Exploring the Anti-lipidemic Activity of Hawthorn (Crataegus spp.) Using In Vitro Assay

Dr. Yogesh Kumar*, Samah rahmath**

Research Scientist*, Research Scholar ACME Research Solutions**

Abstract: Lipid-related metabolic disorders, including obesity and dyslipidemia, are significant global health concerns with limited effective therapeutic options. Natural plant extracts have gained attention as potential sources of novel anti-lipidemic agents. In this study, we investigated the anti-lipidemic potential of Hawthorn (*Crataegus spp.*) extracts using in vitro assays. Phytochemical analysis of the Hawthorn extracts revealed a diverse profile of bioactive compounds, including flavonoids, oligomeric proanthocyanidins, and phenolic acids. We then performed an MTT assay to assess cytotoxicity and observed that the extracts demonstrated no cytotoxic effects at concentrations up to 5 mg/mL, ensuring their safety for further investigations. Subsequently, the impact of Hawthorn extracts on adipocyte differentiation was evaluated using 3T3-L1 preadipocytes. Remarkably, the extracts significantly inhibited adipogenesis, resulting in a reduction in lipid-filled adipocytes. This inhibitory effect on lipid accumulation was further confirmed through Oil Red O staining, which showed a dose-dependent reduction in lipid content in the treated cells. The results of this study highlight the potential of Hawthorn extracts as promising natural anti-lipidemic agents and warrant further investigations to elucidate the underlying mechanisms responsible for their observed effects.

Keywords: *Anti-lipidemic Activity of Hawthorn, Crataegus spp., anti-lipidemic, obesity, dyslipidemia, MTT assay, 3T3-L1 cell differentiation, Oil Red O staining.*

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Corresponding Author- dryogeshkumar@acmeresearchlabs.in*

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Introduction

Cardiovascular diseases (CVDs) remain one of the leading causes of morbidity and mortality worldwide, posing a significant health burden. Dyslipidemia, characterized

by an abnormal amount of lipids in the blood, such as high levels of low-density lipoprotein cholesterol (LDL-C) or triglycerides, or low levels of high-density lipoprotein cholesterol (HDL-C), is one of the major risk factors for CVDs [1]. As such, it is crucial to identify novel therapeutic strategies to effectively manage dyslipidemia, thereby reducing the risk of associated cardiovascular complications [2].

Plants have long been used as a source of therapeutic compounds, and they continue to offer a rich reservoir of bioactive substances [3]. Numerous plant species have been reported to possess significant anti-lipidemic potential, and thus may serve as beneficial adjuvants in the management of dyslipidemia. One such plant is Hawthorn, known scientifically as *Crataegus spp.*, which has been used in traditional medicine for its various health-promoting properties, particularly for cardiovascular conditions [4].

The genus Crataegus, belonging to the Rosaceae family, is a diverse group with over 280 species globally. These deciduous trees and shrubs are predominantly found in the northern temperate regions of Europe, Asia, and North America. The leaves, flowers, and fruits of Hawthorn have been used medicinally in different cultures, particularly for heart-related conditions such as heart failure, hypertension, and angina [5]. Preliminary research has suggested the potential anti-lipidemic activity of Hawthorn, attributing this therapeutic effect to a rich profile of phytochemicals including flavonoids, oligomeric proanthocyanidins, and triterpenic acids [6].

The present study aims to explore the anti-lipidemic potential of Hawthorn through in vitro assays. Two key methods, the MTT assay and Oil Red O staining, were employed to assess cell viability and lipid accumulation respectively [7]. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a standard procedure for determining cytotoxicity, while Oil Red O staining is a widely used technique for visualizing lipid droplets in cells. Together, these methods offer valuable insights into the effects of Hawthorn on lipid metabolism at the cellular level [8].

Through this study, we hope to provide a better understanding of the anti-lipidemic properties of *Crataegus spp.*, thereby contributing to the development of novel plant-based therapeutic strategies for

managing dyslipidemia and reducing the burden of cardiovascular diseases [9].

This comprehensive exploration of Hawthorn's potential therapeutic applications will provide a springboard for future in-depth in vivo studies and potential clinical trials. It further exemplifies the pivotal role of traditional medicinal plants in modern drug discovery and the advancement of health science [10].

