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Research Article

THERAPEUTIC POTENTIAL: *UNDARIA PINNATIFIDA* AND *MORINGA OLEIFERA* EXTRACTS AS MODULATORS OF ADIPOGENESIS IN 3T3-L1 ADIPOCYTES

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ABSTRACT

Objectives: The objectives of the study are to investigate the effects of ethanolic extract of *Undaria pinnatifid*a (UPEA) and methanolic extract of *Moringa oleifer*a (MOM), alone and in combination, on the differentiation and viability of 3T3-L1 pre-adipocytes.

Methods: 3T3-L1 pre-adipocytes were treated with varying concentrations of UPEA, MOM, and their 1:1 combination. The impact on pre-adipocyte differentiation was assessed by measuring lipid accumulation. Cell viability was determined using a standard viability assay to evaluate cytotoxicity across a range of concentrations.

Results: UPEA and MOM, individually and in combination, reduced lipid accumulation in 3T3-L1 cells in a dose-dependent manner, thereby inhibiting differentiation into adipocytes. The combination treatment (1:1 ratio) demonstrated similar efficacy in reducing differentiation. Viability assays revealed minimal cytotoxic effects, with cell viability ranging from 89% to 99% across all tested concentrations.

Conclusion: UPEA, MOM, and their combination exhibit potent anti-adipogenic effects while maintaining low cytotoxicity in 3T3-L1 pre-adipocytes. These findings suggest their potential as therapeutic agents for conditions related to adipogenesis, warranting further studies on their biological activities and therapeutic mechanisms.

Keywords: Adipocytes, Obesity, Cell lines, Toxicity, Staining.

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INTRODUCTION

The cytotoxic effects of ethyl acetate extracts of *Undaria pinnatifida* (wakame), *Moringa oleifera* (drumstick tree), and their combination (ethanolic extract of *U. pinnatifida* [UPEA] and methanolic extract of *M. oleifera* [MOM]) were investigated on 3T3L1 cell lines, a widely used model for cytotoxicity assays. *U. pinnatifida* is known for containing fucoidan, while *M. oleifera* boasts a diverse phytochemical composition, making both plants intriguing candidates for therapeutic research.

Previous studies have highlighted the potential anticancer properties of fucoidan and fucoxanthin, found in *U. pinnatifida* and certain New Zealand seaweed species, respectively. Fucoidan has demonstrated promising anticancer activity against pancreatic cancer cells [1] while fucoxanthin has shown cytotoxic effects against various human cancer cell lines [2].

In addition, the benefits of anti-inflammatory compounds have been explored, with fucoxanthin showing promise in mitigating inflammation in lipopolysaccharide-stimulated macrophages [3,4]. Moreover, *M. oleifera* has garnered attention for its potential health benefits, with research indicating neuroprotective and its anti-inflammatory properties [5].

The current study builds upon this existing knowledge by assessing the cytotoxicity profile of ethyl acetate extracts of *U. pinnatifida*, *M. oleifera*, and their combination on 3T3L1 cells. Previous research suggesting low toxicity of these extracts on 3T3L1 cells provides a foundation for further investigation into their therapeutic potential [6].

Understanding the cytotoxicity profile of these extracts is essential for evaluating their safety and efficacy as potential therapeutic agents, contributing to the growing body of literature on their biological activities and therapeutic effects [7,8].

Further research has indicated the potential of seaweed bioactivities in industrial production and their therapeutic implications [7,8]. In addition, studies have elucidated the enzymatic conversion of brown seaweed biomass to bioethanol, showcasing its potential in sustainable energy production [9].

Furthermore, a thorough analysis of the pharmacology, phytochemistry, ethnopharmacology, genetics, and cultivation of *M. oleifera* leaves has shed light on the plant's several medicinal uses [10,11].

In conclusion, the integration of findings from various studies contributes to a comprehensive understanding of the potential health benefits of *U. pinnatifida*, *M. oleifera*, and their combination. This study could open doors for the creation of novel therapeutic interventions for various health conditions [16-18].

