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Original Research

An Insight into Biochemical Characterization and Explorations of Antioxidant, Antibacterial, Cytotoxic, and Antidiabetic Activities by *Trachyspermum ammi* Nanosuspensions

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Abstract

Background: *Trachyspermum ammi* is a frequently utilized traditional medicinal plant renowned for its pharmacological attributes, particularly in the realm of treating infectious diseases. This current study aims to comprehensively assess the *in vitro* properties of freshly prepared nanosuspensions derived from *Trachyspermum ammi* extracts, with a focus on their cost-effective potential in various areas, including antioxidant, antibacterial, cytotoxic, and antidiabetic activities. **Methods**: Biochemical characterization of *T. ammi* nanosuspensions by high-performance liquid chromatography (HPLC) and Fourier-transform infrared spectroscopy (FTIR) analyses. **Results**: HPLC analysis revealed the presence of kaempferol and sinapic acid in various amounts at 11.5 ppm and 12.3 ppm, respectively. FTIR analysis of *T. ammi* powder revealed the presence of alcohols and amines. The assessment of antioxidant activity was conducted using a DPPH scavenging assay, indicating that the nanosuspensions exhibited their highest free radical scavenging activity, reaching 14.9%. Nanosuspensions showed 3.75 $\pm$ 3.529.5% biofilm inhibition activity against *Escherichia coli.* The antidiabetic activity was accessed through antiglycation and α- amylase inhibition assays, while nanosuspension showed the maximum inhibition activity at 25.35 $\pm$ 0.912133% and 34.6 $\pm$ 1.3675%. Hemolytic activity was also evaluated, and *T. ammi* nanosuspension showed 22.73 $\pm$ 1.539% hemolysis. **Conclusions**: This nanotechnology approach has established a foundation to produce plant-based nanosuspensions, offering a promising avenue for the biopharmaceutical production of herbal nanomedicines. These nanosuspensions have the potential to enhance bioavailability and can serve as a viable alternative to synthetic formulations.

Keywords: bioavailability; nanosuspension; antioxidant; hemolysis; antidiabetic; nanomedicines

1. Introduction

Various drugs have been used in the treatment of infectious diseases, yet most of them cause adverse effects on the living tissues in the human body [1]. In the modern era, a variety of chemical drugs, aspirin, clopidogrel, diclofenac, enoxaparin, ibuprofen, naproxen, and warfarin are used for therapeutic purposes. However, excessive use of these drugs increases the risk of cardiac failure, hepatic dysfunction, hemorrhage, respiratory failure, etc. [2]. Thus, due to their adverse side effects, these drugs are strongly prohibited due to hypersensitivity and autoimmunity [3]. Therefore, there is an urgent need for alternatives to chemical drugs to treat infectious and inflammatory diseases. Medicinal plants are a rich source of bioactive compounds and can be an alternative option due to their high bioavailability and minimal side effects [4]. The efficacy or safety of nanosuspensions from many synthetic plants has not previously been evaluated and remains a challenge in green chemistry [5]. Medicinal plants can be used to treat cardiovascular diseases, prostate cancer, myocardial infarction, and liver cirrhosis. Furthermore, they also activate the natural killer cells in the human body and provide long-term immunity for boosting the immune system [6]. Herbal medications are safe and reliable treatment options that are more effective in treating specific ailments than other medications. They are known to be fully natural unless blended with additional chemical components [7].

Trachyspermum ammi L. (Apiaceae) is the most valuable aromatic medicinal plant, which exhibits pharmacological potential for the upcoming exploration of novel

herbal medicine [8]. It is naturally produced in countries in southwest Asia, the Mediterranean region, Afghanistan, Iran, Egypt, Iraq, India, and Pakistan [9]. It is used in the treatment of different diseases, abdominal illnesses, poisoning, nausea, appendicitis, hypertension, cardiovascular health issues, constipation, and mosquito bites, while it is used for constipation in traditional Asian and African communities [10]. Its roots, seeds, and leaves are used in the treatment of gastrointestinal disorders, stomach pain, tumors, and piles. Antihelmintic qualities are found in the leaves of ajwain plants, thereby making them valuable for treating helminth infections in animals. Its seeds have antivomiting, diuretic, anti-asthmatic, and anti-dyspnea properties [11]. T*rachyspermum ammi* L. is a rich source of phenolics, antioxidants, flavonoids, polyphenols, and volatile aromatic compounds. These compounds have inhibitory action against infectious diseases and are commonly used for formulating various nutraceuticals [12]. It has been reported that *T. ammi* oil showed antimalarial, antimicrobial, antibacterial, anti-inflammatory, antioxidant, cytotoxic, nematicidal, and anthelmintic activities [13]. Different studies revealed that green nanoparticle synthesis of *T. ammi* was performed through different noble metals, such as gold (Au), selenium (Se), magnesium (Mg), and zinc (Zn) [14]. These plant-based noble metallic nanoparticles have shown different biological activities. In comparison to the ethanolic extract of *T. ammi*, green-synthesized nanoparticles showed a decreasing trend in the above-stated activities. Ethanolic extract, crude extract, and aqueous extract of *T. ammi* showed higher potential for different biological activities, compared to its nanoparticles and nanosuspension [15].

