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REGULATORY PERSPECTIVE FOR ANIMAL EXPERIMENTATION DURING PRECLINICAL DRUG DEVELOPMENT OF ANXIOLYTIC

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ABSTRACT: The study focusses on the preclinical development of anxiolytic herbal drug Gotu Kola, which is used as an anxiolytic. The drug's anxiolytic activity was tested on wrister albino rats, following module 4 of the CTD. Animal experiments were conducted, including open field tests, elevated plus maze tests, and light and dark apparatus tests. Dopamine and acetylcholine esterase estimation were performed. Results showed that a single dose of Gotu Kola and a combined dose of caffeine and Gotu Kola alleviate anxiety. Similar effects were observed in the elevated plus maze test and open field test. Mandhukhpqrni and it's combination with caffeine effects in open field test. Combined administration increases the acetylcholine esterase and dopamine level in brain. The study adheres to regulatory authorities' rules and regulations.

***KEYWORDS*:** Gotu kola, Preclinical study, CTD.

INTRODUCTION

Experiments in vivo come after this in vitro work. Pharmacodynamics and pharmacokinetics (absorption, distribution, metabolism, and excretion) are also included in Good Laboratory Practices (GLP).[1] safety, toxicity, dosage, and efficacy studies. Each component of GLP is curated in accordance with regulatory compliances and safety topics from the International Conference on Harmonization.[2]The pre-clinical stage is crucial as it is the first safety valve before in vitro testing and helps in determining the mechanism of action for the Lead compounds.[3]The aim should be to ensure sufficient safety and efficiency – which is a prerequisite for regulatory authorities to approve the progression to the clinical phase of a new drug.[4] The schedule for the pre-clinical research in relation to clinical trial must be decided upon by taking into consideration the characteristics of the new drug, the disease which it is intended for, the duration and exposure of the clinical trial subject to the drug and the route of administration.[5]

Anti-anxiety Medications:

Anti-anxiety medications, often known as anxiolytics, are prescribed to treat and reduce anxiety caused by a variety of anxiety disorders. These medicines can become habit-forming and have a speedy onset of action. As a result, they are usually prescribed only for short-term use. For those who have previous histories of substance abuse or addiction, they are not advised. Gotu kola was utilised in the traditional Ayurvedic medicinal system to treat mental illness, anxiety, sadness, memory loss, and sleeplessness. Anxiolytics are nootropic supplements that are anti-anxiety or anxiety-preventing prescription medications. The non-clinical research papers for Module 4 on anxiolytics therefore employ the drugs mandhukhparni or gotu kola.

Gotu Kola

Centella asiatica (L.) Urb. (Apiaceae), also known as gotu kola, pennywart and pegaga in Malaysia, is a perennial with kidney shaped-leaves commonly found and cultivated in Asian countries [6]. Ayurveda and traditional Chinese medicines have relied on it for thousands of years to cure everything from skin diseases to memory loss. The plant's antipyretic, ant seizure, antidepressant, wound-healing, anti-inflammatory, antioxidant, and neuroprotective properties are just a few examples of the various ways it has been studied for its therapeutic potential [7].Its active components, which include pentacyclic triterpenes like madecassic acid, asiaticoside, and madecassoside, are responsible for a significant portion of its pharmacological actions. [8,9]**.** The versatility of its therapeutic functions, especially for the treatment of mental disorders, has sparked an interest in identifying agents that target various mechanism and pathways for the improvement of cognitive functions and performance **[**[10,11]**]**.

A typical drug development process from herbal medicine broadly includes the following aspects:

1. Isolation of bioactive ingredients and synthetic modifications of them.
2. Evaluation of safety and efficacy.
3. Regulatory approval of the therapeutic agent in case of a new drug.
4. Clinical Trials. The drug should be subjected to drug standardisation followed by biological activity, preclinical studies, safety studies, etc.
5. The standardisation protocols of various single and compound formulations are appended at appropriate places.

