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ORIGINAL ARTICLE

In vitro Evaluation of *Hydrilla verticillata* for Anti-Adipogenesis Activity on 3T3 L1 Cell Lines

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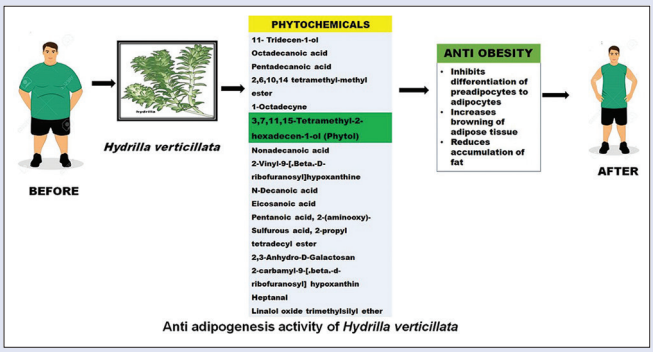
ABSTRACT

Background: Obesity is a metabolic disorder that has reached pandemic proportions worldwide. Phytol, a diterpene isoprenoid alcohol found as an integral part of chlorophyll of plants, algae, and guts of ruminant animals, is proven for its lipid-lowering activity. This compound is largely present in the Indian aquatic medicinal plant, *Hydrilla verticillata*. The research is focused to screen the anti-adipogenesis activity of *H. verticillata* extract on 3T3 L1 cell lines. **Materials and Methods:** The ethanolic extract of *H. verticillata* was prepared and characterized by gas chromatography–mass spectroscopy and Fourier transform infrared spectroscopy analysis. *In vitro* cytotoxic effect of *H. verticillata* extract on 3T3 L1 cell line was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. The ethanolic solvent extract of this plant is screened for anti-adipogenesis activity by performing *in vitro* pancreatic lipase inhibition and Oil Red O staining assay. **Results:** Phytochemical analysis and characterization of the extract showed the presence of a large amount of phytol in *H. verticillata*. *In vitro* pancreatic assay of the crude extract showed maximum lipase inhibition activity at a minimal concentration of 125 µg/mL, similar to the standard anti-obesity drug, orlistat. The half-maximal inhibitory concentration (IC_{50}) of the extract on 3T3 L1 cell line was 840.91 µg/mL. Cells treated with one-third IC_{50} showed minimal lipid accumulation, which is determined by Oil Red O staining. **Conclusion:** This study confirms that the ethanolic extract of *H. verticillata* possesses anti-adipogenesis activity, which can be used to control obesity-related treatments.

Key words: Adipose tissue, lipase inhibition activity, obesity, Oil Red O staining, phytol

SUMMARY

- The present study revealed the anti-adipogenesis activity of the aquatic plant *Hydrilla verticillata* on 3T3 L1 cell lines
- Characterization studies, Fourier transform infrared spectroscopy and gas chromatography–mass spectroscopy, confirmed the successful extraction of phytol from *Hydrilla verticillata* using ethanol
- Differentiation of 3T3 L1 cell lines treated with hydrilla extract significantly inhibited the lipid accumulation and also highly reduced the lipase activity.
- This study paves a way to find a cure for obesity and also prevent its occurrence.



Anti adipogenesis activity of *Hydrilla verticillata*

Abbreviations used: FTIR: Fourier transform infrared spectrophotometer; GC-MS: Gas chromatography–mass spectroscopy; NIST: National Institute of Standards and Technology; NCCS: National Centre for Cell Science; PL: Pancreatic lipase; PNPB: p-Nitrophenyl butyrate; MTT: (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO: Dimethyl sulfoxide DMEM: Dulbecco's Modified Eagle Medium; MDI: Methylisobutylxanthine, dexamethasone, insulin; PBS: Phosphate-buffered saline; FBS: Fetal bovine serum; TBT: Tributyltin chloride; GI: Gastrointestinal.

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INTRODUCTION

Obesity is a complex disorder caused due to excessive accumulation of fat in the body. Body mass index (BMI) is an index of total body fat mass with respect to an individual's weight and height. For a person, a BMI of 30 and above is defined as obese. The epidemic of obesity has drastically increased and created a major havoc on public health. Obesity is considered to be a silent killer leading to many other diseases, such as cardiovascular disease, diabetes, hypertension, and central nervous disorders. Obesity is fatal in most of the conditions; it is estimated that 600 million adults (12%) and 100 million children are affected worldwide. As per the World Health Organization reports, the number of people affected with obesity has tripled from 1975 to date.[1] The main cause of obesity is proven to be the consumption of high-fat diet, stress, lack of energy balance, etc.

The currently available therapy for obesity is targeted toward the reduction of food intake either by reducing appetite or by decreasing the cravings. Although these are found to be effective, they pose certain side effects such as cardiovascular disease and diabetics.[2,3] Recent

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studies conducted by researchers have proven that compounds capable of stimulating the browning of adipocytes are potent anti-obesity drugs and this has been well established in mice models.[4]

Pertaining to the concept of stimulation of browning, many compounds were screened. Among these, Among the compounds responsible for stimulation of browning, phytol was found to stimulate the browning of mice inguinal white adipose tissue due to modulation in the adenosine monophosphate-activated protein kinase signaling pathway.[5] Furthermore, the phytol exerts anti-obesity effect by inhibiting β-hydroxy β-methylglutaryl-CoA reductase enzyme and thereby reducing cholesterol levels.[6] Phytol, a branched-chain fatty alcohol, is a chlorophyll constituent of the plants and is liberated in ruminant animals as a gut product fermentation of ingested plant materials.

