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Original Article

Inhibition of LDL Oxidation and Foam Cell Development of Tannin Methanol Extract from *Citrus limon* and Honey Formulation on Cell lines

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ABSTRACT

Background: *C. limon* rich in Vitamin C was shown to have many health benefits having therapeutic properties. Honey was considered as an natural sweetener said to be immune booster. Hence the formulation of *C. limon* and honey formulation was studied to prevent oxidation and foam cell inhibition. **Objectives:** The present study was aimed to isolate tannins from *Citrus limon* (*C. limon*) fruit juice with a honey mixture to evaluate the antilipidemic and antioxidant activity in cell lines. **Materials and Methods:** Tannins were isolated from *C. limon* fruit juice with a honey mixture with two different solvents, i.e., methanol and water and analysed for antioxidant and LDL oxidation inhibition activity. Further tested on RAW 264.7 and THP-1 cells by employing *in-vitro* assays for cytotoxicity, foam cell development, inhibiting proliferation and, apoptosis. The tannin methanol extract was characterized using HPLC and GC-MS. **Results:** The content of total tannins present was found to be 453.96 mg tannic acid equivalent (TAE)/g methanol extract, whereas 415.87 mg TAE/g in tannin aqueous extract. The highest antilipidemic and antioxidant activity were seen in tannin methanol extract. It was seen that 240 µg/mL tannin methanol extract efficiently inhibited oxidation of low-density lipoproteins (55.89% at 5 hr), thus preventing foam cell development, inhibiting proliferation and, apoptosis in cultured RAW 264.7 and THP-1 cells. GCMS revealed certain compounds. **Conclusion:** This study showed that *C. limon* with honey tannin methanol extract exhibits potential for antiatherosclerosis activity henceforth, considering the medicinal properties of the active phytochemicals, which can be used as a source of naturally occurring nutraceuticals, revealed the potential to prevent oxidation and foam cell formation.

Key words: *Citrus limon*, Tannin methanol extract, Antioxidant activity, LDL oxidation inhibition, Foam cell inhibition.

INTRODUCTION

Oxidation of low-density lipoprotein (ox-LDL) is caused due to raised blood plasma LDL levels with increased cell permeability.[1] The free radicals like superoxide and nitric oxide trigger ox-LDL.[2] Circulating lipoproteins are taken up by macrophage, which in turn gets converted to foam cells by LDL oxidation.[3,4] These foam cells accumulate in the arterial walls causing plaque formation leading to inflammation and narrowing of the arterial lumen in atherosclerosis by preventing the blood flow causing a heart attack.[5] Therefore atherosclerosis is essentially caused because of free radicals and oxidized LDL.

Ayurveda and Siddha are the conventional and the oldest Indian system of medicine in the world, as reported.[6] Some plants have anti-atherosclerotic effects, mentioned in Ayurveda, such as Guggulu, *Emblica officinalis, Allium sativum,* etc.[7,8] Statins are used to treat atherosclerosis, but they have an increased risk of toxic effects such as myotoxicity with muscle symptoms, hepatotoxicity,[9] myopathy, type 2 diabetes mellitus, neurocognitive effects, renal toxicity.[10] Medicines from plant sources have multiple treatment modes with enhanced suitability with lesser side effects. Also, fruits, vegetables, and phytochemicals are rich in antioxidants which can ameliorate cardiovascular diseases.[11] Herbal medicine, rich in nutraceuticals, needs to be implemented for the treatment modules.[12] Most research is aimed to search for natural products from plants possessing anti-atherosclerotic properties for the treatment of atherosclerosis.

Citrus limon (L.) tree belongs to the family Rutaceae, with evergreen leaves. The fruit is edible with a yellow color. Its natural fruit juice is a rich source of Vitamin C, has an impressive range of benefits and immense nutritional properties, and is also

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rich in essential oil.[13] Studies have shown that tannins are present in the maximum amount in fruit juice. Previous studies reported that *C. limon* is the rich source of naturally occurring tannins and is present in all parts of the plant, used for many therapeutic purposes.[14] Recent studies have indicated that *C. limon* has several pharmacological actions related to anticancer, antiulcer, antioxidative, hepatoprotective and, anti-hyperglycemic activity.[15] The previous report has shown the antioxidant and antidiabetic activity of *Citrus hystrix* and *Citrus maxima* in fresh fruit juice.[16]

In addition, honey acts as a natural sweetener, consumed in combination with different foods or by itself which have immense health benefits having antioxidant and anti-inflammatory properties.[17,18] Reports say that honey contains more than 180 components with minerals, vitamins, and amino acids.[19]

Both *C. limon* fruit juice and honey formulation have not been identified for the valuable biological activities of tannins in present modern medicine. Hence in the present study, tannin from *C. limon* fruit juice with honey in combination was looked at its antioxidant and inhibitory property on foam cell progression, which is identified as cardioprotective activity.