MATERIAL AND METHODS

Animals

Subjects Adult male Sprague Dawley rats (200-250 g) were procured from the National Institute of Nutrition, Hyderabad, India. They were housed in four per cage (640 × 410 × 250 mm height), under controlled conditions (22 ± 1 °C and 12 h light/dark cycles, light on at 7:00 AM) with free access to food and water. All experimental procedures were approved by the Institutional Animal Ethical Committee and executed in strict compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

Drugs

1. Centella Asiatica - Procured Online - was diluted with saline to the appropriate concentration (20% w/v).

2. Caffeine – Sigma Aldrich - dissolved in sterile saline solution

Route of administration

During the study, the drugs was administered by per oral route. Centella Asiatica (100, 250 and 500 mg/kg), Caffeine (1, 2.5, 5 mg/kg). All drugs were dissolved in sterile water when prepared for administration in animals.

Open Field Test[12]

The open field apparatus had walls measuring 60 cm and a size of 60 x 60 cm, built out of an acrylic box. With the exception of the white floor, the entire construction was painted black. The lines created sixteen squares (15 x 15 cm) that were uniformly distributed throughout the floor. The apparatus's four central squares made up the central portion. Each animal was positioned at the bottom right corner of the test equipment in the novel test scenario, and a video camera that was 100 cm from the arena was used to record the test for five minutes. The arena was cleaned with cotton soaked in 70% alcohol at each interval between the experiment stages.

1. Locomotion: The number of lines crossed by the rat over 10 min interval
2. Rearing: The frequency of rats standing on their hind limbs
3. Self-grooming: rapid cleaning movements of the forelegs towards the face and/or the body both complete and no complete grooming interrupted at some point along the body was counted together. (A full grooming session usually begins with the rat scratching its face, progresses down its body, and ends at the tip of its tail.)
4. Central latency, the time delays to enter the central part of the apparatus. The rat received a point for entering the central section when it positioned its head and two front paws in the appropriate spot.

Elevated Plus Maze Test [13]

The rats were brought to the testing room and allowed to acclimate for a period of at least 30 minutes before the start of the experiment. The room was maintained at a controlled temperature and lighting conditions. Each rat was gently placed in the center of the elevated plus maze apparatus, which consisted of two open arms and two closed arms elevated above the floor. The arms were arranged in a plus-shaped configuration, and the maze was made of black Plexiglas. The experimenter started the timer and observed the rat's behavior for a predetermined period, usually around 5 minutes. During this time, the rat's movements and choices were recorded using a video camera or manually by an observer. The rat's behavior in the maze was analyzed based on specific parameters, including the time spent in the open and closed arms, the number of entries into each arm, and the frequency of stretched attend postures (exploratory behaviors). At the end of each trial, the rat was gently removed from the maze and returned to its home cage. The maze was thoroughly cleaned with a suitable disinfectant to ensure that any residual olfactory cues were removed. The entire procedure was repeated for each rat in the study group, with appropriate intervals between trials to minimize any potential carry-over effects. The collected data were then

statistically analysed to evaluate the rat's anxiety-like behavior, with a focus on the preference for the closed arms (indicative of anxiety) or open arms (indicative of reduced anxiety).

Light- Dark Apparatus Test [14]

The test apparatus consisted of two chambers connected by a small opening: a brightly illuminated "light" chamber and a dimly lit "dark" chamber. The light chamber was brightly lit, while the dark chamber provided a sheltered environment. Each rat was gently placed in the light chamber facing away from the opening. The experimenter started the timer and observed the rat's behaviour for a predetermined period, typically around 5 minutes. During the test, the rat's movements and choices were recorded. The behaviour of interest included the time spent in the light chamber, the number of transitions between the light and dark chambers, and the latency to enter the dark chamber for the first time. At the end of each trial, the rat was removed from the apparatus and returned to its home cage. The test apparatus was thoroughly cleaned between trials to eliminate any olfactory cues. The entire procedure was repeated for each rat in the study group, with suitable intervals between trials to avoid any potential carry-over effects. The collected data were then analysed statistically to assess the rat's anxiety-like behaviour, focusing on measures such as the preference for the dark chamber (indicative of reduced anxiety) or the latency to enter the dark chamber (indicative of increased anxiety).

