Original Article

In vitro Evaluation of Anti-obesity Potential of *Phyllanthus Fraternus* Leaves

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ABSTRACT

Obesity has been an important health concern for over a decade, causing serious health issues worldwide. Treatments available for obesity include FDA-approved drugs such as Lorcaserin, Orlistat, Bupropion, combinations of Phentermine and Topiramate, and Sibutramine; however, these have adverse effects on health. To address the said issue, the current study was conducted to evaluate the anti-obesity potential of *Phyllanthus fraternus* leaves. These leaves are a rich source of different phytochemicals (e.g., alkaloids, saponins, terpenoids, tannins), and the plant has been shown to exhibit medicinal properties; therefore, it can be used for treating obesity disorders. The crude extract of plants was prepared in three different solvents (e.g., methanol, hydro alcohol, and isopropyl alcohol). Lipid inhibition was determined using lipase inhibition assay, and amylase assay was carried out to determine if the plant extract had anti-diabetic properties. An oil red staining was carried out to determine lipid accumulation in which the cells were incubated with plant extract for 48 h. To determine if the plant extract was toxic to 3T3 cells, an MTT assay was carried out to assess cell viability. Through lipase inhibition assay, we depicted potent anti-obesity properties, isopropyl alcohol extract exhibited 67.45% inhibition at the concentration of 500µg/ml. Methanol extract showed the highest percent of α amylase inhibition i.e., 90.03% at a concentration of 1,000 µg/ml. The MTT assay concluded that the plant extracts were not cytotoxic to the cells at a concentration range between 20µg/ml to 100µg/ml, and the percentage of viable cells was 98-63%. The results obtained from the current study revealed that the plant exhibits potent anti-obesity properties. Thus, this plant extract is a potential source as an alternative treatment to treat obesity.

Keywords: Anti-obesity, Lipase inhibition assay and plant extract, MTT assay, *Phyllanthus fraternus*

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1. Introduction

Obesity is defined as a complex disorder where there is abnormal or excessive fat accumulation that may impair health (1). Body mass index (BMI) is a simple measure of weight for height, this is commonly used to classify overweight and obesity in individuals. It is defined as a person's weight in kilograms divided by the square of his height in meters. When BMI is greater than or equal to 25, it is considered to be overweight, and when BMI is greater or equal to 30, it is considered to be an obese condition. Prevalence rates of obesity in India are around 55.5% (2). Both overweight and obesity have become a substantial problem among the socio-economic individuals living in India, particularly in urban areas, uneducated, and socioeconomically developing states. Analysis reveals that there is an alarming increase in obesity. From 1975-2016, the incidence of obesity among children aged between 5-19 increased four times from 4% to 18% worldwide [WHO]. Consequently, it is posing a great challenge to healthcare providers to select the most effective treatment before the disease becomes a widespread epidemic (3). Treatments available for obesity include FDA-approved medicines such as Lorcaserin, Orlistat, Bupropion, combinations of Phentermine and Topiramate, and Sibutramine; however, these have adverse effects on health (4). The main side effects of medicines such as Orlistat are diarrhea, flatulence, abdominal pain, oily or liquid stool, and liver toxicity. Side effects of Bupropion include headache, nausea, and insomnia. Effects of Phentermine and Sibutramine include adverse cardiac events. To treat individuals with minimum adverse effects, medicinal plants can be used as an alternative or adjuvant therapy. They are considered a main source of therapeutic agents from ancient times to cure and treat various diseases and disorders (5). The plants have been used by humans since prehistoric periods. There is an increasing interest in medicinal plants in modern healthcare as they are affordable compared to conventional medicine, help in stabilizing the immune system, accelerate natural healing, are easily ingestible, and most importantly have the least side effects (6). Although medicines produced from chemical products alleviate pain instantly and effectively cure an illness, they have various side effects (7). *Phyllanthus* species contain a rich diversity of phytochemicals such as tannins, terpenes, alkaloids, and glycosidic compounds, and these species also serve as rich natural sources of antioxidants due to the presence of polyphenolic components (8). Some recent investigations have also reported the Geno protective activity against physio-chemical mutagens (e.g., UV radiations) (9). Other compounds that are present include Eellagic acid, estradiol, carilagin, Gallic acid, and Methyl salicylate (10). *In vitro* studies of combinations of different species of *Phyllanthus* have shown anti-aging, antioxidant, anti-collagenase, and anti-elastase activities, which make it beneficial for use in cosmetic industries (11). Different species of *Phyllanthus* have shown immunomodulatory, antiviral, antibacterial, anti-hyperglycemia, and hepatoprotective properties (12). Although studies have been reported for different species of this plant, anti-obesity studies for *Phyllanthus fraternus* have not yet been reported. Thus, the present study aimed to evaluate the anti-obesity potential of *Phyllanthus fraternus* using a standard anti-obesity assay and *in vitro* model system.

