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Pancreatic Lipase Inhibition and Cytotoxic Profiling of GC-MS Characterized *Pisonia grandis* R.Br Leaves Ethanol Extracts Using Cell Line Models and EtBr Staining

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ABSTRACT

This new millennium has seen the rise of obesity to the position of most frequent metabolic illness on the globe. It causes serious health problems in industrialized countries. In previous research, methods to reduce obesity were suggested, including lowering preadipocyte differentiation and proliferation and reducing lipogenesis. Many different medicinal uses have been associated with the tropical herb *Pisonia grandis* for quite some time. The current study used 3T3-L1 pre-adipocytes in a lab setting to test how well *P. grandis* can block pancreatic lipase at different amounts of 12.5, 25, 50, 100, and 200 µM. In addition to the topics mentioned above, the following were covered: phytoconstituents analyzed using Gas Chromatography-Mass Spectrometry; apoptosis with Ethidium bromide staining, and MTT cell assay. One of the most impressive findings was the comparatively high anti-lipase activity of the ethanol extract of *P. grandis* leaves (61.56%). Adipocytes (3T3-L1) were exposed to doses ranging from 12.5 to 200 µg/mL for a duration of 24 hours. The effects of *P. grandis* at 100 µg/mL (72.82% at 24 h) and 200 µg/mL (32.95% at 24 h) were explored. The exposure significantly reduced the cell viability of 3T3-L1 adipocytes. The GC-MS study found that this leaf ethanol extract has propanoic acid, 2-mercapto-, methyl ester, hexanoic acid, ethyl ester, n-hexadecanoic acid, 5-methylpyrrolidin-2-one, and 9-octadecenoic acid. This information shows that *P. grandis* successfully reduces fat cell formation and boosts cell death in 3T3-L1 preadipocytes and adipocytes.

Keywords: Pancreatic Lipase, *Pisonia grandis*, GC-MS,3T3-L1 preadipocytes.

INTRODUCTION

Based on a review of Body Mass Index (BMI) figures, the World Health Organization[1] argues that obesity is a global epidemic concern. Since then, the alarming rise in obesity rates has emerged as a major issue in public health. Those who are overweight are more likely to suffer from metabolic disorders, cardiovascular problems, malignancies, and inflammation-based pathologies[3,4]. Cancer, osteoarthritis, stroke, and sleep apnea are among the long-term health problems that it increases the likelihood of acquiring.

The World Health Organization (2017) reports that globally, there are 297 million women

and 205 million men classified as obese, resulting in a total of over 600 million individuals. The World Health Organization reported that obesity and overweight contribute to a minimum of 2.8 million deaths each year. Obesity is widely recognized as a significant health concern and the leading cause of morbidity and mortality globally. Numerous comorbidities, including diabetes, fatty liver, cancer, atherosclerosis, and hyperlipidemia, are associated with it[6-9].

Although several factors contribute to a positive energy imbalance, the precise mechanisms by which this imbalance manifests as obesity remain unclear. Excessive caloric intake and insufficient physical activity have been associated in certain studies with the obesity epidemic. Food records from four consecutive NHANES (National Health and Nutrition Examination Surveys) studies, which included 39,094 persons, have been linked to the rising obesity rate in the United States[11]. Researchers tracked how adults' food intake and calorie density changed over time. Two indicators of physical inactivity—television use and automobile ownership—are strongly correlated with the rising obesity rate in England, according to data from the Central Statistical Office. Based on their analysis of NHANES data, the researchers discovered that the obesity rate rose by 2% for every hour that people watched television.

Obesity is a chronic disease that results from a complex interaction of genetic, behavioural, and environmental factors related to socioeconomic status and lifestyle[14]. Some medical disorders and pharmaceuticals, such as steroids, insulin, antiepileptics, antipsychotics, or antidepressants, or some medical diseases, like Cushing syndrome, hypothyroidism, or hypothalamic abnormalities, can lead to obesity, which is known as an iatrogenic problem.

Pisonia grandis is a tropical plant species belonging to the Nyctaginaceae family that is native to Southeast Asia and the Indo-Pacific area. Variously known as the "Pisonia tree" or the "Lettuce tree," this species has long been used by traditional healers to alleviate various ailments, such as wounds, gastrointestinal issues, and fever. Plants are ideal research subjects because of their adaptability and potential medical applications. *P. grandis* has a long history of use in many cultures, which suggests it may have medicinal properties. Phytochemical studies confirm that it can reduce inflammation and help with diabetes by showing it contains useful compounds like flavonoids, terpenoids, and phenolic acids. Thepossible health benefits of *P. grandis* encourage this study to look into how it affects fat cells, 3T3-L1 cell toxicity, and its chemical properties.