Recently, phytol and its derivatives have been proven to own many important pharmacological roles in humans and other animals. It also has been established as a cholesterol-lowering agent. Phytol-treated animals maintained uniform levels of cholesterol.[7]

Several studies have been carried out to study the antioxidant, toxicity, cytotoxicity, and teratogenicity properties of phytol.[8] Phytol can be obtained synthetically or naturally from various sources. It is higher in *Hydrilla verticillata*, commonly called water thyme, an aquatic weed profusely spread in several parts of Asia to all continents.

H. verticillata is a submerged, indigenous Indian freshwater weed that spreads rapidly and can survive in water depths ranging from a few centimeters to 14 m depth and grows up to 2.5 cm in 1 day. Its mechanism of vegetative reproduction causes dense surface mats that hinder recreation, navigation, and water intake operations.[9] These aquatic macrophytes are important in the trophic level of the food chain and the biogeochemical cycle. Their excessive presence in a water body is detrimental, reduces biodiversity, decreases the dissolved oxygen concentration, and also creates conditions for water to become stagnant favoring the breeding ground for mosquitoes.[10] In spite of this socio-economic impacts of this plant, it is credited with various medicinal applications. It was reported that the antioxidant, antimicrobial, and other biological activities may be due to the various phytochemicals present in this plant.[11] It has nearly about 50%–60% protein content and major phytochemicals such as terpenoids, flavonoids, and organic acids.[12,13] Antimicrobial compounds of *H. verticillata* have the potential to serve as biopreservatives and bioinsecticides, with potential use for the development of genetically modified crops with increased disease resistance.[14] This plant weed exhibits antitumor activity and also antibacterial properties and is being used to improve gastrointestinal (GI) function, blood flow, and control blood glucose level. It also detoxifies and decalcifies the pineal gland and is used for stress relief purposes. It has a sedative effect on the sympathetic nervous system and facilitates the process of muscle contraction in the cell. Moreover, this herb supports the immune system and provides more nutrition, which results in healthy skin and hair.[11]

3T3 L1 is a mouse embryo tissue-derived adipocyte used to study the cellular mechanism involved in the obesity-related diseases. 3T3 L1 cells have the ability to differentiate into an adipocyte-like cell and accumulate lipid in suitable condition. Among other cell lines, 3T3 L1 is easier to carry out and economically feasible to culture. The homogeneous response of the cell lines is very helpful in monitoring changes in experimental conditions very easily.[15]

The research is focused to assess the ethanolic extract of *H. verticillata* for anti-adipogenesis activity *in vitro* in 3T3 L1 cell lines.

MATERIALS AND METHODS

Materials

All chemicals used in the analysis are of analytical grade and were procured from Sigma-Aldrich and Janaki Scientific Company, India. The drug orlistat 60 mg (Meyer's Reeshape, USP) capsule was bought from a local pharmacy, Chennai, India. 3T3 L1 cell lines used for anti-adipogenesis assay were acquired from National Centre for Cell Science, Pune, India. The *H. verticillata* plant was taken from the lake near Sriperumbudur, Tamil Nadu, India.

Extraction of *Hydrilla verticillata*

The *H. verticillata* plant collected from the lake was identified according to the guidelines described by the literature.[16,17] The plant materials were washed rigorously using distilled water and sodium hypochloride for proper sterilization. The leaves were then dried well and made into powder form and stored at 4°C for later use. The extract of the plant was obtained by Soxhlet extraction method. 20 g of dried powder was extracted with 250 mL ethanol, and the obtained extract is stored and used for further analysis.[18]

Characterization of *Hydrilla verticillata* extract

Phytochemical test

To confirm the presence of diterpenoids, preliminary phytochemical analysis (Salkowski test) was performed. In a test tube containing 1 mL of *Hydrilla* extract, 1 mL of chloroform was added followed by 1 mL of concentrated sulfuric acid, shaken well, and observed for reddish brown layer formation at the interface.[19]

Fourier transform infrared spectroscopy analysis

Fourier transform infrared spectroscopy (FTIR) is an analytical technique for identifying chemical characterization/functional groups present in a molecule. The infrared light absorbed gives the spectral fingerprint representing the chemical information of the molecule. FTIR absorption spectra were recorded in the transmission mode with a range of wavelength (400–4000 cm^{-1}) at 4 cm^{-1} spectral resolution.