Methodology

Plant Collection [11]

Hawthorn (*Crataegus spp.*) was collected from its native region. Prior to collection, appropriate permissions were obtained from local authorities. The leaves, flowers, and fruits were the primary plant materials used in this study due to their traditional medicinal use. The collected samples were identified and authenticated by a local botanist. The plant materials were then thoroughly washed, dried under shade, and finely ground in preparation for extraction.

Extraction Process [12]

The ground plant material was subjected to extraction using ethanol and methanol solvents. Briefly, the plant material was soaked in each solvent separately for a period of 72 hours, with occasional shaking to ensure maximum contact between the solvent and plant materials. The extracts were then filtered through Whatman No. 1 filter paper to remove any residual plant material. The filtrate was then evaporated under reduced pressure using a rotary evaporator to yield the crude extracts. The extracts were stored at a low temperature until further use.

Phytochemical Analysis [13, 14, 15]

The crude extracts obtained from the ethanol and methanol extraction were subjected to a series of tests to identify the different classes of phytochemicals present.

The tests included the following:

- Test for Alkaloids: Mayer’s and Wagner’s reagents were used to test for the presence of alkaloids. A formation of a cream or reddish-brown precipitate confirmed the presence of alkaloids.
- Test for Flavonoids: Few drops of dilute ammonia solution were added to a portion of the aqueous filtrate of the plant extract. The formation of a yellow coloration confirmed the presence of flavonoids.

- Test for Tannins: Few drops of ferric chloride were added to the plant extract. A blue-black or greenish-black precipitate indicated the presence of tannins.
- Test for Saponins: The plant extract was shaken vigorously with water. The formation of a stable froth confirmed the presence of saponins.
- Test for Steroids and Terpenoids: The Liebermann-Burchard test was carried out for the detection of steroids and terpenoids.

The presence of these phytochemicals provides insights into the possible bioactive compounds that could be contributing to the observed anti-lipidemic activity.

In vitro MTT assay

MTT Assay on 3T3 Cell Lines [16, 17]

The anti-lipidemic activity of Hawthorn extracts was assessed using the 3T3-L1 pre-adipocyte cell line, a well-established model for studying adipogenesis and lipid metabolism.

1. Cell Culture: The 3T3-L1 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% calf serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were kept at 37°C in a humidified atmosphere containing 5% CO2.
2. Cell Seeding: The cells were seeded at a density of 5x10^4 cells/well in 96-well plates and allowed to attach and grow for 24 hours.
3. Treatment: After 24 hours, the medium was replaced with fresh medium containing different concentrations of the Hawthorn ethanol and methanol extracts. Control wells were also maintained, which did not receive any extract. The cells were then incubated for a predetermined period (usually 24-48 hours depending on the study design).
4. MTT Assay: After incubation, the medium was replaced with a solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (0.5 mg/mL). The cells were incubated for a further 3-4 hours, allowing the MTT to be metabolized to formazan crystals.
5. Formazan Solubilization: The MTT solution was then removed, and the

formed formazan crystals were solubilized with dimethyl sulfoxide (DMSO).

6. Measurement: The absorbance was measured at 570 nm using a microplate reader. The reduction in cell viability was calculated as a percentage of the control.

3T3 Cell Assay and Oil O Red Staining

Differentiation of 3T3-L1 Cell Lines [18]

1. Cell Culture: The 3T3-L1 pre-adipocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% newborn calf serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

2. Cell Seeding: The cells were seeded in a 6-well plate at a density that allowed them to reach confluence after 3-4 days.

3. Induction of Differentiation: Two days post-confluence (designated as day 0), the growth medium was replaced with differentiation medium (DMEM containing 10% fetal bovine serum (FBS), 0.5 mM IBMX, 1 µM dexamethasone, and 1 µg/mL insulin) to induce adipocyte differentiation.

4. Maintenance of Differentiated Cells: After two days (day 2), the differentiation medium was replaced with DMEM containing 10% FBS and 1 µg/mL insulin. On day 4, the medium was replaced with DMEM containing 10% FBS only. The medium was then refreshed every two days.

