

Lipid lowering effect of *Eurycoma longifolia* Jack aqueous root extract in hepatocytes.

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Abstract

An aqueous root extract of *Eurycoma longifolia* Jack, or natively known in Malay as Tongkat Ali, is traditionally used to overcome a plethora of health conditions, especially in andrological related maladies. There is compelling evidence that corroborates the therapeutic effects of this plant extract as an efficacious concoction. Recently, the potential for the plant extract to be used for the regulation of fat metabolism has also been proposed. Results from *in vivo* and human trials were unable to determine if a decrease in lipid deposits was caused by *E. longifolia* root extract or that it was merely a side effect of the reported physiological changes. Therefore, this study adopted an *in vitro* model to investigate whether *E. longifolia* root extract possesses lipid lowering and/or absorption inhibitory potentials. The human hepatic cell line WRL-68 (normal and fat fed) was treated with various concentrations (1, 2, 4 and 8 µg/ml) of *E. longifolia* root extract. The results showed reductions in intracellular fatty acids build-up of up to ~72%, in treated cells when compared to untreated control cells. However, treatments with *E. longifolia* did not show any significant inhibition of fatty acids absorption when compared to the respective control cells. This study, therefore, unraveled a novel potential of intracellular lipid lowering effect of *E. longifolia*. The findings warrant further studies to elucidate the intracellular signaling mechanisms that may explain the phenomenon.

Keywords: *Eurycoma longifolia* Jack; fat metabolism; lipid lowering; metabolic disease; Tongkat Ali

1. Introduction

The root of *Eurycoma longifolia* Jack, locally known as Tongkat Ali (TA), has a long recorded ethnobotanical history as a health concoction. The root extract of TA has been shown to possess a myriad of inhibitory activities such as anti-malarial, anti-ulcer, anti-pyretic, anti-cancer, and anti-proliferation (Kuo *et al.*, 2003; Nurhanan *et al.*, 2005; Park *et al.*, 2014; Zakaria *et al.*, 2009; Solomon *et al.*, 2014). Nevertheless, *E. longifolia* root extract is more renowned for its potential for enhancing male sexuality (Chan *et al.*, 2009; Solomon *et al.*, 2014; Kotirum *et al.*, 2015). *In vivo* experiments reported an elevation in testosterone concentrations following a 14-day administration of the aqueous extract of TA (Solomon *et al.*, 2014; Chan *et al.*, 2009). Alongside, sperm concentration, total and progressive mortality and vitality were also shown to be increased. Additionally, an improvement in sexual behavior was also reported when rats were treated with TA root extract (Ang and Sim, 1997; Ang and Sim, 1998a; Ang and Sim, 1998b).

Since modulation of testosterone concentrations in men was correlated to the supplementation of TA root extract, Hamzah and Yusof (2003) previously studied its

effect on body composition and muscle strength and size in men. After a 5-week regime of intense strength training, augmentation of lean body mass and strength in the treated group was reported in comparison to the placebo group. Interestingly, a decrease in the percentage of body fat was also observed in the two test groups. Similarly, when administered with TA root extract, reductions in body weight and omentum fat have also been reported by Solomon, *et al.* (2014). Therefore, this study investigates whether TA root extract has lipid-lowering and/or absorption inhibitory potentials of the supplementation.

2. Materials and methods

2.1 Cell culture

The human hepatic cell line WRL-68 was obtained from the American Type Culture Collection (ATCC, USA). The cells were maintained by regular sub-cultivating in 1X growth media of Dulbecco's modified Eagle medium, DMEM (Gibco, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 u/ml of penicillin, 100 u/ml of streptomycin, and 20 mM of HEPES buffer at 37° C in a humidified incubator in the presence of 5% CO₂ (Gan *et al.*, 2015).