Dopamine Estimation [15]

Preparation of standard solution

A 1000 µg/ml standard solution of Dopamine Hydrochloride (DPH) was prepared by dissolving 0.1 grams of DPH in 100 ml of distilled water. To generate a standard curve, a series of standard solutions with concentrations ranging from 0.05 to 7.5 µg/ml were prepared. To prepare the stock solution of 1.5x10-2 M potassium ferricyanide, 1.2347 grams of potassium ferricyanide were dissolved in 250 ml of distilled water in a standard flask. 1.0137 grams of ferric chloride were dissolved in 250 ml of distilled water.

Procedure

The dopamine level in rat brain was estimated by admixing of homogenized supernatant liquid (1 ml) with 1 ml of ferric chloride (1.5x10-2) and 1 ml of potassium ferricyanide (1.5x10-2) in 25 ml distilled water. It was kept aside for 30 min. The developed color was estimated using the UV-Visible spectrometer at 735 nm. Blank 1 ml ferric chloride + 1 ml potassium ferric chloride in 25 ml distilled water. Kept aside for 30min. Take above solution absorbance at 735 nm**.**

Acetylcholine Esterase Estimation: [16]

Preparation of Solutions

0.1M Phosphate buffer was prepared using K2HPO4 and NaOH, PH 7. 39.6 mg of DTNB with NaHCO3 was dissolved in 0.1 M phosphate buffer (PH 7).21.67 mg of acetylthiocholine is dissolved in 1 ml of distilled water.

Procedure

In this experiment, rat brains are quickly dissected and homogenized in 0.1M Phosphate buffer (pH 8) after being placed in ice-cold saline. A 0.4 ml aliquot of the homogenate is mixed with phosphate buffer and DTNB in a cuvette. The mixture is thoroughly mixed by bubbling air, and the absorbance is measured at 412 nm as the basal reading. Subsequently, acetylthiocholine is added as a substrate, and the change in

absorbance is recorded over time. This allows for the determination of the change in absorbance per minute, providing valuable information about the enzymatic activity, particularly of acetylcholinesterase, in the brain tissue.

Statistical Analysis

The results obtained were assessed by ANNOVA test followed by Post hoc Bonferroni Test in the software Graph Pad Prism. The mean was expressed with SEM as the value of $P < 0.05$ was considered significant.

REGULATORY AUTHORITY IN INDIA

Regulatory Bodies

The main agencies responsible for enforcing these rules are the Central and State Drugs Standard Control Organisation. Established under the Directorate General of Health Services, MoHFW, of the Government of India, the CDSCO is the country's highest drug controller. The Drug Controller General of India (DCGI) is in charge of the CDSCO. The DCGI is the central licensing authority under the CT Rules, and it is in charge of granting permissions and licenses for clinical trials carried out in India.

State authorities for licensing

The state government appoints the State Licensing Authority (SLA) in line with the DCR to carry out the CT Rules' provisions, which include inspecting properties and confirming clinical trial sites' compliance.

Legal Framework

The Indian legal framework and regulation under which clinical trials and medical research have to be carried out mainly comprises of the following:

- Drugs and Cosmetic Act, 1940 and Drugs and Cosmetics Rules, 1945
- New drugs and Clinical Trial Rules, 2019
- National Ethical Guidelines for Biomedical and Health Research involving Human Participants, 2017
- Good Clinical Practice Guidelines for Clinical Research in India issued by the CDSCO
- Good Clinical Laboratory Practices.