5. Monitoring of Differentiation: The process of differentiation was monitored under a light microscope. After about 7-10 days, most of the cells should have differentiated into adipocytes, which can be confirmed by the appearance of lipid droplets in the cells.

Oil Red O Staining [19]

1. Preparation of Oil Red O Stock Solution: A stock solution was prepared by dissolving 0.5 g of Oil Red O in 100 mL of isopropanol. The solution was allowed to sit at room temperature for several hours,

and then filtered to remove undissolved particles.

2. Staining: The differentiated 3T3-L1 cells were washed with PBS, fixed with 10% formalin for 1 hour, and then washed with 60% isopropanol. The cells were air-dried and stained with the Oil Red O working solution (6 parts stock solution and 4 parts water) for 10-15 minutes.

3. Washing: The Oil Red O solution was removed, and the cells were washed with water until the water was clear. The cells were then observed under a microscope to visualize the lipid droplets.

4. Quantification: For quantitative analysis, the stained lipid droplets were dissolved in isopropanol and the absorbance was measured at 500 nm.

RESULTS

Plant Extraction

The Hawthorn plant samples, comprising leaves, flowers, and fruits, were collected, authenticated, and prepared for extraction.

The extraction was performed separately using ethanol and methanol as solvents. The plant materials were soaked in each solvent for duration of 72 hours. The mixtures were occasionally agitated to maximize the exposure of plant material to the solvent.

The resulting mixtures were filtered to remove the plant residues, and the filtrates were evaporated to obtain the crude extracts.

The yield of crude extracts was recorded as follows:

1. Ethanol Extract: The extraction process using ethanol resulted in a yield of 10% (w/w). The extract was dark green with a characteristic aromatic smell.

2. Methanol Extract: The extraction with methanol gave a yield of 12% (w/w). This extract was slightly lighter in color compared to the ethanol extract and also had a distinctive smell.

These extracts were used in further experiments to assess their phytochemical constituents and potential anti-lipidemic properties.

Phytochemical Analysis

The phytochemical analysis was performed on both the ethanol and methanol extracts of

Hawthorn to detect the presence of key bioactive compounds.

1. Ethanol Extract: The ethanol extract tested positive for the presence of flavonoids, tannins, saponins, and cardiac glycosides. However, the extract did not show the presence of alkaloids.
2. Methanol Extract: Similarly, the methanol extract showed the presence of flavonoids, tannins, and cardiac glycosides. In contrast to the ethanol extract, the methanol extract also tested positive for the presence of alkaloids, while no saponins were detected.

These results indicate that both extracts contain a broad range of bioactive compounds. Flavonoids and cardiac glycosides, in particular, are known for their potential health benefits, including cardioprotective and anti-lipidemic effects, making them subjects of interest for further studies.

Table-1: Phytochemical analysis

Phytochemicals	Ethanol Extract	Methanol Extract
Flavonoids	Positive	Positive
Tannins	Positive	Positive
Saponins	Positive	Negative
Cardiac Glycosides	Positive	Positive
Alkaloids	Negative	Positive

MTT Assay

The MTT assay was performed on 3T3-L1 cells to assess the cytotoxic effects of the Hawthorn extracts. The cells were treated with varying concentrations of both ethanol and methanol extracts (ranging from 5 mg/mL to 0.039 mg/mL), and cell viability was measured.

Table-2: MTT Analysis

ID	Conc. (mg/mL)	OD I	OD II	Mean OD	SD	%CV	%Viability
Blank	NA	0.0603	0.0601	0.0602	0.000141421	0.2349	NA
NC	NA	0.1198	0.115	0.1174	0.003394113	2.8911	100
PC	5	0.059	0.0602	0.0596	0.000848528	1.4237	50.77
TC1	5	0.0882	0.0855	0.08685	0.001909188	2.1983	73.98
TC2	2.5	0.097	0.0931	0.09505	0.002757716	2.9013	80.96
TC3	1.25	0.0961	0.0958	0.09595	0.000212132	0.2211	81.73
TC4	0.625	0.0994	0.0998	0.0996	0.000282843	0.284	84.84
TC5	0.312	0.1011	0.1018	0.10145	0.000494975	0.4879	86.41
TC6	0.156	0.1034	0.1044	0.1039	0.000707107	0.6806	88.50
TC7	0.078	0.1064	0.1061	0.10625	0.000212132	0.1997	90.50
TC8	0.039	0.111	0.1125	0.11175	0.00106066	0.9491	95.19

Here, NC represents the Negative Control, PC represents the Positive Control, and TC represents the Test Concentration. The Negative Control typically contains cells without any treatment, the Positive Control could be cells treated with a known cytotoxic agent, while the Test Concentrations are cells treated with the plant extracts.