MATERIALS AND METHODS

Sample preparation

C. limon fruit was collected from the Bangalore market, India. The fruit juice from the lemon was extracted and filtered, and honey was collected from the Nilgiris biosphere, India. Both were mixed in a 1:1 v/v ratio. Methanol and water extraction were carried out (1:20 v/v) sample and solvent ratio incubated in a shaker incubator overnight. The supernatant was separated and lyophilized to get a dried sample.[20]

Extraction and estimation of total tannin

Tannin-rich extracts were prepared according to the method described within.[21] The isolated extract was dried, and the percentage yield was calculated.[22] Estimation was carried out with a few modifications,[23,24] and the tannin concentrations were calculated based on the standard and expressed as mg TAE/g dry weight.

$$\%\text{Yield} = (\text{Final weight})/(\text{Initial weight}) \times 100$$

LDL Isolation and LDL oxidation inhibition assay

Low-density lipoprotein (LDL) isolation was done by density gradient ultracentrifugation technique from human serum. Human blood was collected from a healthy volunteer and added into EDTA tubes. Plasma was separated from other components in the tube at 3000 rpm for 15 min by centrifugation. Further, potassium bromide was added to the plasma to alter the density to 1.3 g/mL. In the 40 mL ultracentrifuge quick seal tubes, 30 mL of saline was layered with a density of 1.006 g/mL on 10 mL of adjusted plasma followed by centrifugation (Beckman ultracentrifuge, USA) at 65,000 rpm for 90 min. LDL was separated with a syringe and dialyzed against 0.1 M phosphate-buffered saline (PBS) overnight to remove the salts and stored at -20°C.[25] The experiments were carried out following the Institutional Ethical Committee (Reg No: 606/03/C/CPCSEA).

Different concentrations with the two-fold dilution of tannin methanol and aqueous extracts (15 - 240 µg/mL), ascorbic acid, were incubated with LDL (100 µg/mL) for 30 min in the total volume of 2 mL. To start the reaction, one mL of 10 µM copper sulfate was added. Addition of 0.5 mL equal proportion of 2-thiobarbituric acid and trichloroacetic acid taken at 2-time periods, namely., 5 hr and 20 hr incubation and mixing. Then the tubes were placed in boiling water for 30 min. By cooling the mixture, the pink color developed. The absorbance was read at 532 nm (Spectramax 13x plate reader, USA).[25,26] Percentage inhibition was calculated.

$$\%\,\text{Inhibition} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{control}}} \times 100$$

Nitric oxide scavenging assay (NO)

Two mL of tannin methanol and aqueous extract at different concentrations were added to 2 mL of sodium nitroprusside (Prepared in 0.1 M PBS at pH 7.4), following incubation at 37°C for 150 min. Later, 4 mL of Griess reagent was added to the tubes. Pink coloration was formed due to the reaction with naphthyl ethylenediamine in the reagent. Absorbance was checked at 546 nm along with standard as ascorbic acid.[27] Inhibition percentage and IC_{50} (µg/mL) value was calculated to determine nitrite scavenging activity.

Superoxide radical scavenging assay (SO)

Different concentrations of 0.5 mL tannin extract from *C. limon* fruit juice and honey mixture (1 mg/mL stock) were mixed with 1 mL of nicotinamide adenine dinucleotide and nitroblue tetrazolium (100 mM phosphate buffer, pH 7.4) solution. To begin the reaction, 100 µL of phenazine methosulphate solution was added, followed by incubation at 25°C for 5 min, and read at 560 nm.[28] Standard was used as ascorbic acid, percentage inhibition, and IC_{50} (µg/mL) was calculated.

2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH)

50 µL of different sample concentrations were taken in separate tubes, and the volume was made up to 100 µL with methanol in each tube.[29] Standard was used as ascorbic acid. Further, 1 mL of DPPH solution was added and incubated for 30 min in the dark. The absorbance was read at 517 nm.[30] Percentage inhibition and IC_{50} (µg/mL) value was calculated for the extracts.

Cell lines and stock solutions

Dulbecco's Modified Eagle's medium (DMEM) supplemented with fetal bovine serum, 100 µg/mL streptomycin, 100 µg/mL penicillin was treated to cultured RAW 264.7 cells (ATCC® TIB-71™; *Mus musculus*) and THP-1 cells (ATCC® TIB-202, USA) in 5% CO_2 incubator at 37°C for targeted cells per 96 microtiter well plate for 24 hr. One mL dimethyl sulfoxide (DMSO) was used to prepare 10 mg/mL tannin methanol extract stock.

