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# Evaluation of anti-adipogenic activity of *Vacha* rhizome (*Acorus calamus* Linn.) in 3T3-L1 cell line

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Obesity increases the risk of physical and mental health problems like cardiovascular diseases, stroke, insulin resistance, diabetes, cancer, and depression. *Acorus calamus* Linn rhizome is one such medication used in Ayurveda and other traditional systems to treat obesity. Purpose of this study is to evaluate anti-adipogenic activity of both an ethanolic, aqueous extract of *A. calamus* Linn. rhizome in 3T3-L1 mouse derived cell line. The ethanolic and aqueous extract of this *A. calamus* Linn. rhizome was prepared with the help of Soxhlet apparatus. Cytotoxicity has been evaluated with the help of MTT assay. Qualitative and quantitative analysis of lipid deposition in the cell line treated with extract was conducted with O Red oil staining with the help of microscope and Image J software, respectively. Anti-adipogenic activity was evaluated through differentiation and gene expression analysis (RTPCR) for PPARγ and GLUT4 mRNA expression. Viability assay revealed that the 50 µg/mL showed cell proliferation without any cellular toxicity. The 3T3-L1 adipocytes treated with ethanolic extract of *A. calamus* Linn. rhizome exhibited significant decrease in the lipid accumulation as evidenced by oil red O staining and subsequent quantification with Image J software. Down regulation of PPARγ and up regulation of GLUT4 genes via RT-PCR in treated group showed the anti-adipogenic activity. This demonstrates the ethanolic extract of AC is effective in reducing adipogenesis *i.e.,* it has antiadipogenic activity. An ethanolic extract of *A. calamus* Linn. thus establishes it as a promising herb for the treatment of obesity and associated disorders.

**Keywords**: 3T3L1 cell line, *Acorus calamus*, Anti-Adipogenesis, Herbal extract, *Lekhan*, Obesity, Traditional medicine, *Vacha*

**IPC Code:** Int Cl.[26]: A61K 36/00

The World Health Organization reports that 13% of adults are obese and 39% of adults are overweight globally. The prevalence of obesity has nearly tripled since 1975 and is predicted to rise further by 2030. Obesity has close associations with metabolic syndrome, a cluster of pathological conditions including metabolic disorders, pose a major risk to overall human health[1]. Focusing on adipocytes and creating effective medications that target the body's cells is essential to addressing the global effects of metabolic disorders. To overcome the side-effects of invasive and non-invasive anti-obesity procedures and medicines, respectively needs to study and utilize complementary and alternative natural herbal source. In Charaka Samhita, *Acorus calamus* Linn. has been included in '*lekhaniya mahakashaya*' group, a group of scraping agents[2]. *Vacha* is Sanskrit synonym of *A. calamus* Linn.

*Vacha* (*A. calamus* Linn.) is a semi-aquatic herb of the Araceae family distributed throughout the world. It contains phytoconstituents like monoterpenes, betasitesterol, triterpenoid, flavons, xanthoneglycosides, saponine, essential oil which contain salfa and beta asarone. Sesquiterpenes, asarone and saponnin present in rhizome show effect against hyperlipidaemia in rat experiment. Particularly α and β- asarone are effective in suppressing the immune system, preventing cancer, antilipidemic, and treating diabetes. *Vacha* has number of proven pharmacological activities like antihypertensive effect, nootropic effect, anti-depressant, anti-convulsant, anti-diabetic, anticancer, etc.[3]. Some studies has been conducted on inhibition of adipogenesis in 3T3-L1 cells by isolating molecule or principle like saponin, α and β asarone

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**Abbreviations**: AC: *Acorus calamus*, MTT: 3-(4,5-dimetrhylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, DEPC: Diethyl Pyrocarbonate, DEX: Dexamethasone, RT-PCR: Reverse transcriptase polymerase chain reaction, PPARγ: Peroxisome proliferator-activated receptor gamma, GLUT4: Glucose transporter protein type-4, DMSO: Dimethyl Sulfoxide, IBMX: isobutyl methyl xanthine, IT: Indomethacin, DMEM: Dulbecco's Modified Eagle Medium, ELISA: Enzyme-linked immunosorbent assay, PBS: Phosphate buffer saline, ΔΔCT: Delta delta cycle threshold, BAT: brown adipose tissue, WAT: White adipose tissue.