The nanotechnology approach is applied to formulations of herbal medicines for therapeutic purposes [16,17]. These nanomedicines have gained special attention in targeting tumors and cancerous cells [18]. Nanosuspensions are defined as nanoparticles, which are made of pure drug, with no matrix material, and an average diameter below 1 µm (typically in the range of 200–500 nm). Nanotherapeutics have improved absorption, minimal toxicity, healing effects, and created better pharmacokinetics [19]. Improving and increasing the bioavailability and solubility of drugs is a major challenge in medicine [14]. However, nanosuspensions have been formulated and designed for medical applications through advancements in nanotechnology, especially in targeting the delivery of nanosuspensions of natural products [20]. The major advantages of nanosuspensions are in the pharmaceutical technicalities of natural product delivery, including increasing dissolution rate and saturated solubility, thereby improving bioavailability for oral administration and enhancing skin surface penetration or cell membrane adhesion for transdermal administration [21]. Techniques such as media milling and high-pressure homogenization have been used commercially for producing nanosuspensions. The unique features of nanosuspensions have enabled their use in various dosage forms, including specialized delivery systems, such as mucoadhesive hydrogels [22].

We hypothesize that nanosuspensions of *T. ammi* extract showed enhanced bioactivities and bioavailability in therapeutic compounds, compared to its ethanolic extract. The main objectives of this study included the synthesis and the *in vitro* evaluation of the antioxidant, antibacterial, cytotoxic, and antidiabetic activities of *T. ammi* extract nanosuspensions. Structural characterization and identification of functional groups in bioactive compounds were accomplished using high-performance liquid chromatography (HPLC) and fourier-transform infrared spectroscopy (FTIR) analyses.

2. Materials and Methods

2.1 Chemicals and Reagents

The following chemicals and reagents were procured from the specified sources: Ethanol, acetone, and acetonitrile from Sigma Aldrich (Schnelldorf, Germany); Lot No. S240697, DPPH (2,2-Diphenyl-1-picrylhydrazyl), and alpha-amylase from Sigma Aldrich, PVA (polyvinyl alcohol) from Appli. Chem (Boca Raton, Florida, USA); Lot No. P28C013, bovine serum albumin from Merck (Germany), and Folin–Ciocalteu reagent from Sigma Aldrich; Lot No. 11-0700SAJ.

2.2 Collection and Identification of Plant Materials

Trachyspermum ammi (Ajwain) seeds were randomly collected from the local market in Faisalabad and verified by the Department of Botany, University of Agriculture, Faisalabad, Pakistan.

2.3 Preparation of Plant Extract

T. ammi seeds were initially grounded into fine powder form, and extraction was carried out using the Soxhlet apparatus. Petroleum ether was used to remove the fatty substances and 95% ethanol was used as the solvent for further extraction. The extract was separated using standard protocols of filtration. Finally, the extract was kept in the desiccator for further use in subsequent experiments [23].

2.4 Preparation of Nanosuspensions

Nanosuspensions of *T. ammi* were prepared using the nanoprecipitation method. By following this method, 11.25 mL of ethanol and acetone (1:3) were used to dissolve 1.5 g of the plant extract. The resultant solution was injected into 15 milliliters of water using polyvinyl alcohol (1.5% by volume). The resultant emulsion was diluted in 30 mL of an aqueous solution of PVA (0.2% W/V), and it was continuously stirred at 500 rpm for 3 hours. Then, following the evaporation, the solvent and nanosuspensions were produced. Finally, the freshly prepared nanosuspension was stored at –18 °C to keep it frozen and in dry form [23].

2.5 Antioxidant Profile

We assessed the antioxidant profile of *T. ammi* using the methods described below. Primarily, we employed colorimetric and scavenging techniques to estimate the antioxidant levels.

2.5.1 Total Flavonoid Contents

Quantification of the total flavonoid content in the sample was carried out using the colorimetric method. Specifically, 38 µL of both the extract and nanosuspension were combined and incubated at ambient room temperature for 10 minutes. Subsequently, 19 µL of a 10% AlCl3 solution was introduced to the mixture and further incubated for 5 minutes. Absorbance measurements were conducted at 510 nm by a microtiter plate reader (Synergy HTX, BioTek, Santa Clara, CA, USA), and the obtained results were compared to a standard catechin calibration curve [24].

2.5.2 Total Phenolic Contents

The Folin-Ciocalteu assay was employed to quantify the phenolic content in the sample. Briefly, a mixture of diluted reagent (10%; 25 µL) and test samples (125 µL) was prepared and incubated at room temperature for two hours. Subsequently, absorbance was measured using a microtiter plate reader (BioTek, U.S.A.) at a wavelength of 765 nm, and the obtained results were compared with a standard gallic acid calibration curve [25].