2. Materials and Methods

2.1. Sample collection

Dried leaves of *Phyllanthus fraternus* were obtained from KLE Ayurveda College, Belagavi. The study was conducted on a 3T3 cell line obtained from National Centre For cell Sciences NCCS, Pune.

2.2 Preparation of plant extract

Leaves were powdered and weighed. About 30 g of leaf powder was mixed with three different solvents. i.e., 70% Methanol, 70% Isopropyl alcohol, and Hydroalcoholic. This mixture was soaked in the respective solvents for 72 h and then filtered using Whatman filter paper no. 40. After this, a rotary evaporator was used for the effective separation of solvents from the sample via distillation. After enough solvent was collected, the remaining sample from the round bottom flask was subjected to a boiling water bath, and a dried form of plant extract was obtained.

2.3 Lipase Inhibition Assay

The lipase enzyme solution was prepared by dissolving 6 mg of enzyme in a 10 ml buffer solution. This enzyme was prepared immediately before use. For standard, 60 mg of orlistat was dissolved in 12 ml Dimethyl Sulfoxide (DMSO). A total of 96-well plate was used for the assay, as three different solvents and six different concentrations were studied. Around 25µl of plant extract was added to each well (triplicates of each sample with decreasing concentration). About 50µl of enzyme solution was added to each well further 100µl of buffer was added to each well followed by the addition of 25µl of p-Nitrophenyl Butyrate (PNPB) substrate. Blank readings were obtained by adding enzyme, buffer, and PNPB (triplicates). Standard readings were obtained by adding 200µl of the standard drug (triplicates). The plate was incubated at 37^0C, and Absorbance was measured at 400 nm using an ELISA plate reader (13).

% Inhibition was measured by using the formula,

% Inhibition = [(Ab-At)/Ab] x 100

Where Ab was the absorbance of the blank, and At was the absorbance of the test.

2.4 Alpha amylase assay

Plant extract dilutions: 1mg/ml solution of plant extract was prepared in sodium phosphate buffer, and it was serially diluted to obtain concentrations of 1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, and 31.25µg/ml. Around 250µl of plant extract was added to each well (triplicates) according to reducing concentrations, and 250µl of sodium phosphate buffer was added to the well. About 250µl of amylase solution was added, followed by the addition of 250µl starch solution. After adding all the chemicals to the wells, the plates were incubated at 37^0C for 20 min. 20µl of 1M HCl was added to all the wells to stop the reaction. Further, 100µl of iodine solution was added to all the wells, followed by incubation for 20 min. After incubation, absorbance was measured at 625nm using an ELISA plate reader (14). The control consisted of starch, enzyme, and buffer, and standard Metformin was used. % Inhibition was calculated by,

% Inhibition = [(Ac-At)/Ac] x 100

Where Ac was the absorbance of the control, and At was the absorbance of the test