MATERIAL AND METHODS

Collection of plants

P. grandis R.Br.leaves are gathered from the surrounding areas of Coimbatore, Tamil Nadu, India. India's BSI (Botanical Survey of India) in Coimbatore, Tamil Nadu, has verified the plant's authenticity. After collection, we thoroughly cleaned, rinsed, shade-dried, and ground the leaf samples into powder. Extraction was carried out using analytical-grade solvents, adhering to the established protocol. Aqueous and ethanol solvents were utilized for Soxhlet extraction using the coarsely ground leaf powder, respectively. After that, additional analysis was done using the final extracts.

In vitro anti-obese activity in 3T3 L-1 adipocytes
Pancreatic lipase inhibition activity

To quantify the inhibition of lipase by leaf extract and P-nitrophenyl butyrate (NPB) was used to test the pigs' pancreatic lipase activity. Potassium phosphate buffer solution (0.1 mM, pH 6.0) has been employed for preparing lipase solution (1mg/mL). To investigate the lipase inhibitory effect, extract (1 mL) and lipase (1 mL) have been pre-incubated for a minute at 37°C. 0.1 mL of NPB substrate is added to initiate the reaction. A UV-Visible spectrophotometer was used to detect the reaction's p-nitrophenol's inhibitory activity at 405 nm following 15 min at 37°C[19].

Cell lines and culture medium

The 3T3-L1 pre-adipocytes were procured from the National Centre for Cell Science (NCCS) in Pune and incubated at 37°C in a humidified environment containing 95% air and 5% CO_2 in DMEM (Dulbecco's Modified Eagle Medium) with the addition of 10% FBS (fetal bovine serum), 100 µg/mL penicillin, and 100 µg/mL PS (polystyrene). The cells have been cultured in a mixture containing 0.5 mM 3-isobutyl-1-methylxanthine in DMEM for the first day, µM dexamethasone, and 10 µg/mL insulin after they have reached confluence. Following 2 days of encouraging adipogenesis, media changed

to DMEM with 10% FBS (fetal bovine serum), PS (polystyrene), and 10 µg/mL insulin on Day 4. They have cultivated post-differentiation media, consisting of DMEM with 10% FBS and PS alone, from Day 2 to 8. The effects of varying doses of leaf extract on adipogenesis were studied by administering it to 3T3-L1 preadipocytes during differentiation (Day 0-Day 2)[20].

Determination of cytotoxicity

Tetrazolium isn't reduced by dead cells or their metabolites. Quantity of cells and the activity of mitochondria are quantified. A succinate dehydrogenase changes MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) into blue formazan. For the trypsinized monolayer cells, the DMEM/10% FBS concentration was adjusted to 100,000/mL. We used 0.1 mL of the diluted cell solution per well in each of the 96 microtitre plates. The partial monolayer was washed with medium after 24 h, and the liquid above was discarded. Then, 100 µL of solvent extracts of different strengths were applied to microtitre plates. Plates have been examined under a microscope for 24 h during the following 3 days at 37°C with 5% CO_2. Following 72 h, excess of dye solutions had been removed and each well then filled with 50 µL of MTT in PBS(phosphate-buffered saline). The plates were incubated for three hours at 37°C with 5% CO_2 after a gentle shaking. Shake the plates gently with 100 µL of propanol to dissolve the Formazan and remove the supernatant. At 540nm, microplate reader reads. We calculated extract concentration required to 50% inhibit cell growth for each cell line using dose-response curves[21].

Determination of apoptosis

The 3T3-L1 cells that had been exposed to 30 µg/mL of NOV-SAC (nonivamide (NOV) and S-allyl cysteine (SAC)) for 24 h were thereafter harvested and rinsed with 1x PBS-7.2. Citrus acridine orange (AO) and ethidium bromide (EB) were followed by cell staining. Collecting cells after 20 min of dark incubation at 37°C allowed them to be examined using a fluorescence cell imager (ZOE, BioRad, USA).