2.5.3 DPPH Radical Scavenging Assay

The assessment of the antioxidant activity of the *T. ammi* nanosuspensions and extract was conducted using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Specifically, a mixture of 250 µL of DPPH solution and 2.5 µL of the extract and nanosuspension solution was prepared and covered with aluminum foil for approximately 35 minutes. All measurements were performed in triplicate [26]. The free radical scavenging capacity was expressed as a percentage of inhibition and calculated using the following formula.

% DPPH scavenging = [A (control) – A (sample) / A (control)] × 100

2.6 Biofilm Formation Inhibition Assay

The biofilm inhibition assay was conducted using a microtiter plate reader. Nutrient broth, plant extracts, and 100 µL of both *Escherichia coli* and *Staphylococcus aureus* bacteria were incubated aerobically in a 96-well plate at 37 °C for 12 hours. Subsequently, the plates were rinsed three times with sterile phosphate-buffered saline to remove the adhering bacteria. A 100 µL solution of crystal violet stain (50%) was added to the wells of the microtiter plate, and any excess stain was removed by washing with tap water. Following the drying of the plates, the dye was solubilized with 100 µL of glacial acetic acid (33% v/v) in each well. Absorbance at 630 nm was measured using a plate reader [26]. A positive control was established by using nutrient broth containing ciprofloxacin, while nutrient broth with bacterial strains served as the negative control. The fractions/extracts were examined under a microscope to assess their potential to inhibit the formation of microbial biofilms [26].

The following formula calculated percentage inhibition:

% inhibition = 100 × [A (control) – A (sample) / A (control)]

2.7 Cytotoxic Activity (Hemolytic Assay)

Hemolytic activity was assessed to evaluate the cytotoxicity of the *T. ammi* nanosuspensions. The procedure began with the centrifugation of 3 mL of blood samples to separate the cellular components from the plasma. The separated red blood cells (RBCs) were washed three times with 5 mL of sterile chilled isotonic phosphate buffer saline (PBS; pH 7.4), and the plasma was discarded. Then, the washed cells were subjected to another round of centrifugation at 8000 rpm to obtain a normal saline suspension. The concentration of RBCs was determined, resulting in a count of 7.068 × 10.8 cells/mL. For the hemolytic assay, PBS was utilized as the negative control, while a 0.1% Triton X-100 solution served as the positive control. Absorbance was measured at 570 nm using a microtiter plate reader [27]. The following formula was used to calculate the percent hemolytic inhibition:

% hemolysis = A (sample) – A (negative control) / A (positive control) – A (negative control) × 100

2.8 Antidiabetic Evaluation

2.8.1 Alpha-Amylase Inhibition Assay

Alpha-amylase inhibition of *T. ammi* nanosuspensions was evaluated. In a 96-well plate, 30 µL samples were mixed with acarbose for 10 minutes, then treated with 10 µL amylase solution prepared in 0.02 M sodium phosphate buffer (pH 6.9; 0.5 mg/mL). Next, 40 µL of 1% starch solution was added and incubated for 30 minutes. Then, 75 µL of iodine solution and 40 µL (1 M) HCl solution were added to each well. A microtiter plate reader was used to measure absorbance at 630 nm against a blank [28]. The following formula was used to calculate % inhibition:

% inhibition = 1 – A (control) / A (sample) × 100

2.8.2 Antiglycation Potential

A spectrophotometer was used to assess the antiglycation potential of the nanosuspension. Firstly, 450 mg bovine serum albumin and 4.5 g D-glucose were mixed together, and the mixture was kept at 37 °C for 24 hours. Absorbance was measured by spectrophotometer at 440 nm for the emission wavelength and 370 nm for the excitation wavelength [29]. The following formula was used to calculate % inhibition:

% inhibition = [$A_{(440nm)}$ / $A_{(370nm)}$ – $A_{(440nm)}$] × 100

Table 1. Comparison of different assays of *T. ammi* nanosuspensions and extracts.

Treatments	Antioxidant profile			Antidiabetic profile (%)		Biofilm inhibition (%)		Hemolysis (%)
	TPC	TFC	DPPH	Glycation inhibition	α-amylase inhibition	*EC*	*SA*	
NS	253.04	157.06	14.9	25.35 $\pm$ 0.912133	34.6 $\pm$ 1.3675	13.75 $\pm$ 3.529	No Activity	22.73 $\pm$ 1.539
E	103.96	168.28	43	30.91 $\pm$ 2.16547	45.67 $\pm$ 2.9655	36.91 $\pm$ 2.238	No Activity	22.69 $\pm$ 0.834
Control	750.87	244.44	90	56.91333 $\pm$ 2.16269	82.53 $\pm$ 2.644598	0.121 $\pm$ 3.013	0.119 $\pm$ 2.862	90 $\pm$ 2.549

* Results are represented as a percentage or as the mean and standard deviation of measurements taken in triplicate. NS, *T. ammi* nanosuspension; E, *T. ammi* extract; *EC*, *Escherichia coli*; *SA*, *Staphylococcus aureus*; TPC, total phenolic contents; TFC, total flavonoid content; DPPH, 2,2-diphenyl l-picrylhydrazyl. Ciprofloxacin (antimicrobial assay). Metformin (antiglycation assay). BHT (butylated hydroxytoluene).