2.5 Oil red staining

Oil red stain: 0.3 gms of oil red stain was dissolved in 60ml of isopropyl alcohol. Day 1: 1 ml of cell suspension was added to each of the three wells. One well was seeded for negative control and the plate was incubated overnight. Day 2: 1ml of plant extract was added to each well, and the Plate was incubated for 48 h in a CO_2 incubator at 37^0C. On 4^{th} day, the supernatant was removed carefully. All the wells were washed with 500µl of PBS. Around 10% of 200µl formalin was added and incubated for 30 min. Further, all the wells were washed with 500µl and further washed two times with distilled water. 1ml of Oil Red O stain was added to all the wells and was incubated for 30 min, and the wells were observed under a microscope to visualize the stained cells. After observation, 300µl of isopropyl alcohol was added to all the three wells and 100µl of the preparation was transferred to 96-well plates (triplicates). Absorbance was determined using an ELISA reader at 510nm (15).

2.6. MTT assay

Tryphan blue assay was conducted in triplicates to determine the number of viable cells. Cells were counted and seeded in 96-well plates. Approximately 5,000 cells per well were seeded along with 150µl of Dulbecco's Modified Eagle Medium DMEM media. The plate was incubated overnight in a CO_2 incubator. After overnight incubation, different concentrations (20, 40, 60, 80, and 100µg/ml) of plant extracts were added, and only media acted as a negative control. The plate was incubated for 48 h in a CO_2 incubator. A 50 µl of MTT dye was added, and the plate, was covered with aluminum foil and further incubated at room temperature for 4 h. The supernatant was discarded slowly without disturbing the formazan crystals. 100µl of DMSO was added to each well to dissolve the formed crystals. Absorbance was measured at 570nm in an ELISA plate reader (16). Percentage viability was determined using the formula: Abs of Test / Abs of Blank x 100.

Percentage viability = Absorbance of Test / Absorbance of Blank x 100

3. Results

3.1. Total yield of plant extract

The total yield of plant extract was found to be 8.07g, 9.2g, and 9.5g from *P. fraternus* leaves utilizing solvents such as hydro alcohol, isopropyl alcohol, and methanol, respectively.

3.2. Lipase Inhibition Assay

Lipase inhibition assay is based on the assumption that dietary fat will not be directly absorbed by the stomach until it is treated with pancreatic lipase, it is widely used in the case of anti-obesity therapy. Six different concentrations of all three extracts (methanol, hydro alcohol, and isopropyl alcohol) were serially diluted to get the concentrations of 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, 31.25µg/ml, and 15.6µg/ml. Further, these were treated with PNPB, lipase enzyme, and buffer, and incubation was done at 37^0C. Absorbance was measured at 400nm using an ELISA plate reader. When compared to orlistat, among all the three extracts, isopropyl alcohol extract showed a high percent of lipase inhibition, as depicted in Figure 1.

3.3. Alpha Amylase Inhibition Assay

This assay is used to determine the activity of amylase enzyme through the starch iodide method. Amylase acts on starch and will break down to give monosaccharides. Iodine will react with the monosaccharides to produce a blue color. Six different concentrations of all three extracts (methanol, hydro alcohol, and isopropyl alcohol) were obtained by serially diluting it, and concentrations of 1000µg/ml 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, and 31.25µg/ml was achieved. Furthermore, the extracts were treated with starch, iodine, and hydrochloric acid. Measurement of absorbance was performed at 625nm. Metformin was used as a standard, and among all the three plant extracts, methanol extract exhibited the highest percent of inhibition (Figure 2).