2.2 Cell viability assay

Cytotoxicity of TA root extract and palmitic acid against WRL-68 cells were determined using cell viability assay in a 96-well cell culture plate. Briefly, culture media containing increasing concentrations of TA root extract and palmitic acid were independently provided to the preformed WRL-68 cell monolayer. Fresh media containing MTS solution (Promega, USA), at the concentration recommended by the manufacture, were replaced after 24 h post addition of TA root extract or palmitic acid. The cell culture plate was maintained at a culture condition for an additional two hours prior to quantitation of the formazan level, which was converted in each well with an Epoch spectrophotometer (BioTek Instruments, USA). The toxicity of the TA root extract and palmitic acid were derived from the comparison made with the viability of cells provided with only culture media, respectively.

2.3 Fatty acid treatment

A starting solution of 200 mM palmitic acid was prepared by dissolving the palmitate (Sigma, USA) in absolute ethanol. The starting solution was subsequently mixed with 10% bovine serum albumin (BSA) (Sigma, USA) by shaking overnight at room temperature to generate 4 mM palmitic acid stock solution. A sub-confluent monolayer of WRL-68 cells was provided with 150 μ M BSA-bound-palmitate in 1X serum free growth medium over 24 h to induce cellular lipid uptake (Luo *et al.*, 2012).

2.4 Preparation of TA root extract

The TA standardised extract (Physta[®], MY), batch number TA150101, was obtained from Biotropics Malaysia Berhad. The certificate of analysis was appended as supplementary data (S1). The powder (2 g) was allowed to dissolve in 35 ml of double distilled water overnight at room temperature and the solution was then centrifuged at 13,000 rpm for 30 min at 4° C (Satorious, DE). The centrifugation was performed to remove any undissolved particles and amorphous polysaccharides. The clear supernatant was separated and subjected to freeze drying. The extraction yield was measured and expressed in percentage. The extract was then reconstituted with double distilled water to a 50 mg/ml stock and stored at -20° C for further analyses. Additionally, the stock extract was passed through a 0.2 μ m syringe filter (Satorious AG, DE) for sterilization prior to cell culture treatments.

2.5 Treatment with TA root extract

Generally, the TA concoctions with final concentrations of 1, 2, 4 and 8 μ g/ml of TA root extract were prepared from TA stock in a 1X serum-free growth media. Control sets were provided with 1X serum-free growth media without

the fortification of the TA root extract. To measure the lipid lowering effect of the TA, studies were performed on induced fatty liver cells. Meanwhile, different concentrations of TA concoctions were introduced to the liver cells 24 h before or during the palmitic acid induction to investigate if the extract possessed prophylactic and/or anti-fatty acid absorption activities, respectively.

2.6 Oil-Red-O staining

Control and treated WRL-68 cells were stained with Oil-Red-O at the end of every assay to examine the amount of intracellular lipid accumulation. Briefly, cells were washed with cold phosphate-buffered saline and fixed with 10% paraformaldehyde (Sigma, USA) for 1 h. One hundred microliters (100 μ l) of 0.5% of Oil-Red-O (Sigma, USA) in 60% ethanol were added to the cells for 1 h after the removal of the fixing solution (Yao *et al.*, 2011). The cells were then rinsed with distilled water and images were photographed (Olympus DP73, JP). Finally, the intensity of the stain was quantified by dissolving the lipid stain with dimethyl sulfoxide (DMSO), a universal solvent (Sigma, USA), and the intensity of the red-colored dye was read at 510 nm on the Epoch spectrophotometer (BioTek Instruments, USA).

2.7 Statistical analyses

Histograms are expressed as mean \pm standard error of the mean. The comparisons between groups were made using the Student's t-test with the statistical computer package SPSS version 17.0 software for Windows (IBM, USA) (Dashti *et al.*, 2014). The significant level was set at $p < 0.05$.