GC-MS Analysis of *P. grandis*

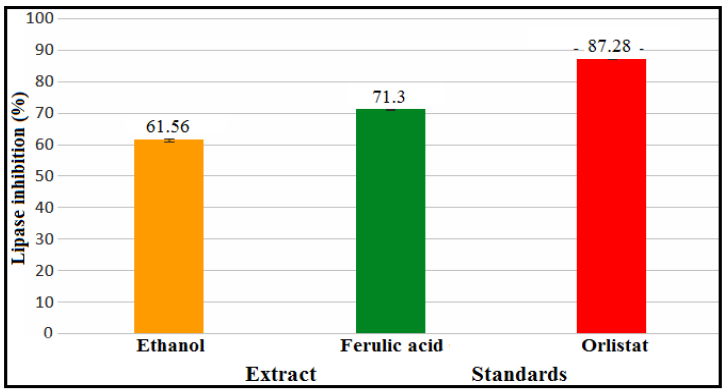
Instruments and chromatographic conditions: The gas chromatograph, gas chromatography–mass spectrometry, and AOC–20i autosampler are all part of this Perkin Elmer GC Klarus 500 system: The electron impact mode was employed at 70eV with helium (99.999%) as the carrier gas in a capillary column composed of Elite-1 fused silica (30×0.25mm i.d. Ï1EM df, 100% dimethyl polysiloxane). The injection volume was 0.5EI and the flow rate was 1 mL/min (10:1). The injector should be set to 250°C and the ion source at 280°C. An initial temperature of "110°C (isothermal for 2min)" was set for the oven. Following that, it increased by "10°C/min to 200°C," "5-280°C," and 9°C at 280°C, where it become isothermal. At 70 eV and 0.5 s intervals, mass spectra ranging from 40 to 550 Da were analyzed. The unknown component was identified by comparing its mass spectra to those of components recognized by NIST (National Institute of Standards and Technology). Molecular mass and structure calculations were performed on all components of the test sample.

RESULT AND DISCUSSION

***In vitro* anti-obesity activity**

Pancreatic lipase inhibitory activity

The inhibitory activities of pancreatic lipase of *P. grandis* leaf extract were reported in Fig. 1. All the tested leaf extracts were active against pancreatic lipase. *P. grandis* leaf ethanol extract (61.56%) showed relatively high anti-lipase activity. However, the ethanol extract was less potent than orlistat (87.28%), a well-known anti-lipase agent, in inhibiting pancreatic lipase. Similar to orlisat, the leaf extract of *P. grandis* possesses strong lipase inhibitory action. Intestinal fat absorption occurs after exposure to pancreatic lipases. Hydrolysis of dietary triacylglycerols into monoacylglycerols and fatty acids is catalyzed by pancreatic lipase, an enzyme that is essential for absorption. Of all the medications tested, orlistat had one of the most potent direct lipase interactions. It is derived from Streptomyces toxytricini, a naturally occurring lipase inhibitor[22]. Covalent bond formation between orlistat and the lipase's serine active site is the mechanism by which it inhibits[23]. According to Karamadoukis *et al.,*[24], there are some unpleasant gastrointestinal side effects, although it is clinically approved for the treatment of obesity, Bloating, diarrhea, dyspepsia, and flatulence are side effects of lipase inhibitors like orlistat[25].



Values are mean of triplicate determination (n=3) ± Standard deviation, Statistically signification at p<0.05 where a>b>c

Fig. 1. Pancreatic lipase inhibition potential of ethanol extracts of *P. grandis*

The impact of *P. grandis* on the viability of 3T3-L1 adipocytes in culture

3T3-L1 adipocytes were subjected to progressively increased concentrations of the substance (12.5-200µg/mL) for a duration of 24 h in order to ascertain the effect of *P. grandis* on cell proliferation. See Fig. 2 and Table 1 for details. Administration of 100 µg/mL to *P. grandis* (72.82% at 24 h) and 200 µg/mL (32.95% at 24 h) treatment. Drastically decreased the 3T3-L1 adipocytes' cell viability. There was a dose-dependent inhibition of cell viability observed in the treatment with *P. grandis*. On the other hand, the level of inhibition was below the half-life (IC_{50}). Our findings points to an IC_{50} cell inhibition value of 155.74 µg/mL for the *P. grandis* ethanol extract. WHO[26] states that because obesity is associated with an increased risk of serious health issues, it has emerged as major public health concern in recent decades. This syndrome is characterized by an accumulation of adipose tissue and an increase in adipocyte size and number, also known as hypertrophy and hyperplasia[27,28]. Adipocyte hypertrophy results from excessive consumption of lipids that form energy, such as triglycerides[29].