Cytotoxicity test using MTT assay

RAW 264.7 and THP-1 cells (50,000 cells/well) with log-spaced tannin methanolic extract (1.560, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, 600, 800, 1000 µg/mL) concentration were incubated with ox-LDL 100 µg/mL in 5% CO_2 incubator for 24 hr. Cells only with ox-LDL were treated as control. 100 µL of MTT prepared in PBS pH 7.4 was added and incubated for 4 hr. Later, the supernatant was discarded, followed by the addition of 100 µL of DMSO, and the plates were shaken gently. The absorbance was read at 590 nm.[31] The percentage inhibition and IC_{50} (µg/mL) values were calculated.

Foam cell inhibition assay

RAW 264.7 and THP-1 (100 µL of 3 x 10^5 cells) were seeded in the plate. To this, 50 µg/mL ox-LDL was added with 100 µL of different extract concentrations (1.56, 3.13, 6.25, 12.50, 25 µg/mL), without extract as control and standard as simvastatin were incubated for 48 hr. Followed by fixing the cells for 15 min in paraformaldehyde, subsequently stained with 1% oil O red for 30 min.[32] Foam cells get stained with oil O red which was observed under a microscope 60X (Lawrence and mayo) by rinsing the cells with particle-free water, followed by incubation for 10 min with 250 μL of isopropanol. The absorbance was read at 492 nm.[33] Percentage inhibition of foam cells were measured. IC_{50} (μg/mL) values were calculated.

Cell proliferation assay

Treating 8.0 x 10^5 cells (RAW 264.7 and THP-1 cells) with different concentrations of 100 μL of extract (0.78, 1.56, 3.13, 6.25, 12.50, 25 μg/mL) by inducing 20 μg/mL ox-LDL for 6 days in CO_2 incubator.[34] Simvastatin was read as a standard. To this, 100 μL of MTT was added by incubating in a 5% CO_2 incubator for 5 hr. Later, the supernatant was discarded, followed by adding 100 μL of DMSO, incubated for 30 min. The absorbance was read at 550 nm spectrophotometrically. Cell proliferation was measured by percentage inhibition of the cells. IC_{50} (μg/mL) values were calculated.[35,31]

Apoptosis study by trypan blue assay

100 μg/mL of ox-LDL and tannin methanol extract at different concentration (0.78, 1.56, 3.13,6.25, 12.50, 25, 50 μg/mL) was incubated with 5.0 × 10^5 cells (RAW 264.7 and THP-1) in 96 well plate, for 24 hr. The cells were stained with 20 μL of trypan blue dye for 15 min and washed with 1X PBS. Further, lysing the cells with 100 μL of sodium dodecyl sulfate (1%) to observe the dead cells stained with trypan blue. The absorbance was measured at 590 nm. Inhibition percentage and IC_{50} (μg/mL) values were calculated.[36,37]

High-Performance Liquid Chromatography (HPLC)

In the present experiment, tannin methanol extract was analyzed using HPLC (Waters model no. 486; Waters Corp., Milford, MA, USA) with tannic acid as standard. Isocratic elution mode was performed with acetonitrile and water mixture (70:30) with a C_{-18} column having a 1 mL/min flow rate. The standard and sample (1 mg/mL) were prepared in the mobile phase. 20 μL was injected, and the elution was observed at 270 nm for tannic acid present in sample.[38]

GC-MS analysis

A sample containing tannin methanol extract was subjected to GC-MS analysis (Agilent 8890). The non-polar column was used (DB 624) by maintaining the instrument condition with 1.2 mL/min helium gas flow and initial oven temperature 50°C raised to 250°C by introducing 1μL of sample to injecting port. Chromatogram with mass by charge ratio was obtained, and the compounds were identified by the library search (National Institute of Standard and Technology MS Version.2.3 -2017).

Statistical analysis

Presented results were expressed as n=3 mean ± SD, statistically significant was considered $P \leq 0.05$ in all analysis by Tukey's test and Bonferroni posttest (two-way analysis) for LDL oxidation Inhibition assay after ANOVA using GraphPad Prism software Inc (version 5.03) (California, USA). Significance was represented as $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ and $^{ns}P > 0.05$ respectively.

RESULTS

Isolation of phytochemical-rich extracts from *C. limon* fruit juice and honey mixture

Total tannin methanolic extract concentration from *C. limon* fruit juice and honey was found to be highest in the sample, having 453.96 mg TAE /g dry weight. In contrast, tannin aqueous extract showed 415.87 mg TAE /g dry weight, respectively. Also, the percentage yield of tannin aqueous extract was found to be the highest, which was determined to be 19.53% [Table 1].