*in vitro* and *in vivo*[4]. Toxicology study reveals that isolated α and β asarone compounds can cause hepatomas, mutagenicity, genototoxicity, teratogenicity[5]. Here the whole rhizome extract was preferred than isolated molecule to mitigate toxicity of α and β asarone and also applying the concept of 'Entourage effect' of other compound present in whole extract. This research aims to demonstrate the impact of *Vacha* on adipogenesis in 3T3-L1 cell line. Because these cell line function as a pre-adipocyte cell line that can eventually develop into adipocytes, which are mostly used in anti-adipogenesis research.

## Material and Methods

#### Drug collection and extract preparation

The *A. calamus* Linn. rhizome was collected from the Vaidya Gaikwad brother's organic herbal farms, Pal, Maharashtra. Drug Identification was done at Botanical Survey of India, Pune. The identification no. is BSI/WRC/Iden.Cer. /2022/ 0903220021656. The medicine was standardized at Sudhatatva Pharmacy, Dr. D. Y. Patil College of Ayurveda and Research Centre, Pune (India) by Dr. D. Y. Patil College of Ayurveda and Research Centre, Pimpri, Pune, Maharashtra. The aqueous and ethanol extract of AC rhizome was obtained through the Soxhlet apparatus using 50 g of dried powder of rhizome.

#### Cell line collection

The 3T3-L1 mouse derived cell line had been procured from National Centre for Cell Science, Pune (Dated 19 March 2022, Passage 7).

#### Cell culture

3T3-L1 pre-adipocyte cells were used as a model to investigate the anti-adipogenic effect of ethanol extract of AC rhizome. Cells were cultured in the high glucose DMEM medium (Gibco) with 10% Fetal Bovine Serum and 1% antibiotic solution (Gibco).

#### Viability assay[6]

Passage 13 cells were seeded in 96-well cell culture plates with a cell density of $1 \times 10^3$/well and maintained for 24 h at 37ºC and 5% $CO_2$.Cell viability was assessed by MTT assay. Then, cells were treated with ethanolic and aqueous extract of *Vacha* (*A. calamus* Linn.) at the concentration of 1, 10, 20, 50, and 100 µg/mL for 48 h. Then, the culture media was removed, and 50 µL of MTT solution was added to the wells. The plate was incubated for4 h at 37ºC with 5% $CO_2$. Further, 100 µL of DMSO (Hi Media, India) was added to each well in order to dissolve the purple formazan crystals. Absorbance was measured at 560 nm on spectrophotometer.

#### Adipogenic differentiation

The 3T3-L1 cells were seeded in a 24 cell culture plate and allowed to become 70-80% confluent. Then, cells were treated with aqueous and ethanolic extract of *A. calamus* Linn along with an adipogenic differentiation cocktail consisting of 1.0 µM dexamethasone (DEX), 0.5 mM isobutyl methyl xanthine, and 1 µM insulin in a 10% high glucose medium till day 3. On day 3 onwards, adipogenesis maintenance medium (DMEM with 10 µL/mL insulin) was added into the well along with ethanol extract of *A. calamus* Linn.

#### Oil red O staining

The medium was removed and cells were fixed in the 4% of formalin for 30 min. Further, cells were stained with diluted oil red O stain for one hour. Cells were washed with distilled water thrice and lipid droplets were visualized under microscope. The extent of lipid accumulation was assessed by the quantitative estimation by Image J software.

#### Gene expression analysis

Cells were treated with extract as described in the above section. The cells were dissolved in the Trizol (Gibco). Total RNA was isolated and reverse transcribed into cDNA a high-capacity cDNA synthesis kit (Applied Biosciences). Quantitative real-time PCR (Applied Biosystem) was used to analyze the gene expression levels (ABI, USA). Table 1 listed the primers used for PPARγ and GLUT4 gene expression investigation. The ΔΔCT technique was used to calculate the relative gene expression (Table 1).