Drugs and Cosmetics Act, 1940 and Drugs and Cosmetics Rules, 1945[17]

The DCA and its implementing regulations, the DCR, are the main laws that govern the pharmaceutical business in India. The DCA and DCR are designed to guarantee that consumers can obtain standard-quality medications and cosmetics. Regarding clinical trials, it is necessary to make sure that the DCA and DCR requirements are met throughout the manufacturing and supply chains of new pharmaceuticals and investigational new drugs, in addition to adhering to the CT Rules. The DCA and DCR will control the sale and distribution after a marketing authorisation has been obtained.

New Drug and Clinical Trial Rule 2019 [18]

This is the second schedule under Cosmetics Act, 1940 (D&C Act) called NDCT Rules there under deals with the preclinical study, before using the animals, the applicants required to take a permission from two committees

- Institute Animal Ethics Committee, IAEC.
- CPCSEA, the committee for the purpose of control and supervision of experiment in animal.

The review committee for gene manipulation, under Department of Biotechnology, under ministry of science and technology deals with the microorganism if the product is of biotechnology and if there is a problem using the microorganism in the environment, this committee is there to look after such issues.

The CT Rules under Rule 2(j) define clinical trial as follows: ―

Clinical trial in relation to a new drug or investigational new drug means any systematic study of such new drug or investigational new drug in human subjects to generate data for discovering or verifying its—clinical or; ii. Pharmacological including pharmacodynamics, pharmacokinetics or; iii. Adverse effects, with the objective of determining the safety, efficacy or tolerance of such new drug or investigational new drug.

Exemption from Applicability of CT Rules

The CT Rules is not applicable to academic clinical trials and biomedical and health research. An academic clinical trial is the clinical trial of a drug already approved for a certain claim and initiated by any investigator, academic or research institution for a new indication or new route of administration or new dose or new dosage form, where the results of such a trial are intended to be used only for academic research purposes and not for seeking approval of the CLA or regulatory authority of any country for marketing or commercial purposes.

Good Clinical Practice Guidelines Issued by CDSCO [19]

GCP is an ethical and scientific standard for conducting clinical trials. It aids with the design, conduct, recording, analysis and reporting of clinical trials. In India, the CDSCO has formulated the CDSCO-GCP which has been subsequently adopted by the Drugs Technical Advisory Board (DTAB‖) – the highest statutory decision making body for drug laws in India. The CDSCO-GCP is modelled on the Ethical Guidelines for Biomedical research on Human Subjects issued by the ICMR and various international standards such as ICH -GCP, WHO-GCP, USFDA-GCP etc Previously, CDSCO-GCP compliance was not backed by regulatory requirements and most pharmaceutical companies did not follow GCP principles. The CDSCO amended Schedule Y under the DCR in 2005 to make CDSCOGCP compliance mandatory. Subsequently, the CT Rules followed suit and also mandates compliance with CDSCOGCP. It should be followed for carrying out biomedical research in India at all stages of drug development. Compliance with internationally recognized standards is not sufficient to fulfil this requirement, biomedical research carried out in India will have to be in line with the India-specific requirements laid down in the CDSCO-GCP. The CDSCO-GCP primarily ensures protection of the trial subjects and the authenticity of data generated in clinical trials. The ethical principles contained in the CDSCO-GCP are in accordance with the principles laid down for research on human subjects in the Declaration of Helsinki developed by the World Medical Association. These cover principles of essentiality, confidentiality, informed consent, risk minimization etc. which are more or less along the same lines as various other international standards. The CDSCO-GCP provides general guidance for study design, recruitment of trial subjects, data handling and management and analysis of trial results. An Independent Ethics Committee is responsible for checking the suitability of the protocol, reviewing the methods and documents submitted by the sponsors with regards to subject recruitment and also check the authenticity of the informed consent documents signed by the trial subjects prior to participating in the trial.

