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In-vitro Anti-adipocytic activity of *Katuki* (*Picrorhiza kurroa* Royle ex Benth)

Research Article

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Abstract

When we look back at human evolution, we observe that humans are closely bonded with nature. We highly depend on resources derived from nature for our day-to-day lives. Whether in good health or disease, we greatly rely on plant, animal, and mineral extracts. With the advent of time, as humans evolved, we started using artificially prepared chemical formulations and modern drugs - for prevention and fighting against diseases. In *Ayurveda*, plants, animals, and minerals are used to treat diseases. *Katuki (Picrorhiza kurroa Royle ex Benth)* is an *ayurvedic* herb that belongs to the Plantaginaceae family and is primarily found in the Himalayan region. *Katuki* has multi-factorial use in the treatment of various ailments. It is widely used as hepatoprotective, preventing and curing liver damage caused by hepatotoxic agents. Though *Acharya Charak* has mentioned *Katuki* in *Lekhniya Mahakashaya*, it is scarcely used for treating *Sthoulya* (Obesity). Hence, through a research study, here is an attempt, to prove the activity of *Katuki* in the inhibition of fat cell formation using the 3T3-L1 cell line. Material and Methods**:** *Katuki* Rhizomes was purchased from, Dehradun, Uttarakhand. A modern drug, Orlistat was used as a standard drug. The 3T3-L1 cell line was procured from NCCS, Pune. Cytotoxicity of *Katuki* was studied using XTT Assay. Oil Red O staining was used to study the anti-adipocytic activity of *Katuki*. Result: *Katuki* was found to be safe for 3T3-L1 cells. *Katuki* was tested in four different concentrations. After the test results, it was found that the fat cell formation on the differentiated 3T3-L1 cell line treated with *Katuki* was significantly inhibited. Discussion and Conclusion**:** The test drug *Katuki* has shown noteworthy anti-adipocytic activity on the 3T3-L1 cell line in all the studied concentrations. However, the anti-adipocytic effect of *Katuki* was not dose-dependent.

Keywords: *Katuki, Picrorhiza kurroa,* Anti-Adipocytes, 3T3L1 cell line, Lekhan, Obesity.

Introduction

For centuries, humans have been using natural resources to live a healthy life. Natural resources, such as plants, animals, and minerals, are not only useful for living a healthy life but also for different diseased conditions. In the traditional Indian medicine systems, such as *Ayurveda*, *Siddha, and Unani*; plants are used as medicine in different forms. Even being surrounded by plants makes you feel relaxed, happy, and healthy.

Nowadays, humans are suffering from modern lifestyle disorders. Obesity is ranked at the top of all. It further leads to many diseases, such as Diabetes, Heart Disease (Atherosclerosis), Hypertension, and Non-alcoholic Fatty Liver. To deal with obesity, very few modern medicines are available, and these medicines also have multiple side effects, which further lead to other disorders.

Katuki (Picrorhiza kurroa Royle Ex. Benth) is found in the alpine Himalayan range spread from Kashmir to Sikkim, at an altitude of 2500 to 4500 meters. *Katuki* is also known as *Katurohini, Kutki, Matsyashakala, Tiktarohini and Matsyapitta* (1). *Katuki* is one of the medicinal plants that can be used in the treatment of obesity. In the ancient *Ayurvedic* text, *Charak Samhita,* it is included in *Lekhniya Mahakashaya* (2).

The process of reducing or scraping unwanted *dhatus* (tissues) and *malas* (metabolic wastes) from the body is called *Lekhan* (3). It helps in reducing *kapha* and *meda* (fat) by eliminating excessively accumulated *dhatu* in the body.

Though it is said to remove *meda* (fat) from the body, its extensive use in the treatment of obesity is not researched properly yet. Whether *Katuki* is helpful in the reduction of fat cells or not, will be studied on the 3T3-L1 cell line using the Oil Red O (ORO) stain. The 3T3-L1 cell line, used for the study, is pre-adipocytes, which will be converted into mature adipocytes to observe the absorption of fat cells after treatment with *Katuki*.

