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ORIGINAL ARTICLE

Antioxidant, Anti-proliferative, and Anti-atherosclerotic Effect of Phytochemicals Isolated from *Trachyspermum ammi* with Honey in RAW 264.7 and THP-1 Cells

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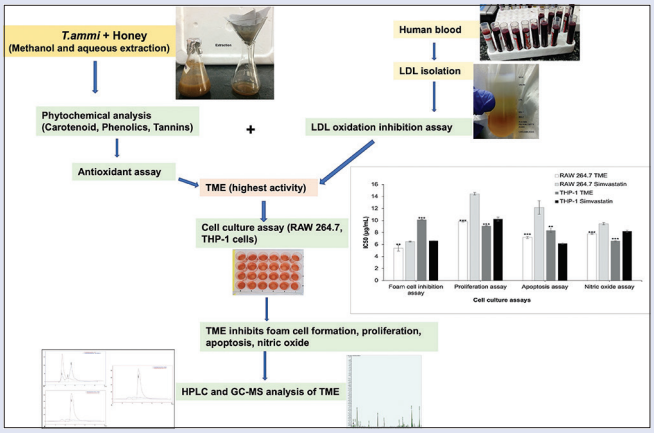
ABSTRACT

Background: *Trachyspermum ammi* is a common spice with vital medicinal properties used in Ayurveda to treat many ailments. Furthermore, natural Honey is said to have high levels of antioxidants and anti-inflammatory properties. While the combination of both *T. ammi* and Honey is unexplored in anti-atherosclerotic activity. **Materials and Methods:** Assays guided isolation of carotenoids, phenolics, and tannins were performed by methanol and aqueous extraction from *T. ammi* seed powder and honey mixture. Phytochemical screening for antioxidant activity (superoxide radical scavenging activity, nitric oxide radical scavenging activity, and 2, 2-diphenyl-1-picrylhydrazyl scavenging activity) and antilipidemic activity by inhibition of low-density lipoprotein (LDL) oxidation were investigated. Sample showing highest activity with lowest IC_{50} value from antioxidant and antilipidemic activity was further evaluated on RAW 264.7, THP-1 cells for foam cell inhibition activity, anti-proliferation, antiapoptotic activity, and nitric oxide assay. The sample was characterized using high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). **Results:** Out of the phytochemicals screened, tannin methanol extract (TME) showed the highest antioxidant activity with IC_{50} value having 7.11 μg/mL in superoxide scavenging assay, the IC_{50} value of 1.46 μg/mL by nitric oxide scavenging assay, and followed by IC_{50} value of 20.83 μg/mL in 2, 2-diphenyl-1-picrylhydrazyl activity. The results showed that it also efficiently inhibited LDL oxidation at 240 μg/mL (71.29% at 5 h)., thus inhibiting foam cell development, preventing proliferation, apoptosis, and nitrate production in cultured RAW 264.7 and THP-1 cells. HPLC analysis showed the presence of tannin in the extract. TME was characterized in GC-MS, which showed 13 prominent compounds. **Conclusion:** The scientific evidence in the present study showed that the TME from *T. ammi* seed powder and honey act as an antioxidant and antiatherogenic properties in inhibiting foam cell formation. Hence, this phytochemical can be used for the treatment of atherosclerosis.

Key words: Antioxidant activity, antiproliferative activity, foam cell inhibition, low-density lipoprotein, medicinal plants

SUMMARY

- The use of phytochemicals isolated from plant products is needed nowadays, considering the side effects of presently available treatment for atherosclerosis. In the present study, phytochemicals (carotenoids, phenolics, tannins) were isolated from *Trachyspermum ammi* seed powder and honey mixture in methanol and aqueous extraction, evaluated for antioxidant and antilipidemic activity by inhibiting low-density lipoprotein (LDL) oxidation
- Tannin by methanol extraction showed pronounced effects as antioxidant activity and inhibition of oxidized LDL. Hence, further TME, the highest active sample, was evaluated for inhibiting foam cell formation, anti-proliferation, antiapoptotic, and nitric oxide activity on RAW 264.7 cells and THP-1 cells
- TME showed prominent results in preventing foam cell formation, antiproliferative, antiapoptotic, and nitic oxide assay on RAW 264.7 and characterized using high-performance liquid chromatography and gas chromatography-mass spectrometry. Hence, TME from *T. ammi* seed powder and honey mixture has potential as a natural antilipidemic and antioxidant nature in preventing free radical formation and foam cell inhibition.



Abbreviations used: LDL: Low-density lipoprotein; Ox-LDL: Oxidized low-density lipoprotein; *T. ammi*: *Trachyspermum ammi*; VLDL: Very-low density lipoprotein; HDL: High-density lipoprotein; IC_{50}: Inhibitory concentration 50; SO: Superoxide radical scavenging activity; NO: Nitric oxide radical scavenging activity; DPPH: 2, 2-diphenyl-1-picrylhydrazyl; TME: Tannin methanol extract; TAE: Tannin aqueous extract; PME: Phenolic methanol extract; PAE: Phenolic aqueous extract; CME: Carotenoid methanol extract; CAE: Carotenoid aqueous extract; AA: Ascorbic acid; PBS: Phosphate buffer saline; MTT: 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; HPLC: High-performance liquid chromatography; DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's Modified Eagle's medium; LPS: Lipopolysaccharide; GC-MS: Gas chromatography–Mass spectrometry; OD: Optical density; rpm: Rotation per minute; RT: Retention time; SD: Standard deviation.