Gas chromatography–mass spectroscopy analysis

Gas chromatography–mass spectroscopy (GC-MS) analysis is a well-known established hyphenated technique for the analysis of volatile chemical species. The chemical components present in the *Hydrilla* extract was analyzed using a Clarus 680 GC (PerkinElmer) instrument. The temperature during the analysis was increased at a rate of 10°C/min from 50°C to 250°C to evaporate the sample components. For the analysis, 2 μL volume of sample was injected in the 10:1 split mode and run for 30 min. Both the injection port and detector temperature were maintained at 250°C, and 99.999% pure inert helium gas with a flow rate of 1 mL/min was used as a mobile phase carrier gas. MS was performed in the electron impact mode, a gas phase ionization source, and the mass spectrum was plotted by recording the ion signal as a function of the mass-to-charge ratio in the range 45–450 AMU. The ionization is done using electrons at 70 eV; the temperatures of the inlet and source were 200°C. The obtained mass spectrum pattern of the sample was verified with the U.S. National Institute of Standards and Technology online library (Version 11).[18]

Pancreatic lipase inhibition assay

The inhibition of the pancreatic lipase (PL) activity was measured using the spectrophotometric assay using p-nitrophenyl butyrate (PNPB) as a substrate. The working solution of PNPB was prepared by adding 8.403 μL of stock solution in 10 mL by acetonitrile. The solution of the standard drug was prepared by dissolving one capsule content of orlistat

in 12 mL of dimethyl sulfoxide (DMSO). Orlistat and extract (25 µL) were incubated with 50 µL of enzyme solution (6 mg of porcine PL enzyme dissolved in 10 mL of buffer solution), 100 µL of pH 7.2 buffer solution (100 mM phosphate-buffered saline [PBS], 150 mM sodium chloride, and 0.5% Triton-X-100), and then 25 µL of PNPB solution for 30 min at a temperature of 37°C. The absorbance values were noted at 400 nm using ELISA reader.[20]

$$\%\,\text{Pancreatic Lipase Inhibition} = \frac{\text{Absorbance of blank} - \text{Absorbance of test}}{\text{Aborbance of blank}} \times 100$$

In vitro studies on 3T3 L1 cell lines

In vitro studies were carried out on 3T3 L1 cell lines. The cell line was cultured in media consisting of glucose incorporated Dulbecco's modified Eagles medium (DMEM) enriched with 5% fetal bovine serum (FBS) and antibiotic solution. The 3T3 L1 cells were grown in 25 cm^2 T flasks until they reach 100% confluency at 37°C with 5% CO_2.[20]

Cytotoxicity studies by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cytotoxic effect of *H. verticillata* extract in the 3T3 L1 cells was evaluated using (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Varying *Hydrilla* extract concentrations from 62.5 to 1000 µg/mL were prepared using DMEM with a supplementary formulation of 2% inactivated FBS. The trypsinized 3T3 L1 monolayer cells were cultured in DMEM containing 10% FBS at a concentration of 1.0×10^5 cells/mL. 0.1 mL volume of cell suspension containing nearly 10,000 number of cells per well was seeded into 96-well microplates, and the monolayer formed after 24 h was washed with the medium and added with 100 µL volume of varying concentrations of *Hydrilla* extract. After 1 day of incubation under 5% CO_2 atmospheric condition and at 37°C, the solutions in the wells were removed and washed with PBS. 50 µL aliquot of MTT in PBS solution was then added to each well, respectively, and incubated following the previous step condition for 4 h. Formazan formed was solubilized using 100 µL of DMSO, and the cell viability was recorded by reading the absorbance at 540 nm.[21,22] The percentage of growth inhibition of 3T3 L1 cells was determined by:

$$\text{Growth Inhibition}\,(\%) = 100 - \left[\left[\frac{\text{Mean absorbance of test group}}{\text{Mean absorbance of control group}}\right] \times 100\right]$$

Anti-adipogenesis assay by Oil Red O staining

The anti-adipogenesis studies were carried out on the 3T3 L1 cell lines by evaluating the quantitative effect of *Hydrilla* extract on the intracellular accumulation of lipid droplet using Oil Red O staining method. 3T3 L1 cell lines were grown in methylisobutylxanthine dexamethasone, insulin (MDI) induction medium, a differential medium to induce differentiation of cells. The assay was carried out in six-well plates seeded with cells. After cells reached 70% confluency, the medium was discarded, and tributyltin chloride, orlistat, and extract were mixed proportionally and made up to 1 mL using MDI induction medium and labeled as day 0. On day 3, the MDI induction medium was removed from the cells and was replaced with test components (reconstituted in insulin medium). On day 6, insulin medium was removed from the cells and fresh DMEM was added. On day 7–10, fully differentiated adipocyte-like cells were observed by staining.

Before fixation, the cells were washed with PBS solution twice and incubated with 10% formalin for a period of 30 min. The formalin was discarded and the cells were again washed twice using distilled water. The freshly prepared diluted Oil Red O working solution (0.5% (w/v) Oil Red O in isopropanol) was added and incubated for 60 min at room temperature to stain. The stained cells were extracted with the solvent isopropanol, and the absorbance was measured at 500 nm using a microplate reader.[20]

$$\text{Lipid Inhibition}\,(\%) = 100 - \left[\left[\frac{\text{Mean absorbance of test group}}{\text{Mean absorbance of control group}}\right] \times 100\right]$$

In this study, untreated 3T3 L1-treated cell lines were used as control, standard drug orlistat-treated cell lines as positive control, tributyltin chloride (TBT) an inducer of adipogenesis-treated cell lines as negative control, and 1/3rd, 1/5th, and 1/10th half-maximal inhibitory concentration (IC_{50}) of *Hydrilla* extract as test groups.

