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Original Article

Investigation of Anti-obesity Potential of *Simarouba glauca* Leaves Extract Using *in-vitro* Method

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ABSTRACT

The current study aimed to evaluate the anti-obesity potential of *Simarouba glauca* leaf extract by using a 3T3-l1 cell line. Various assays were performed to check anti-obesity such as lipase inhibition assay, amylase inhibition assay, oil-red-o staining, and MTT assay to check the anti-obesity potential of leaves extract. The leaves were shade-dried and crushed into powdered form. Three different extracts such as ethanol, methanol, and hydroalcoholic extract were prepared by using the Soxhlet extraction method. The pure form of the extract was formed after Rota evaporation. Later anti-obesity activity was determined by using different assays. The ethanol extract of leaves showed higher anti-obesity potential on 3T3-l1 cell lines for lipase inhibition assay, amylase inhibition assays, oil red o staining, and cytotoxicity assay. *Simarouba glauca* is traditionally used for the prevention and treatment of many diseases such as cancer, dysentery, fever, and malaria.

Keywords: Simarouba glauca; Obesity; Lipase Inhibition Assay; Amylase Inhibition Assay; Oil-Red-O Staining; MTT Assay

1 INTRODUCTION

Obesity, defined as a body mass index (BMI) of more than 30, is a medical condition encountered daily by physicians throughout the world. According to the World Health Organization (WHO), 1.9 billion adults in the world were either overweight or obese in 2018, and obesity has increased three times since 1975 [1]. Overweight and obesity contributed 8.4% of the risk factor of the burden of diseases in us in 2015 (Australian Institute of Health and Wellness). There is evidence that obesity is strongly linked with a higher acquisition of disability. Further, obese are more likely to have poor physical health and intellectual fitness. It has a widespread negative impact on numerous labor market outcomes, consisting of high absenteeism, increased presentism, activity dissatisfaction, and a higher rate of job discrimination [2]. There is increasing empirical evidence that obesity triggers the probability of various non-communicable diseases (NCDS), such as type 2 diabetes, asthma, cardiovascular disease (CVD), high blood pressure cancer, poor mental health, and sleep apnea [3]. More weight gain from early childhood to maturity is always related to heart disorders [4]. Furthermore, the chance of various patterns of arthritis, such as rheumatoid arthritis, osteoarthritis, and psoriatic arthritis, is related to increased body weight [5]. Nature has blessed us abundantly with many herbs and we are using those in our daily life without knowing their medicinal importance. Phytochemicals obtained from these medicinal plants are also important for pharmacological research and drug development. *Simarouba glauca* also known as paradise tree has a long history of herbal medicine in many countries and belongs to the family *Simaroubaceae*. The *Simaroubaceae* family includes 32 genera and more than 170 species of trees and brushes of pan-tropical distribution. It was widely used for the treatment of cancer hence it is known as the tree of solace of cancer. Studies on anti-obesity have limited reports,

hence we took an opportunity to explore its anti-obesity potential on *in-vitro* model systems.

2 METHODOLOGY

2.1 Collection of plant material

The leaves of *Simarouba glauca* were collected from NITM-ICMR Belagavi, Karnataka, India followed by its identification and authentication. Plant leaves were washed and shade-dried before further analysis. The leaves of *Simarouba glauca* were shade dried then crushed into fine powder and stored at 4°C. The course powder is then subjected to Soxhlet extraction using the standard procedure by Jensen et al., 2007 [6]. 70g of course powder of *Simarouba glauca* leaves was weighed and filled into stitched muslin cloth and tied. Later cloth containing plant material is slowly inserted into the thimble also known as the extraction chamber. About 1000ml of solvent is added to the round bottom flask. The round bottom flask is placed on the electric heating mantle. Place the thimble on the round bottom flask and the condenser is placed on the thimble. The condenser was always circulated with water throughout the experiment which is maintained at 4°C. The soxhlet extraction was conducted for three different solvents ethanol, methanol, and hydro-alcohol (1:1 ratio i.e., 500ml ethanol and 500ml water) temperature of the heating mantle is applied based on the solvent boiling point. Up to 14 cycles the apparatus is allowed for solvent extraction. After extraction approximately 800ml of extracted solvent is obtained which is collected and stored at 4°C. To separate the excess solvent obtained during soxhlet extraction solvent is treated under rotary evaporation.

2.2 Screening of anti-obesity potential of Simarouba glauca extract against 3T3 cell line

2.2.1. 3T3-l1 cell line

3T3-l1 cell line was cultured in DMEM media presented with 10% FBS (fetal bovine serum), PBS (phosphate buffer saline), and antibiotics like Streptomycin, Kanamycin, Amphotericin, at 5% CO_2 at 35°C.