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INTRODUCTION

Low-density lipoprotein (LDL) plays a vital role in atherosclerosis. Macrophages take up LDL present in the blood, get transformed into foam cells. Foam cell proliferation, accumulation, and apoptosis result in plaque formation by hindering the flow of blood in the arteries, which is the cause of atherosclerosis.[1] Phytochemicals such as carotenoids, tannins, phenolics, and flavonoids are rich in plants and their derivative source, which can offset free radical activity and protect atherosclerosis's progression.[2]

A very known spice is *Trachyspermum ammi* (*T. ammi*), used in Ayurveda to treat respiratory ailments, bronchial pneumonia, stomach disorders, etc.[3,4] In addition, one of the available natural sweeteners is Honey. Polyphenols from Honey are used as therapeutic agents in the prevention of cardiovascular diseases like atherosclerosis.[5] A recent report shows that Honey has antioxidant activity. It also prevents cardiovascular diseases. Hence, dietary antioxidants are the most effective source to fight against atherosclerosis with a wide range of benefits.[6,7] Few findings reported that Honey was able to prevent lipid oxidation and increase the free radical scavenging activity.[8,9]

T. ammi and many plants have been identified for their therapeutic effects, such as antioxidant, antihyperlipidemic, antidiabetic, antimicrobial, antihypertensive, and hepatoprotective properties.[10-12]

There are no scientific reports present on the anti-atherosclerotic activity of *T. ammi* seed powder with honey combination because of its wide range of therapeutic purposes in the traditional system of medicine. Hence, there is a need to report the importance of the scientific evidence of its activity. Therefore, the *T. ammi* and Honey were selected. The present study is aimed to isolate phytochemicals from *T. ammi* seed powder with the honey combination was looked at antioxidant and its inhibitory property on atherosclerosis progression in RAW 264.7 and THP-1 cell lines. The evaluation of oxidized LDL (ox-LDL) induced foam cell prevention, proliferation, apoptosis, and nitric oxide assay was performed, which was not reported with this combination so far. With this knowledge, these natural products can be integrated into the therapeutic regimen for the treatment modes.

MATERIALS AND METHODS

Chemicals

Folin–Ciocalteu's reagent, gallic acid, potassium bromide (KBr), bovine serum albumin, potassium sodium tartrate, butylated hydroxyanisole, nicotinamide adenine dinucleotide (NADH), 2-thiobarbituric acid (TBA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, penicillin, streptomycin, phosphate buffer saline (PBS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), nitroblue tetrazolium (NBT), trypan blue, lipopolysaccharides (LPSs), gallic acid, caffeic acid, vanillin and trichloroacetic acid (TCA) were procured from Sigma-Aldrich (St. Louis, USA). Phenazine methosulphate (PMS), sodium nitroprusside, Griess reagent were procured from fisher scientific (USA). Dulbecco's Modified Eagle's medium (DMEM), RPMI, trypsin ethylenediaminetetraacetic acid (EDTA), and fetal bovine serum (FBS) were procured from Gibco (USA). Oil Red O was purchased from Loba Chemie Pvt Ltd (Mumbai, India). Paraformaldehyde, isopropanol were purchased from Sisco Research Laboratories Pvt. Ltd (Mumbai, India).

Plant material and extract preparation

The seed of *T. ammi* was collected from the Bangalore market, India. They were oven-dried at 50°C for two days and powdered.[13] Furthermore, Honey was collected from the Nilgiris biosphere, India. Both were mixed in combination were selected based on their medicinal use in Ayurveda to check for antioxidant and anti-atherosclerotic properties. Two different solvents (methanol and water) were extracted in (1:20 w/v) sample and solvent ratio followed by mixing. The sample was incubated in a rotary shaker overnight, and the supernatant was collected and lyophilized to get a dried sample for further carotenoid, phenolic, and tannin extraction.[14]

Extraction and estimation of total carotenoids

5 g previously dried sample from *T. ammi* seed powder and honey mixture (methanol and aqueous extract) were crushed for 30 min with 100 mL of an acetone-hexane mixture (3:7), kept in shaker incubator for 5 h. Later, the liquid is separated at 5000 rpm for 5 min by centrifugation to obtain carotenoids rich extract. The extract's carotenoid stock solution (1 mg/mL) was read at 661.5, 645, 470, and 450 nm. The amount of total carotenoid present was calculated.[15] The percentage yield of the extract was calculated.[16]

%Yield = (weight after extraction and drying)/(weight of sample before extraction) ×100

Chlorophyll a (μg/mL) =OD661.5 × 11.24– OD645 × 2.04.

Chlorophyll b (μg/mL) =OD645 × 20.13–OD661.5 × 4.19.

Total carotenoids (μg/mL) = (OD470 × 1000– [Chlorophyll (a) ×1.9 + Chlorophyll (b) ×63.14])/214.

Extraction and estimation of total phenolics

Extraction was done by modifying the method described within.[17] Blending 3 g of the previously extracted sample with 12 mL of methanol for 24 h in a rotary shaker. After 24 h, the resultant was centrifuged for 5 min at 5350 rpm, and the supernatant was removed containing phenolic compound-rich extract isolated was dried. The percentage yield of phenolic was calculated as mentioned previously.[18] Folin–Ciocalteu's reagent with a stock solution of 1 mg/mL was used to estimate the content of phenolic present in the extract and expressed as mg gallic acid equivalents/g dry weight.[19]

Extraction and estimation of total tannin

Tannin-rich extract was prepared, the percentage yield was calculated, which was obtained by drying the extracts using a rotary evaporator.[20] Estimation was carried out, and the tannin concentration was calculated based on the tannic acid standard and expressed as mg tannic acid equivalent/g dry weight.[21]

Superoxide radical scavenging assay

One mL of NBT solution and 1 mL of NADH were prepared in 100 mM phosphate buffer at pH 7.4. Both were mixed with 0.5 mL of different concentration phytochemical extracts (1 mg/mL stock) (100, 200, 300, 400, 500 μg/mL concentration). 100 μL of PMS solution was added, followed by incubation at 25°C for 5 min, and read at 560 nm with ascorbic acid as a standard.[22] Percentage inhibition was calculated below, where A_C represents the absorbance of control without sample and A_S represents the absorbance of the sample. IC_{50} value (μg/mL) was calculated.