Inhibition of LDL oxidation assay

The preliminary cause of plaque formation in atherosclerosis is because oxidation of LDL. The inhibitory activity of tannin methanol and aqueous extract was studied by inducing LDL to prevent oxidation. The results demonstrated that the action of tannin methanol extracts isolated from *C. limon* fruit juice and honey mixture for its significant scale of LDL oxidation inhibition, having 55.89% ± 0.63 after 5 hr, furthermore, 53.70% ± 0.20 at 20 hr for 240 μg/mL concentration with a significant difference at $P < 0.001$. Similarly, tannin aqueous extract was observed to have a lesser inhibition percentage of 47.81% ± 0.14 at 5 hr with the lowest inhibitory activity with significance at $P < 0.001$ [Figure 1].

Antioxidant activity by NO, SO and DPPH

Of the different concentrations of tannins screened, methanolic tannin extract against control displayed the lowest IC_{50} value of 4.01 ± 0.36 μg/mL with the highest activity in NO scavenging assay by their ability to scavenge nitrate radical. In contrast, tannin aqueous extract indicated IC_{50} value of 4.66 ± 0.08 μg/mL, which exhibited significance at $P < 0.001$. Whereas with respect to SO and DPPH assay, there was no significant change in tannin aqueous extract, which illustrated the lowest IC_{50} value. However, this difference with control as ascorbic acid was not significant at $P > 0.05$. At the same time, tannin methanol extract showed a significant difference at $P < 0.05$ [Figure 2].

Tannin methanolic extract showed the highest biological activity in all the above experiments compared to aqueous extract since it was further studied in THP-1 and RAW 264.7 cell lines. **MTT assay**

In a two-fold dilution series, ox-LDL was treated with cell lines in the presence of tannin methanol extract at different concentrations

Table 1: Percentage yield and concentration of tannin methanol and aqueous extract from *C. limon* fruit juice and honey formulation.

Tannins from *C. limon* with honey	% Yield	Total Tannins (mg TAE /g dry weight)
Methanol extract	16.04	453.968
Aqueous extract	19.53	415.87

TAE: Tannic acid equivalent

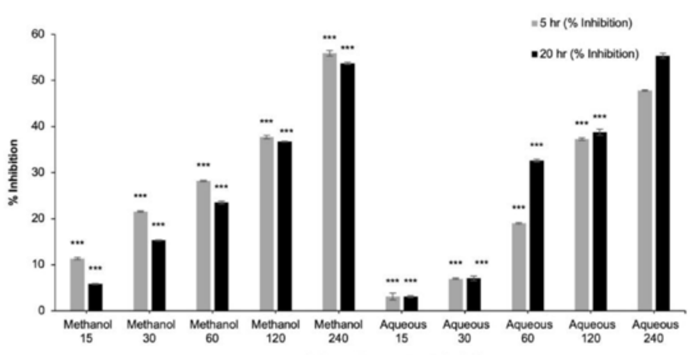

Figure 1: Inhibition of LDL oxidation activity of tannin methanol extract from *C. limon* fruit juice and honey mixture at 5 hr and 20 hr. (n = 3) ± SD; results in the column differ significantly at $P \leq 0.05$ after the Bonferroni posttest by ANOVA ($^{***}P < 0.001$; samples vs. ascorbic acid respectively)

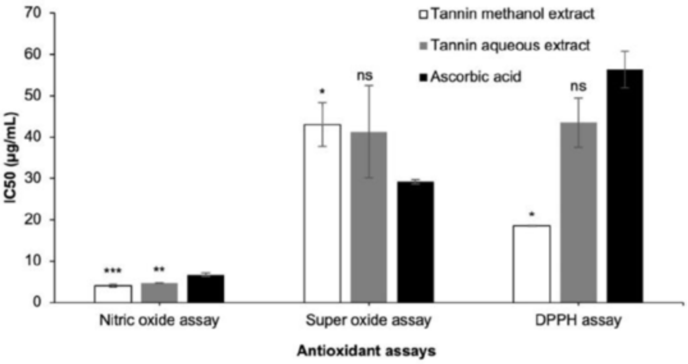

Figure 2: Antioxidant activity of tannin methanol extract of *C. limon* fruit juice and honey mixture by superoxide, nitric oxide, and DPPH radical scavenging activity. (n = 3) ± SD; results in the column differ significantly at $P \leq 0.05$ after Tukey's test through ANOVA ($^{*}P < 0.05$; $^{**}P < 0.01$; ($^{***}P < 0.001$; $^{ns}P > 0.05$; samples vs. ascorbic acid respectively)