3. Results and discussion

A clear aqueous extract from TA root was prepared as mentioned in the methods section. The total extract yield resulting from the centrifugation was $91.98 \pm 0.12\%$. Subsequently, the TA root extract was investigated for its potential toxicity. Increasing concentrations of TA root extract starting from 0.25 up to 64 μ g/ml were mixed in the cell culture media. The media were then used to culture the human hepatic cell line WRL-68 and the cell viability assay was conducted 24 h thereafter. The viability of WRL-68 cells cultured in the TA root extract up to 64 μ g/ml demonstrated $\sim 100\%$ viability, as compared to those without (see Figure 1). Thus, the results revealed no toxic effects for the various tested TA root extract concentrations.

On the other hand, increasing concentrations of palmitic acid starting from 50 μ M up to 800 μ M in culture media resulted in a gradual loss of WRL-68 cell viability (see Figure 1). When cultured with media containing

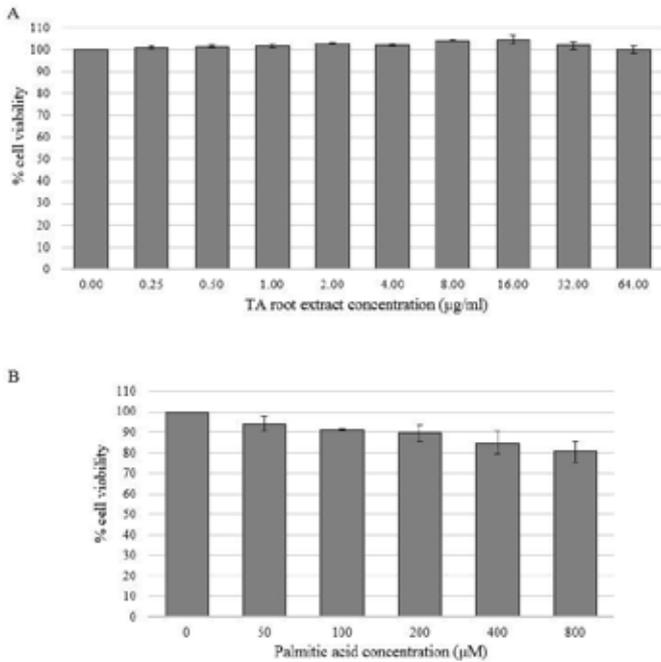


Fig. 1. Cell viability of human hepatic cell line WRL-68 when cultured with (A) TA root extract and (B) Palmitic acid.

50 µM of palmitic acid, the cell viability of WRL-68 was ~95%, in comparison to those cells cultured in palmitic acid-free media (control sets). Culturing the cell line with media containing 100 and 200 µM of palmitic acid decreased the cell viability to ~90% when compared to the control sets. The plummeting trend of cell viability continued with the presence of higher concentration of palmitic acids. A further reduction of 5% of cell viability was observed in the culture with the presence of 400 µM of palmitic acid, and the cell viability was reduced to ~80%, when the cells were cultured in media containing 800 µM of palmitic acid. Similarly, significant changes in hepatic cell morphology and even cell death were also observed by others when media containing high concentrations of palmitic acid were used (Feldstein *et al.*, 2004). Therefore, culture media containing 150 µM palmitic acid was used to induce WRL-68 cellular fatty acid uptake.

In order to investigate the lipid-lowering potential of the TA root extract, WRL-68 was first induced to store a fatty acid in the cell. Cells with increased fatty acid build-up were then treated with 1, 2, 4, or 8 µg/ml of TA root extracts. The concentration of the cellular fatty acids was assessed 24 h post-treatment, and comparisons were made with those without TA root extract as control (see Figure 2). From the images, a markedly reduced amount of cells with intracellular lipid droplets (stained red) were observed in those cells treated with TA root extract. Moreover, reduced intensity of the red dye was also observed in cells, especially in those treated with 2 and 4 µg/ml of TA root extract. Accordingly, intracellular fatty acids were extracted with DMSO and subjected to

a color intensity measurement. It is worth mentioning, that the average amount of fatty acids quantified in the control sets was regarded as a 100% build-up. Results revealed that treatment with TA root extract at concentrations of 1 to 8 µg/ml diminished the fatty acid build-up when compared to the control sets (see Figure 3). Therefore, these observations have demonstrated