%Inhibition = $(A_C – A_S)/A_C \times 100$

Nitric oxide radical scavenging assay

Quantification of nitric oxide scavenging activity of different phytochemical extracts was performed by adding 2 mL sodium nitroprusside to different tubed with samples at different concentrations (100, 200, 300,400, 500 μg/mL) and incubated at 37°C for 2.5 h. The incubated solution was diluted with 1.5 mL of Griess

reagent. Pink coloration was formed, and absorbance was measured at 546 nm along with standard as ascorbic acid.[23] Nitrite radical scavenging activity was calculated to determine percentage inhibition and IC_{50} (μg/mL) value.

2,2-diphenyl-1-picrylhydrazyl assay

The phytochemical extracts were quantified for free radical scavenging activity by 2, 2-diphenyl-1-picrylhydrazyl, which was determined as given below with a few modifications.[24] 50 μL of different phytochemical concentrations (Tris-HCL 50 mM, pH 7.4), mixed with 450 μL of Tris-HCL (50 mM, pH 7.4). One mL of DPPH (0.1 mM in methanol) was added to all the tubes. The control contained all the reagents except the sample. Standard was used as ascorbic acid, which was taken in separate tubes - followed by vigorously shaking the mixture and allowed to incubate in the dark for 30 min at room temperature. The absorbance was read at 517 nm. Percentage inhibition and IC_{50} (μg/mL) value was calculated as previously mentioned to determine the scavenging activity.[25]

Low-density lipoprotein Isolation

Isolation of LDL was done by density gradient ultracentrifugation technique from human serum. The human blood sample was collected from healthy volunteers and added into EDTA tubes. The sample was run at 3000 rpm for 15 min, and plasma was separated from the tube with other components. Plasma density was altered to a density of 1.3 g/mL with the KBr addition. Density gradient was formed by layering 30 mL of saline with a density of 1.006 g/mL over 10 mL of adjusted plasma into 40 mL ultracentrifuge quick seal tubes. The tubes were sealed using solder after filling it with the sample, followed by placing the layered tubes carefully in the centrifugation rotor chamber by bolting them. The sample was run in density gradient ultracentrifuge (Beckman ultracentrifuge, USA) at 65,000 rpm for 1.5 h. Components in the plasma was separated according to density after ultracentrifugation. LDL was identified and collected using a syringe, and salts were removed by performing dialysis. LDL sample was stored at −20°C.[26] The experiments were carried out by following the rules set by the Institutional Ethical Committee of animal care and use (Reg No: 606/03/C/CPCSEA).

Low-density lipoprotein oxidation inhibition assay

Different concentration of phytochemical extracts (15, 30, 60, 120, 240 μg/mL) were incubated with LDL (100 μg/mL) for 30 min in a total volume of 2 mL. Ascorbic acid was treated as a standard. One mL of 10 mM copper sulfate was added to start the reaction. 0.5 mL of equal proportion of TBA (1.0%) and TCA (2.5%) was added into the tubes taken at 2-time periods (5 h and 20 h). Incubated were mixed thoroughly and then placed in a 95°C water bath for 30 min. The pink color developed after cooling the mixture. Absorbance was read at 532 nm (Spectramax 13X plate reader).[26,27] Percentage inhibition was calculated as previously mentioned.

Cell lines and stock solutions

Supplementing DMEM with FBS, streptomycin (100 μg/mL) and penicillin (100 μg/mL) was treated to culture 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 h. Tannin methanol extract (TME) 10 mg/mL stocks was prepared using 1 mL DMSO.

Cytotoxicity test using 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide assay

Ox-LDL 100 μg/mL was treated independently with RAW 264.7 and THP-1 cells (50,000 cells/well) with TME (1.560, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, 600 μg/mL concentration) and incubated in 5% CO_2 incubator for 24 h. Cells treated only with ox-LDL devoid of the sample are treated as control. 100 μL of MTT was added (prepared in 5 mg MTT in 10 mL of PBS) and incubated for 4 h. The supernatant was discarded, followed by adding 100 μL of DMSO, and the plates were gently shaken. The absorbance was read at 590 nm (Spectramax 13X plate reader). The percentage inhibition and IC_{50} (μg/mL) values were calculated as mentioned before.[28]

Foam cell inhibition assay

100 μL of 3×10^5 cells (RAW 264.7 and THP-1) were seeded into 96 well plates with DMEM in a CO_2 incubator for 24 h. To this 50 μg/mL ox-LDL was added with 100 μL of different concentrations of TME (1.56, 3.13, 6.25, 12.50, 25 μg/mL) incubated for 48 h. Simvastatin was used as a standard. Cells were fixed for 15 min paraformaldehyde (2%), prepared in 1 X PBS, succeeding staining and incubation for 30 min with 1% Oil O red stain prepared in 60% isopropanol. Later stain was rinsed for 2 s in water and observed under a microscope 60X (Lawrence and mayo). Uptake of ox-LDL by the cells would take Oil O red stain with a distinct red. Isopropanol (100%) was added to cells treated with the sample to quantify oil red stain uptake by cells with incubation for 10 min. Absorbance was read at 492 nm spectrophotometrically.[29] The percentage inhibition of foam cells was measured as previously mentioned. IC_{50} (μg/mL) values were calculated.