Statistical analysis

All data expression is done using mean ± standard deviation. Significant results were reported by one-way analysis of variance follow-up test by Tukey's HSD *post hoc* multiple comparisons with the help of GraphPad Prism Scientific Software (San Diego, California). $P < 0.05$ is considered as statistically significant.

RESULTS

Characterization of *Hydrilla verticillata* extract

Phytochemical test

In this study, the ethanolic extract of *H. verticillata* was obtained by the Soxhlet extraction method. Salkowski test was performed for the preliminary phytochemical test to confirm the presence of phytol. The reddish brown ring formation [Figure 1] indicates that the phytol compound is present in the extract.

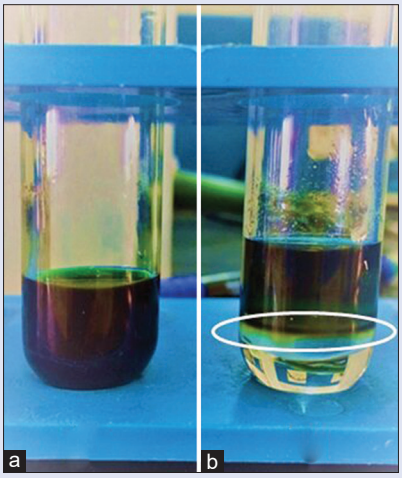

Figure 1: Confirmatory test for phytol. (a) Crude ethanolic extract of *Hydrilla verticillata*, (b) appearance of reddish brown ring

Table 1: Structural features of the *Hydrilla verticillata* extract by Fourier transform infrared spectroscopy spectrum

Peak number	Wave number (/cm)	Functional groups
1	3900-3200	OH stretching
2	2921	Alkyl C-H stretching
3	2301	Alkynes stretching
4	546	Aryl halides
5	478	Alkyl halides

Fourier transform infrared spectroscopy analysis

The spectra obtained from the FTIR analysis are shown in Figure 2, and the characteristic absorption of the various functional groups in the crude ethanolic extract of the weed *H. verticillata* is represented in Table 1. The FTIR spectrum of *Hydrilla* extract showed a characteristic peak at 3900, 2921, 2301, 546, and 478 cm^{-1}, indicating the presence of hydroxyl groups, alkyl groups, alkynes, and other groups, respectively.

Gas chromatography–mass spectroscopy analysis

The presence of bioactive molecules in the ethanolic extract of *H. verticillata* was found through the mass spectrum, as shown in Figure 3. It shows the time spent in the column and the response peaks for the various bioactive analytes present in the extract. The parameters used to assess these compounds including retention time, molecular formula, molecular weight, and concentration (%) are summarized in Table 2. Based on abundance, the four important phytochemicals present in the plant ethanol extract were 11-tridecen-1-ol (18.966%), phytol (15.567%), 2,6,10-trimethylundeca-1,3-diene (11.217%), and 1-octadecyne (6.491%). Figure 4 shows the mass spectra of the four most prevalent compounds identified by comparison of the mass spectra and measured retention indices results with the NIST library. Table 3 shows the bioactivity of the phytochemicals found in the plant ethanolic extract by GC-MS.[18]

Pancreatic lipase inhibition assay

The graph [Figure 5] shows the percentage inhibition of PL by ethanolic extract of *H. verticillata* and standard drug (orlistat). *Hydrilla* extract and orlistat showed maximum inhibitory activity at 125 µg/mL.

In vitro cytotoxicity study by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

In vitro cytotoxicity testing is a quantitative colorimetric assay mainly carried out using MTT and is done to assess the cellular metabolic activity. In viable cells, the enzyme mitochondrial dehydrogenase cleaves the MTT and produces formazan as a purple color product, and the concentration of this formazan directly relates the number of viable cells and indirectly the cytotoxicity. The cytotoxicity effects of *Hydrilla* extract were tested against the 3T3 L1 cell lines at concentrations of 62.5–1000 µg/mL. The difference in percentage survival and cytotoxic effect of the 3T3 L1 cell lines treated with different concentrations of *Hydrilla* extract is presented in Figure 6. From this study, it is observed that the difference in percentage survival of adipocytes treated with 250 µg/mL of the extract shows significant decrease when compared to control group. There is a gradual decline in percentage survival of cells as concentration of extract increases. The IC_{50} of the extract on 3T3 L1

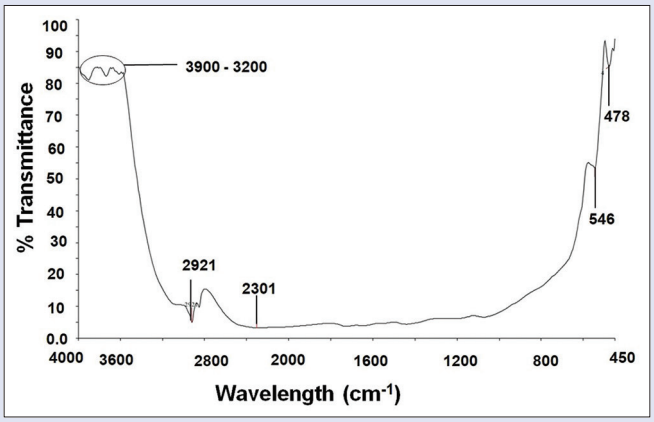

Figure 2: Fourier transform infrared spectrophotometer spectrum of ethanolic extract of *Hydrilla verticillata*