2.9 Biochemical Characterization

2.9.1 Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy was performed to analyze the structure of the *T. ammi* powder. In chloroauric solution, the substance was centrifuged at 10,000 rpm for 15 minutes. Pellets were washed out thrice with 20 mL of unionized water. The FTIR analysis was carried out by the FTIR system (Agilent Cary 630, Agilent Technologies, Santa Clara, CA, USA) in diffuse reflect array mode with a resolution of 4 cm [30].

2.9.2 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography was performed using a Flexar HPLC system (200, Shelton, WA, USA) to identify different compounds. *T. ammi* seeds were dried and hydrolyzed. Then, 0.5 g of dried contents were mixed with 20 mL of ethyl alcohol and 1 g/L of BHT (butylated hydroxytoluene). Subsequently, the obtained mixture was combined with 10 mL of 1 M HCl. Saponification was conducted for 15 minutes, and the resulting mixture was employed for HPLC (high-performance liquid chromatography) analysis. A test sample of 20 µL was injected, and the analysis was conducted at 280 nm. Various compounds were identified based on their respective retention times [31].

2.10 Statistical Analysis

ANOVA was used to evaluate the data in order to compare the means of two populations at once and determine whether certain formulation parameters were significant. $p < 0.05$ was chosen as the significance threshold. Finally, data were presented as average, standard deviation, or triplicity percentage measurements.

3. Results

This study aimed to investigate the cost-effective synthesis and bioactivities of *T. ammi* nanosuspensions and its ethanolic extract. The results demonstrated that *T. ammi* nanosuspensions exhibited higher total phenolic contents (TPCs) at 253.04 GAE/100 g compared to the extract, which had TPCs of 153.06 GAE/100 g. Nanosuspensions exhibited significant biofilm inhibition against *Escherichia coli*, with a maximum inhibition of 3.75 $\pm$ 3.52%. The DPPH free radical scavenging activity of the nanosuspensions reached its peak at 14.9%. Furthermore, the nanosuspensions displayed a higher hemolytic potential of 22.73 $\pm$ 1.54% compared to the extract, which showed a hemolytic potential of 22.69 $\pm$ 0.83%. In terms of antiglycation and alpha-amylase inhibition, the nanosuspensions exhibited promising inhibitory potential, with values of 25.35 $\pm$ 0.91% and 34.6 $\pm$ 1.37%, respectively.

3.1 Antioxidant Potential

Table 1 shows the antioxidant potential of *T. ammi* nanosuspensions. The results revealed that nanosuspension showed high total phenolics, compared to the ethanolic extract of *T. ammi*, at 253.04 and 103.96 mg GAE/100 g, respectively. The nanosuspension showed a total flavonoid content (TFC) of 157.06, and the extract contained 168.28 mg CE/100 g TFCs, respectively. The nanosuspension showed a maximum free radical activity of 14.9%, and the extract showed 43% accordingly. Moreover, according to statistical analysis, the TPC, TFC, and DPPH tests indicated extremely significant differences ($p < 0.01$).

3.2 Biofilm Formation Inhibition Potential

Recently, two bacterial strains, i.e., *E. coli* and *S. aureus,* were tested against the nanosuspension and ethanolic extracts of *T. ammi* seeds, and the ethanolic extract and nanosuspension exhibited amazing biofilm inhibition, either by removing the biofilm or lowering biofilm formation against *E. coli*. The ethanolic extract exhibited an inhibition of 36.91 $\pm$ 2.238%, which is higher than the nanosuspension, i.e., 13.75 $\pm$ 3.529%. The biofilm inhibition strategy exhibited significant changes, according to the statistical analysis ($p < 0.05$). Moreover, the nanosuspension and ethanolic extract did not show any activity against *S. aureus*, which is a stabilizer and dose. Fig. 1 shows the trend of inhibition in the given sequence $E_{inh.}$ *E. coli* > $NS_{inh.}$ *E. coli* > $PC_{inh.}$ *E. coli.*

3.3 Cytotoxic Activity (Hemolytic Assay)

Table 1 shows that the *T. ammi* nanosuspension exhibited 22.73 $\pm$ 1.539% hemolysis, which was higher than the extract (22.69 $\pm$ 0.834%). However, according to statistical analysis, the hemolytic assay did not demonstrate a significant difference ($p > 0.05$).