METHODS

Materials required

Cell lines

3T3-L1–Mouse embryo fibroblast cell line (NCCS, Pune).

Cell culture medium

- DMEM- high Glucose - (#AL111, HiMedia)
- Adjustable multichannel pipettes and a pipettor (Benchtop, USA)
- Fetal Bovine Serum (#RM10432, HiMedia)
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Reagent (5 mg/mL) (# 4060 HiMedia)
- DMSO (#PHR1309, Sigma)

- D-PBS (#TL1006, HiMedia)
- 96-well plate for culturing the cells (From Corning, USA)
- T25 flask (# 12556009, Biolite - Thermo)
- 50 mL centrifuge tubes (# 546043 TORSON)
- 1.5 mL centrifuge tubes (TORSON)
- 10 mL serological pipettes (TORSON)
- 10–1000 uL tips (TORSON).

Equipments

- Centrifuge (Remi: R–8°C)
- Pipettes: 2–10 µL, 10–100 µL, and 100–1000 µL.
- Inverted microscope (Biolink)
- 37°C incubator with humidified atmosphere of 5% CO_2 (Healforce, China).

Assay controls

i. Medium control (medium without cells)
ii. Negative control (medium with cells but without the experimental drug/compound)
iii. Positive control (medium with cells and 5% DMSO).

***In vitro* analysis on 3T3L1 cells**

Cytotoxicity assay -MTT assay

This calorimetric assay, known as the MTT assay, measures cell proliferation and cytotoxicity by reducing the yellow-colored, water-soluble MTT tetrazolium dye to formazan crystals. Live cells produce mitochondrial lactate dehydrogenase, which breaks down MTT into insoluble formazan crystals that turn purple when dissolved in the right solvent. The intensity of which may be detected spectrophotometrically at 570 nm and is proportional to the number of live cells [13].

Cytotoxicity activity of UPEA and MOM

In a 96-well microplate, 200 µL of 3T3L1 cell suspension was implanted at volume of 20,000 cells in each well and maintained for 24 h. These cells received treatment with UPEA, MOM, and UPEA+MOM (1:1) in different concentrations (12.5, 25, 50, 100, and 200 µg/mL). After 24 h incubation, media was removed, and cells were treated with 0.5 mg/mL MTT reagent. After 3 h, 100 µL of DMSO was added to the plates, which were then covered with aluminum foil to protect them from light. The plates were then gently shook to dissolve the formazan crystals, and they were allowed to incubate for a further 3 h at room temperature.

The following formula was used to determine the percentage of cell viability after the absorbance was measured at 540 nm.

$$\% \text{ of cell viability } = \frac{\text{Absorbance of control } - \text{ Absorbance of test}}{\text{Absorbance of control}} \times 100$$

With the use of the linear regression equation, Y=Mx+C, the IC_{50} value was found. Here, the viability graph was used to determine the values of Y=50, M, and C [14].

Preparation of Adipocyte differentiation medium

50 mL of 3-isobutyl-1-methylxanthine and 1mM of dexamethasone were dissolved in DMSO to create stock media and standard cell culture components (dexamethasone, isobutyl-methyl-xanthine, indomethacin, and insulin). Insulin was then added.

Preparation of test samples UPEA and MOM

The concentration of test compounds (25 ug/mL) was evaluated to check their anti-adipogenesis activity by enzyme-linked immunosorbent assay (ELISA) on 3T3L1 cells. Concentrations of the following substances used to treat the cells:

Pre-adipocyte 3T3 L1 cell line differentiation into adipocytes

A 6-well plate was seeded with 2000 µL of cell suspension (1×105 cells per well). Cells were given a 12-h window to grow. As samples were introduced to several wells, orlistat was used as the positive control, negative control (just medium), and UPEA (25 µg/mL), MOM (25 µg/mL), and UPEA+MOM (25 µg/mL 1:1 ratio). Plates were kept in an environment of 5% CO_2 and incubated for 14 days at 37°C. The culture media was changed every other day to nourish the cells. Oil Red O staining was used to observe differentiated adipocyte cells based on lipid accumulation following a 14-day incubation period.