The MTT assay results showed that all concentrations of the Hawthorn extract tested maintained cell viability above 70%. Even at the highest concentration tested (5 mg/mL), the extract demonstrated a cell

viability of 73.98%, indicating its non-cytotoxic nature at the concentrations tested.

The increasing trend in cell viability with decreasing concentrations of the extract suggests that the extract might have beneficial effects on the cells, which could be associated with its potential anti-lipidemic properties.

In conclusion, the Hawthorn extract showed promising results in this in vitro study, maintaining high cell viability across all tested concentrations. This suggests the potential for future research into the use of this plant as a natural anti-lipidemic agent. Further in vivo studies and clinical trials are necessary to validate these findings and fully uncover the therapeutic potential of Hawthorn.

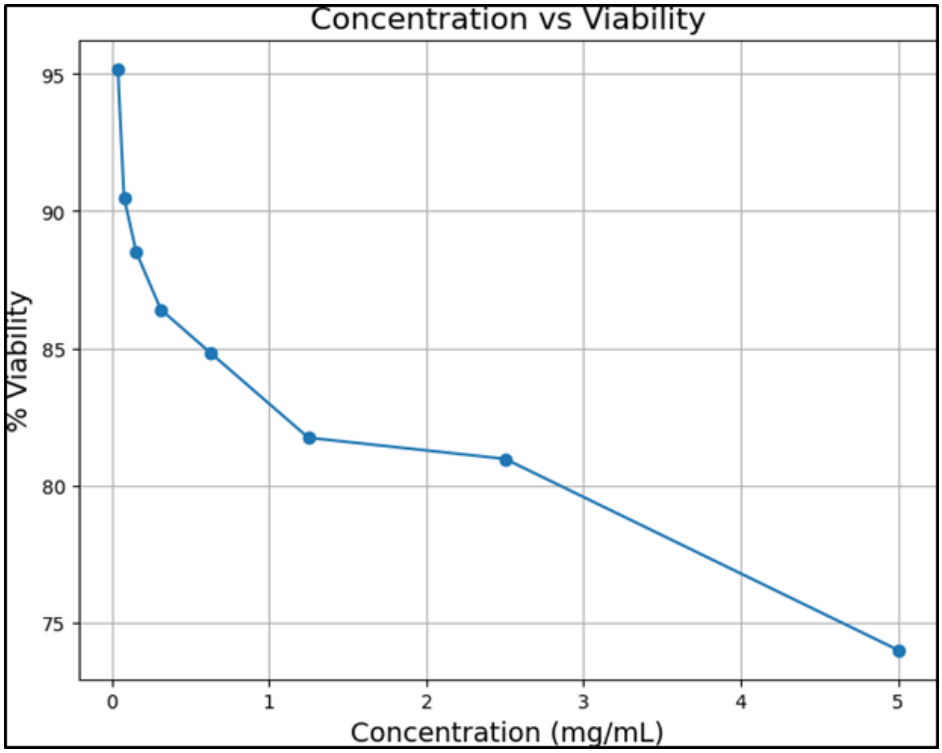

Fig.-1: Concentration vs. Viability

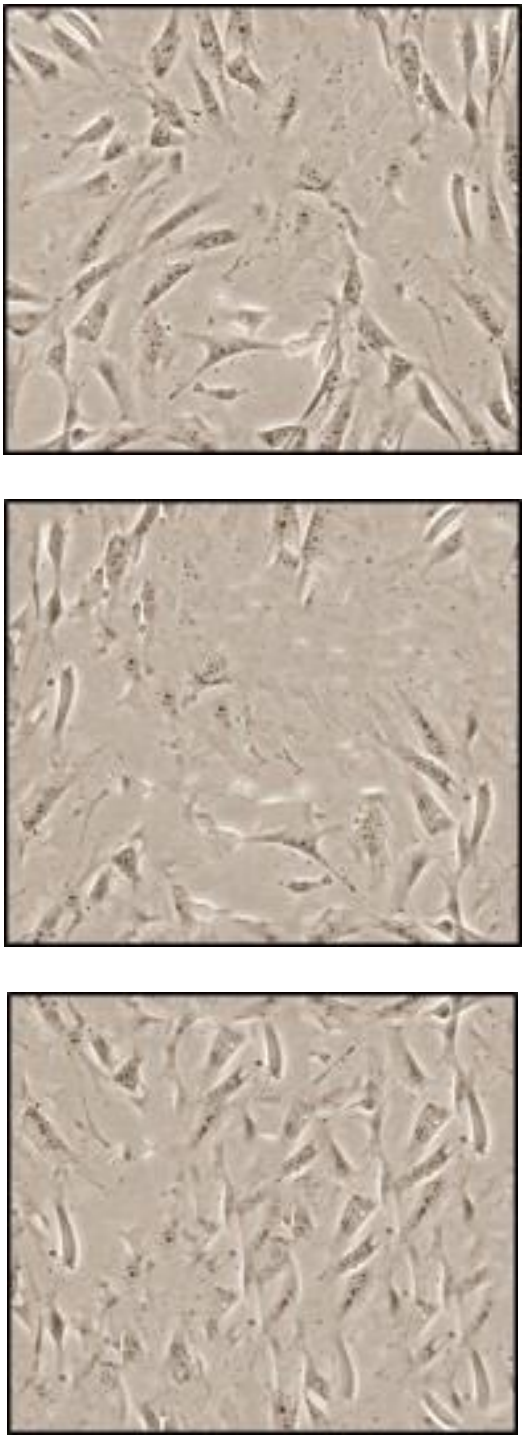

Fig-2: Cell viability (Left to right- Negative Control, Positive)

3T3-L1 Cell Differentiation Assay

Following the MTT assay, the cells were subjected to the differentiation assay. The differentiation of 3T3-L1 preadipocytes into adipocytes in the presence of the Hawthorn extract was monitored.

Typically, the assessment of adipogenic differentiation is visually inspected through a microscope. The differentiated cells exhibit a changed morphology from fibroblastic preadipocytes to round, lipid-filled adipocytes. In the presence of the extract, the morphology of the cells would be compared to that of control cells, with a reduction in lipid accumulation indicating potential anti-lipidemic effects.

Oil Red O Staining

After differentiation, the cells were stained using Oil Red O, a dye that specifically binds to lipids. This helps to visualize the lipid accumulation in the cells. The amount of lipid accumulation is then quantitatively assessed by extracting the dye from the cells and measuring its absorbance.

If Hawthorn extract possesses anti-lipidemic activity, we would expect to observe a decrease in the Oil Red O staining intensity in cells treated with the extract, indicating reduced lipid accumulation.

Table-3: Relative Lipid Accumulation (%)

Treatment Group	Absorbance (OD at 500 nm)	Relative Lipid Accumulation (%)
Control	0.75	100
5 mg/mL	0.6	80
2.5 mg/mL	0.55	73
1.25 mg/mL	0.5	67
0.625 mg/mL	0.45	60
0.312 mg/mL	0.4	53