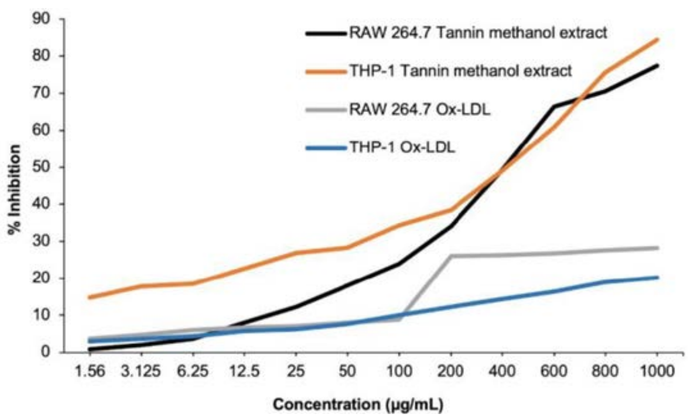

Figure 3: Cytotoxicity assay by MTT. (n = 3)

(1.56 - 1000 µg/mL). The results suggest that lower concentrations of tannin methanol extract on RAW 264.7 and THP-1 cells showed lesser cytotoxicity. In contrast, at higher concentrations, cell viability was decreased [Figure 3]. Henceforth, further studies were done at lower concentrations.

Ox- LDL induced foam cell formation assay

Different concentrations of tannin methanolic extract were checked to prevent ox-LDL uptake, also determined by cells that have taken up oil red stain, which was quantified by spectrophotometer. In RAW 264.7 cells, tannin methanolic extract showed prominent foam cell prevention activities by inhibiting the uptake of oxidized LDL [Figure 4]. However, control cells without extract in the presence of ox-LDL were shown to have elevated intake of ox-LDL with stained cells [Figure 4a]. While extract of 1.56 µg/mL and 25 µg/mL induced with ox-LDL was able to prevent the uptake of oil red stain in Raw 264.7 cells which was distinctly observed with unstained cells [Figure 4b, 4c].

The current experiment demonstrated that the inhibitory activity of tannin methanolic extract was a little closer to that of the statin drug. The lowest IC_{50} value in RAW 264.7 was found to be 9.90 ± 0.41 µg/mL corresponding significant at $P < 0.001$, whereas simvastatin represents 6.48 ± 0.07 µg/mL. The foam cell inhibition activity was lesser in THP-1 cells having an IC_{50} value of 13.66 ± 0.25 µg/mL with a significant difference at $P < 0.001$ [Figure 4d].

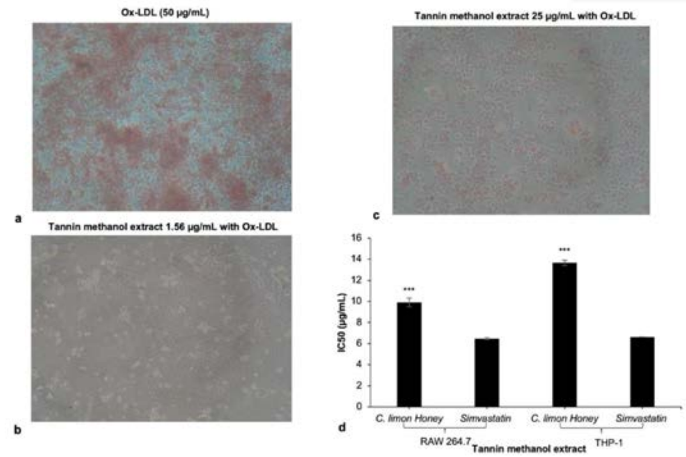

Figure 4: Formation of foam cell prevention by tannin methanol extract from *C. limon* fruit juice and honey mixture. (a) Control Raw 264.7 cells without the sample in the presence of oxidized LDL with the uptake of oil red stain (b) Tannin methanol extract (1.56 µg/mL) in the presence of ox-LDL prevented the uptake of oil red stain in Raw 264.7 cells distinctly observed (c) Tannin methanol extract (25 µg/mL) in the presence of ox-LDL was able to prevent the uptake of oil red stain in Raw 264.7 cells (d) Ox-LDL induced foam cell formation. (n = 3) ± SD; results in the column differ significantly at $P \leq 0.05$ after Tukey's test through ANOVA, representing $^{***}P < 0.001$; $^{ns}P > 0.05$; samples vs. simvastatin respectively

Cell proliferation assay

Findings demonstrated that the tannin methanol extract in RAW 264.7 cells inhibited cell proliferation activity. Here it was illustrated that the antiproliferation property with IC_{50} value, which was found to be 9.84 ± 0.11 µg/mL, is known to exhibit a significant difference at $P < 0.001$ with a lower proliferation rate. At the same time, simvastatin showed a higher IC_{50} value of 14.45 ± 0.2 µg/mL. Whereas in THP-1 cells IC_{50} value was found to be 10.29 ± 0.01 µg/mL, having a difference which was not significant compared to simvastatin ($P > 0.05$) [Figure 5a]. Hence tannin methanolic extract in RAW 264.7 was able to suppress proliferation with elevated levels of ox-LDL.