Table 1: UPEA and MOM treatment in 3T3 L1 cell line

S. No	Test compounds	Cell line	Concentration treated to cells
1	Control	3T3L1	No treatment
2	UPEA	3T3L1	1 (25 µg/mL)
3	MOM	3T3L1	1 (25 µg/mL)
4	UPEA+MOM	3T3L1	1 (25+25 µg/mL)
5	Positive control	3T3L1	Adipogenesis medium

UPEA: Ethanolic extract of *Undaria pinnatifida*, MOM: Methanolic extract of *Moringa oleifera*

Table 2: Percentage of cell viability after treatment of ethanolic extract of *Undaria pinnatifida*

Concentration of samples (µg)	Percentage of cell viability
Blank	000
Untreated	100
Camptochetin (Toxic control)	41.52
12.5	76.26
25	89.45
50	96.18
100	98.51
200	99.15

Table 3: Percentage of cell viability after treatment of methanolic extract of *Moringa oleifera*

Concentration of samples (µg)	Percentage of cell viability
Blank	000
Untreated	100
Camptochetin (Toxic control)	41.52
12.5	69.14
25	84.02
50	89.71
100	94.11
200	98.31

Table 4: Percentage of cell viability after treatment of *Undaria pinnatifida* and methanolic extract of *Moringa oleifera* (1:1 ratio)

Concentration of samples (µg)	Percentage of cell viability
Blank	00
Untreated	100
Camptochetin (Toxic control)	41.52
12.5	87.26
25	89.45
50	97.33
100	98.76
200	99.64

Table 5: IC_{50} values of UPAE and MOM

Sample code	IC_{50} (µg/mL)
UPEA	412.8
MOM	333.06
UPEA+MOM	436.7

UPEA: Ethanolic extract of *Undaria pinnatifida*, MOM: Methanolic extract of *Moringa oleifera*

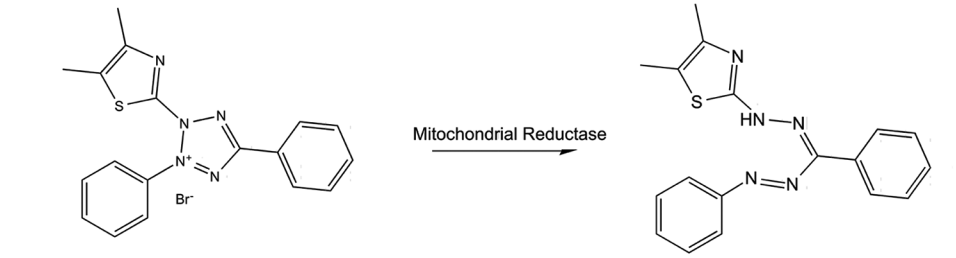

Fig. 1: Formazan crystal formation from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

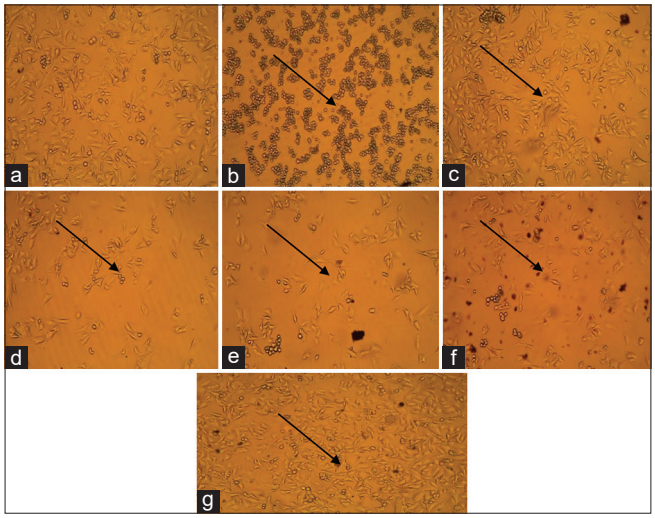

Fig. 2: Inverted microscopic observations of 3T3L1 cells treated with ethanolic extract of *Undaria pinnatifida* (UPEA) (a) Untreated cells (b) Cells treated with toxic control camptothecin (c) cells treated with UPEA with 12.5 µg/mL (d) cells treated with UPEA with 25 µg/mL (e) cells treated with UPEA with 50 µg/mL (f) cells treated with UPEA with 100 µg/mL (g) cells treated with UPEA with 200 µg/mL