RESULT

A. Mandukaparni and its combination with caffeine alleviates anxiety as seen in the Light-dark apparatus

Various doses of caffeine (1-5 mg/kg) and Mandukaparni (100-500 mg/kg) were administered orally to rats. The entries and time spent were analysed. As shown in Figure 1A and 2A, it was found that Mandukaparni in the dose of 500 mg/kg showed significant increase in both no. and time spent in the light chamber. Similarly, caffeine 5 mg/kg caused significant increase in the number and time spent in the light chamber. The subeffective doses which were not significant independently, showed significant effect when given in combination. One-way ANNOVA and post hoc Dunnett Test was used. $P<0.05$ was considered significant.

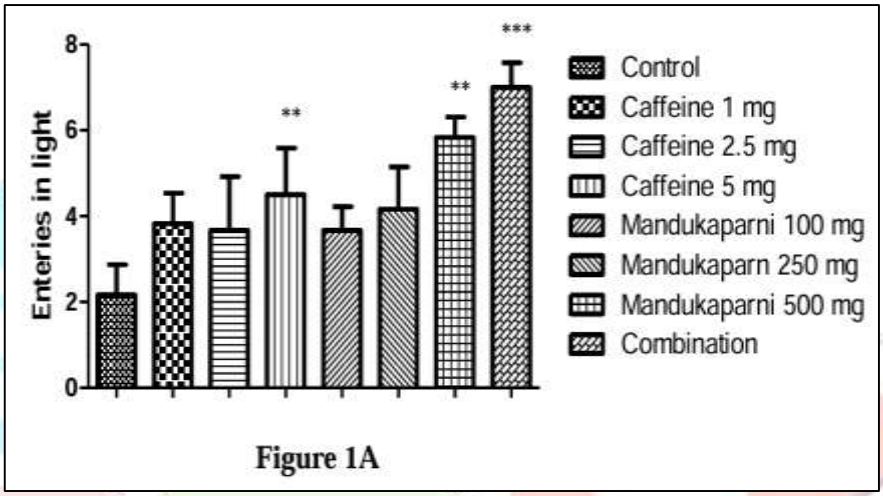

Figure 1A

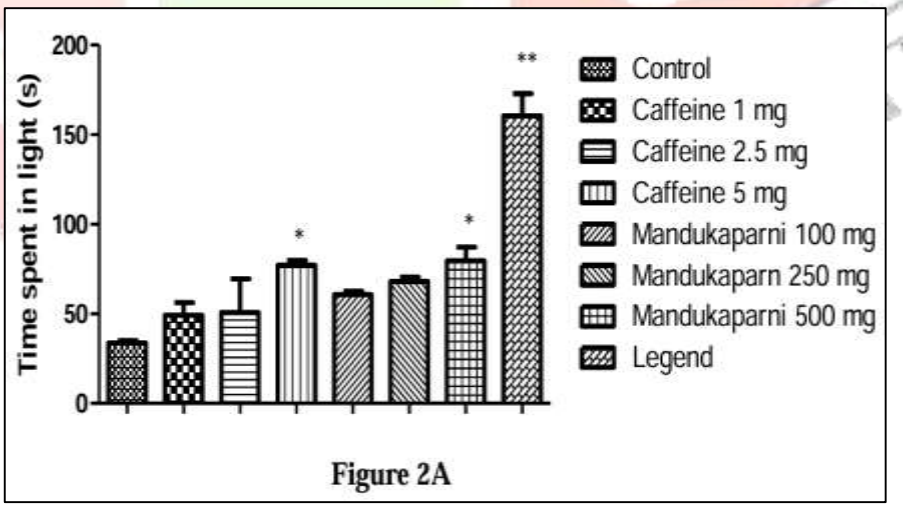

Figure 2A

Figure 1 A and 2A represents, effect of combination dose of Mandukparni and caffeine shows anxiolytic effect. Data were analyzed by Two way ANOVA followed by post hoc Dunnet test. $P<0.05$ was consider as statistically significant (*$P<0.05$;**$P<0.001$)

B. Mandukaparni and its combination with caffeine alleviates anxiety as seen in the Elevated Plus Maze apparatus

Various doses of caffeine (1-5 mg/kg) and Mandukaparni (100-500 mg/kg) were administered orally to rats. The entries and time spent were analysed. As shown in Figure 1B and 2B, it was found that Mandukaparni in the dose of 500 mg/kg showed significant increase in open arm entries and time spent in open. Similarly, caffeine 5 mg/kg caused significant increase time spent in light. the number and time spent

in the light chamber. The subeffective doses which were not significant independently, showed significant effect when given in combination. One-way ANNOVA and post hoc Dunnett Test was used. P< 0.05 was considered significant.

