MATERIALS & METHODS
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Animals (Wistar rats) were obtained from the Central Animal House, BLDEU’s Sri BM Patil Medical College Hospital & Research Center, Bijapur, India, were used in the study. They were housed in quarantine room individually in polypropylene cages for one week of acclimation before the experiment started. The study were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) vide letter dated 01/06/2011(letter enclosed).

All rats were housed in polypropylene cages (30x22x14cm) and fed with commercial pellet rat (manufactured by VRK Nutritional solutions, Sangli, Maharashtra) chow and water and standard laboratory conditions are maintained with 12 :12 h light : dark cycle with a room temperature of 28±4°C.

The experiments were performed as per norms laid by Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA).

**Caralluma fimbriata extract (30% dry extract):**

The *Caralluma fimbriata* extract (30% dry extract) was donated by Digvijay Pharmaceuticals (I) Ltd, Thane (W), Maharashtra, India.
(i) Experimental Design:

A total of 150 animals (Wistar rats) were included in the study.

To evaluate effect of *Caralluma fimbriata* extract on appetite, body weight and lipid profile

a. Given as pre-treatment to rats fed cafeteria diet/hypercalorie diet.

90 rats weighing 180-240g of either sex were randomly divided into 3 groups (n=30 each):

i) Control

ii) Cafeteria diet (CD)

iii) Cafeteria diet + *Caralluma fimbriata* extract (CFE) group.

b. Given after induction of obesity by Cafeteria diet in rats.

60 rats were fed cafeteria diet to induce obesity (diet induced obesity) from the time of weaning and included in the study at the age of 19 weeks (149) and were randomly divided into:

iv) Obese and

v) Obese +CFE treated groups (n=30 each).

Rats in the control group were fed pellet chow, while rats in the cafeteria diet (CD), CD + CFE treatment, obese and obese + CFE treatment groups received both pellet chow and cafeteria diet. CFE was administered in the dose of 100 mg/kg bw/day p.o. for 50 days.
**Dose calculation:**

Kamalakannan et al, (2010) studied the antiobesogenic and antiatherosclerotic properties of CFE in Wistar rats. Wherein they used three doses 25, 50 & 100mg/kg bw/day. CFE showed dose-dependent appetite suppressant and antiobesogenic effects on rats fed cafeteria diet\(^{(147)}\). Hence, I selected the dose of 100mg/kg bw/day for the present study.

(ii) **Hypercalorie/Cafeteria diet\(^{(147,148)}\):**

It consisted of 3 variants;

i) condensed milk + bread + peanuts + pellet chow (4:1:4:1),

ii) chocolate + biscuits + dried coconut + pellet chow (3:2:4:1), and

iii) cheese + boiled potatoes + pellet chow (4:2:1).

The different variants were fed on alternate days throughout the treatment period.
4.2. METHODS

Phytochemical studies of Caralluma fimbriata extract:

a) Qualitative analysis:

Extract was qualitatively tested for the presence of biochemical constituents by Harborne & Kokate\(^{149,150}\) method.

Test for Alkaloids

Mayer’s test: 5 ml aqueous extract was added with 2 ml of 1% HCl. Mayer and Wagner’s reagent was then added to mixture. Turbidity of the resulting precipitate was taken as an evidence for the presence of alkaloid.

Test for Glycosides

To 1ml of extract, 1ml of Fehling’s solution was added and heated. Orange precipitate indicates the presence of glycosides.

Test for Flavonoids

Ferric chloride test: To 1 ml of aqueous extract, 1 ml of 10% lead acetate solution was added. The formation of a yellow precipitate was taken as a positive test for flavonoids.

Test for Tannins

Lead acetate test: About 2 ml of the aqueous extract was stirred with 2 ml of distilled water and few drops of FeCl₃ Solution were added. Formation of green precipitate was indication of presence of tannins.

Test for Sterols

Liebermann Burchard Reaction: A small amount of extract of sample and a few crystal of sodium nitrate were taken in a dry test tube and heated gently for a minute. It was cooled and 0.5 ml of concentrated sulphuric acid was added.
Test for Saponins

**Foam Test:** 5 ml of aqueous extract was shaken vigorously with 5 ml of distilled water in a test tube and warmed. The formation of stable foam was taken as an indication of the presence of saponins.

Test for Proteins

**Biuret Test:** Test solution was treated with 10% sodium hydroxide solution and two drops of 0.1% copper sulphate solution and observed for the formation of violet/pink color.

Test for Free Amino Acids

**Ninhydrin Test:** Test solution when boiled with 0.2% solution of Ninhydrin would result in the formation of purple color suggesting the presence of free amino acids.