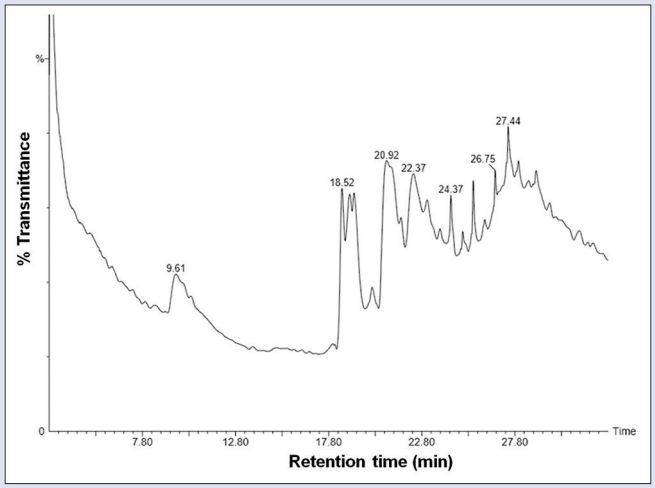

Figure 3: Gas chromatography–mass spectroscopy spectrum of ethanolic extract of *Hydrilla verticillata*

Table 2: Phytochemical compounds identified in the ethanolic extract of *Hydrilla verticillata* by gas chromatography-mass spectroscopy

RT (min)	Area (%)	Name of the compound	Molecular formula	Molecular weight (g/mol)
18.50	18.966	11- Tridecen-1-ol	$C_{13}H_{26}$	198
18.91	15.567	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	$C_{20}H_{40}O$	286
18.975	6.491	1-Octadecyne	$C_{18}H_{34}$	250
19.160	11.217	2,6,10-Trimethylundeca-1,3-diene	$C_{14}H_{26}$	194
19.280	5.981	1,6;3,4-Dianhydro-2-deoxy-.Beta.-D-lyxo-hexopyranose	$C_6H_8O_3$	128
20.891	4.839	Nonadecanoic acid	$C_{19}H_{38}O_2$	298
20.916	3.042	Octadecanoic acid	$C_{18}H_{36}O_2$	284
21.011	2.266	2-Vinyl-9-[.Beta.-D-ribofuranosyl] hypoxanthine	$C_{12}H_{14}O_5N_4$	294
21.046	3.813	N-Decanoic acid	$C_{10}H_{20}O$	172
21.191	2.893	Eicosanoic acid	$C_{20}H_{40}O_2$	312
21.286	3.164	Pentadecanoic acid	$C_{15}H_{30}O_2$	242
23.752	1.724	Pentanoic acid, 2-(aminooxy)-	$C_5H_{11}O_3$	133
24.352	4.206	Sulfurous acid, 2-propyl tetradecyl ester	$C_{17}H_{36}O_3S$	320
24.987	1.703	2,3-Anhydro-D-galactosan	C_6H_8O	144
25.533	4.465	2-carbamyl-9-[.beta.-d-ribofuranosyl] hypoxanthin	$C_{11}H_{13}O_5N_5$	295
26.753	2.048	Heptanal	$C_7H_{14}O$	114
27.428	3.484	Linalol oxide trimethylsilyl ether	$C_{13}H_{26}O_2Si$	242

RT: Retention time

cell line was 840.91 µg/mL. More dead cells were observed in the highest concentration of *Hydrilla* extract-treated cells.

Anti-adipogenesis assay by Oil Red O staining

Anti-adipogenesis effect of *Hydrilla* extract (test) and control on the 3T3 L1 cell lines for day 1 and 4 is given in Figure 7. There is a significant decrease in differentiation in the standard drug (orlistat) group on day 4 compared to day 1. A higher rate of differentiation was observed in the TBT used a negative control group. 1/3rd IC_{50} extract-treated adipocytes showed the least differentiation compared to other concentrations of *Hydrilla* extract. The results of staining on the anti-adipogenesis effect of *Hydrilla* extract and other control groups on 3T3 L1 are shown in Figure 8. TBT group shows a large number of lipid droplets compared to all other groups. Orlistat-treated group shows a lesser amount of lipid accumulation compared to control and TBT. 1/3rd IC_{50} extract-treated cell lines show lesser accumulation compared to control and 1/5th and 1/10th IC_{50} concentrations of the extract.

DISCUSSION

Stimulating the browning of adipose tissues is one of the key mechanisms to prevent adipogenesis and thereby reducing obesity.[23,24] In this study, the influence of ethanol solvent extract of aquatic plant *H. verticillata* on the regulation of adipogenesis is primarily studied. The phytoconstituents present in the *H. verticillata* extract exhibited anti-obesity effects by the inhibition of lipid accumulation in the adipogenesis process.