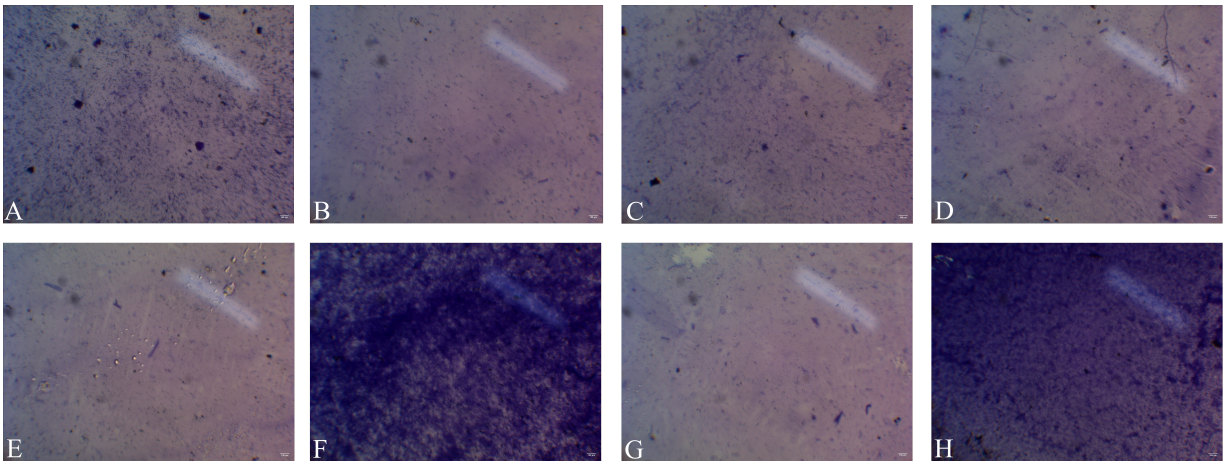

Fig. 1. Qualitative biofilm inhibition. (A) inhibition of *E. coli* by extract fraction (min). (B) Inhibition of *E. coli* by nanosuspension fraction (max). (C) Inhibition of *S. aureus* by extract fraction (min). (D) Inhibition of *S. aureus* by nanosuspension fraction (max). (E) Inhibition of *E. coli* by positive control. (F) Inhibition of *E. coli* by negative control. (G) Inhibition of *S. aureus* by positive control. (H) Inhibition of *S. aureus* by negative control.

Table 2. Fourier-Transform Infrared Spectroscopy (FTIR) spectrum chart indicating the identified functional groups in powdered *T. ammi* seeds.

Sr. No.	Peak Indemnification	Characteristic Absorption	Identified Functional Groups	Compounds Class
1	High	3281	O-H stretching	Alcohol
2	High	3008	O-H stretching	Alcohol
3	High	2922	N-H stretching	Amines
5	Medium	1418	O-H stretching	Alcohol
6	Medium	1228	C-O stretching	Alkyl aryl ether
7	Medium	1146	C-O stretching	Aliphatic ether
8	Low	1023	C-N stretching	Amine

Sr. No., Serial Number.

3.4 Antidiabetic Evaluation

3.4.1 Antiglycation Assay

The high flavonoid content and significant antioxidant capabilities of the seeds contribute to the antiglycation effect of the *T. ammi* nanosuspension. The nanosuspension showed an antiglycation activity of $25.35 \pm 0.912133\%$ and the extract exhibited an antiglycation activity of $30.91 \pm 2.16547\%$ (Table 1). Thus, according to statistical analysis, the antiglycation experiments differed significantly ($p < 0.05$).

3.4.2 Alpha-Amylase Inhibition Assay

The results showed that the nanosuspension only inhibited $34.6 \pm 1.3675\%$ alpha-amylase, whereas the extract inhibited $45.67 \pm 2.9655\%$ (Table 1). The alpha-amylase inhibition experiments differed significantly ($p < 0.05$), according to the statistical analysis.

3.5 Biochemical Characterization

The structural characterization of the *T. ammi* seed extracts, as well as the identification of the bioactive functional groups present in the sample, were performed by HPLC and FTIR.

3.5.1 Fourier-Transform Infrared Spectroscopy (FTIR)

Fig. 2 shows the infrared spectroscopy analysis of the chemical compounds, such as acids, polyphenols, and a variety of other known and unknown moieties identified in the test sample. Table 2 shows the absorption values of different functional groups predicted to be present in the powder of the *T. ammi* seeds by FTIR. Alcohols were detected because of a prominent peak at 3281 cm^{-1}, 3008 cm^{-1}, and 1418 cm^{-1}. The presence of amine salts in the sample was indicated by a band at 3000 cm^{-1}, 2922 cm^{-1}, and 2853 cm^{-1}. The band at 805 cm^{-1} indicates the presence of alkanes, while the two medium bands at 1228 cm^{-1} and 1146 cm^{-1} determine the presence of an aliphatic ether.

3.5.2 High-Performance Liquid Chromatography (HPLC)

Fig. 3 shows the bioactive compounds that were identified through HPLC analysis. Kaempferol and sinapic acid were found in *T. ammi* ethanolic seed extracts in various amounts at 11.5 ppm and 12.3 ppm, respectively.