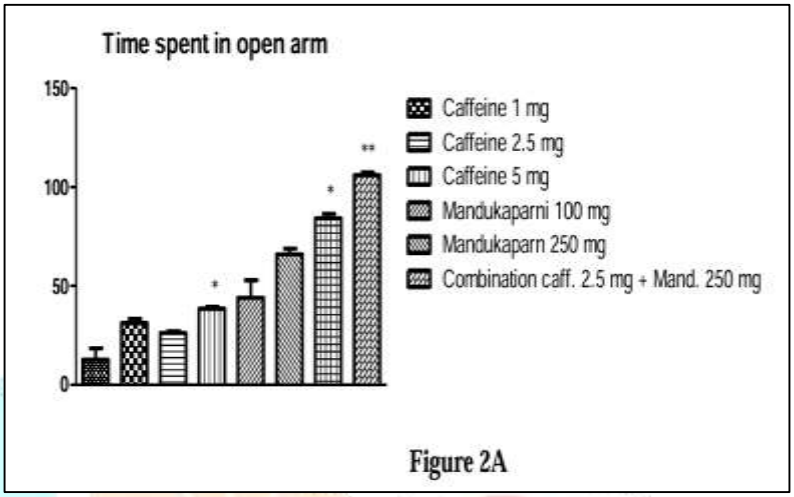

Figure 2A

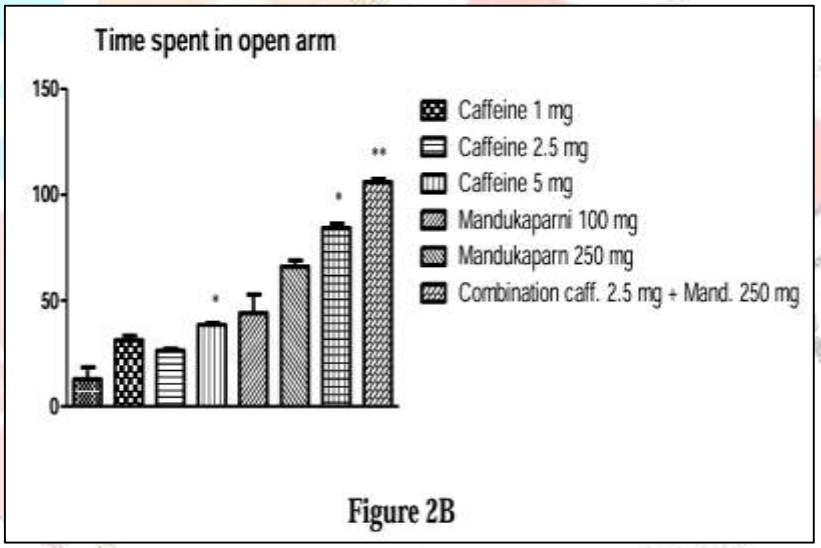

Figure 2B

Figure 2A and 2B represents, effect of combination dose of Mandukparni and caffeine shows anxiolytic effect. Data were analyzed by two way ANOVA followed by post hoc Dunnet test. (*P<0.05;**P<0.001)

C. Mandukaparni and its combination with caffeine – effect in open field test.

Various doses of caffeine (1-5 mg/kg) and Mandukaparni (100-500 mg/kg) were administered orally to rats. Ambulation, Grooming, Rearing was analysed in animals. As shown in Figure 3A, 3B, 3C it was found that Mandukaparni in the dose of 500 mg/kg showed significant increase in ambulation and time spent in central zone. Similarly, caffeine 5 mg/kg caused significant increase time spent in central zone. The sub effective doses which were not significant independently, showed significant effect when given in combination. This indicates the motor component in the anti-anxiety effect of the drugs. Considering the nature of caffeine, which is a CNS stimulant, this is quite possible. One-way ANNOVA and post hoc Dunnett Test was used. P< 0.05 was considered significant.