In the Salkowski test, reddish-brown color formation at the interface confirmed that the diterpenoid compound phytol is present in the

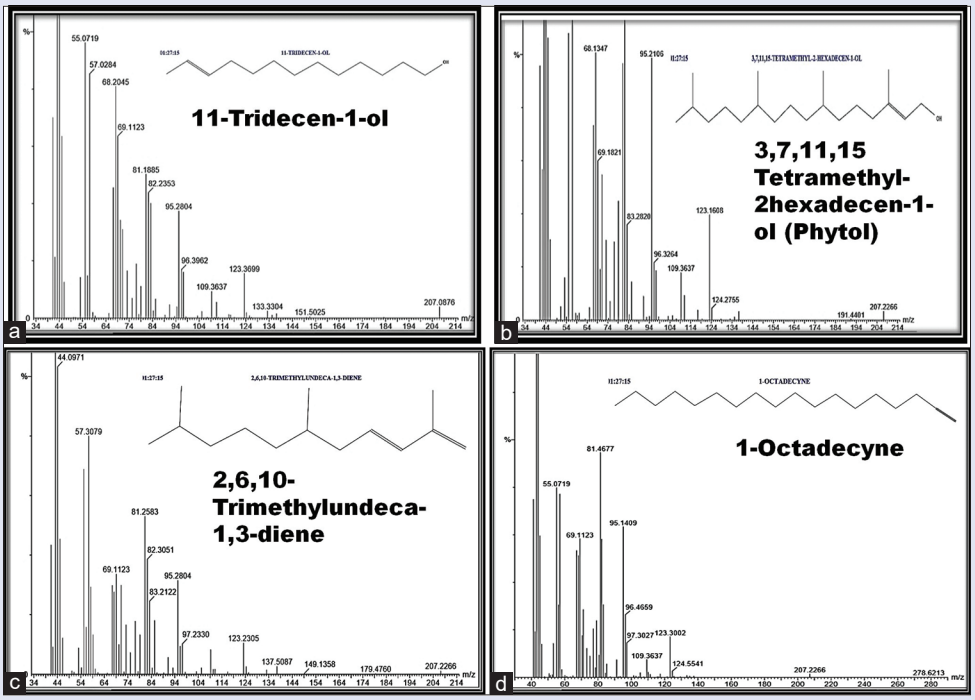

Figure 4: Mass spectrum of most prevalent compounds in ethanolic extract of *Hydrilla verticillata*: (a) mass spectrum of 11-tridecen-1-ol, (b) mass spectrum of 3,7,11,15-tetramethyl-2hexadecen-1-ol (phytol), (c) mass spectrum of 2,6,10-trimethylundeca-1,3-diene, (d) mass spectrum of 1-octadecyne

Table 3: Activity of different compounds identified in the ethanolic extract of *Hydrilla verticillata*

Name of the compound	Biological activity
11- Tridecen-1-ol	NA
3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	Antimicrobial, anti-inflammatory, antioxidant, diuretic
Octadecanoic acid	Antimicrobial, antioxidant hypertriglyceridemia
Pentadecanoic acid	Antimicrobial
2,6,10,14-Tetramethyl-methyl ester	Antimicrobial
1-Octadecyne	NA
1,6;3,4-Dianhydro-2-deoxy-Beta-D-lyxo hexopyranose	Antimicrobial
Nonadecanoic acid	Anticancer
2-Vinyl-9-[beta-D-ribofuranosyl] hypoxanthine	Antiviral
N-Decanoic acid	Antifungal
Eicosanoic acid	NA
Pentanoic acid, 2-(aminooxy)-	Antihelminthic
Sulfurous acid, 2-propyl tetradecyl ester	NA
2,3-Anhydro-D-galactosan	Antidiabetic
2-Carbamyl-9-[.beta.-d-ribofuranosyl] hypoxanthin	Neuroprotective, cardioprotective, anti-inflammatory, and immunomodulatory activities
Heptanal	Flavoring agent
Linalol oxide trimethylsilyl ether	Flavoring agent

NA: No activity

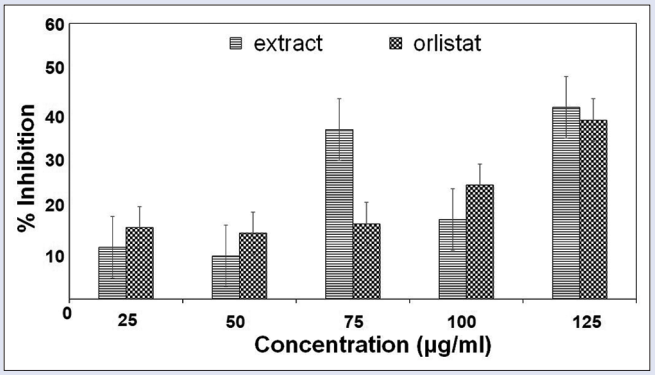

Figure 5: Graph showing the percentage inhibition of pancreatic lipase by ethanolic extract of *Hydrilla verticillata* and orlistat

