentration relative to the control group. However, CSN1S2 nanogel at dose 500 mg/kg was also observed to increase the GABA level (mean±S.E.) but not as much as the higher dose as shown in Figure 1.

Effect of CSN1S2 on Serotonin levels

The current investigation revealed an elevation in brain serotonin levels within the control group (mean±S.E.), above the typical concentration of serotonin in brain tissue (108 µg/gm).[22] The brains of mice, treated with both doses of nanogels of CSN1S2 (mean±S.E. and mean±S.E. respectively) exhibited a reduction in the concentration of serotonin significantly ($p<0.001$) when placed in comparison with the control group animals. However, CSN1S2 nanogel at the dose of 750 mg/kg has having more effective inhibitory effect on brain serotonin levels as shown in Figure 2. The potentiality of the CSN1S2 to reduce the brain serotonin level was observed to be well comparable to that of diazepam (mean±S.E).

DISCUSSION

In the present research, the nanogels of CSN1S2 are formulated targeting their compatibility and easy permeability into the nasal mucosa for better bioavailability of CSN1S2 to the central nervous system. The formulations also passed all the evaluations

Table 1: Anxiolytic activity of CSN1S2 in elevated plus maze test.

Treatment Group	Number of entries		Average time spent	
	Close arms	Open arms	Close arms	Open arms
Saline	4.16±0.30	2.16±0.16	268.33±2.17	17.33±0.33
Diazepam	9.17±0.30*	7.00±0.36**	195.83±2.82**	91.67±3.904*
CSN1S2 nanogel 500 mg/kg	5.83±0.307**	3.17±0.30**	229.83±2.857**	53.83±1.641**
CSN1S2 nanogel 750 mg/kg	7.66±0.33**	6.50±0.22*	205.50±2.24**	78.83±2.03*

The observations are shown in Mean±SEM, $n=6$ mice in each group. Following Dunnett's multiple comparison test, the statistical analysis the statistical analysis in represented with p value=$p<0.05$*, $p<0.001$** comparing with the control group observations.

Table 2: Anxiolytic activity of CSN1S2 nanogel in Open field test.

Treatment Group	Total no. of squares crossed	Time spent in central square	No. of rearings in central square
Saline	53.16±1.424	12.00±0.577	1.50±0.223
Diazepam	34.33±1.229**	149.00±2.250**	7.833±0.307*
CSN1S2 nanogel 500 mg/kg	53.50±1.176*	88.166±1.815*	2.50±0.223**
CSN1S2 nanogel 750 mg	37.66±1.68**	139.50±6.25**	4.50±0.22*

The observations are shown in Mean±SEM, $n=6$ mice in each group. Following Dunnett's multiple comparison test, the statistical analysis the statistical analysis in represented with p value=$p<0.05$*, $p<0.001$** comparing with the control group observations.

Table 3: Anxiolytic activity of CSN1S2 in light and dark test.

Treatment Group	Time spent in light chamber	No. of entries in dark chamber	No. of rearing in bright	No. of rearing in dark chamber
Saline	14.00±0.36	2.33±0.21	1.33±0.21	11.66±0.421
Diazepam	30.83±0.47**	3.66±0.21*	6.166±0.30*	21.166±0.60*
CSN1S2 nanogel 500 mg/Kg	25.00±0.36**	2.00±0.25**	3.83±0.30**	14.16±0.47*
CSN1S2 nanogel 750 mg/Kg	27.83±0.30*	2.66±0.21*	5.33±0.33*	18.833±0.30**

The observations are shown in Mean±SEM, n=6 mice in each group. Following Dunnett's multiple comparison test, the statistical analysis the statistical analysis in represented with p value=p<0.05*, p<0.001** comparing with the control group observations.