Cell proliferation assay

Treating RAW 264.7 macrophages and THP-1 cells (8.0×10^5 cells/well) with different concentrations of 100 μL of TME (1.56, 3.13, 6.25, 12.50, 25 μg/mL), and simvastatin as a standard, in the presence of ox-LDL (20 μg/mL) for 6 days in CO_2 incubator.[30] 100 μL of MTT was added to evaluate anti-proliferation, further incubated at 37°C in 5% CO_2 for 5 h to check the viability. 100 μL of DMSO was added after discarding the supernatant, incubated at 37°C for 30 min followed by gently shaking the plate. The absorbance was read at 550 nm. Percentage inhibition of cell proliferation was measured. IC_{50} (μg/mL) values were calculated.[28,31]

Apoptosis study by trypan blue assay

Cell apoptosis assay was performed using the trypan blue uptake method induced with ox-LDL. 5.0×10^5 cells seeded in 96 well plates, incubated with 100 μg/mL of ox-LDL and TME at different concentrations (1.56, 3.13, 6.25, 12.50, 25 μg/mL) for 24 h, followed by incubation for 15 min by treating with 20 μL of trypan blue dye prepared in 0.05% PBS. The cells were washed with 1 X PBS, and then 100 μL of 1% sodium dodecyl sulfate was added. The absorbance was read at 590 nm. In addition, the percentage inhibition of cell apoptosis was measured as before. IC_{50} (μg/mL) values were calculated.[32,33]

Nitric oxide assay

The production of nitric oxide was measured in cell culture using Griess reagent by treating with the sample extracts. RAW 264.7 cells and THP-1 (2×10^4 cells/well) were seeded into 96-well plates with 1 μg/mL LPSs incubated for 24 h with the sample and simvastatin. After incubation, 100 μL of Griess reagent was added to 100 μL of the supernatant media and incubated for 15 min. Absorbance was read in a microplate reader at 540 nm.[34] Percentage inhibition and IC_{50} (μg/mL) values were calculated.

High-performance liquid chromatography analysis

High-performance liquid chromatography (HPLC) (Waters model no. 486; Waters Corp., Milford, MA, USA) analysis for caffeic acid, tannic acid, and vanillin as standards was checked for sample extract.

Mobile phase elution was in isocratic mode with acetonitrile and water mixture (70:30). C-18 column with a 1 mL/min flow rate was adjusted as a modified method. The standard 1 mg/mL caffeic acid and sample (1 mg/mL) was dissolved in the mobile phase, and 20 µL of each sample was introduced into the injection port. The elution was observed at 230 nm for caffeic acid, 254 nm for gallic acid, and at 270 nm for tannic acid present in the sample by peak detection.[35]

Gas chromatography-mass spectrometry analysis of tannin methanol extract

TME from *T. ammi* seed powder and honey mixture isolated from HPLC was subjected to gas chromatography-mass spectrometry (GC-MS) study (GC-Agilent 8890, MS-7000 D Instrument). Column DB 624 capillary standard non-polar column was loaded. The instrument condition was maintained with 1.2 mL/min of gas flow; carrier gas used in the column was purity grade helium. Oven condition was held at 50°C initially and increased to 250°C at 37 min by Injecting 1 µL of the sample for analysis. The m/z ratio was plotted on a graph, and the compound was found using the library search in the database of the National Institute of Standard and Technology MS Version. 2.3-2017.

Statistical evaluation

Results were carried out in triplicates (n=3), corresponding to mean ± standard deviation. GraphPad Prism software Inc (version 5.03) (California, USA) was performed for statistical analysis, with significance at $P \leq 0.05$ in all the analyses after Tukey's test (one-way analysis). Bonferroni posttest (two-way analysis) was performed for LDL oxidation Inhibition assay through ANOVA (significance was represented as ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns: non-significant; standard vs. samples).

RESULTS

Phytochemicals and antioxidant studies

The concentration of TME was higher, having 666.66 ± 0.006 mg/g. Similarly, the concentration of tannin aqueous extract was 638.0 ± 0.004 mg/g, followed by carotenoids and phenolics [Table 1].

The TME observed the lowest IC_{50} value having 7.11 ± 0.09 with significance at $P < 0.001$ in SO assay. Similarly, 1.46 ± 0.32 and 20.83 ± 0.03 µg/mL had significance at $P < 0.01$, which showed the highest activity in NO assay and DPPH activity. Ascorbic acid showed lesser activity when compared to TME; this may be due to the antioxidant attributes present in the sample, which plays a role in scavenging the free radicals. SO, NO, DPPH resulted in differences in their IC_{50} values, maybe because of the sample and their methods. In the antioxidant activity, when evaluated between methods of the sample, TME was observed higher activity when compared to other samples [Figure 1].

Low-density lipoprotein isolation and oxidation inhibition activity

The desired separation of LDL was observed in the density gradient ultracentrifugation. The plasma density was altered, resulting in very-LDL floating on top of the tube, followed by LDL separation. Subsequently, LDL fraction with slight orange color was suctioned from the tube with a syringe [Figure 2]. The ability to prevent oxidation of LDL activity was measured in tannins, carotenoids, and phenolics (methanol and aqueous extract). Different concentrations (60, 120, 240 µg/mL) were measured to determine the inhibition capacity of LDL oxidation at 5 h and 20 h incubation time. Even here, similar to the antioxidant assay, the activity of TME showed the most significant scale of LDL oxidation inhibition which was recorded to be 71.29% ±0.14 after 5 h, 62.13% ±0.20 at 20 h in 240 µg/mL concentration having significantly higher oxidation inhibition at $P < 0.001$. The TME concentration with the highest dose was considerably showing higher percentage inhibition, whereas all the samples were highly significant at $P < 0.001$. On the other hand, phenolic aqueous extract indicated with lowest percentage inhibition at 20 h in 60 µg/mL concentration with lesser activity followed by the carotenoid aqueous extract. Henceforth, antioxidant activity and the role of antilipidemic ability were prevalent in TME, which was examined for further cell culture studies in RAW 264.7 and THP-1 cells [Figure 3].