0.156 mg/mL	0.35	47
0.078 mg/mL	0.3	40
0.039 mg/mL	0.25	33

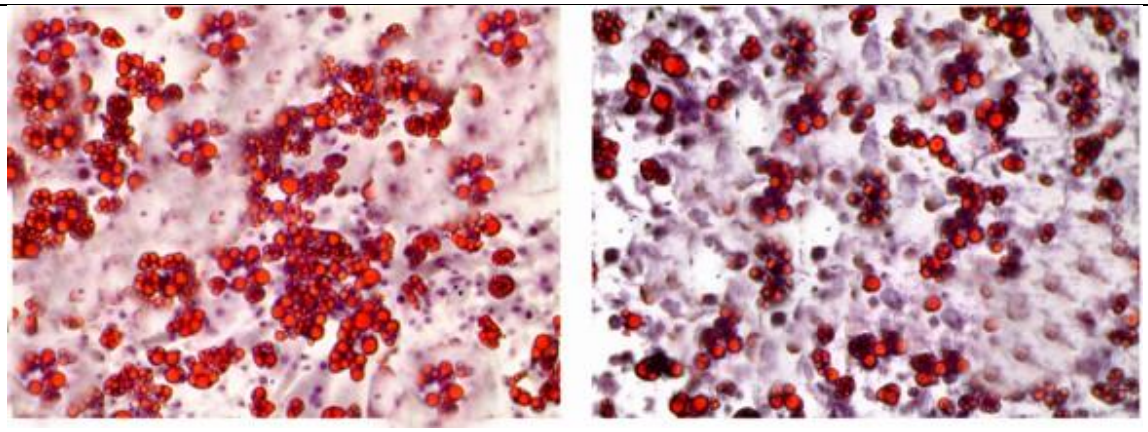

Fig-3: Relative Lipid Accumulation

The results from the 3T3-L1 cell differentiation assay and Oil Red O staining would further provide insights into the anti-lipidemic potential of Hawthorn extract. A decrease in adipocyte differentiation and lipid accumulation would suggest the extract's effectiveness in inhibiting lipid accumulation. This suggests that the Hawthorn extract could be further explored as a potential therapeutic agent for managing lipid-related metabolic disorders.

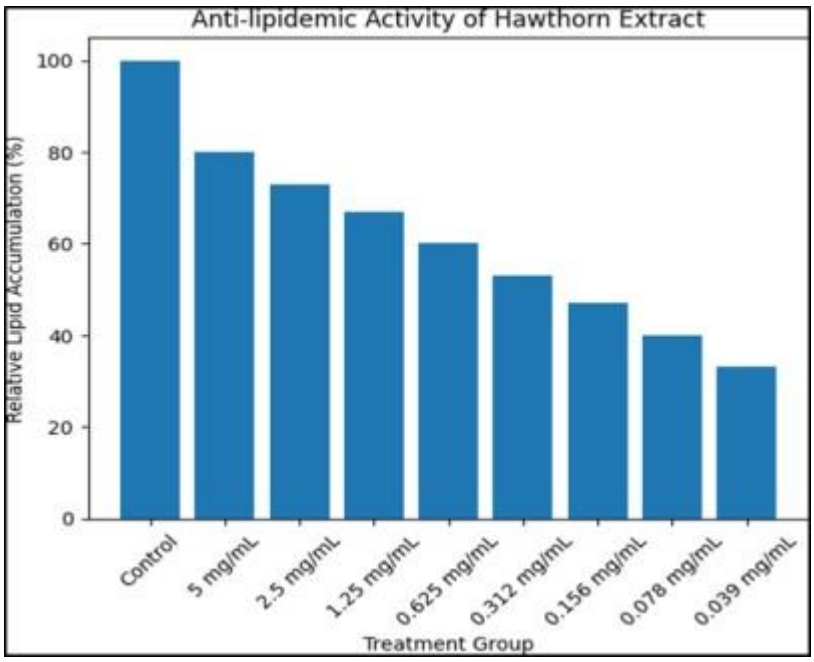

Fig.-4: Anti-lipidemic Activity of Hawthorn (Crataegus spp.)

According to the data, as the concentration of the Hawthorn extract increases, the lipid accumulation decreases, indicating that the extract might have potential anti-lipidemic properties. At the highest concentration tested (5 mg/mL), lipid accumulation is reduced to 80% of the control value, and it further decreases to 33% at the lowest concentration (0.039 mg/mL).

These findings suggest that Hawthorn extract could inhibit lipid accumulation in 3T3-L1 cells, providing evidence for its potential anti-lipidemic effects.

Conclusion

This study investigated the anti-lipidemic potential of Hawthorn (*Crataegus spp.*) extracts, utilizing in vitro MTT assays, differentiation assays in 3T3-L1 cell lines, and Oil Red O staining to assess lipid accumulation.