#### Statistical analysis

All the experiments were performed in triplicates. Data was expression as mean±SD. The statistical

Table 1 — The forward and reverse primer used for PPARγ and GLUT4 gene expression.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| PPRAγ | AGACCACTCGCATTCCTTTG | ATCGCACTTTGGTATTCTTGG |
| GLUT4 | TTCCCTGGTTCATTGTGGC | AAGATGAAGAAGCCAAGCAGG |
| B-Actin | TGGTGGGAATGGGTCAGAA | TCTGGGTCATCTTTTCACGG |

difference in between the groups were determined by one way Anova followed by Tukey's multiple comparison test. The $p<0.05$*, and $p<0.01$* was considered as statistically significant.

## Results

#### Extract of AC obtained through Soxhlet apparatus

Weight of the empty petri plate (47.54 g) along with ethanol extract of AC-50.18 g and its extractive value has been found 5.26 g.

While extractive value of aqueous extract of AC was 49 g.

#### Culture of 3T3 L1 cells

The 3T3-L1 cells were sub cultured till passage 13 in a T75 flask in a high glucose DMEM (Gibco) with 10% fetal Bovine Serum (FBS, Gibco) and 1% antibiotic solution (Gibco). As seen in (Fig. 1), the cells exhibited fibroblast-like morphology.

#### Viability assay

It was discovered that there was no toxicity in cells following treatment with both extracts of *A. calamus* Linn. at concentrations of 1, 10, 20, 50, and 100 µg/mL (Fig. 2 a & b). Out of these concentrations 50 µg/mL had shown relatively good absorbance with the control group. The drug showed good proliferation at every concentration which reveal that it is safe at all concentrations.

When compared to other concentrations and the control group, the alcoholic extract at 50 µg/mL demonstrated good proliferation with less error and less cell toxicity. Therefore, 50 µg/mL was used for further analysis.

#### Anti-adipogenic activity of alcoholic extract of *Acorus calamus* Linn. (*Vacha*)

At a concentration of 50 µg/mL, cells were subjected to treatment with both extracts. Staining was performed using a 0.3% oil red O stain (Fig. 3 to Fig. 5) In the control group, there was no discernible reduction in lipid droplets, while the group treated with the aqueous extract exhibited a minimal decrease in lipid droplets compared to the control group. Notably, the alcoholic extract-treated group displayed a significant reduction in lipid droplets

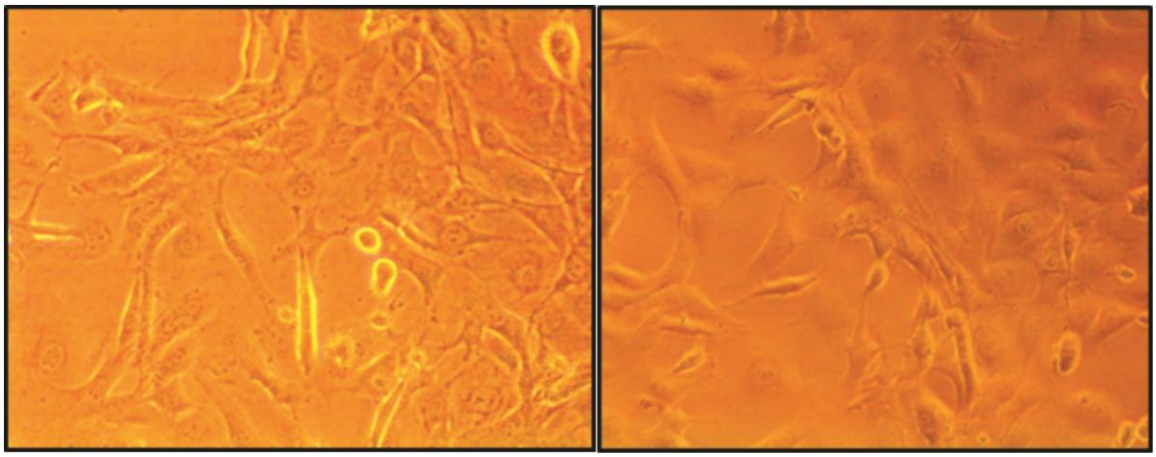

Fig. 1 — 3T3 L1 cells at 75% confluence. Images are taken on bright field microscope