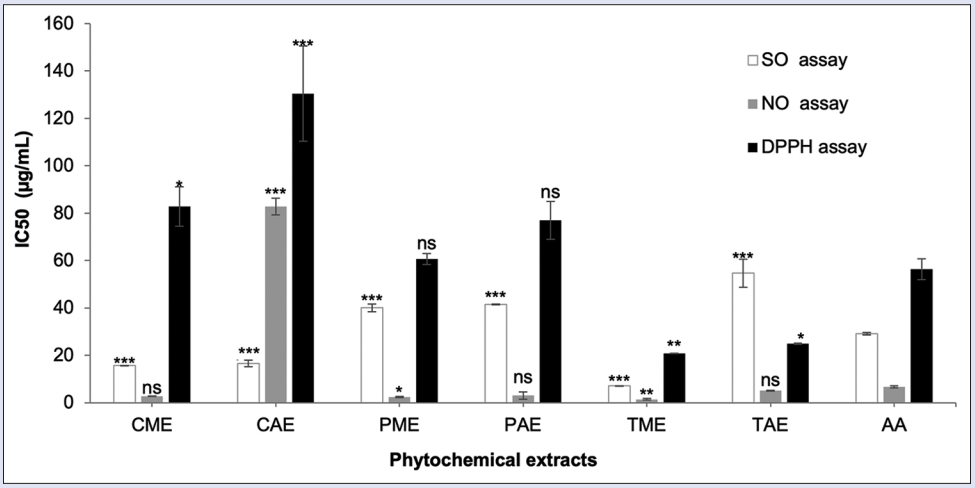

Figure 1: Antioxidant activity of phytochemical extract from *Trachyspermum ammi* seed powder and honey mixture. Data correspond to the mean value ± standard deviation; from triplicate measurements (n = 3). Results within a column followed by asterisk differ significantly at $P \leq 0.05$ after Tukey's test by ANOVA (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns: Non-significant; Ascorbic acid vs. samples). TME: Tannin methanol extract; TAE: Tannin aqueous extract; PME: Phenolic methanol extract; PAE: Phenolic aqueous extract; CME: Carotenoid methanol extract; CAE: Carotenoid aqueous extract; AA: Ascorbic acid; SO: Superoxide radical scavenging assay; NO: Nitric oxide radical scavenging assay; DPPH: 2, 2-diphenyl-1-picrylhydrazyl scavenging assay

3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide cytotoxicity assay

The results suggest that the different concentrations of TME with 100 µg/mL ox-LDL treated in RAW 264.7 and THP-1 cell line, which was observed lesser cell cytotoxicity at lower concentrations with increased inhibition of ox-LDL. Whereas in the presence of ox-LDL without TME, cell toxicity increased, and viability of the cells was decreased [Figure 4]. Henceforth, a lower concentration ranging from 1.56-50 µg/mL was performed for further studies.

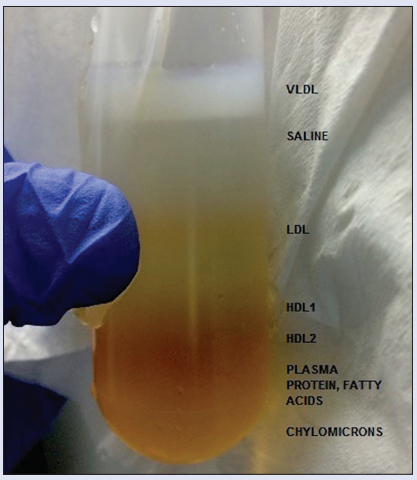

Figure 2: Isolation of LDL from human blood plasma using ultracentrifugation. VLDL: Very-low density lipoprotein; LDL: Low-density lipoprotein; HDL1 and 2: High-density lipoprotein representing two layers

Foam cell inhibition assay

Among the RAW 264.7 and THP-1 cells, the lowest IC_{50} value was observed to be 5.40 ± 0.50 µg/mL corresponding significance at $P < 0.01$ in RAW 264.7 for TME and hence the activity was found to be highest in inhibiting the Ox-LDL uptake, which was observed under a microscope by the uptake of oil red stain.

Control with ox-LDL devoid of sample in RAW 264.7 cells showed the build-up of LDL with the uptake of oil red-stain. TME showed foam cell prevention activity by preventing the uptake of ox-LDL intern reducing the intake of stain in RAW 264.7 with noticeable results [Figure 5]. However, the activity of TME in THP-1 cells was found to be less with an IC_{50} value of 10.15 ± 0.07 µg/mL in TME with significance at $P < 0.001$ and 6.62 ± 0.02 µg/mL in simvastatin [Figure 6]. RAW 264.7 cells decreased the ability to form foam cells in the presence of 25 µg/mL TME and ox-LDL for 48 h by the reduced number of stained cells compared with THP-1 cells. TME ameliorates foam cell prevention with the lowest IC_{50} value [Figure 5].

Oxidized low-density lipoprotein-induced cell proliferation assay

In the Proliferation assay, TME exhibited the highest anti-proliferation activity in THP-1 cells, which were found to be 9.12 ± 0.09 µg/mL, followed by macrophages 9.84 ± 0.09 µg/mL, displaying significant