Fig. 1: Lipase Inhibition Assay concentration v/s percentage inhibition

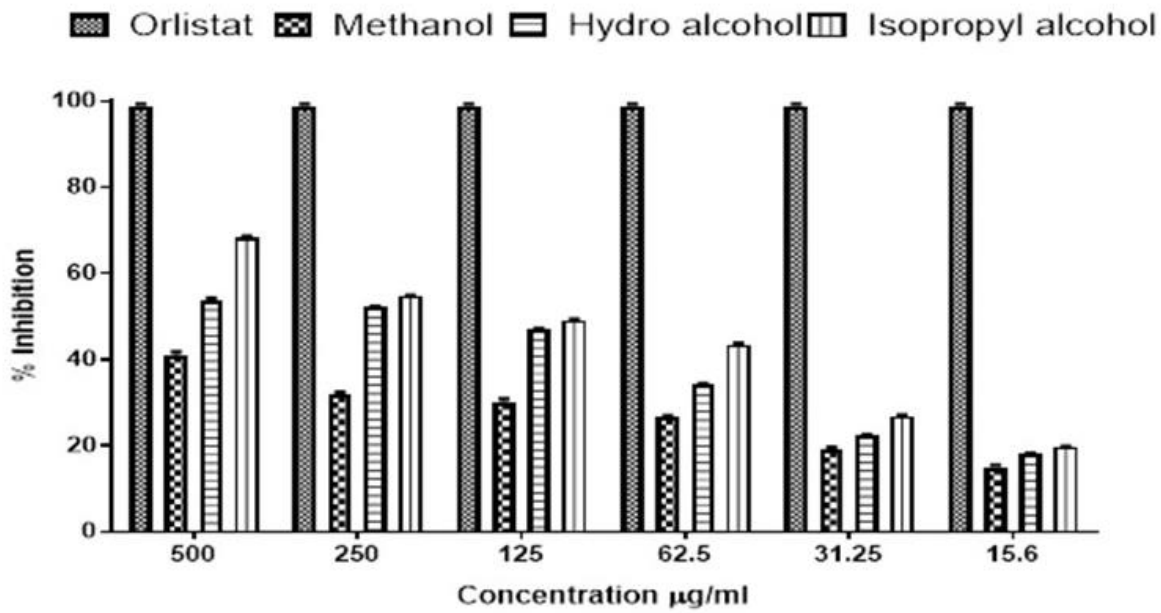
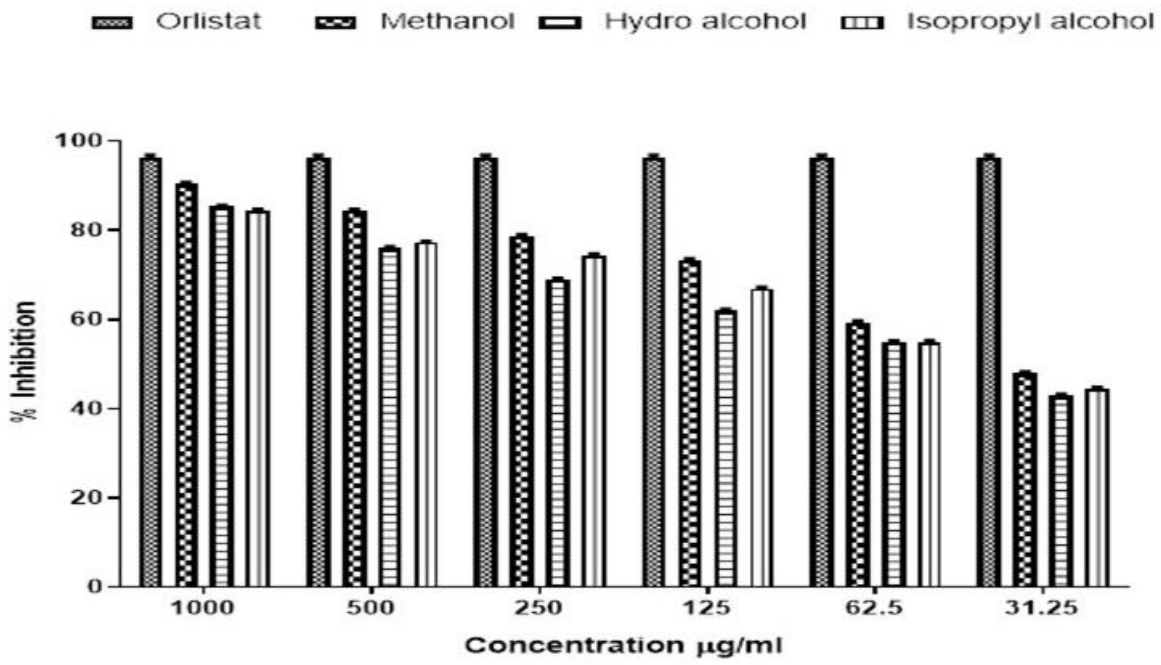