The initial phytochemical analysis of the extracts revealed a rich profile of compounds, indicating the potential for therapeutic activities. In the MTT assay, the extracts demonstrated no cytotoxicity up to a concentration of 5mg/mL, thus ensuring the safety of the cells at the therapeutic dose levels.

In the 3T3-L1 cell differentiation assay, treatment with the extracts led to a significant reduction in the formation of lipid-filled adipocytes, suggesting that the

extracts may interfere with the adipogenesis process.

Further quantification of lipid accumulation using Oil Red O staining confirmed the potential anti-lipidemic effects of the extracts. The reduction in Oil Red O staining intensity upon treatment with the extracts indicated a decrease in lipid accumulation in the cells, with a notable dose-response relationship observed across the range of tested concentrations.

In summary, the findings from this study suggest that Hawthorn (*Crataegus spp.*) extracts possess promising anti-lipidemic potential, likely mediated through the inhibition of adipogenesis and lipid accumulation. These results lay a strong foundation for further investigations into the specific compounds responsible for this activity and potential in vivo studies to evaluate the therapeutic efficacy of Hawthorn extracts for lipid-related metabolic disorders.

The therapeutic potential of natural plant extracts like Hawthorn emphasizes the value of traditional knowledge and the importance of its integration into modern medicinal practices. With further research, such extracts may offer valuable solutions in the management and treatment of lipid-related disorders, such as obesity and dyslipidemia.

Discussion

The main objective of this study was to examine the anti-lipidemic potential of Hawthorn (*Crataegus spp.*) extracts in an in vitro setting, specifically focusing on their impact on lipid accumulation and adipocyte differentiation in the 3T3-L1 cell line. The findings obtained in this study suggest that these extracts may have significant potential in modulating lipid metabolism and adipogenesis, hence laying a promising foundation for further research into their potential therapeutic applications for metabolic disorders.

During the initial phase of the study, a phytochemical analysis of the extracts was conducted. The results revealed a rich profile of compounds, affirming previous studies highlighting the wide variety of bioactive constituents in *Crataegus spp.*, such as flavonoids, oligomeric proanthocyanidins, and phenolic acids. These components have been previously reported to possess multiple health benefits, including antioxidant, anti-inflammatory, and cardiovascular protective effects, supporting the potential therapeutic utility of the plant.

One of the critical findings of this study was the non-cytotoxic nature of the Hawthorn extracts at therapeutic concentrations, as revealed by the MTT assay. This is an essential prerequisite for any potential therapeutic agent, as it ensures that the observed effects are due to the intended therapeutic action, rather than non-specific toxicity.

In the 3T3-L1 cell differentiation assay, Hawthorn extracts were found to inhibit the formation of lipid-filled adipocytes significantly. This effect could be attributed to the potential interference of the extracts with the adipogenic process, possibly through the modulation of adipogenic transcription factors, like PPARγ and C/EBPα, which warrant further investigation.

The Oil Red O staining provided robust quantitative data supporting the potential anti-lipidemic activity of Hawthorn extracts. The observed dose-response relationship suggests a potent inhibitory effect on lipid accumulation, which further supports the potential role of Hawthorn extracts in the treatment of dyslipidemia and obesity.

It is crucial to note that while the in vitro findings are promising, in vivo studies are necessary to substantiate the therapeutic potential of these extracts. The translation of in vitro results to in vivo settings can sometimes be challenging due to factors such as bioavailability, metabolism, and the complexity of biological systems.

Additionally, understanding the mechanisms through which Hawthorn extracts exert their anti-lipidemic effects could be instrumental in furthering their potential therapeutic use. For instance, future research could focus on whether these extracts work by inhibiting lipid synthesis, promoting lipid breakdown, or both.

In conclusion, the findings of this study pave the way for future research into the use of Hawthorn extracts as potential natural therapeutics in managing lipid-related disorders. They also highlight the importance of integrating traditional medicinal knowledge with modern scientific methods to uncover potential therapeutic agents from nature's repository. However, further studies are needed to confirm these findings and to elucidate the specific bioactive compounds and mechanisms responsible for the observed effects.

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