Cell apoptosis assay

Here in this study, LDL oxidation-induced apoptosis was performed according to trypan blue uptake assay. Cells that are ruptured and damaged will uptake stain, which was read spectrophotometrically. Apoptosis activity of tannin methanol extract by RAW 264.7 was found to be higher IC_{50} value of 11.33 ± 0.91 µg/mL ($P > 0.05$), while THP-1 cells displayed IC_{50} value of 8.99 ± 0.40 µg/mL with significance at $P < 0.01$. While the activity of simvastatin was found to be 6.17 ± 0.10 µg/mL for the THP-1 cells. Hence RAW 264.7 cells exhibited the highest antiapoptotic property compared with the others [Figure 5b].

HPLC

HPLC analysis of tannin methanolic extract resulted in the separation of different compounds with peaks having a retention time of 2.91, 3.96, 4.50, and 5.68 were identified for tannic acid as a standard, in contrast with an area of 1691.89, 3362.1191.19, and 392.16, respectively [Figure 6].

GC-MS

Comparing the compounds with the library, which showed several peaks identified [Figure 7]. The tannin methanol extract was found to have 24 compounds were recognized using the library. The peak at the retention

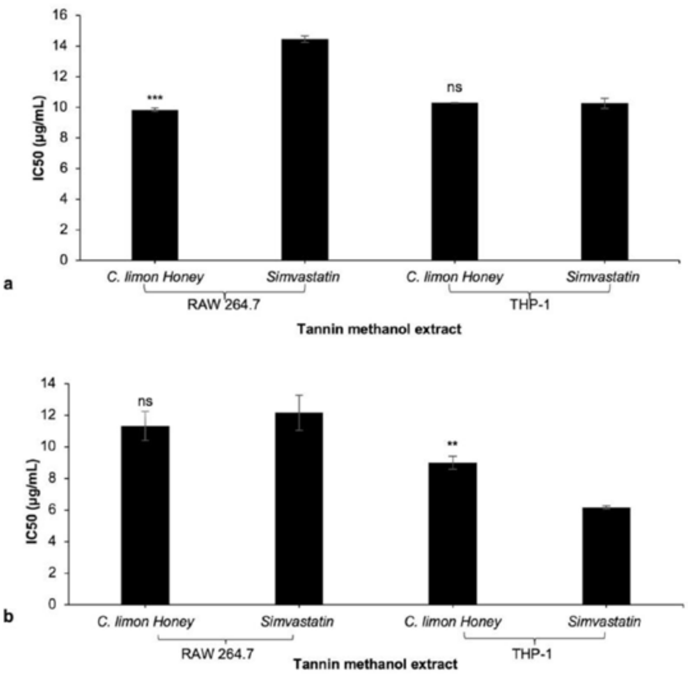

Figure 5: Cell culture assay (a) Antiproliferation in the presence of tannin methanol extract and simvastatin (b) Antiapoptotic in the presence of tannin methanol extract and simvastatin as standard. ($n = 3$) ± SD; results in the column differ significantly at $P \leq 0.05$ after Tukey's test through ANOVA, representing $^{**}P < 0.01$; $^{***}P < 0.001$; $^{ns}P > 0.05$; samples vs. simvastatin

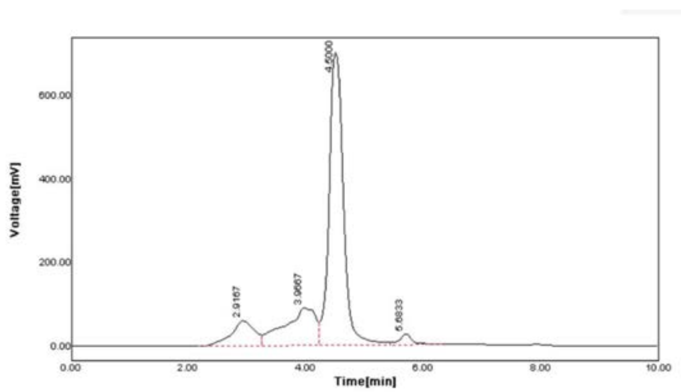

Figure 6: HPLC of tannin methanol extract from *C. limon* fruit juice and honey formulation.

time with the match factor, run time, and compound name was noted accordingly [Table 2].