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Materials and Methods

Material

Plant Identification, Collection, Authentication, and Powder Preparation

Katuki rhizomes were identified by the taxonomical, morphological, and family characteristics of the plant. *Katuki* Rhizomes were purchased from Dehradun, Uttarakhand, India. The sample of *Katuki* was authenticated by a botanist at RTMNU, Nagpur and the specimen sample was vouchered as 10449. The sample was air-dried avoiding direct sunlight. The dried sample was ground to fine powder, and they were sieved with a 20mm sieve for further use. The powder was stored in a zip lock polythene bag to protect it from moisture or any other contamination. A weight-by-volume solution was made by dissolving a fine powder of *Katuki* in DMEM.

Figure 1: Rhizome of *Katuki (Picrorhiza kurroa Royle ex Benth.)*

Figure 2: Powder of *Katuki (Picrorhiza kurroa Royle ex Benth)*

Standard Drug Orlistat

Orlistat is an anti-obesity drug used for the treatment of obesity. It acts as a gastro-intestinal lipase inhibitor, which forms a bond with the enzyme's active site in gastric and pancreatic lipases. It prohibits their activity and prevents the absorption of dietary fat (4).

In-vitro Study

Cell Culture and Maintenance of 3T3-L1 Cell Line

The 3T3-L1 cell line is a pre-adipocytic cell line, isolated from a mouse embryo. The embryo was ordered from NCCS Pune, India, and stored in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 100 U/ml of penicillin-streptomycin antibiotic solution at 37°C at 5% of CO_2. After every two days, the medium was replaced with a fresh medium.

Cytotoxicity Assay

Chemicals and Reagents

- The XTT tetrazolium salt (2,3-bis [2-methoxy 4-nitro 5-sulfophenyl] 2H-tetrazolim 6-carboxanilide)
- PBS (Phosphate-buffered Saline)

XTT Assay for the 3T3-L1 Cell Line (5)

The cytotoxicity of *Katuki* on the viability of 3T3-L1 cells was measured by XTT assay. The purpose of XTT assay is to determine the sub-lethal concentrations (LC50). The assay is based on the ability of metabolically active 3T3-L1 cells to convert the XTT tetrazolium salt into the orange-colored formazan dye. The obtained formazan dye is a water-soluble compound, and its intensity can be read at a wavelength of 450nm with the help of a spectrophotometer. The formazan dye's intensity is directly proportional to the number of metabolically active 3T3-L1 cells.

Intensity of Formazan Dye ∝ Number of 3T3-L1 cells

More the active 3T3-L1 cells in the well, the more the mitochondrial enzymes' activity, and, in turn, the more the concentration of the dye formed. It can then be measured and quantified using a spectrophotometer.

In brief, the 3T3-L1 cell ($2x10^4$ cells/well/500µl) was grown onto a 24-well plate for 24 hours with treatment of different concentrations of *Katuki* drug. After the incubation period, the supernatant was discarded and washed once with Phosphate buffered saline (PBS) to remove the medium. At the end of the incubation period, 500µl of PBS with 20µl of XTT was added to each well and then incubated at 37°C for 3-4 hours. The optical density values were measured at 450 nm and the percentage (%) of cell viability relative to control cells was calculated. The Lethal Concentration 50 (LC50) value of *Katuki* was calculated based in a nonlinear regression (curve fit).

Differentiation of the 3T3-L1 Pre-adipocytic Cell Line (6) (7):

On Day 1, the 3T3-L1 cells were cultured at 37°C under a humidified 5% CO_2 atmosphere in Complete Medium, which contains DMEM with glucose, 10% FBS, and 100 unit/ml penicillin-streptomycin. A day after the confluence, pre-adipocytes of 3T3-L1 (designated as Day 2) were developed in Differentiation Medium-1 (DM1), which contains 10% FBS, 10 µg/ml insulin, 500µm isobutyl-methylxanthine (IBMX), and 5.2µm dexamethasone (Dexa). DM1 was added every three days regularly. After three days (Day 4), the DM1 was substituted with Differentiation Medium-2 (DM2), which contains 10% FBS and 10 µg/ml insulin. Again, the same method was followed for regular changing of DM2 for the next three days. After another three days (Day 7), the medium was switched to the Complete Medium (CM) for the next 3 days (till Day 10), which was then processed for the Oil Red O (ORO) staining as shown in Figure 3 & 4.