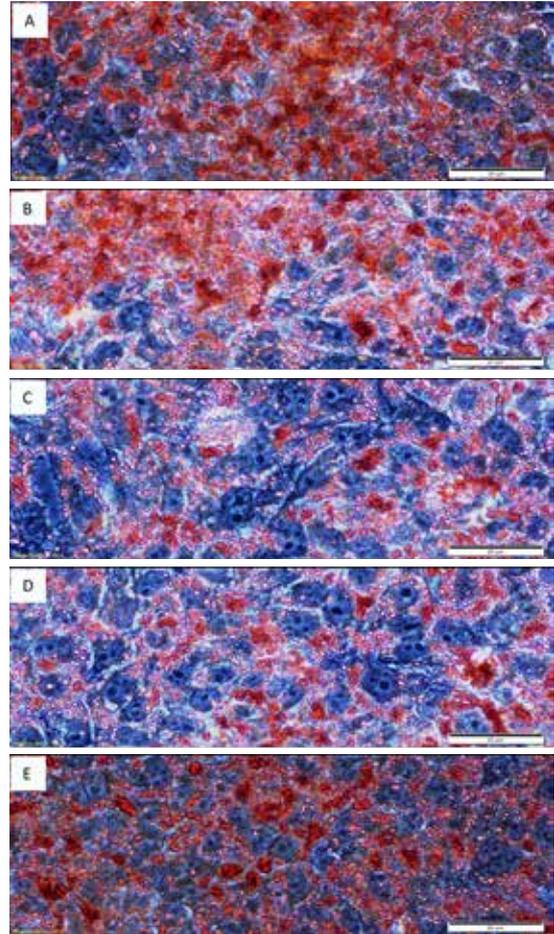


Fig. 2. Lipid lowering effect of TA root extract treatment on fat induced-human hepatic cell line WRL-68.

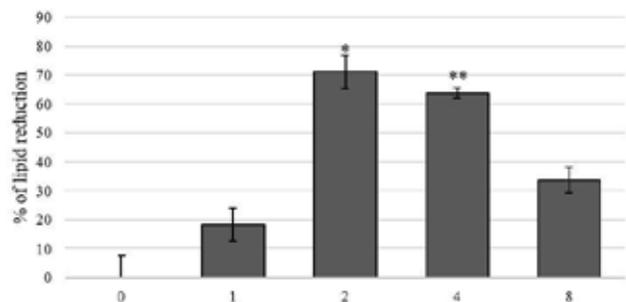


Fig. 3. Percentage reduction of fatty acids in human hepatic cell line WRL-68 upon supplement of TA root extract at various concentrations.

the lipid-lowering potential of TA root extract.

To the best of our knowledge, this is the first observation which demonstrates the intriguing lipid-lowering effect of this medicinal plant without the presence of testosterone in cell milieu. It was observed that a 24 h treatment of 1 $\mu\text{g/ml}$ of TA root extract resulted in 18.2% ($p = 0.3261$) fewer fatty acids detected than in the control cells. Meanwhile, treatment with 2 and 4 $\mu\text{g/ml}$ of TA root extracts resulted in 71.2% ($p = 0.0126$) and 63% ($p = 0.0092$) drops in cellular fatty acids, respectively. Lastly, treatment with 8 $\mu\text{g/ml}$ of the same extract resulted in 33.7% ($p = 0.0922$) when compared to the control cells. The basis for the reduced lipid-lowering potential at 8 $\mu\text{g/ml}$ of TA root extract remains unclear and requires further investigation.