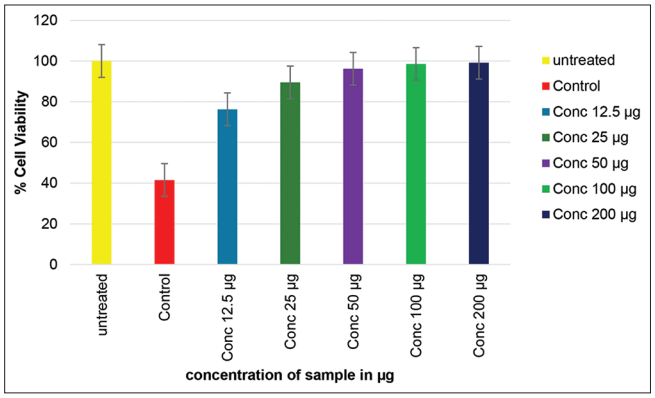

Fig. 3: Percentage cell viability of ethanolic extract of *Undaria pinnatifida* extract treated 3T3-L1 cells

Table 6: Oil red O staining of 3T3L1cell lines treated with ethyl acetate extract *Undaria pinnatifida* (UPEA) and methanolic extract of *Moringa oleifera* (MOM)

Sl. No	Test compounds	Cell line	Concentration treated to cells
1	Cell control	3T3L1	No treatment
2	UPEA	3T3L1	1 (25 µg/mL)
3	MOM	3T3L1	1 (25 µg/mL)
4	UPEA+MOM	3T3L1	1 (25+25 µg/mL)
5	Positive control	3T3L1	Adipogenesis medium

Oil red O staining of differentiated cells of UPEA and MOM

By using Oil Red O staining, differentiated adipocytes with accumulated lipids were identified. For 20 min, differentiated adipocytes were fixed in 4% paraformaldehyde. Times, wash with 1–1.5 mL PBS. After dissolving the oil red O coloring stock solution in ddH_2O at a ratio of 3:1, it was left to stand for 10 min. Filtered oil red O staining solution was placed into a container and promptly capped.

After staining the cells for 60 min with an oil O red working solution, the cells were cleaned with deionized water to get rid of any leftover dye. After that, the dish was mixed with 1 milliliter of isopropanol for 5 min. Monolayers that were stained were examined under a microscope. After that, 96-well plates were filled with 200 µL aliquots of the extracted dye, and the absorbance at 520 nm was determined.

RESULTS AND DISCUSSION

***In vitro* analysis on 3T3L1 cells**

Cytotoxicity activity of UPEA and MOM -MTT assay

Cytotoxicity activity of UPEA

The extracts UPEA with maximum antioxidant potential is selected for cytotoxicity assay on 3t3L1 cell lines. Cytotoxicity assay was performed for UPE proportion of living cells. At concentrations of 12.5 µg/mL UPEA exhibited 76.261% viability, 25 µg/mL exhibited 89.45%, 50 µg/mL exhibited 96.18%, 100 µg/mL exhibited 98.51%, and 200 µg/mL exhibited 99.15% in comparison with camptothecin (toxic control) with 41.52% of cell viability.

Fig. 3 exhibit the pictures taken using inverted microscopy of 3t3L1 cell lines following with 12.5, 25, 50, 100, and 200 µg/mL with UPEA along with control and camptothecin (toxic control). Images of 3t3L1 cell lines treated with control shows no change, cells treated with toxic control showed the cell lysis and debris, representing the death of nearly 60% of cells. The concentration of UPEA from 12.5 µg/mL to 200 µg/mL improved the percentage of cell viability, and the cells treated with 200 µg/mL demonstrated 99% cell viability.