Experiment Designing and Evaluation of Anti-adipocytic Activity

A fine powder of *Katuki* was dissolved in Dulbecco's Modified Eagle's Medium (DMEM) in a weight-by-volume solution. For evaluation of anti-adipocytic activity, the 3T3-L1 cells were cultured in 1 x 10^4 cells/well in a 48-well tissue culture plate. The cells harvested in wells were divided into the following batches:

i. Control (Cells only)
ii. Cells + Adipocytic Differentiation Media (Insulin + IBMX+ Dexa)

Figure 3: Flow chart of 3T3-L1 differentiation protocol

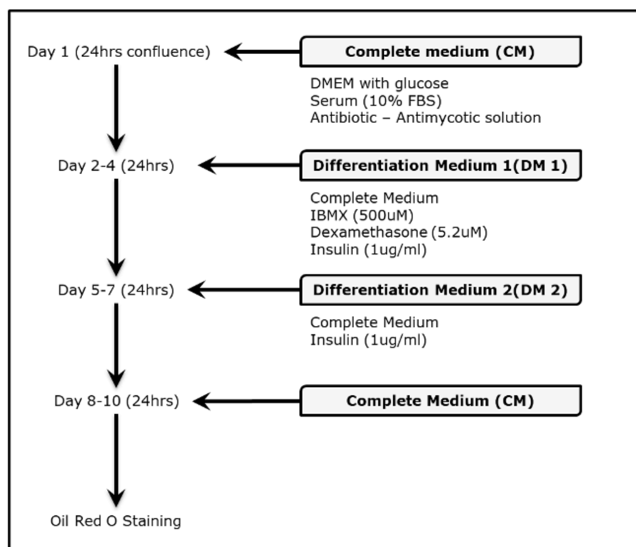
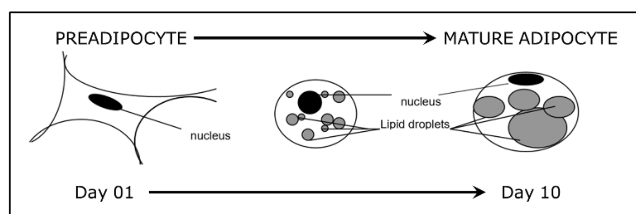


Figure 4: Pre-adipocytes converting to mature adipocytes



iii. Cells + Adipocytic Differentiation Media + Orlistat 50 µg/ml (Standard)
iv. Cells + Adipocytic Differentiation Media + *Katuki* (Concentration of 250 µg/ml)
v. Cells + Adipocytic Differentiation Media + *Katuki* (Concentration of 500 µg/ml)
vi. Cells + Adipocytic Differentiation Media + *Katuki* (Concentration of 1000 µg/ml)
vii. Cells + Adipocytic Differentiation Media + *Katuki* (Concentration of 2000 µg/ml)

Oil Red O (ORO) Staining

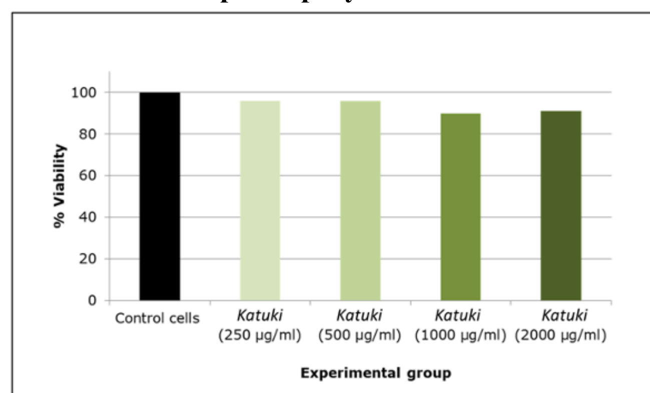
After ten days of observation, the intracellular lipid accumulation in differentiated adipocytes was measured using the Oil Red O (ORO) stain in order to examine the anti-adipocytic activity of *Katuki* in 3T3-L1 cells. After removing the medium, adipocytes were rinsed with PBS thrice, and fixed with 10% formaldehyde solution for 30 minutes at room temperature. Following a PBS rinse, the fixed adipocytes were air-dried on the plate for 10 minutes. After dissolving the lipid droplets in isopropyl alcohol and staining them with ORO stain (5 mg/ml), adipocytes were agitated by shaking the plate gently for 15 minutes. Following ORO staining, the cells were rinsed five times with distilled water to produce the stained 3T3-L1 cell images. After dissolving the stained lipid droplets in 100% isopropyl alcohol, they were added to a 96-well plate at a rate of 200µl/well. The absorbance of each well was quantified using a microplate reader at a wavelength of 520 nm for the quantitative measurement of lipid content as an index of anti-adipocytic activity.