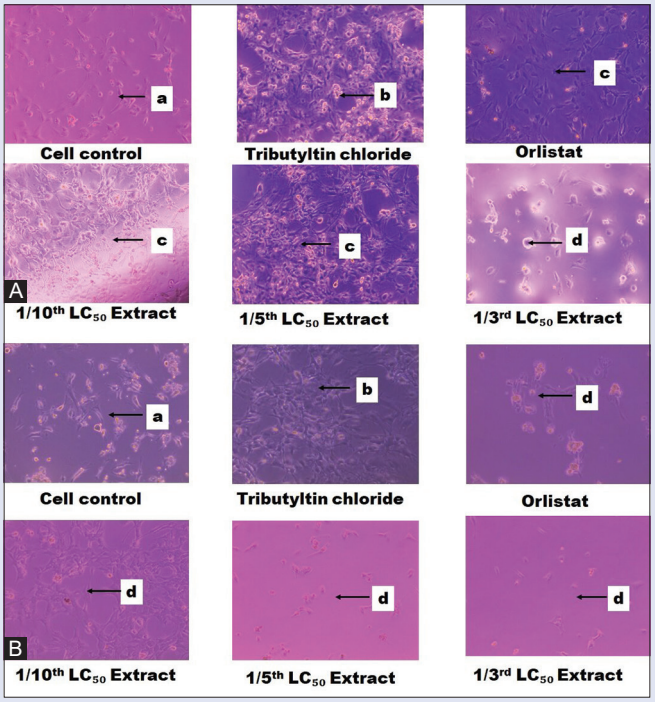

Figure 7: (A) Day 1 – Anti-adipogenesis effect of ethanolic extract of *Hydrilla verticillata* (test), tributyltin chloride (negative control), orlistat (positive control), and control (untreated) on 3T3 L1 cell lines. (B) Day 4 – Anti-adipogenesis effect of ethanolic extract of *Hydrilla verticillata* (test), tributyltin chloride (negative control), orlistat (positive control), and control (untreated) on 3T3 L1 cell lines. (a) Normal differentiated 3T3 L1 cell lines, (b) Increase in differentiated cell lines, (c) Total reduction in number of cells, (d) Dead cells. IC_{50} concentrations (concentration of drug causing 50% growth inhibition) of extract was found by plotting log (inhibitor concentration) versus response (% inhibition) from MTT assay using GraphPad Prism software. IC_{50} concentration of extract was found to be 840.91 µg/mL. $1/10^{th}$ IC_{50} = 84 µg/mL, $1/5^{th}$ IC_{50} = 160 µg/mL, $1/3^{rd}$ IC_{50} = 280 µg/mL. IC_{50}: Half-maximal inhibitory concentration; TBT: Tributyltin chloride

H. verticillata extract. The reddish-brown color is due to the formation of conjugated dienes, a resultant product of the protonation reaction between the hydroxyl group of phytol and sulfuric acid.[19]

Characterization studies, FTIR and GC-MS, also prove the confirmation of phytol presence in the ethanolic extract. In the FTIR analysis, the

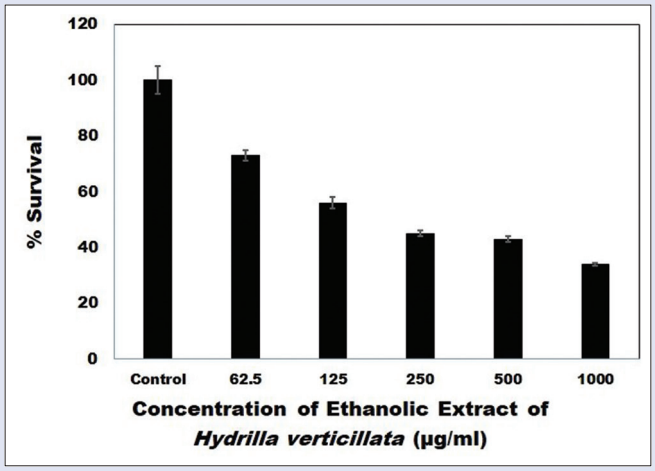

Figure 6: The difference in percentage survival of 3T3 L1 cell lines treated with different concentrations of ethanolic extract of *Hydrilla verticillata*. Data are presented as mean ± SD. $^{**}P < 0.05$ compared with the control group

absorption band at 3900 cm^{-1} corresponds to the stretching absorption of hydroxyl groups in the *Hydrilla* extract, showing the presence of terpenoids and thus representing the presence of phytol. The different peaks observed at 2921, 2301, 546, and 478 cm^{-1} confirmed the presence of symmetric stretching of saturated (sp^3) carbon, C (triple bond) C–H: C–H stretching, aryl, and alkyl halides, respectively.[25]

GC-MS analysis of the *H. verticillata* extract confirmed the presence of various high and low molecular weight phytochemicals that are biologically active. Seventeen bioactive compounds were identified including phytol as one of the most prevalent compounds in the extract.[18]

Inhibiting the PL enzyme present in adipocytes of the GI tract prevents the hydrolysis of the dietary lipids and thereby hinders lipid absorption through the GI lumen. This mechanism is believed to reduce the amount of body fat and hence the body weight. Therefore, adipocytes lipase enzyme can be set as a target enzyme to control obesity.[26,27] In this investigation, the ethanolic extract of *H. verticillata* inhibits lipase activity may, therefore, be recommended to control obesity. *Hydrilla* extract shows maximum lipase inhibition activity at 125 µg/mL due to the presence of a mixture of large amounts of flavonoids, terpenoids, and alkaloids in the plant extract.[28] The effective and specific binding of phytol in the extract to the active site of the enzyme may also increase their inhibitory action. This rationalizes its effectiveness in treating obesity and its related disorders such as hyperlipidemia.