Cytotoxicity activity of MOM- MTT assay

MOM with maximum antioxidant potential is selected for cytotoxicity assay on 3t3L1 cell lines. Along with cytotoxicity assay is performed for MOM to analyze cell viability.

At concentrations of 12.5 µg/mL UPEA exhibited 69.146% viability, 25 µg/mL exhibited 84.02%, 50 µg/mL exhibited 89.71%, 100 µg/mL exhibited 94.11%, and 200 µg/mL exhibited 98.31% in comparison with camptothecin (toxic control) with 41.52% of cell viability.

Images of 3t3L1 cell lines in an inverted microscope after treated with 12.5, 25, 50, 100, and 200 µg/mL of MOM along with control and

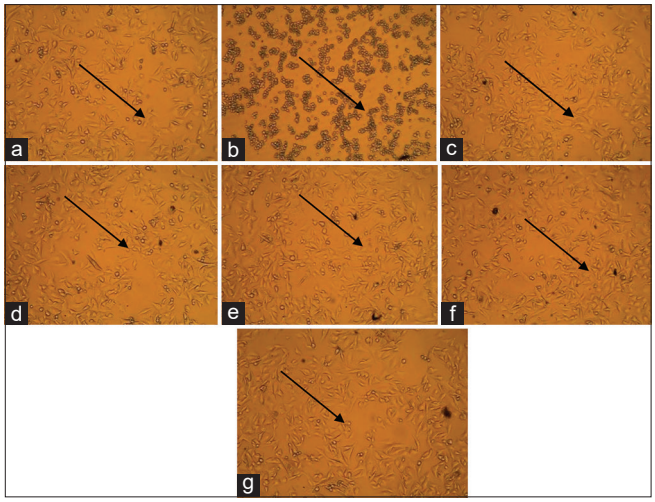

Fig. 4: Inverted microscopic observations of 3T3L1 cells treated with methanolic extract of *Moringa oleifera* (MOM) (a) untreated cells (b) cells treated with toxic control camptothecin (c) cells treated with MOM with 12.5 µg/mL (d) cells treated with methanolic extract of MOM with 25 µg/mL (e) cells treated with methanolic extract of MOM with 50 µg/mL (f) cells treated with methanolic extract of MOM with 100 µg/mL (g) cells treated with methanolic extract of MOM with 200 µg/mL

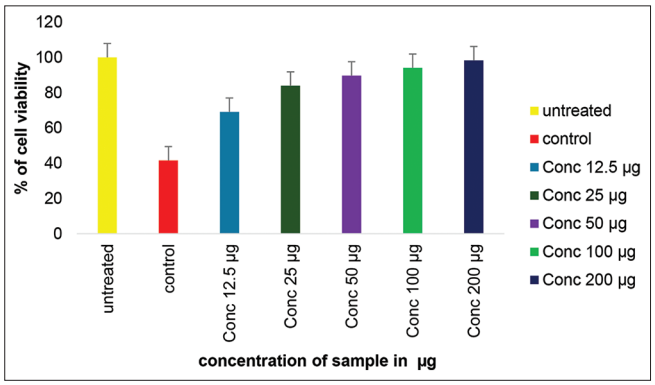

Fig. 5: Percentage cell viability of methanolic extract of *Moringa oleifera* extract treated 3T3-L1 cells

camptothecin (toxic control). The images of 3T3L1 cell lines treated with control show no change, cells treated with toxic control showed the cell lysis and debris, representing the death of nearly 60% of cells. Cells treated with 200 µg/mL showed 98% cell viability, as the concentration of UPEA from 12.5 µg/mL to 200 µg/mL was increased percentage of cell viability was also increased.

Cytotoxicity activity of UPEA and MOM

The extracts UPEA and MOM (1:1 ratio) with maximum antioxidant potential are selected for cytotoxicity assay on 3t3L1 cell lines. Along with cytotoxicity assay is performed for UPEA to check the percentage of cell viability.