Study, Observation, and Results

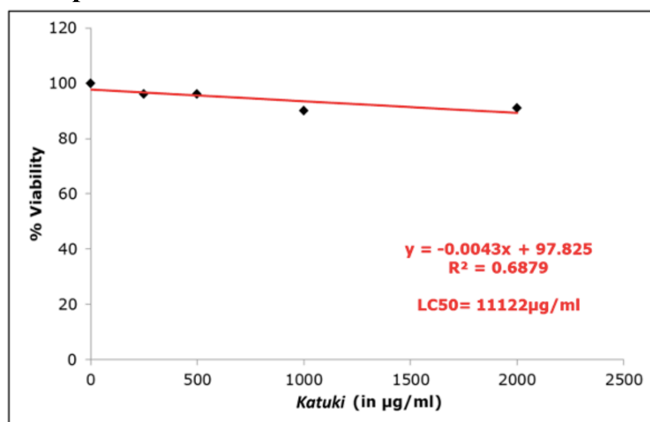
Results of Cytotoxicity Assay

3T3-L1 cells were treated with *Katuki* at a concentration of 250µg/ml, 500µg/ml, 1000µg/ml, and 2000µg/ml for 24 hours respectively. After 24 hours, the effect of *Katuki* treatment on the viability of 3T3-L1 cells was evaluated with the help of XTT assay. The result of the XTT assay shows a non-significant impact of the *Katuki* on the viability of the 3T3-L1 cells (Graph 1). LC50 value of *Katuki* treatment was calculated mathematically by regression analysis of % viability against *Katuki* concentration. The LC50 value for *Katuki* treatment was found to be very high (i.e. 11122µg/ml) (Graph 2). This suggests that *Katuki* is safe to be used for the treatment.

Graph 1: Effects of *Katuki* on viability of the 3T3-L1 preadipocytic cell line



Graph 2: Calculation of the LC50 Value of *Katuki*



Results of Evaluation of the Anti-adipocytic Activity of *Katuki*

Control cells were elongated with some fat granules in a few cells only, while differentiated cells had many fat granules which confirm the differentiation of the 3T3 cells in the adipose tissue. Further, the fat granule inside the differential 3T3-L1 cells reduced significantly with the standard drug Orlistat (50µg/ml) as shown in Figure 5, 6, 7, Graph 3 and Table 1.

Similarly, we found a significant anti-adipocytic activity of *Katuki* in all the studied concentrations, as shown in Figure 8, 9, 10, 11, Graph 3 and Table 1. As the anti-adipocytic effect of *Katuki* was not dose-dependent, hence Effective Concentration 50 (EC50) was not calculated.