Test for Carbohydrate

**Molisch's test:** Test solution was mixed with few drops of Molisch's reagent. This was followed by addition of 2 ml of concentrated sulphuric acid down the side of the test tube. The mixture was then allowed to stand for 2-3 minutes. Formation of red or dull violet colour at the interphase of the two layer to show a positive result for the presence of carbohydrate.

Test for reducing sugar

To few drops of the extract, 2ml of Fehling’s reagent & 3ml of water is added. Appearance of red orange indicates the presence of reducing sugar.

Test for quinones

To 1ml of extract, few drops of concentrated sulphuric acid was added along the sides of the test tube. Appearance of red colour indicates presence of quinones.
**Test for Phenolic Compounds**

Small amount of various extracts were taken separately in water and tested for the presence of phenolic compounds with dilute ferric chloride solution. Violet colour indicates the presence of phenolic compounds.

**Test for Fixed Oils and Fatty Acids**

Few drops of 0.5N alcoholic potassium hydroxide were added to small quantity of various extract along with a drop of Phenolphthalein. The mixture was heated on a water bath for 1-2 hrs. Formation of soaps or particle neutralization of alkali indicates the presence of fixed oils and fats.

**Test for Gums and Mucilage’s**

About 10ml of the extract was added to 25 ml of absolute alcohol with stirring and filtered. The precipitate was dried in air and examined for its swelling properties and for the presence of gum and mucilages.

b) **Quantitative analysis, presence of heavy metals and Microbiological tests:**

Quantitative analysis, presence of heavy metals and Microbiological tests were carried out and certified by Digvijay Pharmaceuticals (I) Ltd, Thane (W), Maharashtra, India. (Certificate of analysis is enclosed as Annexure 1).
**Toxicological studies:**

Toxicological assessment the safety of CFE evaluated by Odendaal AY et al (2013), included 2 in vitro genotoxicity assays\(^{151}\), a repeated dose oral toxicity study, and a developmental study in rats. No evidence of in vitro mutagenicity or clastogenicity surfaced in the in vitro studies at concentrations up to 5000 μg of extract/plate (Ames test) or 5000 μg of extract/mL (chromosomal aberration test). No deaths or treatment-related toxicity were seen in the 6-month chronic oral toxicity study in Sprague-Dawley rats conducted at 3 doses (100, 300, and 1000 mg/kg body weight (bw)/d). The no observed effect level for CFE in this study was considered to be 1000 mg/kg bw/d. A prenatal developmental toxicity study conducted at 3 doses (250, 500, and 1000 mg/kg bw/d) in female Sprague-Dawley rats resulted in no treatment-related external, visceral, or skeletal fetal abnormalities, and no treatment-related maternal or pregnancy alterations were seen at and up to the maximum dose tested. CFE was not associated with any toxicity or adverse events.

Studies carried out by the Department of Pharmacology, St John’s Medical College in Bangalore, India\(^{152}\) and by Jagtap A et al (2006), after observation of 14 days they did not find any toxicity or mortality in the dose of 2g & 5g/kg bw in Wistar rats\(^{153}\). The LD\(_{50}\) was concluded as greater than the highest dose tested (5g/kg bw).
Evaluation of *Caralluma fimbriata* extract in animal models of obesity

**Appetite suppressing activity:**

The appetite suppressing activity of CFE was calculated by monitoring food intake and animal’s body weight (at baseline, every 10 days and at term).

**Biochemical analysis:**

Laboratory parameters included:

(i) Blood glucose (Trinder’s method/Glucose oxidase–peroxidase method),
(ii) Serum lipid profile including cholesterol (by CHOD-PAP method), triglycerides (by GPO-TOPS method/glycerol-3-phosphate oxidase method) and high density lipoprotein (by Precipitation method, Phosphotungstate magnesium acetate reagent).

Which were measured –at baseline, every 10 days and at term.

**In addition, liver function and renal function tests were assessed by analyzing:**

i. Serum glutamic pyruvic transaminase (SGPT) by International Federation of Clinical Chemistry (IFCC) method, kinetic,
ii. Serum glutamic oxalo acetic transaminase (SGOT) by IFCC method, kinetic,
iii. Alkaline phosphatase (ALP) by DGKC-SCE recommended procedure,
iv. Serum creatinine by Modified Jaffe’s method,
v. Uric acid by Uricase TOPS method and
vi. Blood urea levels by Urease/Glutamate dehydrogenase (GLDH) methodology.

These tests were done at baseline and at term.
**Histopathology evaluation:**

Animals in normal control, untreated and treated obese groups are sacrificed at the end of the study and organs like liver, kidney and aorta were removed and sent for histopathological evaluation.

**Data Analysis:** All the values were analyzed by one-way analysis of variance (ANOVA) using the Brown-Forsythe statistic followed by Games-Howell post hoc comparisons tests to study the differences between groups. The level of statistical significance was set at \( p<0.05 \). Data are expressed as Mean ±SEM (standard error of mean).