2.2.2. Trypan blue dye assay

Normal cell line (3T3-l1 cell line) was made in density 1.2 x 10^6 cells/ml in DMEM medium respectively and cell suspension 10 μl was mixed with 10 μl of trypan blue incubated for 1 minute for each cell type. The cell counting was done using a Neubauer chamber by observing under an inverted microscope [7]. Cell type and cell viability were seen.

$$\text{Cells per } \mathbf{ml} = \frac{\mathrm{I}+\mathrm{II}+\mathrm{III}+\mathrm{IV}}{4} \times 2 \times 10 \times 1000$$

2.3 Lipase inhibitor assay

2.3.1. Enzyme preparation

Porcine pancreatic lipase enzyme solution was prepared by dissolving 6mg of the enzyme in 10 ml of buffer solution by gentle vortexing. It will be prepared freshly before use.

4-Nitrophenyl butyrate (PNPB) working solution was prepared by 20μl of PNPB stock solution with 10ml of acetonitrile. The solution of standard drug was prepared by dissolving content of one capsule of orlistat (60mg) in 15ml of DMSO (dimethylsulphoxide). Standard was incubated with 50μl of enzyme solution, 100μl of buffer solution, and 25μl of PNPB solution for 30 min at 37°C. The total assay volume was made to 200μl. Lipase activity was determined by measuring the hydrolysis of PNPB to p-nitrophenol at 400nm using an ELISA plate reader [8]. Percent inhibitory activity is calculated using the following formula.

Percent inhibition = absorbance of blank absorbance of test/absorbance of blank * 100

2.4 Alpha-amylase assay

In a test, tube take 1 ml of PBS solution and mix it with 0.5 ml of different concentrations of samples (50, 100, 150, 200, and 250 μl/ml) with different standard solutions and 200 μl of 5mg/ml starch solution and incubated for 10 minutes at room temperature. Control was taken as a starch solution with and without amylase. The reaction mixture was stopped by adding 400 μl of DNS solution and heating the mixture in a boiling water bath for 5 minutes. Absorbance was measured at 540nm in UV visible spectrophotometer [9].

Plant extract dilutions: 1 mg/ml solution of plant extract in sodium phosphate buffer was made, and it was thereafter serially diluted to obtain concentrations of 1000, 500, 250, 125, 62.5, and 31.25 mg/ml. According to decreasing concentrations, 250 μl of plant extract and 250 μl of sodium phosphate buffer were added to each well (done in triplets). 250 μl of the amylase solution and 250 μl of the starch solution were added. The plates were incubated at 37° C for 20 minutes after the wells had been filled with all the ingredients. To halt the process, 20 μl of 1M HCl was added to each well. Additionally, 100 μl of iodine solution was added to each well, which was then given a 20-minute incubation period. The most used oral diabetes medication worldwide is metformin hence it was used as standard. The percent of enzyme inhibition was calculated using the following formula:

$$\%\text{ of Alpha Amylase Inhibition} = [(AC - AS)/AC] \times 100$$

Where, AC (absorbance of control) and AS (absorbance of sample).

2.5 Oil red O-staining

1 ml of cells were put in all the wells. Cultured dishes were washed two times with 500 μl PBS. Cells were fixed with 200 μl of 10% formalin for 1 hour. Cells were washed with 500 μl PBS and stained with ORO solution (0.5g of ORO in 100ml isopropyl alcohol) for 30 minutes at room temperature. Cells were washed twice with distilled water for minutes. Cells were observed under the microscope. 500μl of isopropyl alcohol was added in all the wells OD was taken at 520nm [10].

2.6 MTT solution preparation

5mg in 1 ml of phosphate buffer saline.

Day-1: Cell suspension was seeded into each well in a 96-well plate final volume was done up to 150μl by adding DMEM media and incubated it for overnight. Day-2**:** Dilution of the test compounds were prepared in DMEM media 100μl of the test compounds of different cone was added to each well and incubated for 24hr in the presence of 5% CO_2 at 37°C in the CO_2 incubator. Day-3: After 24hr, 20μl of 5 mg/ml reagent was added to the wells the plate was kept for 4hr incubation in a dark place at room temperature. The supernatant was carefully removed without disturbing the precipitated formazan crystals and then 100μl of DMSO was added to dissolve the crystals formed. The optical density was measured at a wavelength of 492 nm [11].

3 RESULTS

3.1 Total yield of crude extract

The total yield of crude extract from *Simarouba glauca* leaves by using the solvent, viz. methanol, Ethanol, and hydroalcoholic are 9.08g, 9.62g, and 7.56g respectively with reference to shade-dried plant material.