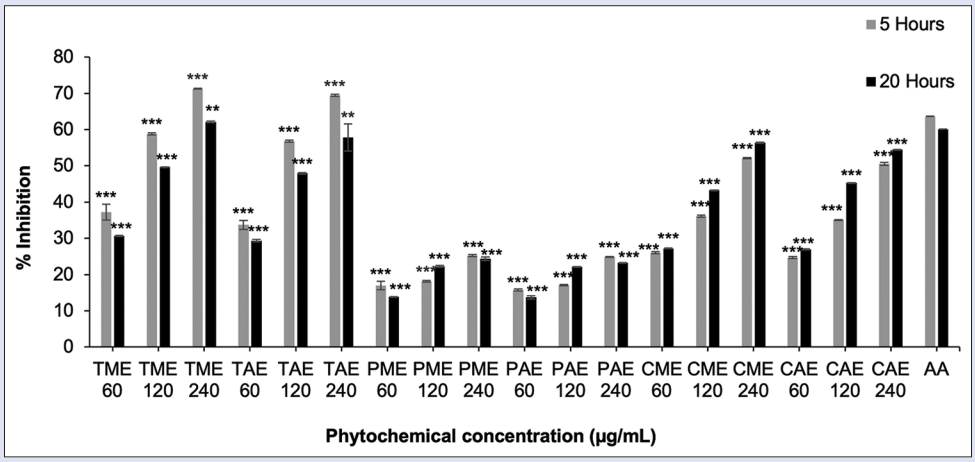

Figure 3: Inhibition LDL oxidation of different phytochemical extracts at a varied concentration (60, 120, 240 µg/mL) from *Trachyspermum ammi* seed powder and honey mixture at 5 and 20 h incubation. Data correspond to the mean value ± standard deviation from triplicate measurements ($n = 3$). Results within a column represent significance at $P \leq 0.05$ after Bonferroni posttest by ANOVA (***$P < 0.001$; **$P < 0.01$; Ascorbic acid vs. samples at different concentrations). TME: Tannin methanol extract; TAE: Tannin aqueous extract; PME: Phenolic methanol extract; PAE: Phenolic aqueous extract; CME: Carotenoid methanol extract; CAE: Carotenoid aqueous extract; AA: Ascorbic acid

Table 1: Estimation and percentage yield of phytochemicals isolated from *Trachyspermum ammi* seed powder and honey mixture

Phytochemicals extracts	Concentration (mg/g)		Yield (%)	
	Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract
Total carotenoids (mg/g dry weight)	0.04±0.00	0.05±0.00	27.2	35
Total phenolics (mg GAE/g dry weight)	71.2±0.001	68.4±0.001	37.4	41.8
Total tannins (mg TAE/g dry weight)	666.6±0.006	638.0±0.00	21.3	20.3

Data correspond to the mean value±SD from triplicate measurements (n=3). GAE: Gallic acid equivalent; TAE: Tannic acid equivalent; SD: Standard deviation

difference at $P < 0.001$ [Figure 6]. TME suppressed proliferation in a concentration-dependent manner in RAW 264.7 and THP-1 cells, respectively. In the case of simvastatin, the activity of THP-1 cells was higher in inhibiting cell proliferation.

Oxidized low-density lipoprotein induced apoptosis inhibition assay

Trypan blue is taken up by cells only when there is damage to the membrane integrity. RAW 264.7 cells were found to be having highest activity in TME with an IC_{50} value of 7.19 ± 0.16 µg/mL ($P < 0.001$), followed by THP-1 cells having 8.36 ± 0.37 µg/mL, which exhibited significance at $P < 0.01$. Here, the activity of simvastatin showed 6.17 ± 0.10 µg/mL for the THP-1 cell line, which was found to be less than that of the RAW 264.7 cell line [Figure 6].

Nitric oxide assay in cell lines

RAW 264.7 inhibited nitric oxide with the sample having the lowest IC_{50} value of 6.58 ± 0.11 µg/mL with the highest activity inhibiting nitrite production, followed by THP-1 cell line, whose activity was not significantly different from RAW 264.7. The sample was compared with simvastatin which showed the lowest IC_{50} value of 8.21 ± 0.18 µg/mL in THP-1 [Figure 6].

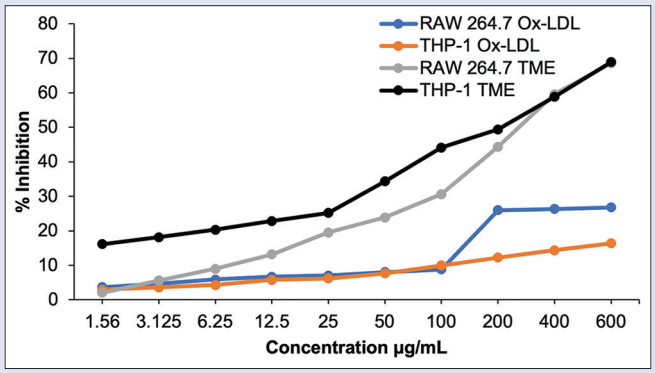

Figure 4: Cytotoxicity assay by MTT on RAW 264.7 and THP-1 cells with sample induced with ox-LDL and ox-LDL alone at varied concentrations. Results are expressed as means from triplicate measurements ($n = 3$). Ox-LDL: Oxidised LDL

High-performance liquid chromatography

TME resulted in the separation of different compounds with peaks having a retention time (RT) of 3.3, 4.5, 5.2 were identified for tannic acid as a standard. Three different standards were run and overlayed, namely, tannic acid, caffeic acid, and vanillin, as shown. The highest peak similarity in HPLC profiles was found between caffeic acid and vanillin. Whereas tannic acid standard overlayed with TME showed similarity to both caffeic acid and vanillin [Figure 7].

Gas chromatography-mass spectrometry

TME isolated from HPLC was subjected to GC-MS, which showed the presence of certain compounds with a high match factor. The chromatogram obtained after the run was compared with a library which showed 13 prominent peaks with RT from 3 to 37 min [Figure 8]. Compound, RT, compound name, formula, match factor, and molecular weight are listed [Table 2]. The peak at RT with the highest match factor was at RT 6.6 min, which was found to be 1H-Pyrrole-2-carboxaldehyde, 1-methyl-with 91.9 match factor. RT at 4.8 min showed the presence of 1H-Pyrazole-4-carboxylic acid with a