The study further investigated the capability of TA root extract to inhibit fatty acid uptake by the cells. First, the hepatic cell line WRL-68 was treated with the same set of TA root concoctions for 24 h prior to the induction of cellular fatty acid accumulation. This was performed to investigate whether the TA root extract has any prophylactic inhibitory effect on fatty acid absorption. Similarly, all treated cells were compared with a control set, which was cultured in media free of TA root extract. The cells were stained for intracellular lipid droplets accumulated after 24 h of induction. Interestingly, the cell images showed a slight red dye reduction in cells that were treated with different concentrations of TA root extract when compared to the control sets (see Figure 4). Furthermore, spectrophotometric measurement of the color intensity showed up to a 22% color intensity reduction, which is associated with fewer fatty acids absorbed into the cells treated with 2 $\mu\text{g/ml}$ of TA root extract onwards (see Figure 5). However, the reductions were statistically insignificant ($p > 0.08$). Therefore, the results showed a weak inhibition efficiency for fatty acid absorption activity by TA root extract when WRL-68 cells were prophylactically treated.

In order to examine if the TA root extract would interfere with the fatty acid absorption during the induction of cellular fatty acid accumulation itself, different concentrations of TA root extract were included during the induction with 150 μM palmitic acid. Similarly, all treated cells were compared with a control set, which was induced without the addition of TA root extract in the induction media. Again, the cells were stained for intracellular lipid droplets accumulated after 24 h of induction. The images of the cells (see Figure 6) and the spectrophotometric readings (see Figure 7) showed no significant differences in both cellular morphology and color intensity between those induced WRL-68 cells with or without the presence of TA root extract. Thus, the results indicate no interference with

fatty acid absorption during the induction of cellular

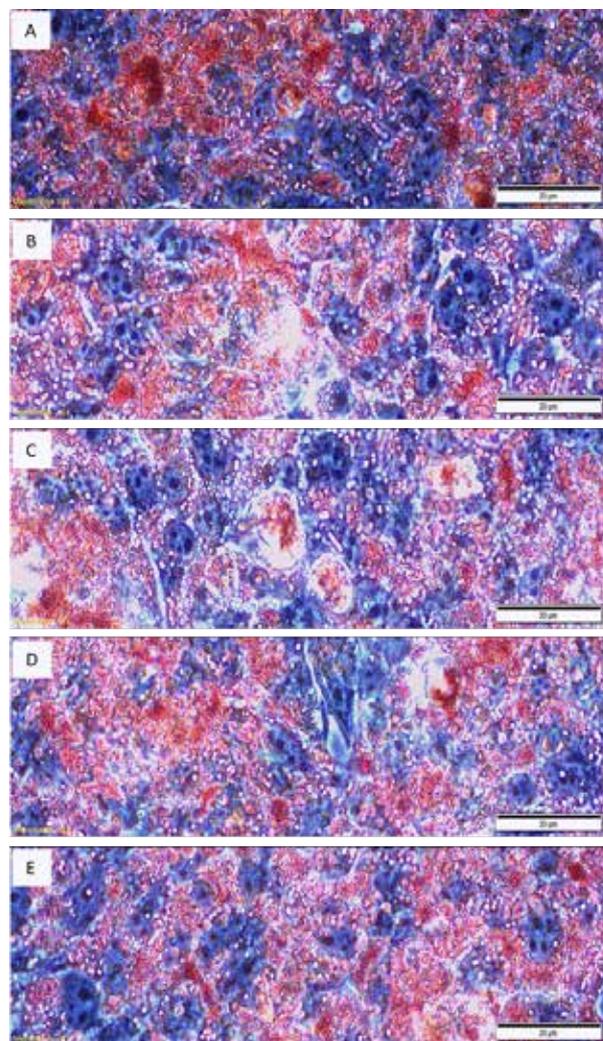


Fig. 4. Prophylactic effect of TA root extract treatment on fat induction of human hepatic cell line WRL-68.