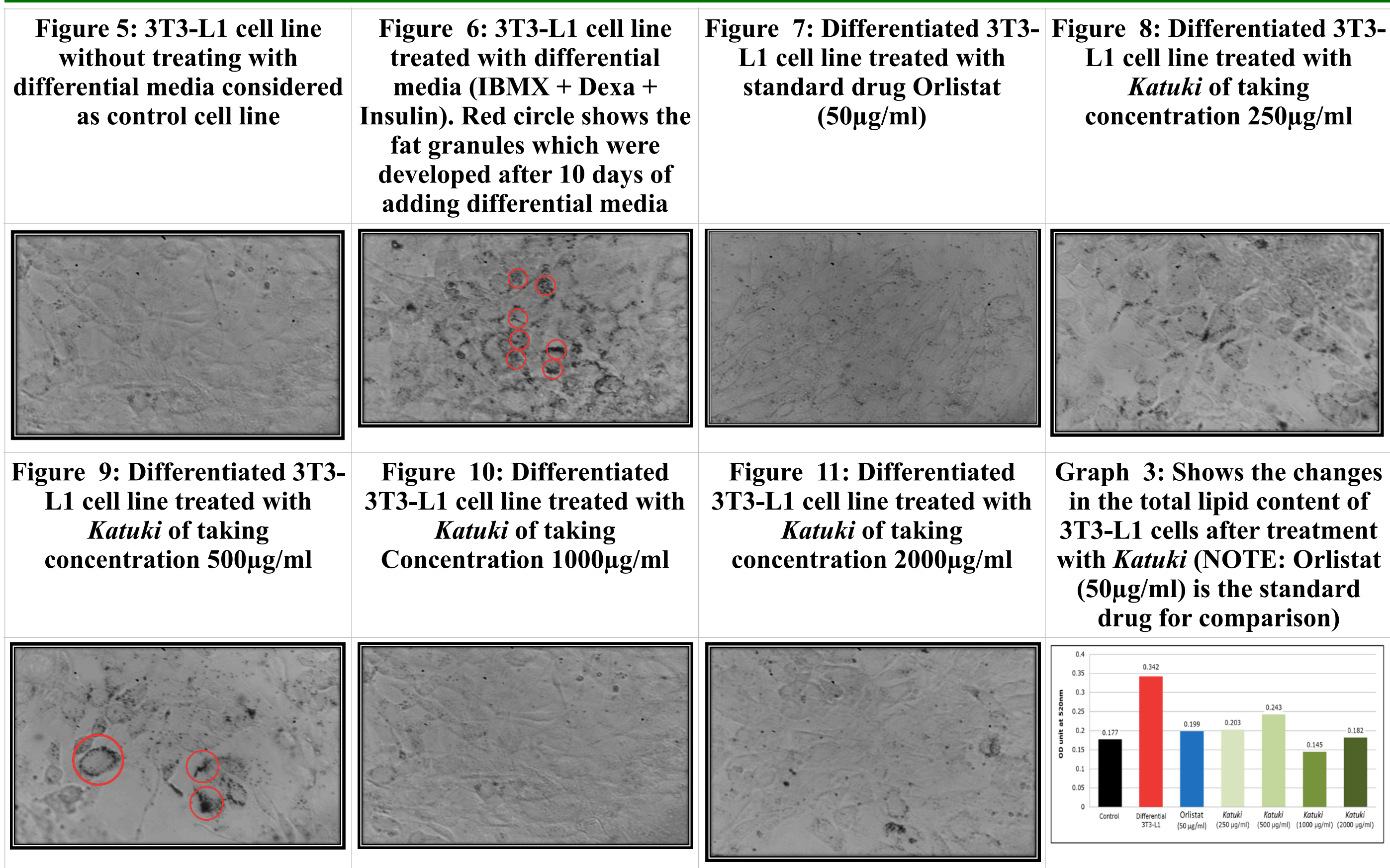


Figure 5: 3T3-L1 cell line without treating with differential media considered as control cell line

Figure 6: 3T3-L1 cell line treated with differential media (IBMX + Dexa + Insulin). Red circle shows the fat granules which were developed after 10 days of adding differential media

Figure 7: Differentiated 3T3-L1 cell line treated with standard drug Orlistat (50µg/ml)

Figure 8: Differentiated 3T3-L1 cell line treated with *Katuki* of taking concentration 250µg/ml

Figure 9: Differentiated 3T3-L1 cell line treated with *Katuki* of taking concentration 500µg/ml

Figure 10: Differentiated 3T3-L1 cell line treated with *Katuki* of taking Concentration 1000µg/ml

Figure 11: Differentiated 3T3-L1 cell line treated with *Katuki* of taking concentration 2000µg/ml