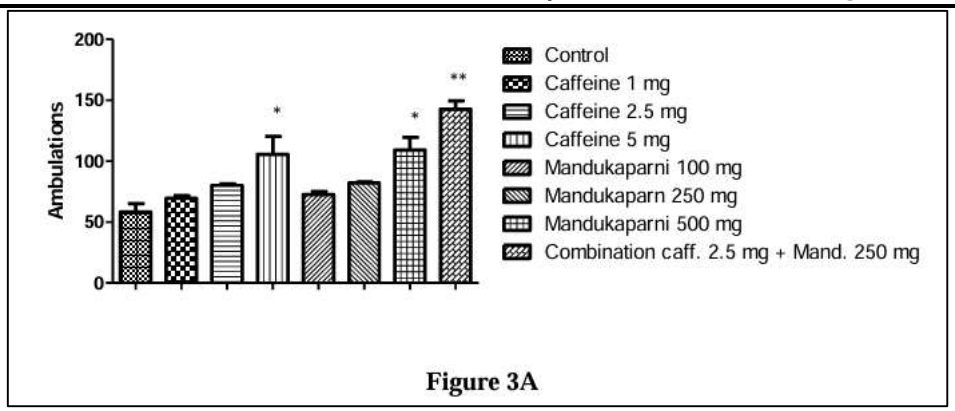

Figure 3A

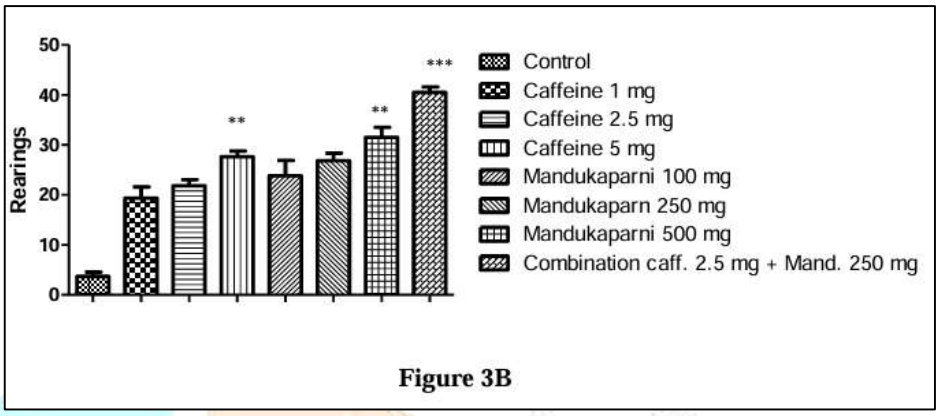

Figure 3B

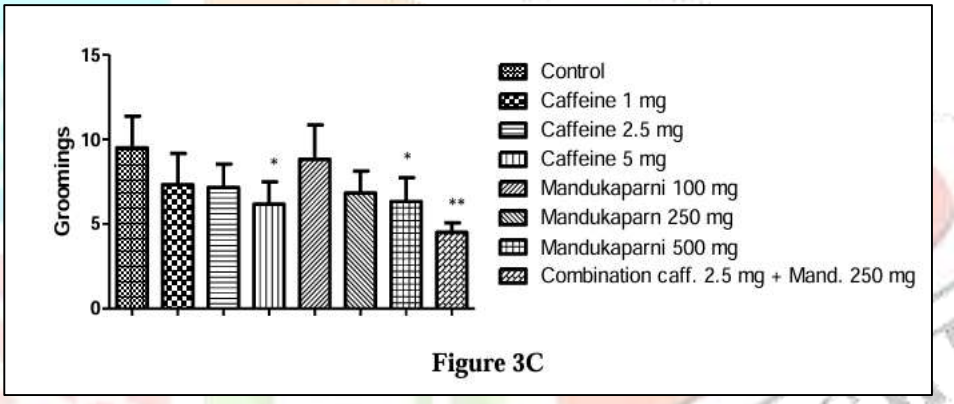

Figure 3C

Figure 3A, 3B and 3C represents, effect of combination dose of Mandukparni and caffeine shows anxiolytic effect. Data were analyzed by two way ANOVA followed by post hoc Dunnet test. P<0.05;**P<0.001)

D. Combined administration increases the Acetylcholine esterase and Dopamine levels in brain.

Brain isolation and further analysis to determine the acetylcholine esterase levels showed an increase when Mandukaparni 250 mg/kg and Caffeine 2.5 mg/kg were administered together. Student T test and Post hoc Dunnett test was used to determine the statistical significance. A value of $P < 0.05$ was considered significant. An increase in dopamine indicates more awareness, Increase in AchE is usually associated with decrease in acetylcholine, thereby pointing to better motor control.

Table no.1: Combined administration increases the Acetylcholine esterase and Dopamine levels in brain.

	Control	Control Mandukaparni 250 mg/kg	Caffeine 2.5 mg/kg	Combination
Acetylcholine esterase (umol/min/mg of tissue)	0.453 X 10^4	0.456 X 10^4	0.4549 X 10^4	0.852 X 10^4 ⇧
Dopamine (ug/ml)	0.450	0.302	0.449	0.489 ⇧

DISCUSSION

After thorough study of various documents, it was understood that for the Module 4 various preclinical studies are required. The objective of preclinical trial is to develop adequate data by testing the new drug on animal subjects and to reasonably decide that it is safe to proceed for human trials. The following studies are mandatory in a preclinical trial.

1. **Animal toxicology study:**

- Systemic toxicity studies
- Repeated-dose systemic toxicity studies
- Dose-ranging study
- Male fertility study