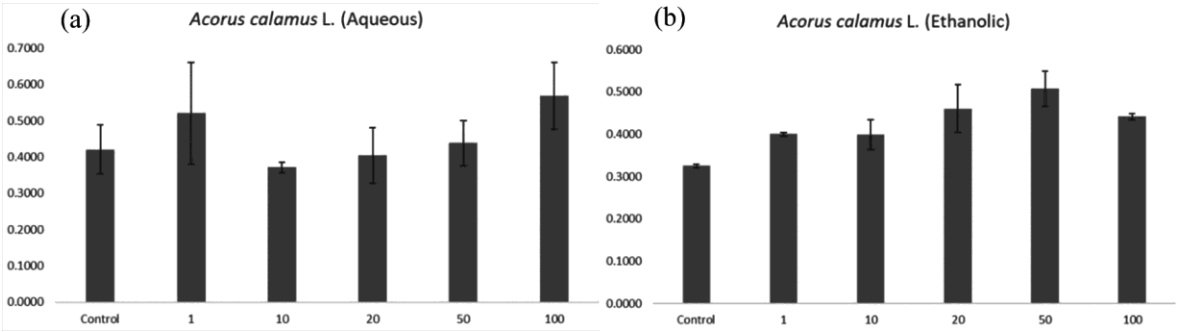


Fig. 2 — (a) Cytotoxicity of aqueous extract of *A. calamus* L. (*Vacha*) rhizome at 1, 10, 20, 50, and 100 ug/mL in 3T3-LI cells. *p<0.05. n-3, data, (b) Cytotoxicity of ethanolic extract of *A. calalmus* L. (*Vacha*) rhizome, at 1, 10, 20, 50, and 100 ug/mL in 3T3-LI cells, *p<0.05. n-3, Data shown are mean ± SD

when compared to both the control and aqueous extract-treated groups. This observation underscores the high potential of the ethanolic extract of *Vacha* in reducing lipid droplets.

The quantitative analysis is highly significant at $p<0.05$ and demonstrates high lipid accumulation in untreated 3T3-L1 adipocytes, whereas exposure to the aqueous extract results in a partial reduction in intracellular lipid content. Notably, the alcoholic extract induces a pronounced decrease in Oil Red O–positive staining, indicating significantly greater efficacy in suppressing lipid accumulation and adipogenic activity.

**Gene expression by AC**

The cells treated with the ethanolic extract 50 µg/mL of *A. calamus* Linn. (*Vacha*) and observed for the PPARγ and GLUT4 genes. They showed the down regulation of PPARγ and up regulation of GLUT4 genes. (Fig. 7 & Fig. 8) the mean fold

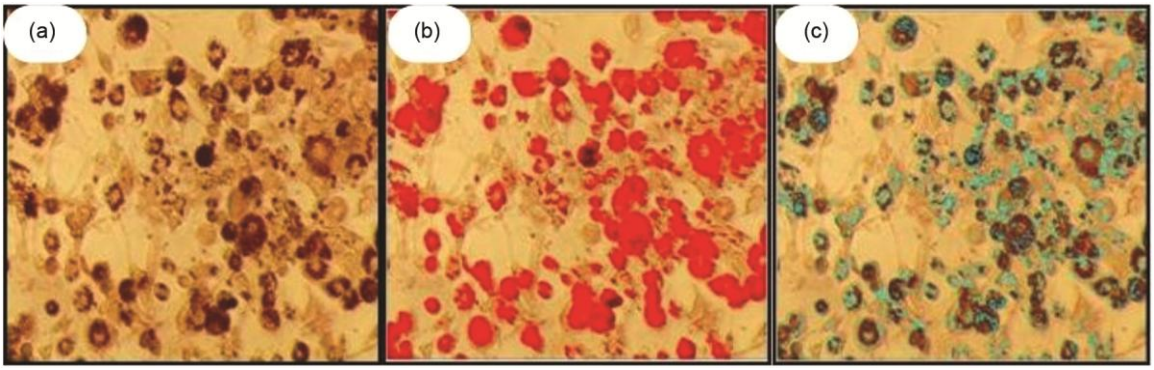


Fig. 3 — Images a, b, c shows the staining of control group containing DI + DPBS + Ethanol + Formaldehyde along with the 0.3% Oil Red O Stain