4. Discussion

T. ammi nanosuspension extracts contain different proportions of phenolic and flavonoid compounds [32].

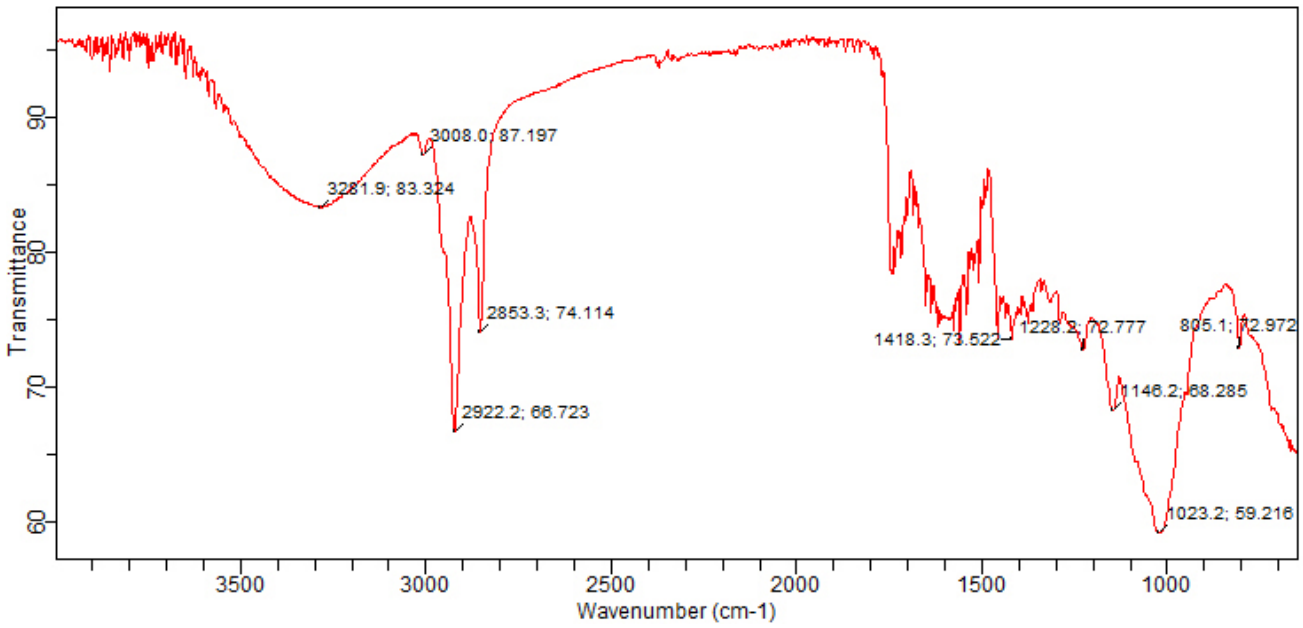

Fig. 2. Fourier-transform infrared spectroscopy (FTIR) chromatogram of powdered *T. ammi* seeds.

Our findings agreed with the previous study, which reported that *T. ammi* extracts contain more flavonoids than nanosuspensions, whereas *T. ammi* nanosuspensions possess higher phenolic contents [33]. Another study investigated the ethanolic extract and nanosuspension of *T. ammi* at higher concentrations (50 g/mL); the acetone extract of *T. ammi* had 99% free radical scavenging, which is quite close to normal rutin (99.12%). Previous studies revealed that *T. ammi* extracts with different proportions of DPPH free radical scavenging potentials followed as such: acetone > methanol > ethanol > chloroform > distilled water > hexane [34].

Different studies revealed the presence of antioxidants in *T. ammi* nanosuspensions, which is consistent with our findings, whereby a study reported that the antioxidants compounds in the ethanolic extract of *T. ammi* showed maximum free radical scavenging activity of 64.40%, compared to the scavenging activity identified in leaves [35]. The current study also agreed with another study, which revealed that nanosuspensions of *T. ammi* have a maximum radical scavenging activity of 43% [36]. Previous studies reported that the extract of *T. ammi* nanosuspensions contained antioxidants like p-pinene, p-cymene, and β-terpinene, which are responsible for its antioxidant properties. This improves the free radical scavenging potential of *T. ammi* nanosuspensions for hydroxyl ions and superoxide radicals and protects the living cells from oxidative damage by increasing glutathione levels [37].

Previous studies reported similar findings to our study, revealing nanosuspensions of *T. ammi*, biogenic selenium nanoparticles (SeNPs), and testing for their toxicological and therapeutic potential in collagen-induced arthritic animals. Another study reported that the tested doses of the SeNPs had no notable deleterious effects on the liver, kidney, spleen, or serum biochemical markers in healthy mice and that less toxicity was induced by the SeNPs, which lessened the severity of the condition [15]. Another study reported that selenium-based bioengineered nanosuspensions of *T. ammi* combined with TAE extract significantly improved their therapeutic efficacy by up to 90% [35]. Another study reported that the administration of biogenic SeNPs of *T. ammi* into the liver tissues boosted the catalase activity to some amount [14].