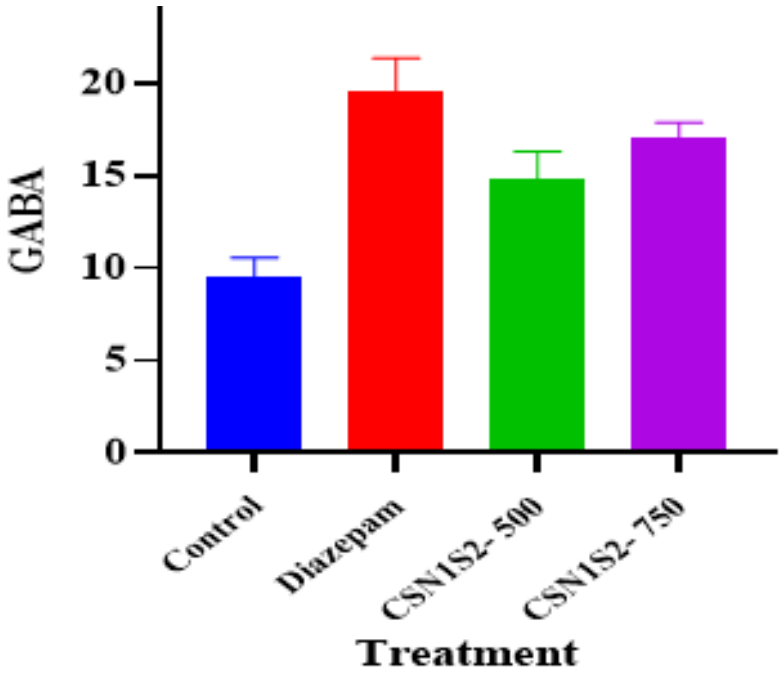

Figure 1: Estimation of the effect of CSN1S2 nanogel on brain GABA concentration. The observations are presented in Mean±S.E.M; (n=6). The observations were obtained comparinfg with the control group. To assess the distinct from the control Dunnett's test was used, considering p<0.001 as the highest significance. The observed values in CSN1S2 nanogel administered groups reflected equivalent values to that of the control group.

for their comfortable appropriate application. The pH, viscosity, and spreadability of the nanogels affect the absorption and residence time of the across the nasal mucosa. The spreadability range of nanogel for intranasal administration is 8.5 gcm/s to 10.3 gcm/s and observed spreadability of the nanogel was 8.96±0.09 gcm/s. pH range for suitable intranasal gel should be 5.5 to 7.0 and the observed pH of the nanogel was 6.8. At the same time the viscosity of prepared nanogel was 4458±31.7 cp. All these observations support the comfortable and easy application of the intranasal nanogels of CSN2S2 without any irritation.[14,15]

The current work showed that CSN1S2 nanogels given to mice via the intranasal route had a substantial anxiolytic effect in 3 standard anxiety animal models: LDT, EPM and OFT.[27-29] Anxious behaviour in mice was considerably reduced in animal studies, indicating that anxiety in mice was reduced following administration with the CSN1S2 nanogels.

The outcomes of this study on the EPM after treatment with goat milk extracted CSN1S2 nanogels revealed anxiolytic activity, as there was significant attenuation of anxiety-like behaviour (increased time spent in open arm and number of entries) in EPM, which is one of the most representative indices of anxiolytic activity.[30] The overall arm entries measure variation in the observed activities, on the other hand the duration of stay in the middle square reflects its decision making and/or risk assumptions.[31]

In the open field test, the observations retrieved from the tests supported the anxiolytic potentiality of nanogels of CSN1S2 proteins of goat milk, due to raised duration of stay at the middle square, frequency of rearing, and number of squares crossed, as compared to the control group, indicating its anxiolytic effect. The anxiolytic effects of CSN1S2 in the EPM and OFT was observed

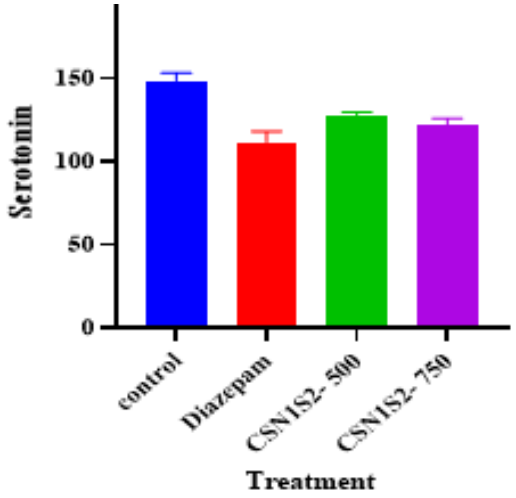

Figure 2: Estimation of the effect of CSN1S2 nanogels on brain serotonin concentration. The observations are presented in Mean±S.E.M; (*n*=6). The observations were obtained comparinfg with the control group. To assess the distinct from the control Dunnett's test was used, considering $p<0.001$ as the highest significance. The observed values in CSN1S2 nanogel administered groups reflected equivalent values to that of the standard group.

to be well comparable, but less than the standard treatments like diazepam.