At concentrations 12.5 µg/mL UPEA and MOM (1:1 ratio) exhibited 87.261% viability, 25 µg/mL exhibited 89.456%, 50 µg/mL exhibited 97.33%, 100 µg/mL exhibited 98.76%, and 200 µg/mL exhibited 99.64% in comparison with camptothecin (toxic control) which showed 41.52% of cell viability.

Fig. 7 depicts the images of 3t3L1 cell lines in inverted microscopy after treatment with 12.5, 25, 50, 100, and 200 µg/mL of UPEA and MOM (1:1) along with control and toxic control. Camptothecin 3t3L1 cell lines

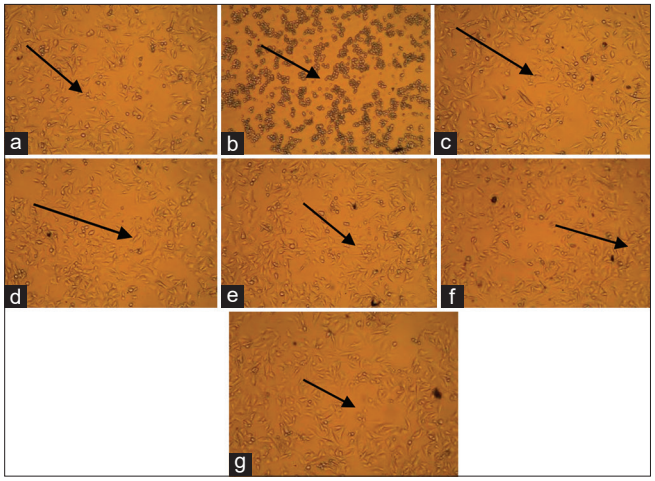

Fig. 6: Inverted microscopic observations of 3T3L1 cells treated with ethanolic extract of *Undaria pinnatifida* (UPEA) and methanolic extract of *Moringa oleifera* (MOM) (1:1 ratio) (a) Untreated cells (b) Cells treated with toxic control camptothecin (c) cells treated with UPEA and methanolic extract of MOM (1:1 ratio) with 12.5 µg/mL (d) cells treated with UPEA and methanolic extract of MOM (1:1 ratio) with 25 µg/mL (e) cells treated with UPEA and methanolic extract of MOM (1:1 ratio) with 50 µg/mL (f) cells treated with UPEA and methanolic extract of MOM (1:1 ratio) with 100 µg/mL (g) cells treated with UPEA and methanolic extract of MOM (1:1 ratio) with 200 µg/mL

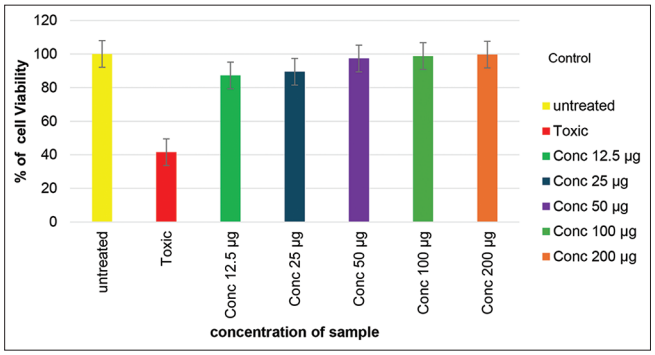

Fig 7: Percentage cell viability of *Undaria pinnatifida* and methanolic extract of *Moringa oleifera* extract treated 3T3-L1 cells

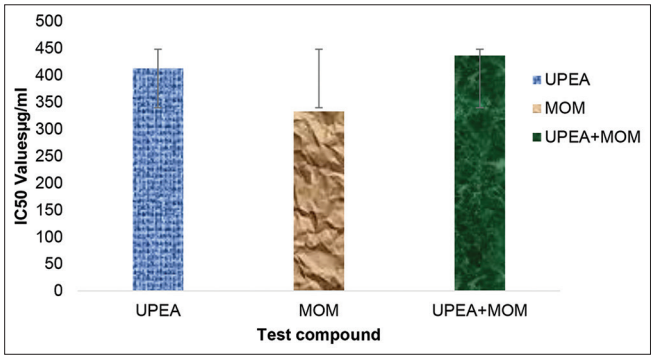

Fig. 8: IC_{50} (µg/mL) *Undaria pinnatifida* and methanolic extract of *Moringa oleifera*

treated with control show no change, cells treated with toxic control showed cell lysis and debris, representing the death of nearly 60% of cells. Cells treated with 200µg/mL showed 99% cell viability, as the