Our research aligns with other prior studies, which also observed the inhibitory effects of *T. ammi* seed nanosuspensions on biofilm formation and the susceptibility of three distinct bacterial strains to foodborne pathogens. This reinforces the effectiveness of *T. ammi* seeds in preventing biofilm formation [36]. Another study reported on the efficacy of gold nanoparticles made from *T. ammi* extracts (TA-AuNPs) against drug-resistant biofilms and found that NPs inhibited the formation of biofilms and the growth of bacterial pathogens, such as *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, E. coli, Klebsiella pneumoniae, Proteus mirabilis*, and *Pseudomonas aeruginosa* [38]. This study revealed that nanosuspensions acted as environmentally friendly and have strong antibiofilm potential, which can be used in a variety of biomedical applications [35]. A prior study highlighted *T. ammi* oils as a valuable reservoir of bioactive compounds with potential efficacy against multidrug-resistant and oxidative stress-related ailments. This finding suggests that the application of *T. ammi* in the food industry holds promise as a natural antioxidant and also in the pharmaceutical sector for the development of innovative antibiotics [39].

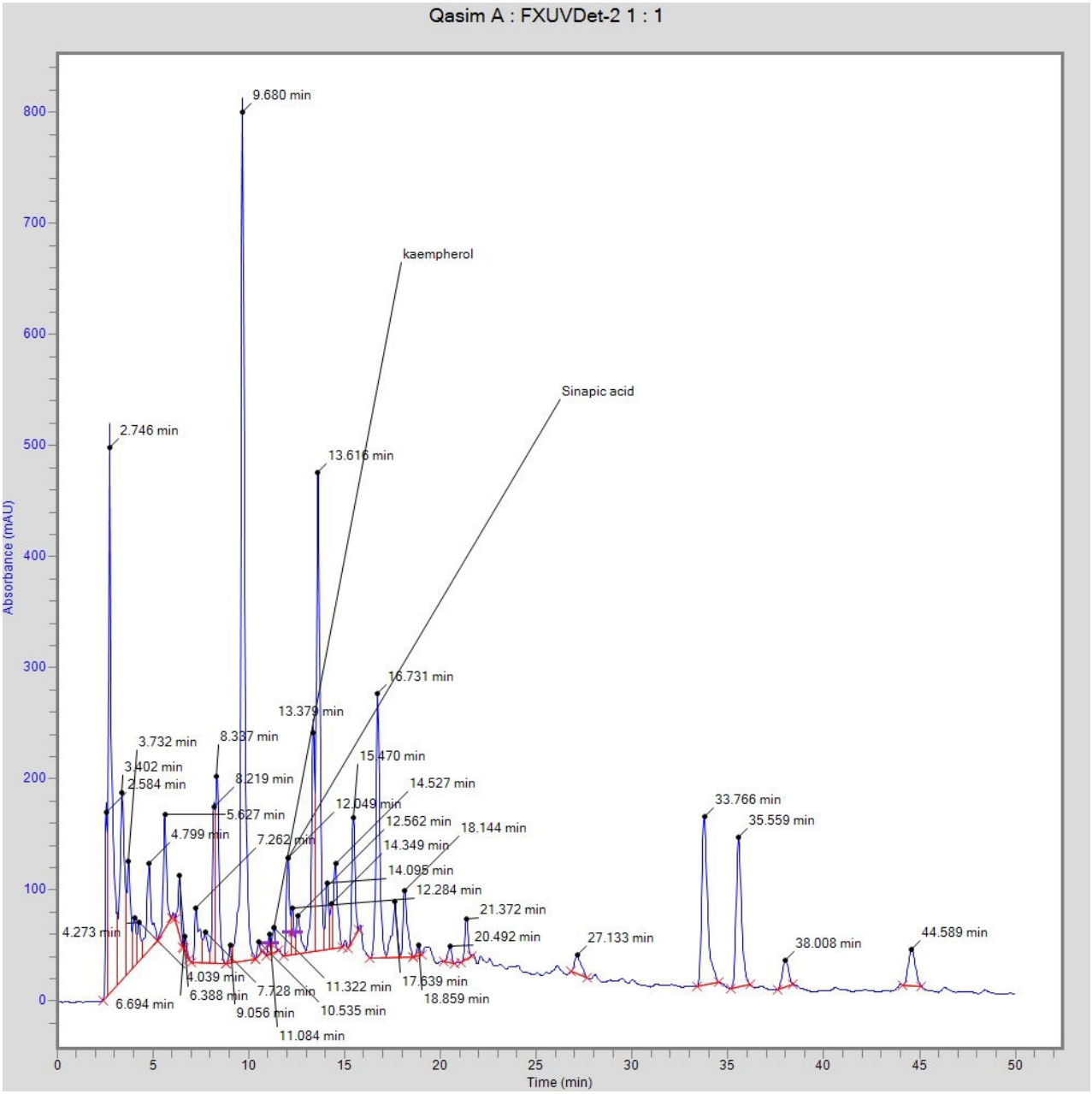

Fig. 3. High-performance liquid chromatography (HPLC) chromatogram of *T. ammi* demonstrating the bioactive compounds.