3.2 Lipase inhibition assay

Lipase inhibition is the most widely used method for the determination of anti-obesity. Orlistat is the only commercial drug used for pancreatic lipase inhibition. *Simarouba glauca* plant extracts (methanol, ethanol, and hydro-alcohol) extracts six concentrations were prepared. Absorbance was taken at 400nm on an ELISA reader absorbance of the blank was 3.70, and ethanol extract showed high lipase inhibition at 500μg/ml as compared to standard orlistat presented in Figure 1 followed by hydro-alcohol and methanol. Further IC_{50} value of plant extracts (methanol, ethanol and hydro-alcohol) and standard was found to be 23.52, 12.56, 14.56 and 1.2 respectively.

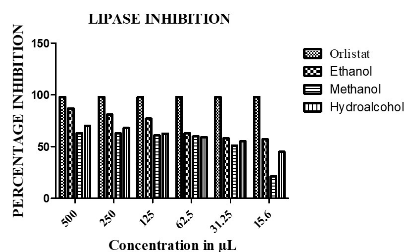

Figure 1: Lipase inhibition assay for extract of *S. glauca*

3.3 Amylase inhibition assay

Alpha amylase inhibition reduces sugar levels. Five different concentrations (50, 100, 150, 200, and 250mg/ml) of each extract were prepared. Metformin was used as positive control and absorbance was taken at 400nm on a UV spectrophotometer. Ethanol extract among methanol and hydro-alcohol extract showed a significant effect of amylase inhibition as seen in Figure 2 IC_{50} value of amylase inhibition assay against plant extracts (methanol, ethanol and hydro-alcohol) and standard was found to be 124.52, 51.56, 72.63 and 1.6 respectively.

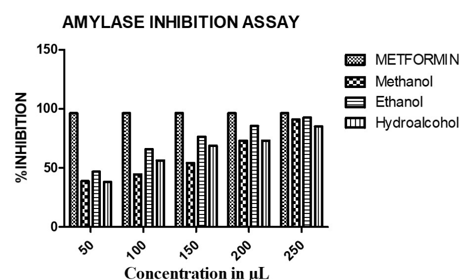

Figure 2: Amylase inhibition assay for extract of *S. glauca*

3.4 Oil Red O-staining activity

Lipid accumulation activity was measured using oil red o staining. High color intensity can be seen because of increased differentiation and triglyceride accumulations. Ethanol extract showed a higher degree of anti-obesity potential represented in Figure 3 whereas hydro-alcohol and methanol showed a lower degree of lipid accumulation.

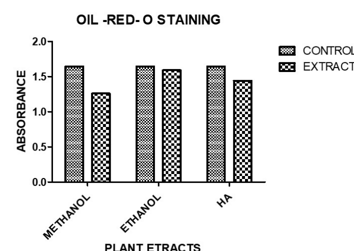

Figure 3: Oil-Red-O staining for extract of *S. glauca*

3.5 MTT assay

The MTT assay is done to determine the cytotoxicity of cells. It is a calorimetric test for assessing the metabolic activities of the cell. Three different types of plant extracts have been used i.e., ethanol, methanol, and hydro-alcohol of each six dilutions were prepared and orlistat is used as standard. Each concentration was taken in triplicates in 96 well plates. Percent inhibition was observed at 570 nm using an ELISA reader. The plant extract of *Simarouba glauca* was exposed for ant proliferative activity using an MTT assay

using different concentrations (200, 100, 50, 25, 12.5, and 6.5 μg/ml) for 48 hours. Ethanol and hydro-alcohol extract showed good anti-obesity potential at 200 μg/ml against standard drug orlistat (Figure 4). IC_{50} value of plant extracts (methanol, ethanol and hydro-alcohol) and standard was found to be 42.54, 10.32, 4.5 and 1.1 respectively.