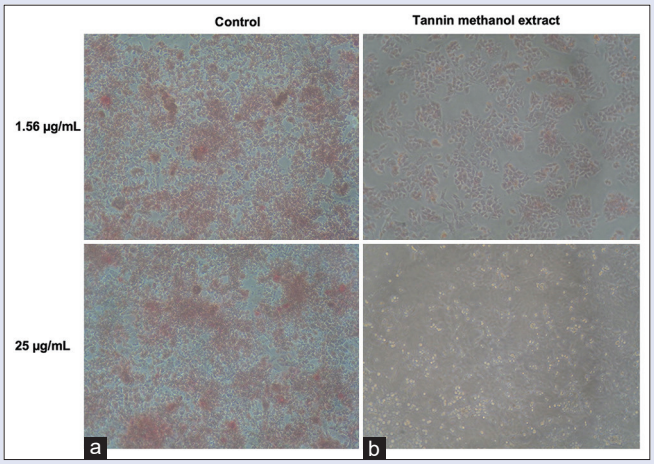

Figure 5: Foam cell inhibition assay (a) Control RAW 264.7 cells treated with ox-LDL resulting in foam cell formation stained with Oil red O taken up by the cells, (b) Cells treated with ox-LDL and 25 µg/mL tannin methanolic extract depicts the absence of foam cells seen distinctly from the unstained cells

Table 2: Chemical profile identified by gas chromatography-mass spectrometry for tannin methanol extract from *Trachyspermum ammi* seed powder and honey mixture

Retention time	Compound	Molecular formula	Match factor	Molecular weight (g/mol)
3.9	1H-Pyrrole, 2,3-dimethyl-	C_6H_9N	78.0	95.14
4.8	1H-Pyrazole-4-carboxylic acid	$C_4H_4N_2O_2$	88.2	112.09
5.8	2-(4-Methyl-1H-1,2,3-triazol-1-yl) ethan-1-amine	$C_5H_{10}N_4$	80.7	98.11
6.6	1H-Pyrrole-2-carboxaldehyde, 1-methyl-	C_6H_7NO	91.9	109.13
7.5	3-(t-Octylamino) propionitrile	$C_{11}H_{22}N_2$	78.0	182.31
11.5	Succinamide, 2-amino-N1, N1, N4, N4-tetraethyl	$C_{12}H_{25}N_3O_2$	68.3	228.33
16.1	Benzonitrile, 4-hydroxy-3-methoxy-	$C_8H_7NO_2$	65.1	149.15
19.3	1-Octanone, 1-(2-furanyl)-	$C_{12}H_{18}O_2$	74.2	194.27
26.8	Acetamide, N-propyl-N-heptyl-	$C_{12}H_{25}NO$	58.6	157.25
28.5	Histidine-2-carboxylic acid, N-t-butyloxycarbonyl, methyl (ester)	$C_{13}H_{19}N_3O_6$	54.1	369.4
33.0	1,3-Dioxolan-2-one, 4,5-bis (methylene)-	$C_5H_4O_3$	72.0	128.13
33.3	1,3-Cyclobutanedione, 2,2,4,4-tetramethyl-	$C_8H_{12}O_2$	59.0	140.18
36.9	N-methylene-n-octadecylamine	$C_{19}H_{39}N$	53.4	283.5

match factor of 88.2; and at RT 5.8 min 2-(4-Methyl-1H-1,2,3-triazol -1-yl) ethan-1-amine was present with a match factor of 80.7 respectively.

DISCUSSION

LDL oxidation and the development of plaque are mainly caused due to the free radicals.[36] Several authors have reported the hypolipidemic, antiatherogenic effects of different plants and their plant products.[37] Herbal medicine, which is rich in nutraceuticals, needs to be implemented for the treatment modules.[38] Of the different phytochemicals screened for antioxidant activity and LDL oxidation inhibition activity, TME showed the highest free radicals scavenging and oxidation inhibition ability. Tannin activity from all the other samples was observed higher, possibly because the contents present was more significant. According to research, the content of tannin present was 8.9% in *T. ammi*.[39] The extract's free radical scavenging ability might be related to its capacity to donate hydrogen and the nature of the extract.[40] Earlier, related studies have shown that the dose of 1000 µg/mL cranberry juice inhibits LDL oxidation by 50.7%, while 2500 µg/mL cranberry juice inhibits by 71.4%.[41] TME was also found to have a higher toxicity inhibition capacity.

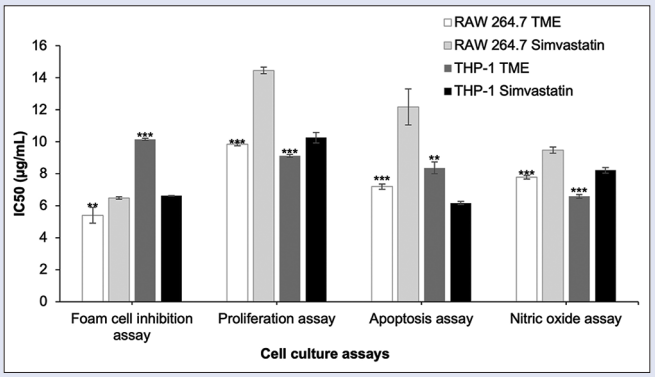

Figure 6: Quantification of ox-LDL induced foam cell inhibition, proliferation, apoptosis, and nitric oxide assay of TME and simvastatin as a standard in RAW 264.7 macrophage and THP-1 cells. Data correspond to the mean value ± standard deviation; from triplicate measurements ($n = 3$). Results within a column followed by asterisk differ significantly at $P \leq 0.05$ after Tukey's test by ANOVA (***$P < 0.001$; **$P < 0.01$; the comparison was made between simvastatin vs. TME sample). TME: Tannin methanol extract

In the present study, TME 25 µg/mL prevented foam cell formation, prominently seen by the oil O red stain method. Oxidation of LDL and uptake by foam cells causing plaque is the major factor in atherosclerosis development. Earlier, 60 µg/mL leaf of *Ocimum basilicum* ethanol extract and the seed extract of *Coriandrum sativum* L. inhibited the development of foam cells.[31,42]