DISCUSSION

In relation to the present study, inhibition of LDL oxidation treatment with tannin methanol extract lowers the progression of atherosclerosis. Previous studies have proven that the highest flavonoid content was found to be 18.55 ± 0.54 mg quercetin equivalent/g of extract.[39] Noticeable results were obtained in *C. limon* with rich phytochemicals, flavonoids, and tannins, a vital diet supplement and plays a significant role in disease prevention.[40] Reports have illustrated that the *C. limon* fruit-dependent free radical scavenging antioxidant activity was prominent in flavonoids.[41] Studies from nitric oxide and DPPH activity

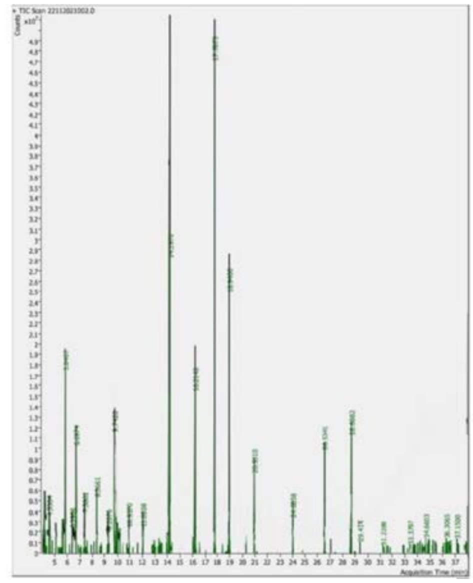

Figure 7: GCMS analysis of tannin methanol extract.

of *C. limon* flower reported having an IC_{50} value of 654.05 ± 23.51 and 335.4 ± 10.39 µg/mL,[39] which had lesser activity when compared to tannin methanol extract of the present study. Studies have proved that citrus essential oils showed DPPH activity between 17.70 - 64.0 %.[42] However, in the present study, *C. limon* fruit juice and honey extract against DPPH activity showed 5.80 - 16.80 % inhibition with lesser IC_{50} value having higher activity when compared to *C. limon* essential oil. Phenolic compounds from honey and other derivatives also indicated antioxidant activity.[43,44]

In Wistar rats, tannin extract treatment reduced LDL oxidation and hyperlipidemia.[45] In addition, lemon oil was shown to be effective in inhibiting oxidation; and these findings revealed that phenolic compounds present in the sample could inhibit oxidation.[46] Previous studies have revealed that honey has antioxidant activity and lowers the risk of cardiac diseases.[47,48] Thus, in agreement with the current study, the earlier reports indicated antioxidant and inhibition of LDL oxidation activity phytochemicals.

Reducing the formation of ox-LDL can prevent the conversion of macrophages to foam cell formation, which reduces the chances of atherosclerosis.[49,50] In the present study, *C. limon* fruit juice on foam cell inhibition activity was displayed to have prominent inhibition capacity on RAW 264.7 cells, thereby regulating the levels of foam cells. Previous investigation has indicated that flavonoids from Citrus can prevent foam cell formation by preventing differentiation to macrophages which takes up ox-LDL.[51] Recent reports have suggested that *Citrus bergamia* could reduce LDL levels in hypercholesteraemic rats, reducing cardiovascular diseases by preventing hyperlipemia and antioxidant activity.[52] In contrast, honey was also discovered for its antilipidemic and antioxidant effects on rat models.[53]

Cells induced with ox-LDL undergo death by apoptosis, causing lesions in atherosclerosis conditions.[54] Ox- LDL, which is toxic to the cultured cells, initiates apoptosis; only damaged cells take trypan blue. In this study, tannin methanol extract can prevent cell apoptosis and proliferation by improving the activity of cells compared to the control. Reports have indicated that anthocyanin of *Hibiscus sabdariffa* exhibited cardioprotective activity against proliferation in cells of vascular smooth muscle.[55] Similarly, polyphenols from Hibiscus leaf prevented

Table 2: Compound detected by GC-MS in tannin methanol extract.