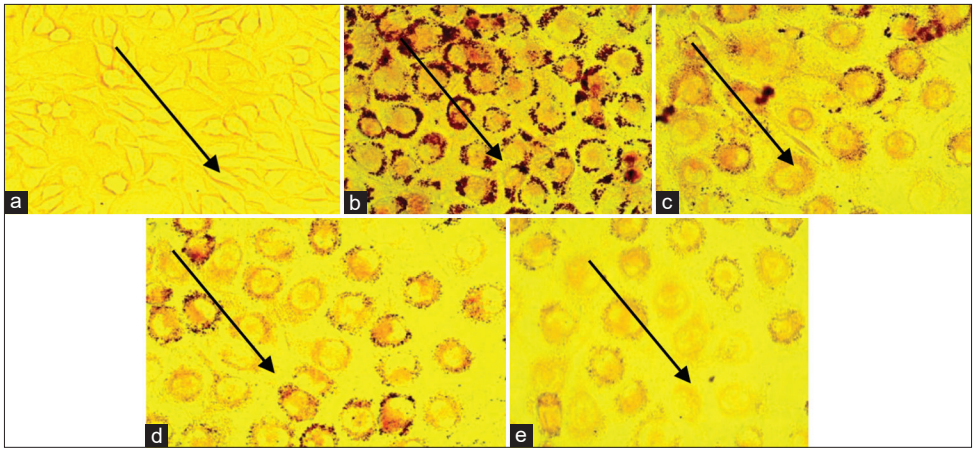

Fig. 9: Morphological/Oil red O staining images of 3t3L1 cells after incubation with extracts for 14 days. (a) Negative control. (b) Positive control. (c) Ethyl acetate extract *Undaria pinnatifida* (UPEA). (d) Methanolic extract of *Moringa oleifera* (MOM). (e) ethyl acetate extract UPEA and methanolic extract of MOM

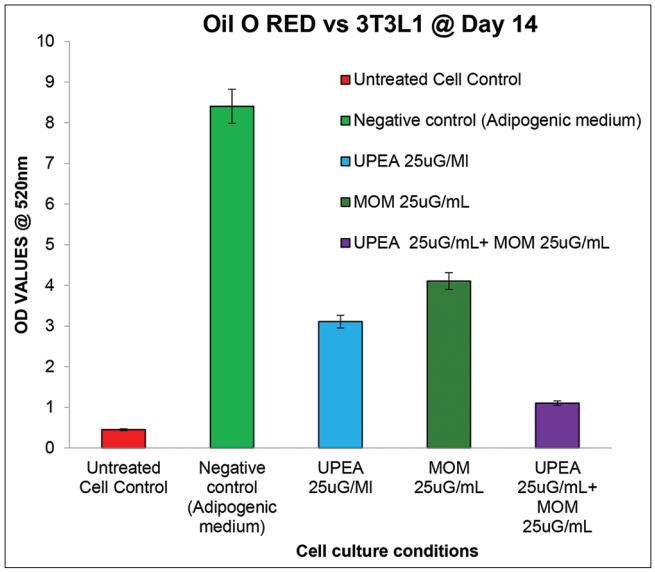

Fig. 10: Overlay bar graph Oil red O staining. Bar graph representation of IC_{50} (0r gL) *Undaria pinnatifida* and *Moringa oleifera*

concentration of UPEA and MOM (1:1) from 12.5 µg/mL to 200 µg/mL was increased percentage of cell viability was also increased.