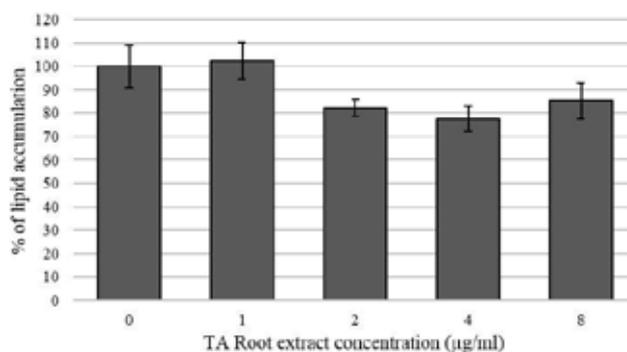


Fig. 5. Percentage accumulation of fatty acids in human hepatic cell line WRL-68 after prophylactic supplementation of TA root extract at various concentrations.

fatty acid uptake in the presence of TA root extract. Administration of the TA root extract was shown to regulate the testosterone level (Hamzah and Yusof, 2003; Chan *et al.*, 2009; Kotirum *et al.*, 2015). In turn, testosterone regulates many physiological processes such as muscle protein metabolism, sexual and cognitive functions, secondary sex characteristics and plasma lipids (Bhasin *et al.*, 2001). Studies involving testosterone replacement in healthy young men have demonstrated a significant increase in overall muscle mass and reduced body fat (Bhasin *et al.*, 2001; Woodhouse *et al.*, 2004; Hamzah and Yusof, 2003). Interestingly, supplementation with complimentary medicine TA root extract has demonstrated similar outcomes to that of the testosterone replacement regime.

In addition, as it is well characterized, increased total

fat is associated with an increased risk of atherosclerotic heart disease, hypertension, and dyslipidaemia (Woodhouse *et al.*, 2004). Therefore, interventions that decrease accumulation of fat in the intraabdominal and intermuscular depots are expected to decrease the cardiovascular risk factors. It is noteworthy that this study has unraveled an additional benefit of TA aqueous root extract to independently reduce intracellular lipids. This phenomenon is most desirable as TA root extract may possess higher fat burning efficacy driven by a testosterone-induced pathway and a TA activated pathway. Work is in progress to further clarify the lipid-lowering potential of TA root extract and to further elucidate the cellular signaling mechanisms that explain the phenomenon.

4. Conclusion

In conclusion, this study provided evidence of intracellular lipid metabolism by TA root extract. The reduction in intracellular lipid droplets was best shown when the induced WRL-68 cells were treated with 2 µg/ml of TA root extract. Moreover, treating the cells prior to or during the induction of cellular fatty acid accumulation with 150 µM of palmitic acid showed no efficacious inhibition of fatty acid absorption activity, albeit the marginal decrease in fatty acid build-up observed in the WRL-68 cells prophylactically treated with 2 µg/ml TA root extract or higher. Nevertheless, this study demonstrates the lipid reduction potential of TA root extract and its availability as a viable alternative medicine, potentially to those with testosterone and fat-related ailments.

Conflicts of interest

All authors and Biotropics Malaysia Berhad declared to have no competing interest relevant to this article. Accordingly, Biotropics Malaysia Berhad has no role in the study design, data collection and analyses, and/or decision to publish or present this research study.