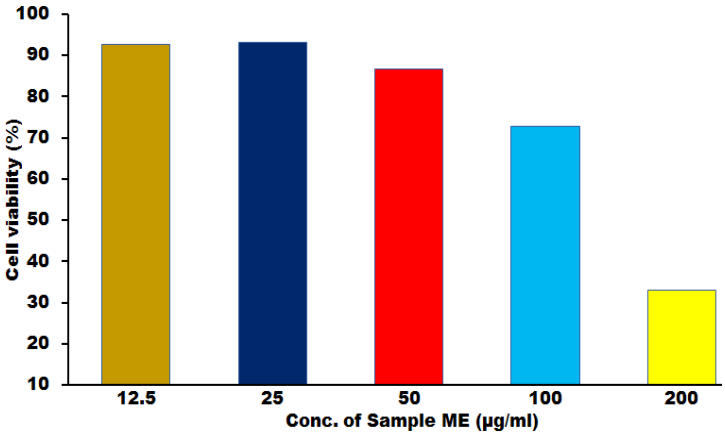


Fig. 2. The effects of *P. grandis on* cell viability in cultured 3T3-L1 adipocytes

Table 1: The effects of *P. grandis on* cell viability in cultured 3T3-L1 adipocytes

SUMMARY-MTT ASSAY (ME)	
Concentration of sample (µg/mL)	% cell viability
Untreated	100
12.5	92.65
25	93.06
50	86.67
100	72.82
200	32.95
	IC_{50} value = 155.74 µg/mL

The Impact of *P. grandis* on Cell Death in Differentiated 3T3-L1 Adipose Tissue Species

We used flow cytometry to determine whether *P. grandis's* many cell death mechanisms apoptosis occurred after 72 h of infection with differentiated 3T3-L1 adipocytes that had been labeled with PI and Annexin V-FITC. Cells treated with *P. grandis* did not change the number of normal cells or apoptotic cells (early and late apoptotic cells) in response to flow cytometric

measurement, regardless of the dosage (Fig. 3). The *in vitro* experiment demonstrated a dose-dependent inhibition of cell survival after *P. grandis* was administered to preadipocytes during the cell proliferative stage. Flow cytometric examination revealed that the ratio of normal to apoptotic cells, including those in the early apoptotic stage, was unaffected by *P. grandis* treatment of differentiated 3T3-L1 adipocytes. Adipocytes treated with *P. grandis* were shown to undergo apoptosis, resulting in a decrease in adipose tissue mass, according to this study.

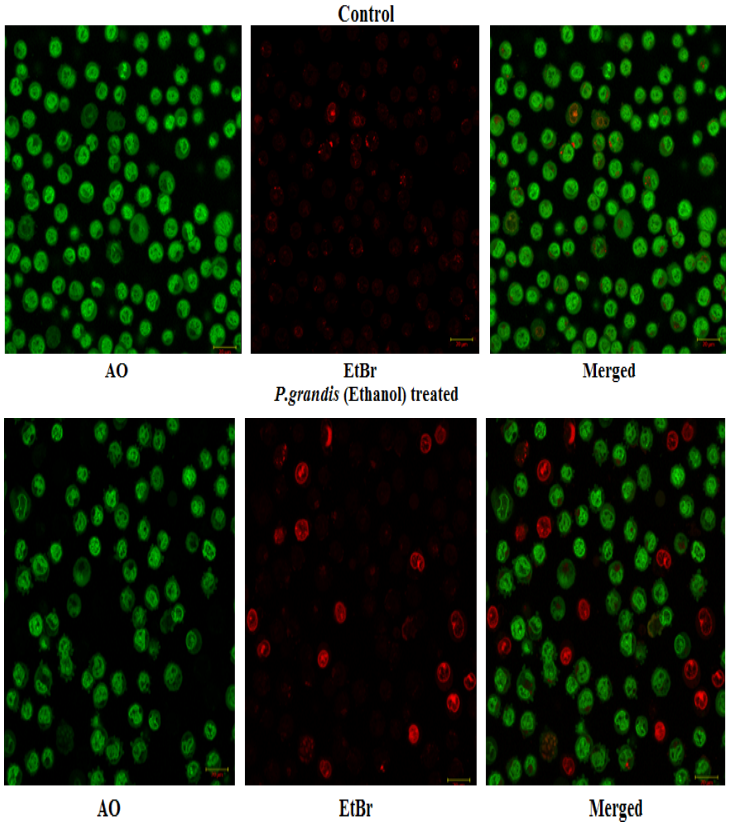


Fig. 3. The Effects of *P. grandis on* apoptosis in differentiated 3T3-L1 adipocytes