Fig. 2: Amylase Inhibition assay concentration v/s percentage inhibition



3.4 Oil Red O Staining

Oil Red O staining was conducted to show the presence of fat and lipids in the tissue sections and compared the degree of adipocyte differentiation in cell culture (Figure 5, A, B, and C). For comparison, all three extracts were added to a 12-well plate, along with one negative control. The wells treated with plant extracts showed a lesser absorbance compared to the control, indicating that the extracts prevent lipid accumulations. In the present study, the Isopropyl alcohol showed the highest, consequently preventing obesity, as shown in Figure 3.

3.5 MTT Assay

The MTT assay is a colorimetric test used to determine cell viability activity. The 3T3 cells were added to each well of 96-well plates and exposed to plant extracts for 72 h by serially diluting them to obtain concentrations of 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml, and 100µg/ml. All three extracts were non-cytotoxic, as the number of viable cells was higher than 50%. The highest percent of viability was exhibited by isopropyl alcohol extract, which was 98.7% at 20µg/ml and 64.9% at 100µg/ml concentration, as presented in Figure 4.

Fig. 3: Oil Red O staining plant extract v/s absorbance

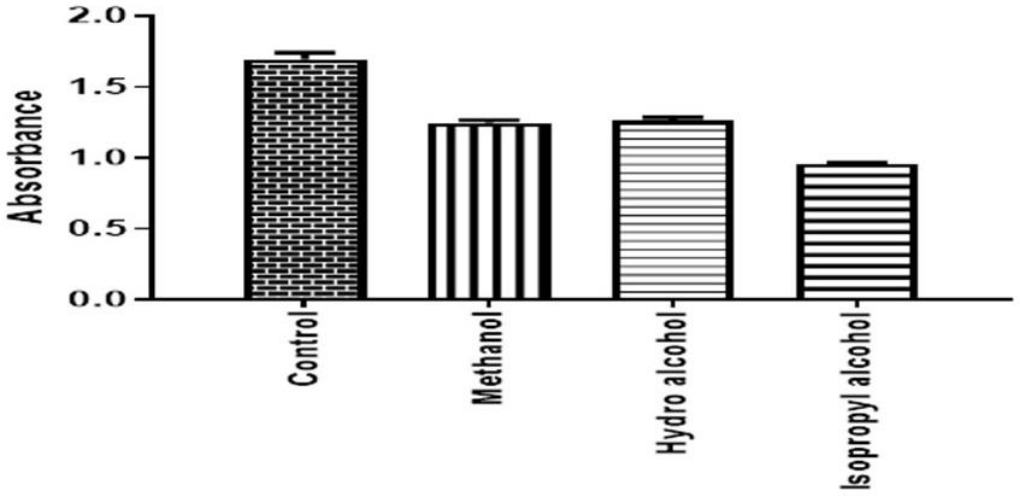
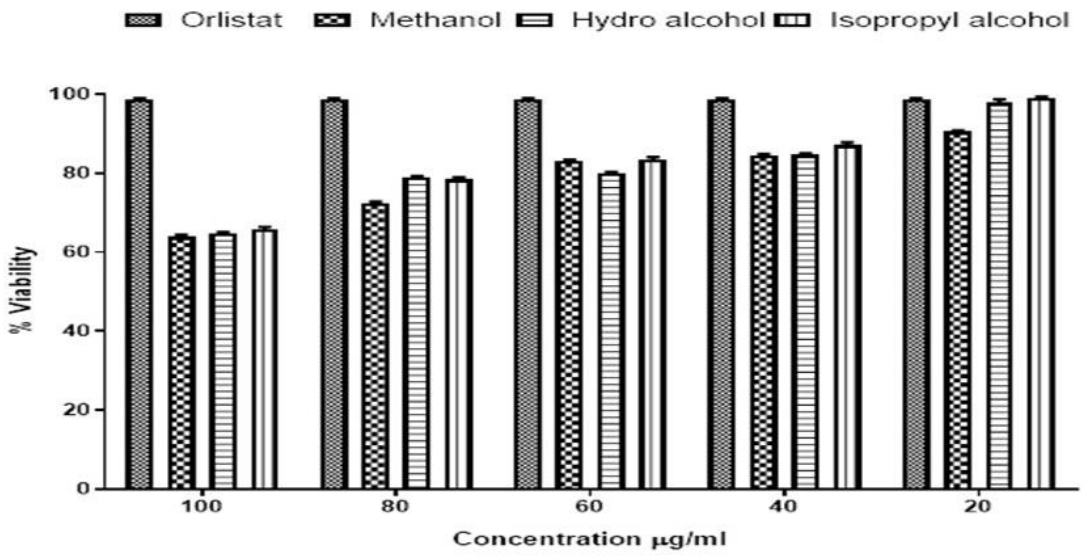