In the light/dark test, the anxiolytic effect was also observed. Despite the transition parameter being strongly reliant on locomotor activity, frequency of transitions between the bright and dark chamber, duration of stay spent in the bright chamber as well as frequency of rearing in the bright chamber are recognised as anxiety indices in this test.[31] Duration of stay in the bright chamber was raised in nanogel administered mice, as were the number of entries and rearings, indicating anxiolytic activity.

The test of brain monoamine level, reduction in serotonin and rise in GABA levels were observed comparing to that of the control group that indicates the anxiolytic potentiality of the nanogels of CSN1S2 protein of goat milk. The effect of the nanogels was in a similar dose dependent manner in relation to the anxiolytic activity observed in this study. CSN1S2 is a protein obtained from goat milk which is also playing vital role in the enhanced synthesis of GABA which is a peptide neurotransmitter. Additionally, the inhibitory effect of GABA on serotonergic in brain is due to the firing on serotonergic neurones simultaneous decreased utilization of serotonin utilization. As in the study it was observed to increase GABA level in brain, it is reducing the serotonin level simultaneously.[32]

CONCLUSION

It can be concluded from the observations that goat milk CSN1S2 protein certainly possesses anxiolytic potentiality at 500 and 750 mg/kg in a dose dependent manner as the results were more significant at the dose 750 mg/kg. This can be a basis for further development of functional foods along with the novel nanogel formulation having anxiolytic activity with other health benefits. This shows that goat milk protein CSN1S2 has many beneficial effects on brain activity and can help in the management of anxiety disorders. For better administration by intranasal routes, the CSN1S2 nanogels can bring an innovative and revolutionary platform to get targeted neurological therapy as in the present study of the anxiolytic activity of CSN1S2. At both dosages (500 and 750 mg/kg), the CSN1S2 protein undoubtedly exhibits anxiolytic property; however, the effects were more pronounced at the latter level. Preclinical efficacy testing of the nanogels were performed in the present study, in future it can be extended to clinical trial to assess its efficacy and safety in human volunteers. Along with the new nanogel formulation that has anxiolytic activity and other health benefits, his could serve as a foundation for the future creation of functional foods.

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ABBREVIATIONS

CSN1S2: α-CN-S1, α-CN-S2; **CSN2:** β-casein; **CSN3:** κ-casein; **CNS:** Central Nervous System; **LDT:** Light-Dark Tests; **EPM:** Elevated Plus Maze; **OFT:** Open Field Test; **M:** Molarity; **mM:** Milimolar; **HCl:** Hydrochloric acid; **GABA:** Gamma Amino Butyric Acid; **mg/kg:** Miligram/Kilogram; **gcm/s:** *Grams centimeter per second;* **cp:** *Centipoise;* ***ANOVA:*** One-Way Analysis of Variance; **SEM:** Standard Error of the Mean.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

In the present research, the animal study was approved by the Institutional Animals Ethics Committee as mentioned under the section on experimental animals and it is not applicable for any other ethical approval as no clinical study was conducted.

SUMMARY

The targeted disease of the present study is anxiety which is the mostly occuring disorder of present modern era where all age groups are suffering from it. From literature survey it was observed the benefits of the proteins of goat milk for the management of anxiety and it has also been noted that nanogels are considered as the best delivery system to CNS. Based on all these findings, an effort has been made to evaluate the antianxiety activity of the nanogels of the most prevalent protein fraction of goat milk; CSN1S2. After isolating it from goat milk, its nanogels were formulated by emulsification diffusion method and subjected to evaluate physical characters like physical appearance, particle size, viscosity, spreadability, Zeta potential, particle size, Zeta potential, along with *in vitro* diffusion profile. The nanogels were screened for antianxiety activity by using Elevated Plus Maze test (EPM), Open Field Test (OFT) and Light and Dark Test (LDT) in mice. To support the observed antianxiety effect of nanogels of CSN1S2, the effect on the brain level of monoamine neurotransmitters like GABA and serotonin was also estimated using flame fluorimeter. The whole study was analyzed and concluded that the nanogels of CSN1S2 has significant (p<0.001) anxiolytic activity in a dose dependent manner. An increase in the brain GABA and decrease in brain serotonin concentration supported the anxiolytic activity of CSN1S2 protein. The research results are with a broad prospective of a better treatment of anxiety.

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