GC-MS analysis

Chemical composition of ethanol extracts from *P. grandis* leaves has been investigated through gas chromatography-mass spectrometry. Retention durations and mass spectra have been compared with databases maintained by National Institute of Standard Technology (NIST) to analyze and identify chemical components. Table 2 and Fig. 4 list discovered compounds along with their molecular weight (m/z), molecular formula, and retention time (RT/min.). Notable substances identified by GC-MS analysis include propanoic acid, 2-mercapto-, methyl ester, hexanoic acid, ethyl ester, n-hexadecanoic acid, 5-Methylpyrrolidin-2-one, and 9-octadecenoic acid.In addition to n-hexadecanoic acid and other compounds with anti-inflammatory, anti-androgenic, and antimicrobial effects, the selected plant extracts also contained squalene, a notable component with anticancer characteristics[30]. 9-Octadecenoic acid (Z)-(CAS) is an Oleic ester, which gives it antioxidant and antimicrobial properties, according to Krishnamoorthy *et al.,*[31].

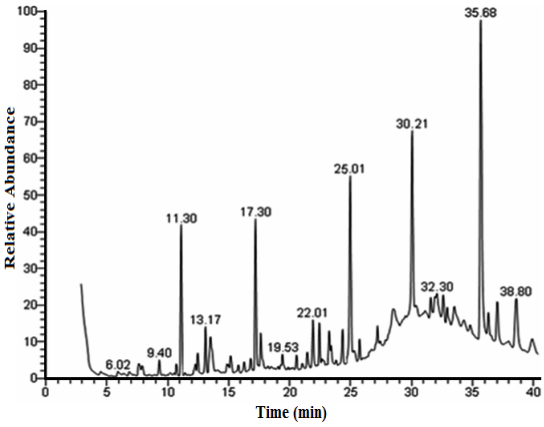
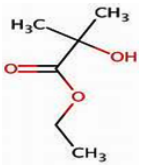
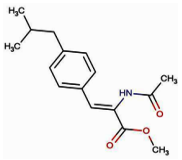
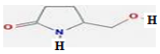


Fig. 4. Gas chromatography mass spectrometry profile of *P. grandis* ethanol extract

Table 2: Gas chromatography mass spectrometry profile of *P. grandis* ethanol extract

Sr. No	Retention Time (RT)	List of the Medicinal Compound	Molecular Formula	Chemical Structure	Nature of compounds	Therapeutic Uses
1	11.30	Propanoic-acid, 2-mercapto-, methyl ester	$C_4H_8O_2S$		Fatty Acid Methil Ester	Antineoplastic, Antiurolithic, Antiobesity, and Antiprotozoal
2	16.47	Hexanoic-acid & ethyl -ester	$C_8H_{16}O_2$		Fatty acid-ester	Antiobesity, Antiprotozoal, Antifibrinolytic, anti-alcoholic, Antipsoriatic,
3	20.52	Hexadecanoic-acid & ethyl ester	$C_{18}H_{36}O_2$		Fatty acid -methyl ester	Antiobesity, Antiprotozoal, Antifibrinolytic
4	25.01	5-Methoxypyrrolidin -2- one	$C_5H_9NO_2$		Alkaloid	Antiobesity, Antineoplastic
5	35.68	9, Octadecenoic-acid & ethyl ester	$C_{20}H_{38}O_2$		Fatty acid Methyl ester	Antiurolithic, Antiobesity, Antiprotozoal, Antifibrinolytic, anti-alcoholic

CONCLUSION

This adaptive plant has various benefits, but this study shows it's *in vitro* anti-obesity activities. *P. grandis* contains many compounds with distinct structures. This study also showed that *P. grandis* cell line research effects, which has never been done before. For obesity-related diseases, the plant's leaf extract, which contains most of the active compounds (9-Octadecenoic acid, ethyl ester), should be studied. Given the global trend toward non-toxic herbal therapies, *P. grandis*-derived pharmaceuticals for obesity-related diseases are needed immediately. This discovery should encourage scientists to find new medical traits in these species as human demand for medicine rises.

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Conflict of interest

Authors reported no conflicts of interest.

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