Fig. 4: MTT Assay concentration v/s percentage viability



4. Discussion

Obesity is the most common menace of the global population, regardless of the age group. It is a common matter of concern because it contributes to the increasing bedevil of metabolic and cardiovascular disorders, cancer, diabetes, and stroke (17). Therapies which have many adverse effects include pharmacotherapy and weight loss surgeries. The ancient practice of using herbal medicine for treating ailments is increasingly emerging in the modern era, and the use of natural products can act as a substitute for treating anti-obesity. To determine the plant extracts with anti-obesity potential, the following assays were conducted, and results were analyzed and discussed accordingly. Pancreatic lipase inhibition assay is extensively used to determine the ability of anti-obesity agents, and it is intended to reduce body fat and body weight. Orlistat is the only drug endorsed by the FDA for the treatment of obesity. The success of naturally occurring compounds that can be used to treat obesity has influenced the research for the identification of novel inhibitors of pancreatic lipase that do not cause undesirable side effects (18). In the present study, isopropyl alcohol extract exhibited the highest inhibition in comparison to the other two extracts, i.e., 67.4% inhibition at 500µg/ml and 20.0% inhibition at 15.6µg/ml. Methanol extract showed the lowest percent of inhibition with 40% inhibition at 500µg/ml concentration. Correspondingly, in a study conducted on *P. chamaepeace* Rid l by Dechakhumphu *et al.,* 2022 (19). Methanolic extracts were prepared using the whole plant. At a concentration of 100µg/ml, a considerable and higher percentage of inhibition was observed. In another study conducted on *P. niruria* by Alias *et al.,* 2017, plant extract was prepared in 80% methanol. 45-50% of lipase inhibition was observed. Amylase is among the principal enzymes that supervise the breakdown of starch into simple sugars. Upon hydrolysis, oligosaccharides and disaccharides are produced, which are further hydrolyzed by α glucosidase to monosaccharides i.e., the enzyme catalyzes the hydrolysis of α-1,4 glycosidic linkages from polysaccharides to yield maltose units hence slows digestion of starch and brings down the rate of glucose absorption (20). Alpha amylase inhibitors are called starch blockers as they avert dietary starch from being absorbed by the body, and this in turn reduces glucose levels. The presence of phytochemicals such as saponins, steroids, and terpenoids are responsible for therapeutic activity, and hence plant extracts can be used potentially to treat diabetes mellitus (21). The present study was carried out using methanol, hydro alcohol, and isopropyl alcohol extracts, out of which methanolic extract showed the highest percentage of amylase inhibition with 90.03% at 1000µg/ml and 47.5% inhibition at 31.25µg/ml. Comparably, in a study conducted by Hashim *et al.,* 2013 (22) to determine anti-diabetic activity, methanolic extract of *P. virgatus was* prepared and screened for α Amylase inhibitory properties, and it was observed that 43.2% and 66.09% of inhibition was observed at 25µg/ml and 50µg/ml, respectively. Oil red O staining is a simple qualitative technique used to inspect lipid droplets-stored triglyceride adipocytes. This staining technique has been routinely used to examine the accumulation of cytoplasmic triglycerides. Adipocytes accumulate surplus energy in the form of triglycerides, which are present inside lipid droplets. The untreated control cells get strongly stained with Oil Red O, which indicates that the cells