Concentration of 200 µg/mL treated with UPEA and MOM (1:1) exhibited 99% cell viability, where 100 and 50 µg/mL concentration treated UPEA and MOM (1:1) showed 98% and 97% no toxic nature in comparison with toxic control camptothecin with 41% of cell viability.

In Fig. 8 IC_{50} values of UPEA and MOM indicating good cell viability at 412.8 µg/mL, 333.06 µg/mL and combination of UPEA and MOM (1:1) showed 436.7 µg/mL respectively.

The results of cytotoxicity study (MTT assay) suggested that UPEA and MOM did not show any cytotoxicity and exhibited more viability at 200 µg/mL. And combination of both extracts showed more cell viability. More potent cell proliferation potency on Mouse embryo fibroblast cells on dose-dependent manner taken as an optimum concentration for adipogenesis differentiation study.

Pre-adipocyte 3T3 L1 cell line into adipocyte differentiation

UPEA and MOM (25 ug/mL), were evaluated to check their anti-adipogenesis activity by ELISA on 3T3L1 cells. Concentrations of the compound to treat the cells:

The UPEA has reduced more than MOM the adipogenesis of 3T3L1 cells at 14 days of exposure. Combination of test compounds showed more activity than individually.

***In vitro* analysis on 3T3L1 cells**

The ethyl acetate extract of UPEA, MOM, and UPEA and MOM in a 1:1 ratio was tested for cell viability using the MTT assay, a calorimetric method, on 3t3L1 cell lines. According to research, fucoidan from *U. pinnatifida* has shown anticancer efficacy against pancreatic cancer cells using the MTT assay [15]. Fucoxanthin-containing extracts from the New Zealand seaweed *U. pinnatifida* have demonstrated anticancer potential when evaluated in nine human cancer cell lines concurrently with pure fucoxanthin [19]. *U. pinnatifida* and *M. oleifera* contain different phytochemicals with biological activity and with good therapeutic effects. Results obtained in cytotoxicity assay of UPEA and MOM proving non-toxic nature of 3T3L1 cell lines. In UPEA, at 200 µg/mL concentration 99% cell viability exhibited almost absence of toxicity, where 100 and 50 µg/mL conc. of UPEA showed 98% and 96% with low toxic nature, MOM exhibited 98% cell viability at 200 µg/mL and 94% and 89% at 100 and 50 µg/mL conc., and UPEA and MOM (1:1) at 200 µg/mL conc. exhibited 99% cell viability almost absence of toxicity, at 100 and 50 µg/mL concentration showed 98% and 97% in comparison with toxic control camptothecin with 41% of cell viability.

CONCLUSION

UPEA and MOM retarded the adipose cell proliferation in dose and time-dependent manner with lower toxicity toward normal 3t3L1 cells. MTT assay was performed, and cell viability was analyzed using both UPEA, MOM and combination of UPEA and MOM in 1:1 ratio on 3t3L1 cell lines. UPEA and MOM proved nontoxic nature, where UPEA at 200 µg/mL showed 99% cell viability, 100 and 50 µg/mL conc. showed 98% and 96%, respectively, MOM at 200 µg/mL exhibited 98% cell viability, 100 and 50 µg/mL conc. of MOM showed 94% and 89%, respectively, and UPEA and MOM at 200 µg/mL UPEA and MOM (1:1) exhibited 99% cell viability, 100 and 50 µg/mL concentration of UPEA and MOM (1:1) was showed 98% and 97%, respectively, which was negligible toxic nature in comparison with toxic control camptothecin with 41% of cell viability. IC_{50} values of UPEA and MOM indicating good cell viability at 412.8 µg/mL, 333.06 µg/mL, and combination of UPEA and MOM (1:1) showed 436.7 µg/mL, respectively.

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The authors declare no conflict of interest related to this study.

AUTHORS' CONTRIBUTIONS

1. Dr. C. Sai Kalyani Yogini - Conceived and designed the analysis, collected the data, performed the analysis; wrote the paper.
2. Dr. K Gnaneswari - Helped in data collection
3. Dr. CH.M. Kumari Chitturi - Supervised in designing the data, performing analysis.

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