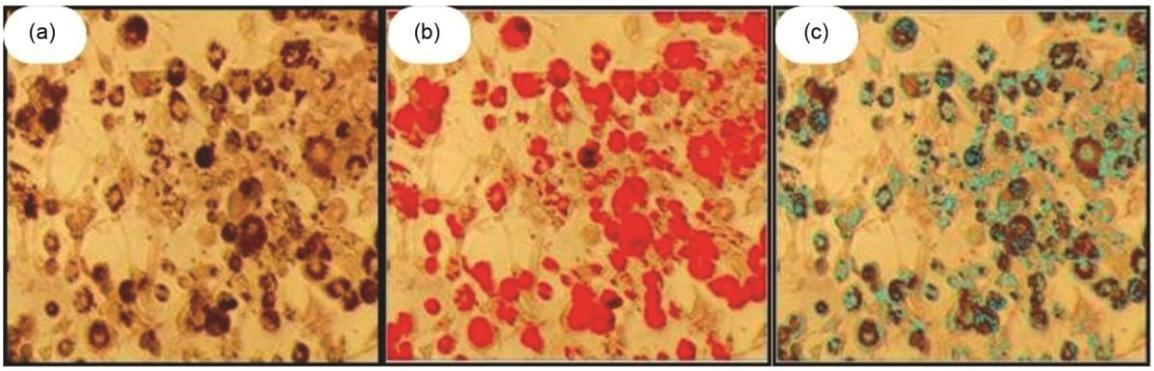


Fig. 4 — Image a, b & c show the deposition of oil droplets in aqueous extract treated group containing DI + DPBS+ Ethanol + Formaldehyde stained with 0.3% Oil Red O stain

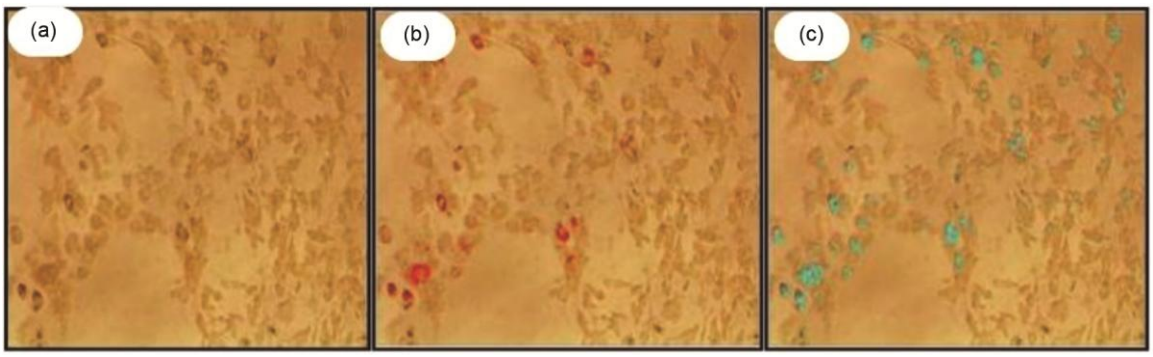


Fig. 5 — Images a, b & c shows the reduced deposition of oil droplets in group, treated with ethanolic extract of *Vacha* containing DI + DPBS + Ethanol + Formaldehyde stained with 3% Oil Red O stain

expression of the values in the control and treated groups is presented. At $p<0.05$, both gene expression levels are highly significant. The mechanism of *A. calamus* Linn. ethanol extract was investigated using corresponding mRNA expression involved within the adipogenic process. mRNA expression of adipogenic genes, including GLUT4 and PPRAγ, has been studied to assess the reduction in fat accumulation (Table 2).