In the anti-adipogenesis study, the lipid accumulation was not observed in the control 3T3 LI cell lines, whereas in the TBT-treated negative control group, TBT induced the differentiation of adipocytes. TBT belongs to the organotin group of compounds that are prevalent in sea foods and pesticides and widely distributed as environmental pollutants. It was proven to be an inducer of adipogenesis showing maximum effect even at 50 nM concentration.[29] On the other hand, the FDA-approved anti-obesity drug, orlistat was selected as the reference drug to compare the anti-adipogenesis activity. It was observed that similar to orlistat, the extract-treated cell lines show significant inhibition in differentiation and also a reduction in lipid accumulation activity, due to the presence of phytol. Zhang *et al.*[5] proved that phytol stimulates the adenosine monophosphate-activated protein kinase, which in turn causes the increase in brown adipogenic markers causing browning and reduction in differentiation. Thus, the *H. verticillata* extract contains a considerable

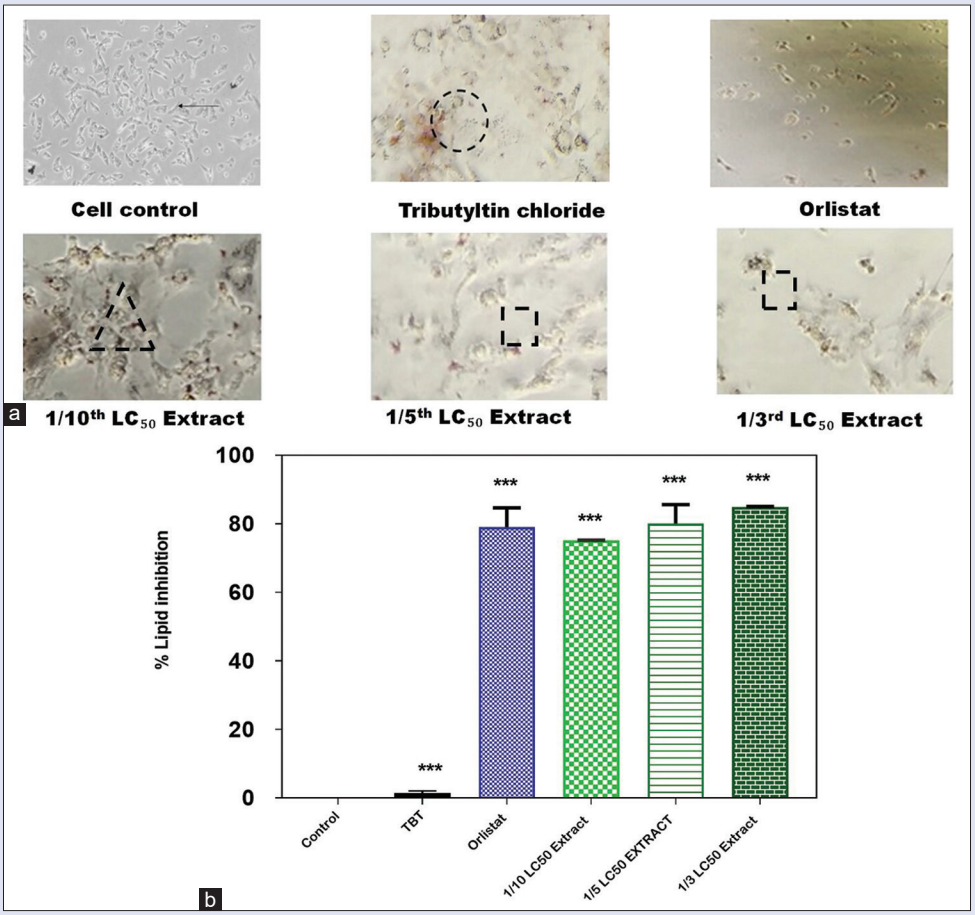

Figure 8: (a) Anti-adipogenesis effect of ethanolic extract of *Hydrilla verticillata* (test), tributyltin chloride (negative control), orlistat (positive control), and control (untreated) on 3T3 L1 cell lines stained with Oil Red O stain. Arrow – Normal differentiated 3T3 L1 stained with Oil Red O stain; circle – Increase in lipid accumulation; square – Reduction in lipid droplets; triangle – Partial reduction in lipid accumulation. (b) Anti-adipogenesis activity of ethanolic extract of *Hydrilla verticillata* (test), tributyltin chloride (negative control), orlistat (positive control), and Control. TBT: Tributyltin chloride

amount of phytol that prevents the differentiation of adipocytes and reduces lipid accumulation proving its possibility as an anti-obesity agent.

CONCLUSION

The findings of this study highlight the anti-adipogenesis potential of the aquatic plant *H. verticillata*. This is mainly due to the presence of phytol, a diterpenoid proven to contain lipid lowering activity. On evaluating the anti-obesity effect of the extract and the standard drug orlistat, the effect was comparable in terms of affecting the lipid accumulation. The differentiation of adipocytes was reduced to a greater extent by the extract without damaging the cells. Thus, the above findings indicate that this plant possesses anti-adipogenesis potential and is considered safe for consumption. *Hydrilla*-based foods could also be included in the daily diet which could prevent the occurrence of obesity. Phytol from the crude ethanolic extract of *H. verticillata* could be subjected to further purification and formulated as a drug to treat obesity. Thus, this study paves an alternate way to find a cure to prevent obesity.

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Conflicts of interest

There are no conflicts of interest.

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