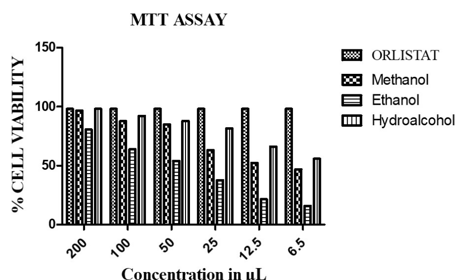

Figure 4: Cytotoxicity study of *S. glauca* plant extract

4 DISCUSSION

Obesity is a serious problem in the world. It is growing day by day. It occurs because of imbalance which is the gain and loss of energy leading to enlarged fat mass. An increase in the amount of fat leads to many health problems such as diabetes mellitus, fatty liver disease, obesity, asthma, hypertension, and osteoarthritis. Factors that cause obesity are a sedentary lifestyle such as lack of physical exercise, intake of high-energy snacks during stress, playing video games, and use of elevators, and cars have led to obesity throughout the world [12]. Current studies have focused on the use of natural resources for reducing obesity with fewer side effects. Limited literature has been reported on the anti-obesity potential of *Simarouba glauca* leaf extracts this study aims to determine whether all three different solvent extracts such as methanol, ethanol, and hydroalcoholic possess an anti-obesity effect or not by using 3T3-l1 cell line by *in-vitro* assays. The plant was collected from ICMR Belagavi, identified according to taxonomical characters as *Simarouba glauca*, and analyzed for different anti-obesity assays. Three solvent extracts were prepared namely ethanol, methanol, and hydroalcoholic. Pancreatic lipase inhibitory properties have been studied on many medicinal plants as an anti-obesity agent reported anti-lipase activity of methanolic extract of various plants using different concentrations with orlistat as a positive control [13]. In the present study ethanol extract showed very high lipase inhibition that is 87% at 500μg/ml. Different concentrations were prepared (500, 250, 125, 62.5, 31.25 and 15.6). Methanol extract showed 63% inhibition at 500μg/ml and hydroalcoholic 70% inhibition. The standard drug orlistat was used it gave 98.1% lipase inhibition. A similar study was done by Cheda et al., in 2016 [14] reported the lipase inhibitory potential of commonly used Indian spices they have taken three different extracts, and 4 concentrations were prepared (1, 3, 10 & 10 μg/ml) significantly inhibit pancreatic lipase which is compared with orlistat standard drug for obesity. Other published extracts showed high anti-lipase activity of more than 30% of cells [15]. Orlistat was used as a positive control it inhibited 42%. In the present study, the anti-obesity potential of *Simarouba glauca* leaf extract has been studied for the alpha-amylase activity of different plant extracts methanol, ethanol, and hydroalcoholic. Among the three extracts ethanol extract showed the highest amylase inhibition at 250 μl that is 92.58% followed by methanol with 90.97% inhibition as compared with standard drugs i.e., Metformin 96.3%. Similarly, in earlier study alpha-amylase inhibitory effect of some indigenous plants with three different concentrations i.e. 5mg/ml, 7mg/ml, and 9mg/ml showed the highest amylase inhibition of 71.93% at 9mg/ml concentration [16]. Lipid accumulation was measured using the oil red O staining technique. 3T3-l1 fibroblast cells were cultured with DMEM media to grow for 6 to 8 days with 10% FBS and antibiotics until proper cell structure was formed. *S. Glauca* plant extracts were dissolved in DMSO it did not affect the cells. Ethanol extract showed the highest absorbance which is 1.59 followed by hydroalcoholic at 1.44 and methanol extract at 1.26. The effect of fat droplets on plant extract was seen. High color intensity showed high lipid accumulation. Authors reported in their study high percentage of lipid accumulation was seen in 3T3-l1 cells that were cultured in a differentiated medium for 12 days in the absence and presence of extract. EGCG treatment prevents lipid accumulation in cells [17]. In vitro, cytotoxicity assay by using a 3T3-l1 mouse fibroblast cell line was performed. Various concentration of *S. glauca* plant extract was prepared (200, 100, 50, 25, 12.5, and 6.25 μg/ml) and positive control orlistat for 48 hours. Each extract was taken in triplicate form. The cell suspension was placed on 96 well plates absorbance was measured at 570 nm by an ELISA reader and the percentage was calculated. Percent inhibition was higher for hydroalcoholic extract followed by methanol extract at a higher concentration of 200 μg/ml. Compared with other extracts most effective cell viability is seen in hydroalcoholic extract. Standard anti-obesity drug orlistat which is commonly used for the treatment of obesity is found to be similar with methanol and ethanol extract. The cells were microscopically examined before performing any experiment. Morphological changes were observed at each step. Media was changed at regular intervals of time with FBS, and an antibiotic (Kanamycin) was added and kept in 5% CO_2 incubator for growth. Researchers reported 3T3-l1 cells treated for 48hrs and 72 hrs incubation period did not alter the cell viability [18].

5 CONCLUSION

The present study suggests that *Simarouba glauca* has shown high potential anti-obesity activity. This plant is

traditionally used for the prevention and treatment of various diseases such as malaria, fever, diarrhea, and gastrointestinal disorders. Among all three *S. glauca* leaf extract ethanol, methanol, and hydro-alcohol ethanol extract showed good effects for anti-obesity compared to the other two extracts with 3T3-l1 cells. Further in-silico and *in vivo* studies can be done to prove the anti-obesity potential of the plant. Alpha amylase activity can be studied to see the molecular mechanism of plants from which glucose homeostasis is regulated by the compounds.

- **Financial interests:** The authors declare they have no financial interests.
- **Conflicts of Interest:** Nil
- **Authors' contributions:** Sweta Gupta conducted experimental work, Shridevi Puranik and Rubeen Nadaf drafted manuscript, Mujeeb M. A, Bhushan Kulkarni, Abhijit Bhatkal, Ravindranath Aladkatti, and Vijay M. Kumbar analysed data, Shridhar Ghagane designed and reviewed manuscript. All authors commented on the manuscript and approved the final manuscript.

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