- Female reproduction and developmental toxicity study
  - ➢ Female fertility study
  - ➢ Teratogenicity study
  - ➢ Perinatal study
- Local toxicity
  - ➢ Dermal toxicity study
  - ➢ Photo-allergy or dermal
  - ➢ photo toxicity Vaginal toxicity test
  - ➢ Rectal tolerance test
  - ➢ Parenteral drugs
  - ➢ Ocular toxicity studies
  - ➢ Inhalation toxicity study
- Allergenicity or hypersensitivity
- Guinea pig maximization test
- Local lymph node assay
- Genotoxicity
- Carcinogenicity

2. Animal pharmacology:

- Specific pharmacological actions
- General pharmacological actions

➢ Cardiovascular system

➢ Respiratory systemFollow-up and supplemental safety pharmacology studies Follow-up safety pharmacology studies

- CVS, CNS, respiratory system studies

➢ Supplemental safety pharmacology

- Studies Urinary, Autonomic Nervous System

➢ (ANS), gastrointestinal and other organ system

Despite our best attempts, it was not possible to conduct all these studies for the said drug. We relied on the literature to decide about the toxicity of the drug, and conducted various behavioural studies to determine the effect of the drug. An additional biochemical estimation was performed too. Overall, the combination of Mandukaparni and Caffeine was found to be useful for managing anxiety.

CONCLUSION

From all the studies, we conclude that a full Module 4 of any new application requires extensive preclinical studies. These are listed in the discussion. We have performed all those, possible in our laboratory settings, indicating the anti-anxiety potential of the drug Gotu Kola also known as Mandukaparni. Its combination with caffeine will be definitely effective as CNS stimulant and anti anxiety in various required functions.

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