The inhibiting proliferation of macrophages is one major step for preventing atherosclerosis. TME was shown to have antiproliferative activity. Reports indicated a similar effect in *Stegnosperma halimifolium* extract with IC_{50} value 400 µg/mL and *Struthanthus palmeri* 200 µg/mL, which exhibited proliferative inhibition activity in RAW 264.7.[43]

Cell apoptosis in plaque advancement causes plaque vulnerability. The plaque's fibrous cap starts to wear out and is prone to rupture, leading to myocardial infarction and cardiac arrest. The effect of phenolic compounds was shown to prevent apoptosis induced with ox-LDL in RAW 264.7 cells.[44]

Nitric oxide levels rise in pathological situations, which produces a toxic molecule known as peroxynitrous acid, which in turn causes oxidative damage.[45] TME showed nitrite reduction with the lowest IC_{50} value in agreement with a similar study in which *Emblica officinalis* fruit methanolic extract showed 5.2 µg/mL IC_{50} value.[46] GC-MS was characterized, which resulted in 13 compounds in TME. Thus TME is more efficient in preventing the uptake of ox-LDL, foam cell inhibition, antiproliferative, antiapoptotic effects.

CONCLUSION

The present study is focused on samples showing the highest and potential action in which TME was observed with increased activity when compared to other phytochemicals, which had the potential to reduce free radicals, inhibit ox-LDL, and anti-atherosclerotic activity. Honey, in

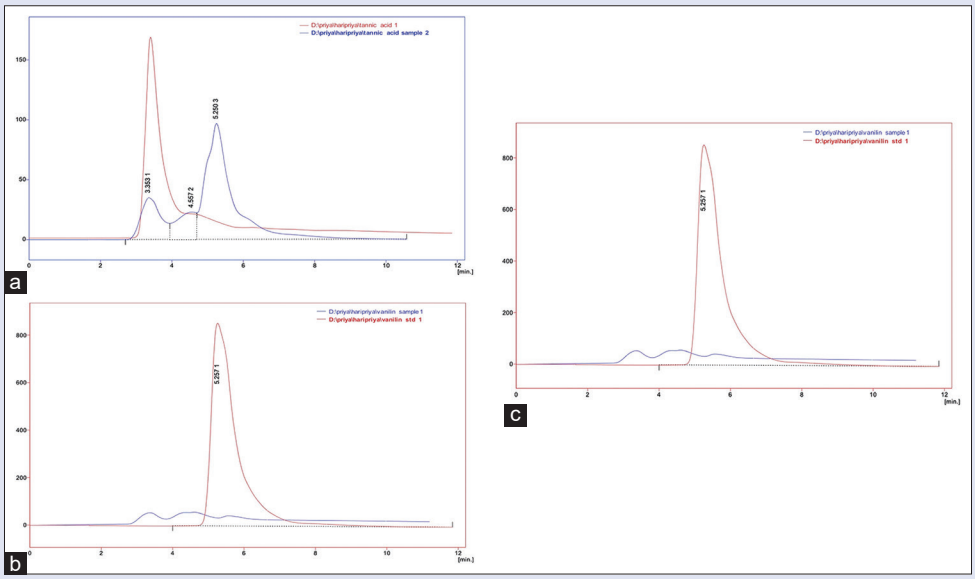

Figure 7: Tannin methanolic extract peaks observed in high-performance liquid chromatography at different wavelengths, overlayed with (a) Tannic acid, (b) Caffeic acid, and (c) Vanillin

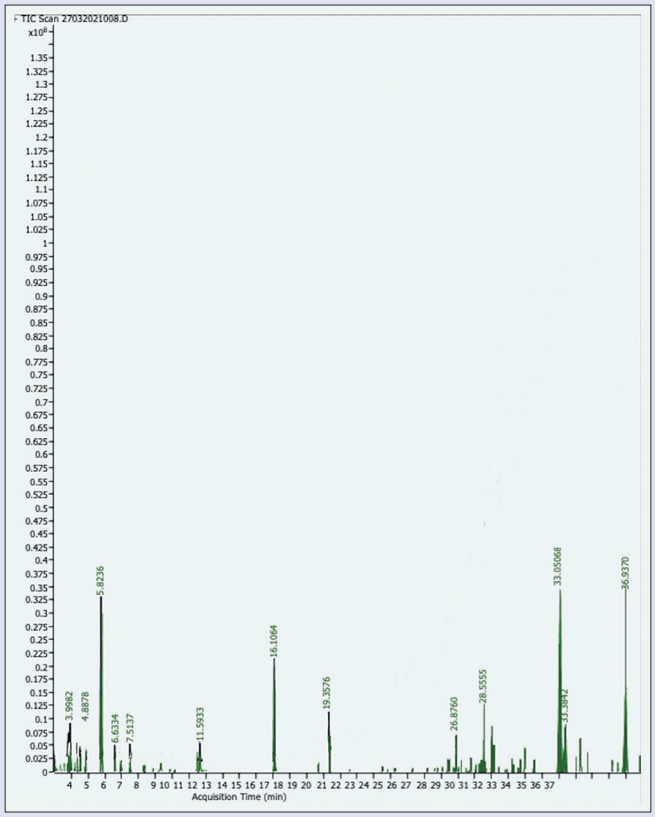

Figure 8: Gas chromatography-mass spectrometry chromatogram of tannin methanol extract isolated from *Trachyspermum ammi* seed powder and honey mixture

combination with various plants, has been used in Ayurveda. However, its use is still missing in allopathic drugs. Hence, a detailed investigation into the plant combination showing antioxidant and anti-atherosclerotic activity in cell lines, with information on the phytochemical profiling, was done. However, deciphering the mode of action of the active compound and the molecular mechanism is needed to be investigated, which should be exploited in future drug development, thus paving the way for newer measures in the treatment of atherosclerosis. Henceforth from the above evidence, it can be proved to prevent preliminary atherosclerosis progression.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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