Graph 3: Shows the changes in the total lipid content of 3T3-L1 cells after treatment with *Katuki* (NOTE: Orlistat (50µg/ml) is the standard drug for comparison)

Table 1: In-Vitro Anti-adipocytic activity of *Katuki*

Sr. No.	Sample	Concentration	% Inhibition of FAT formation
1	Orlistat (Standard)	50 µg/ml	58
2	*Katuki*	250 µg/ml	59
3	*Katuki*	500 µg/m	71
4	*Katuki*	1000 µg/ml	42
5	*Katuki*	2000 µg/ml	53

Statistical Analysis

- Med Calc (Version 10) was used to perform all the statistical analysis.
- A Student T-test was applied for comparison between groups.
- A P-value (of less than 0.05) was considered statistically significant for all the comparisons.

Discussion

Katuki has *tikta rasa* (taste), and its *guna* (quality) is *ruksha* and *laghu*. According to *Acharya Charaka*, one of the *karma* of *tikta rasa is lekhan*. (8) Due to this reason, *tikta rasa of Katuki* pertains *lekhan karma*. According to *Ayurveda, Sthoulya roga* (Obesity) primarily happens due to an increase in *kapha* and *meda dhatu* in the body. As *guna* of *Katuki* is *ruksha* and *laghu, Katuki* helps in reducing *kapha* from the body.

On account of this, *Katuki* is useful in *Sthoulya roga*. However, to prove its utility in reducing fat cells and inhibiting the growth of fat cells in obesity, we studied the activity of *Katuki* on the 3T3-L1 cell line using the Oil Red O staining. Before the study, we differentiated the 3T3-L1 cell line and tested *Katuki* for its toxicity (by calculating the LC50 value).

3T3-L1 cell line

These are pre-adipocytes developed from Murine Swiss 3T3 cells. The main advantage and reason to choose this cell line is that it is easier to culture and cost-efficient to use as compared to freshly isolated mature adipocytes. When differentiating media was used on the 3T3-L1 cell line, it converted into mature adipocytes on day 10.

Cytotoxicity Assay

3T3-L1 cells were treated with *Katuki* at a different concentration for 24 hours. The viability of 3T3-L1 cells was evaluated with the help of an XTT assay. The result of the XTT shows a non-significant impact of the *Katuki* on the viability of the 3T3-L1 cells. LC50 Value for *Katuki* treatment was found to be very high (i.e.11,122 µg/ml). This suggests that *Katuki* is safe to be used for the treatment.

The anti-adipocytic activity of *Katuki*

The anti-adipocytic activity of *Katuki* on the 3T3-L1 cell line using Oil Red O staining and morphological assessment of cells is evaluated. The experiment was conducted in seven groups. The first group that contained only cells (culture) showed no change. The second group, in which cells were treated with adipocytic differentiation medium, showed many fat granules. It confirmed the differentiation of pre-adipocytes into mature adipocytes. In the third group, the differentiated 3T3-L1 cells were treated with the standard drug, Orlistat, at the dose of 50 µg/ml. this group showed 58% inhibition of fat formation. In the

fourth, fifth, sixth, and seventh group, the differentiated 3T3-L1 cells were treated with *Katuki* of concentrations 250 µg/ml, 500 µg/ml, 1000 µg/ml, and 2000 µg/ml respectively. The percentage inhibition of fat formation was 59%, 71%, 42%, and 53% respectively. It clearly showed a significant decrease in adipocytes in comparison with the standard drug. Orlistat.

Particularly, *Katuki* of concentration 500 µg/ml showed the highest percentage of fat inhibition, even more than that of the standard drug, Orlistat. However, the effect of *Katuki* was not dose-dependent therefore, the EC50 value was not calculated.

Conclusion

Based on the study, it is concluded that the test drug *Katuki (Picrorhiza kurroa Royle ex Benth)* shows a significant anti-adipocytic activity on the 3T3-L1 cell line in all the studied concentrations, but the anti-adipocytic effect of *Katuki* was not dose-dependent, and hence, the EC50 value was unable to calculate.

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