Compounds	Retention Time	Match Factor	Molecular Formula
5-Amino-3-methylpyrazole	4.53	71	$C_4H_7N_3$
2-(4-Methyl-1H-1,2,3-triazol-1-yl) ethan-1-amine	5.84	81.2	$C_5H_{10}N_4$
Propane, 1-(ethenylthio)-	6.39	72.3	$C_5H_{10}S$
1H-Pyrrole-2-carboxaldehyde, 1-methyl-	6.69	86.4	C_6H_7NO
2-Hydroxy-gamma-butyrolactone	7.38	93.7	$C_4H_6O_3$
4(1H)-Pyridinone, 2,3-dihydro-1-methyl-	8.36	77	C_6H_9NO
Isopropylphosphonic acid, fluoroanhydryde-, decyl ester	9.26	71.3	$C_{13}H_{28}FO_2P$
2-Formyl-4,5-dimethyl-pyrrole	9.74	83.8	C_7H_9NO
Propanoic acid, 3-amino-3-(2-furyl)-	10.93	66.7	$C_7H_9NO_3$
Methyl 1-methylpyrrole-2-carboxylate	12.02	54.5	$C_7H_9NO_2$
3-Amino-1,2,4-triazine	14.19	71.6	$C_3H_4N_4$
Glycine, N-ethyl-n-propargyloxycarbonyl-, propargyl ester	16.21	62	$C_{11}H_{13}NO_4$
2-Ethyl-2-propyl-N-ethylpiperidine	17.78	61.9	$C_{12}H_{25}N$
Phenol, 2-amino-5-nitro-	18.94	60.5	$C_6H_6N_2O_3$
2,5-di-tert-Butylaniline	20.93	74.2	$C_{14}H_{23}N$
Propionamide, N-propyl-N-(hept-2-yl)-	24	65	$C_{13}H_{27}NO$
Hexanamide, N-(2-butyl)-N-heptyl-	26.53	60.7	$C_{17}H_{35}NO$
9,11-Octadecadienoic acid, methyl ester, (E,E)-	28.68	67	$C_{19}H_34O_2$
Bis[1-methylimidazole-2-yl methyl] amine	29.41	53.4	$C_{10}H_{15}N_5$
10-Hexyl-10H-acridin-9-one	31.22	54	$C_{19}H_{21}NO$
10-Pentyl-10H-acridin-9-one	33.37	50.6	$C_{18}H_{19}NO$
4,4'-bi-4H-pyran, 2,2',6,6'-tetrakis(1,1-dimethylethyl)-4,4'-dimethyl-	34.66	50.3	$C_{28}H_{46}O_2$
2-Fluoro-5-trifluoromethylbenzoic acid, propyl ester	36.3	51.9	$C_{11}H_{10}F_4O_2$
Methanamine, N-(1-[1,1'-biphenyl]-2-ylethylidene)-	37.15	51.5	$C_{15}H_{15}N$

proliferation and oxidation of lipoprotein.[56] Thus, tannin methanol extract effectively prevents the uptake of ox-LDL and inhibition of foam cells by reducing arterial plaque in diseased conditions.

CONCLUSION

The present study indicates that the potent activity in tannin methanolic extract from *C. limon* fruit juice honey mixture exhibited antilipidemic and antioxidant activity with lesser IC_{50} values. Findings also provided evidence that supports the traditional use of *C. limon* fruit juice and honey combination for anti-atherosclerotic activity. The identified compounds by GC-MS may be responsible for inhibiting the development of foam cell formation, antiproliferation, and antiapoptotic activity, which leads to potential research in drug development.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

Ox-LDL: Oxidised low-density lipoprotein; ***C. limon:*** *Citrus limon*; **TAE:** Tannin aqueous extract; **IC_{50}:** Inhibitory concentration 50; **NO:** Nitric oxide radical scavenging activity; **SO:** Superoxide radical scavenging activity; **DPPH:** 2, 2-diphenyl-1-picrylhydrazyl; **PBS:** Phosphate buffer saline; **MTT:** 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; **DMSO:** dimethyl sulfoxide; **DMEM:** Dulbecco's Modified Eagle's medium; **HPLC:** High-performance liquid chromatography; **GC-MS:** Gas chromatography–Mass spectrometry.

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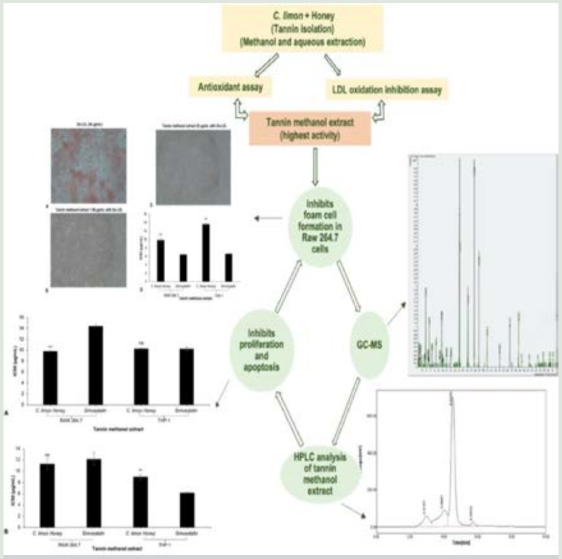
GRAPHICAL ABSTRACT



SUMMARY

- Detection of antioxidant and LDL oxidation inhibition activity was performed in tannins isolated from *C. limon* and honey mixture in methanol and aqueous extraction. Tannin methanol extract was observed to have highest activity which was further evaluated for antiatherosclerotic parameter in RAW 264.7 and THP-1 cells and characterised using HPLC and GC-MS. The tannin methanol extract was found have prominent inhibitor of oxidation of LDL which is the main cause for atherosclerosis progression.

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