accumulated substantial amounts of cytoplasmic triglyceride (23). The cells treated with *P. fraternus* plant extracts showed a suppression of intracellular triglyceride accumulation, which concluded that the plant extract could hold great potential to treat obesity. In the present study, 3T3 cells were treated with plant extracts and incubated for 48 h to determine lipid accumulation. Absorbance was measured at 510nm, and the absorbance obtained from plant extracts was compared with the absorbance of control that contained only cells and media. Isopropyl extract showed relatively least absorbance i.e., 0.94 as compared to control, which had an absorbance of 1.644. Methanol and hydro alcohol extract showed an absorbance of 1.21 and 1.12, respectively, which were also less than the control. As a result, it was concluded that the extract prevented lipid accumulation and could be used to treat obesity disorder. The MTT assay was performed to determine the intracellular cytotoxic effect of Methanolic, Hydroalcoholic, and Isopropyl extracts on *Phyllanthus fraternus* on 3T3 cells. The cells were treated with a range of concentrations of 20, 40, 60, 80, and 100µg/ml. Determination of cell viability was examined after 48 h of incubation of cells treated with the plant extracts. The percentage of viable cells was calculated by measuring the absorbance of the test against the absorbance of the blank (the control group contained only cells and media). Figure 4 depicts the results obtained from the MTT assay. The survival percentage of cells was highest for Isopropyl extract i.e., 98.7% at 20µg/ml and 64.9% for 100µg/ml concentration. The other two plant extracts also showed a considerable percentage of viability, with 64.4% of viability for Hydro alcoholic extract and 63.9% of viability for methanol extract at 100µg/ml. Similarly, in a study conducted by Hashim *et al.,* 2013 methanolic extract of *P. virgatus* was prepared and tested for anti-cytotoxic effect on 3T3 cells at various concentrations, and it was concluded that the plant extract was not cytotoxic. In conclusion, our study suggests that naturally-sourced medications derived from plant leaves, particularly the Isopropyl alcohol and methanolic extracts

exhibit promising properties for addressing obesity and anti-diabetic potential activities. These extracts demonstrated the potential to inhibit key enzymes involved in lipid and carbohydrate metabolism and prevent lipid accumulation. The MTT assay showed that plant extract was non-cytotoxic to cells at a concentration range of 20µg/ml to 100µg/ml. Further research and clinical trials are warranted to explore the full therapeutic potential and safety of these plant extracts in the treatment of obesity. If proven effective and safe, these natural remedies could offer a valuable addition to the existing arsenal of obesity treatments, potentially mitigating the unpleasant side effects associated with conventional medications.

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Authors' Contribution
Rajalaxmi Patil conducted experimental work, Rubeen Nadaf drafted the manuscript, Vijay Kumbar, and Suneel Dodamani analysed data, and Shridhar Ghagane designed and reviewed the manuscript. All authors commented on the manuscript and approved the final manuscript.

Ethics
This article contains no studies with human participants or animals performed by any of the authors.

Conflict of Interest

Rajalaxmi Patil, Rubeen Nadaf, Vijay Kumbar, Suneel Dodamani, and Shridhar Ghagane declare that they have no conflicts of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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