Previous studies reported that the antidiabetic activity of *T. ammi* extracts containing free amino acids (IC_{50} = 207 g/mL) and protein amino acids (IC_{50} = 299 g/mL) and *T. ammi* nanosuspensions had a considerable inhibitory effect on amylase activity [40]. Another study reported that acarbose is a common antidiabetic drug, which is involved in the breakdown of polysaccharides, and thereby reduces hyperglycemia. Free amino acids in the extract were more effective at inhibiting amylase activity [41]. Other studies reported that the flavonoid content and antioxidant potential of *T. ammi* seeds contributed to the antiglycation effect of *T. ammi* nanosuspensions [42]. They also reported that aqueous and ethanolic extracts exhibited a high potential for antiglycation in type 2 diabetes [43].

Another study reported that *T. ammi* seeds inhibited up to 50% of alpha-amylase activity. However, alpha-amylase activity in the *T. ammi* seeds was increased with the passage of time and the nature of alpha-polypeptide amylase, as well as the polyphenol concentration of the *T. ammi* seeds [44] Another study revealed that the *T. ammi* seeds and combinations of medicinal substances inhibited the alpha-amylase activity by 70%, meaning it could be used as a viable alternative to synthetic diabetic medicines [45]. These studies suggested that *T. ammi* seeds are a rich source of polyphenols and antioxidants that could be used for diabetes. Further, *in vivo* studies are needed to investigate the action of novel medicines.

Our findings agreed with a previous study, which reported that *T. ammi* nanosuspensions showed a hemolytic potential of 30 $\pm$ 0.90% [31]. Another study reported that *T. ammi* biogenetic stable suspensions showed a maximum hemolytic potential of 70% [46,47]. Previous studies reported that nanosuspensions-based medicinal substances demonstrated hemolytic activity in the presence of the Triton X100 solution (2%), which was used as the control [48]. These studies suggested that *T. ammi* nanosuspensions showed inhibitory potential in hemolysis. Further studies are needed to investigate the combined action of *T. ammi* nanosuspensions with other medicinal substances. These combinations also provided a foundation for herbal therapeutics.

Infrared spectroscopy is used to determine the structural properties of sample materials by identifying their distinct functional groups. Previous studies investigated the infrared spectra of *T. ammi* oil and found that the stretching vibration of the amine group was allocated to a medium-intensity band at 3429 cm^{-1}. The peak at 2962–2729 cm^{-1} was caused by the CH_3 group stretching, whereas the peak at 1458 cm^{-1} was caused by the C=C aromatic ring stretching [31]. Other studies revealed that out-of-plane alkene C–H stretching caused the peak at 810 cm^{-1}. The aldehydic groups C–H stretching resulted in a medium-intensity peak being identified in the oil samples that were prepared through different extraction techniques. However, the high stretching vibration of the alcoholic group was present in all samples at 1155.36 cm^{-1}, except for the ultrasonicated oil sample [30,47].

High-performance liquid chromatography is used to identify and quantify various biologically active functional groups, which are present in the test material [42]. Other studies have reported that the chromatogram created by the HPLC shows the peaks of several bioactive chemicals that are contained in the sample based on their column retention time, such as kaempferol in the seed extracts of *T. ammi*, yet they do not disclose a quantitative analysis of the phenolic and flavonoid chemicals found in the leaves [49,50]. Another study reported that the phenolic and flavonoid contents were high in the *T. ammi* extract [51]. These studies suggested that *T. ammi* nanosuspensions have a diverse nature of phenolic and flavonoids, which could be used in pharmaceutical industries.

5. Conclusions

This study reflects the cost-effective synthesis and enhanced bioactivities of freshly prepared *T. ammi* nanosuspensions. Biochemical characterization analysis was performed by HPLC and FTIR and revealed the presence of bioactive compounds: chlorogenic acid, gallic acid, kaempferol, alcohols, and amines. The nanosuspension showed a higher quantity of TPCs, compared to the ethanolic extract of *T. ammi*, at 253.04 and 103.96 mg GAE/100 g, respectively. At the same time, the nanosuspension contains TFCs at 157.06, while the extract contains 168.28 mg CE/100 g TFCs. Nanosuspensions demonstrated a maximum free radical scavenging activity of 14.9% and an antibacterial activity against *E. coli* of 3.75 $\pm$ 3.529.5%, alongside biofilm inhibition. The nanosuspension showed a hemolysis of 22.73 $\pm$ 1.539%, whereas its extract only showed hemolysis of 22.69 $\pm$ 0.834%. Finally, the nanosuspension showed a maximum antiglycation inhibition activity of 34.6 $\pm$ 1.3675%. Although there have been a few exceptions, using the nanosuspension can be advised for medicinal purposes. This research also provided the groundwork for plant-based nanosuspension production, which can be used to create herbal nanomedicines with improved bioavailability by replacing synthetic formulations.

Availability of Data and Materials

The datasets used/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

RJ and TA—designed the research study. RJ and TA—performed the research. FH—provided help and advice on experimentation. RJ, MSS, SD, MJA, TAA, MN and FH—visualization, acquiring, analyzing, or interpreting data from the reviewed literature. MJA, TA, MN and FH—wrote the manuscript. SD, TAA, MSS and MJA review and finalize the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Trachyspermum ammi (Ajwain) seeds were used in this study. Verified by Dr. Farooq Ahmad University of Agriculture Faisalabad Pakistan.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2812340.

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