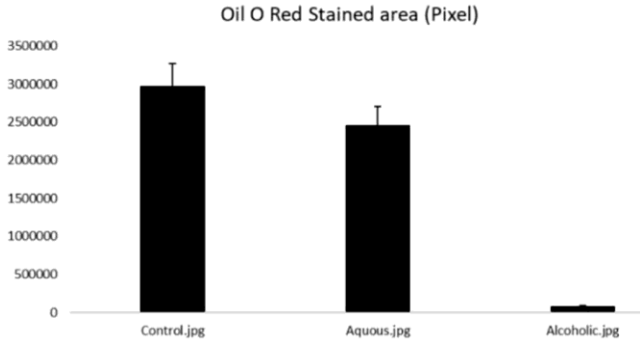


Fig. 6 — Quantification of lipid accumulation in 3T3-L1 adipocytes treated with aqueous and alcoholic by u=image J software. Data shown are mean±SD

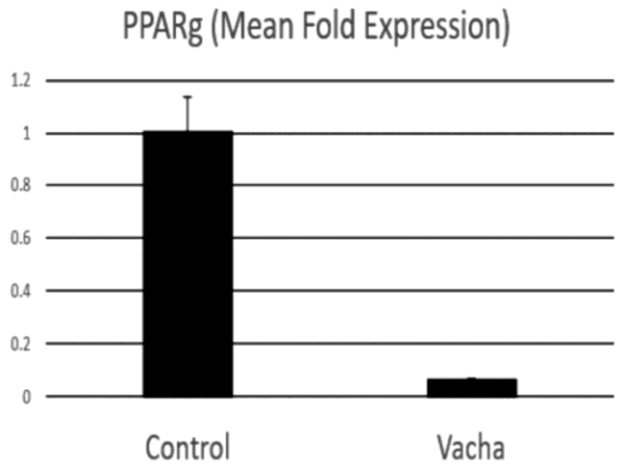


Fig. 7 — The graph shows downregulation of PPARy gene expression when treated with ethanolic extract of *Vacha* (*A. calamus* linn.)

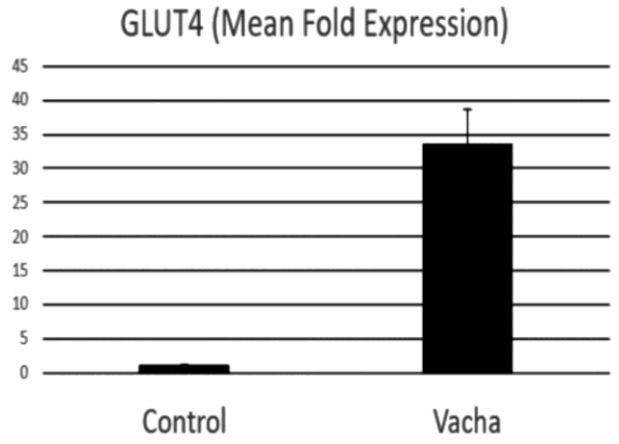


Fig. 8 — The graph shows upregulation of GLUT4 gene expression when treated with ethanolic extract of *Vacha* (*A. calamus* linn.)

## Discussion

*Vacha* (*A. calamus* Linn.) antiadipogenic capability in the 3T3-L1 cell line model is reported in the current work. Long term, low-grade inflammation of adipose tissue is scientifically associated with obesity, metabolic disease & hallmark of insulin resistance[7]. β-Asarone in *Vacha* significantly inhibited intracellular lipid accumulation during adipocyte differentiation[8]. Antioxidants derived from *Vacha* such as polyphenols act through direct ROS scavenging, upregulation of endogenous antioxidant enzymes, and modulation of key signalling pathways like nuclear factor kappa B (NF-κB) and PPARγ, reducing lipid peroxidation, inflammation, and adipocyte dysfunction[8]. Polyphenols, terpenes in it also nullify the toxicity of β-Asarone. Sedentary lifestyles and junk food consumption have contributed to the rise of a number of metabolic disorders in the modern world. Modern medicine has however a number of disadvantages and lingering effects. Herbal medicines that can effectively control chronic disorders without generating complications are in high demand in order to solve this issue. Maintaining balance between adipose tissue growth and preventing the storage of lipid droplets is essential since these disorders usually entail adipose tissue imbalance. Adipose tissue comes in two primary varieties and has a specific purpose in the human body. Extra energy is stored in white adipose tissue (WAT) as triglycerides. Brown adipose tissue (BAT) uses energy as thermo genesis. Obesity can be controlled by encouraging BAT development and inhibiting WAT development[9]. Preventing the differentiation of 3T3-L1 cells can help prevent obesity, which is one of the detrimental impacts of excessive adipogenesis, according to