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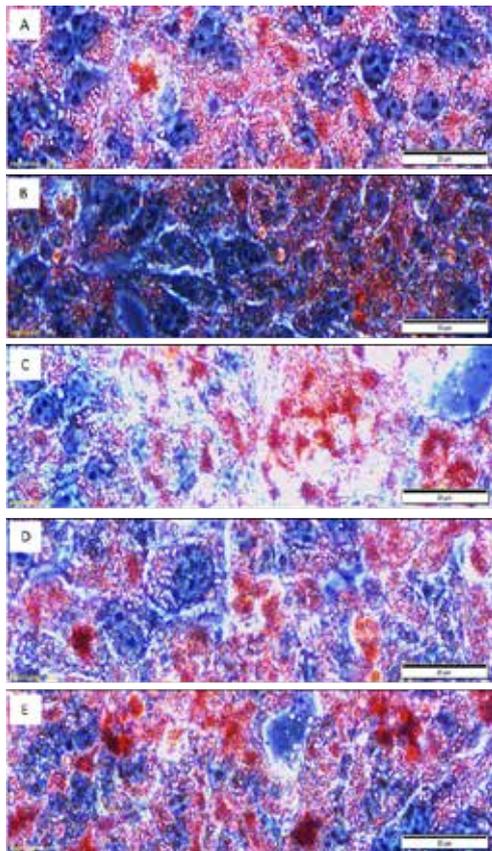


Fig. 6. Effect of TA root extract treatment on fat induction of human hepatic cell line WRL-68.

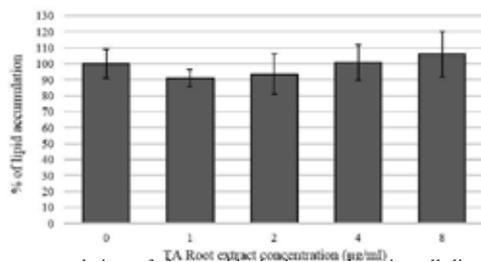


Fig. 7. Percentage accumulation of fatty acids in human hepatic cell line WRL-68 after supplementation of TA root extract at various concentrations during cellular induction of fatty acid accumulation

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تأثير مستخلص مائي من جذور نبات عكازة علي (*Eurycoma longifolia* Jack) على خفض الدهون في خلايا الكبد

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المخلص

يتم استخدام مستخلص مائي من جذور نبات *Eurycoma longifolia* Jack، أو المعروف في لغة الملايو باسم تونكت علي (Tongkat Ali) للتغلب على الكثير من المشاكل الصحية، وخاصة الأمراض المتعلقة بالذكورة. بالإضافة إلى الدليل الذي يثبت التأثيرات العلاجية لهذا المستخلص النباتي كطبخة نوائية فعالة، من الجدير بالذكر أنه قد تم مؤخراً اقتراح إمكانية جديدة لاستخدام المستخلص النباتي في تنظيم التمثيل الغذائي للدهون. ونتائج التجارب على الأجسام الحية والبشر غير قادرة على تحديد ما إذا كان الانخفاض في راسب الدهون ناجماً عن استخدام مستخلص جذور نبات تونكت علي أو مجرد أثر جانبي للتغيرات الفسيولوجية المسجلة. ولذلك، اعتمدت هذه الدراسة نموذجاً في المختبر للتأكد ما إذا كان مستخلص جذور تونكت علي يمتلك القدرة على خفض الدهون و / أو إذا كان يمتلك إمكانات مثبتة لامتناسها في الجسم. تم علاج الخلايا الكبدية البشرية WRL-68 (تغذية عادية ودهنية) بمستخلص جذور نبات تونكت علي بتركيزات مختلفة (1 ، 2 ، 4 ، 8 ميكروغرام / مل). وأظهرت النتائج انخفاض في تراكم الأحماض الدهنية داخل الخلايا بنسبة تصل إلى 72%، في الخلايا التي تمت معالجتها مقارنةً بالخلايا غير المعالجة. ومع ذلك، لم تُظهر المعالجات باستخدام تونكت علي أي تثبيط ملحوظ في امتصاص الأحماض الدهنية مقارنةً بالخلايا في الدراسة المعنية. وبالتالي، فقد كشفت هذه الدراسة إمكانية جديدة لاستخدام تونكت علي وتأثيره على خفض الدهون داخل الخلايا. ويضمن هذا الاكتشاف المزيد من الدراسات لتوضيح آليات إرسال الإشارات الخلوية التي تفسر هذه الظاهرة.