Table 2 — Comprehensive analysis of GLUT4 and PPRAγ genes in 3T3 L1 cells treated with *Acorus calamus* L. by real time rt

| | Mean fold expression value | | |
|---|---|---|---|
| | Control | *A. calamus* | P-value |
| GLUT4 | 1.004381324 | 33.55161859 | 0.011962662 |
| PPRAγ | 1.004381324 | 0.063101478 | 0.009789763 |

numerous research investigations[10]. 3T3-L1 cells are identical in appearance and biochemistry and can generate a homogenous population in situ[11].

The MTT assay was utilized to assess cell cytotoxicity by employing varying concentrations of both extracts. It was observed that cell viability was maintained across all concentrations in both extracts. However, the 50 µg/mL concentration of the alcoholic extract exhibited robust proliferation with minimal error in comparison to the control group and other concentrations. Consequently, subsequent investigations were conducted using the ethanolic extract of AC.

As preadipocytes develop into adipocytes with increasing lipid deposition, the amount of expression of various transcription factors and adipocyte specific genes increases[12]. The process by which the instructions in DNA are translated into functional products, such proteins, is known as gene expression. Numerous transcription factors, include CCA AT/enhancer-binding protein (C/EBP), fatty acid synthase (FAS), PPARγ, and others has a significant impact on adipogenesis[13]. The expression of the PPARγ and GLUT4 genes helped to evaluate antiadipogenic capability of *Vacha*. It has been already proven that the GLUT4 gene was upregulated while the PPARγ gene was downregulated by 50 µg/mL of ethanolic extract of *A. calamus*. The PPARγ gene is upregulated in insulin resistance, obesity, dyslipidaemia, and hypertension, all of which greatly increase the risk of type 2 diabetes, cardiovascular diseases, and cancer[14]. Given that the PPARγ gene aids in adipocyte development and differentiation, its downregulation denotes decreased adipogenesis, or antiadipogenic action. Insulin-resistant diseases including obesity and type 2 diabetes cause GLUT4 expression to decrease in adipose tissue while staying mostly constant in muscle tissue[15]. Increased insulin sensitivity and glucose absorption, which inhibit the development of adipose tissue, are demonstrated by the over expression of the GLUT4 gene. Consequently, it inhibits the adipogenesis.

## Conclusion

In 3T3-L1 cells, downregulation of PPARγ, upregulation GLUT4 expression and decreases in lipid accumulation proves that *Vacha* (*A. calamuas* Linn.) acts as an antiadipogenic agent.

Cumulative effect of phytoconstituents of *Vacha* extract is effective as an antiadipogenic herb. Therefore, the medication can be utilized to treat a variety of metabolic diseases, such as obesity, diabetes mellitus, cardiovascular disease, etc., that include adipose tissue malfunction. As adipose tissue produces excess oestrogen, increasing risk of hormone related cancers and neuropsychological and neural disorders; it needs to study the effect of *A. calamus* Linn. as preventive and curative remedy in above mentioned disorders related with obesity.

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## Author Contributions

AS, JC conception; AS, PS, JC the acquisition, AS, Av.S, RB analysis, Av.S, RB interpretation of data; AS, JC, PS have drafted the work, JC substantively revised it.

## Conflict of Interest

Authors declare that there is no conflict of interest.

## Ethics Statement

Not Applicable

## Informed Consent

Not Applicable

## Data Availability

Data supporting this study findings are included within the manuscript and will be made available by the authors upon reasonable request.

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