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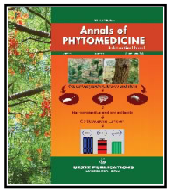

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Comparative analysis of the biological activities of different extracts of *Nigella sativa* L. seeds

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Abstract

Plants provide an abundant supply of metabolites with a wide range of chemical structures that may have medicinal promise. This chemical diversity facilitates the discovery of novel and unusual chemical constituents with their biological properties. This study looked at preliminary phytochemical profiles and tested the antidiabetic, antioxidant, and anti-inflammatory activities of four different extracts (methanol, hexane, ethanol, and aqueous) of *Nigella sativa* L. seeds. Quantitative and qualitative phytochemical analysis was performed using standard methods. The antidiabetic ability was determined using α-amylase and α-glucosidase enzyme inhibition assays. To examine the antioxidant property *in vitro*, DPPH, ABTS, HRSA, and NOS assays were used. The correlation between TPC and antioxidant activity was also evaluated. Using agarose gel electrophoresis, the degree of glycoxidative DNA damage was determined. Anti-inflammatory activity was evaluated using the inhibition of albumin denaturation assay. Aqueous extract of *N. sativa* seeds exhibited the highest values: TPC (228.18 ± 0.13 mg GAE/g DW), TFC (191.64 ± 0.031 µg QE/g DW), DPPH (74.62%), ABTS (49.45%), hydroxyl radical scavenging activity (63.60%), and nitric oxide scavenging activity (79.39%) as compared to the other seed extracts. The IC_{50} values for the extracts in the α-amylase and α-glucosidase assays were in the range of 1.07 mg/ml to 2.97 mg/ml, respectively. Also, there was a significant correlation between TPC and antioxidant activities. Inhibition of protein denaturation was 43.30%, 28.68%, 35.98%, and 57.56% in the methanolic, hexane, ethanolic, and aqueous extracts of *N. sativa* seeds, respectively, compared to aspirin (68.74%) at 1 mg/ml. Furthermore, the presence of extracts had a substantial antioxidant effect, protecting DNA against the oxidative stress agent, H_2O_2. From the results, it is concluded that the phytochemicals present in the *N. sativa* extracts may be responsible for the antioxidant, antidiabetic, and anti-inflammatory activities. This discovery provides a sufficient scientific framework to isolate and purify bioactive molecules for future applications.

1. Introduction

Plants, humanity's oldest friend, serve people not only by shielding them but also by curing them of various maladies. India is known as the "World's Botanical Garden," with over 2200 medicinal and aromatic plant species (Thomas *et al.*, 1999; Chikezie and Ojiako, 2015). About 3000 flowering plants are recognized for their medicinal use in India, and they are divided into three categories: plants of codified knowledge used in systems of medicine such as Ayurveda, Unani, and Siddha; plants of empirical knowledge used in folk medicine; and plants of scientific knowledge (Yadav *et al.*, 2011). Since ancient times, medicinal plants have been a primary source of therapeutic agents to cure human sickness (Prairna *et al.*, 2020). The renewed interest in natural medicines began in the last decade, owing to the widespread assumption that herbal medicine is healthier than synthetic pharmaceuticals (Mehrotra, 2021).

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Isolated phytochemicals from medicinal plants serve as drugs, protective agents, and pesticides (Ezzat *et al.*, 2019). They have shown very promising outcomes with minimal side effects or injury to other parts of the body. They can produce a wide range of bioactive chemicals that may protect against free radical damage (Manoharachary and Nagaraju, 2016; Bosch-Morell, 2020). Plants that contain beneficial phytochemicals may act as natural antioxidants, supplementing the human body's demands. Vitamins A, C, and E, as well as phenolic chemicals found in plants such as lignins, tannins, and flavonoids, all act as antioxidants (Altemimi *et al.*, 2017). These natural antioxidants protect against the damaging effects of reactive oxygen species (Ali *et al.*, 2019), which have been linked to aginge (Hajam *et al.*, 2022), as well as a variety of acute and chronic diseases such as cancer, diabetes, cardiovascular and neurological disorders (Len *et al.*, 2019).

N. sativa, commonly known as black cumin or kalonji, has been used for generations to treat a variety of ailments, including infectious diseases, making it one of Tibbe Nabawi's most essential remedies (Hannan *et al.*, 2021; Rubab *et al.*, 2021). Its seeds have been intensively investigated over the last few decades, with findings indicating that they exhibit a variety of therapeutic characteristics. The seeds are used as stimulants, astringent, diuretic,

bitter, expectorant, emmenagogue, piles, jaundice, paralysis, purgative, anthelmintic, constipating, abortifacient, and for skin ailments in Indian medicine (Balyan *et al.*, 2022). The findings show that both the oil and its active components, particularly thymo quinone (TQ), have repeatable antioxidant effects *via* boosting the oxidant scavenger system, which leads to antitoxic effects generated by a variety of factors (Badary *et al.*, 2003; Ali, 2020). The purpose of this study is to examine the phytochemical profiles and the antidiabetic, antioxidant, and anti-inflammatory potential of four different extracts (methanol, hexane, ethanol, and aqueous) of *N. sativa* seeds. For the first time, we show the correlation between phytochemical content and the antioxidant activities of this plant material in our study.

2. Materials and Methods

2.1 Plant material and extraction

N. sativa seeds were provided by Dr. Aqueela Sattar (Royal College, Mira Road, Thane) with taxonomic recognition by the Institute of Herbal Science, Plant Anatomy Research Centre, Chennai, India (Certificate No. PARC/2019/3911). Seeds were cleaned of any foreign matter, rinsed, and dried in the shade. The air-dried, powdered seeds (50 g) were extracted using a Soxhlet apparatus with solvents separated at a 1:10 (w/v) ratio of plant material powder mass/solvent. Pure solvents of varied polarities, such as methanol, hexane, ethanol, and water, were used in the extraction process. The filtrate was then dried in a rotary evaporator at 50-60°C until all the solvents had evaporated, leaving only the dry extract. The obtained powder was weighed, and the yield was calculated as follows: % yield = Actual yield/Theoretical yield × 100. The extracts were then employed for a variety of applications, including phytochemical screening and biological activity research.

2.2 Phytochemical screening

2.2.1 Qualitative analysis for preliminary phytochemical analysis

The Mayer's test for alkaloids, ferric chloride test for phenols, Fehling's and Benedict's tests for carbohydrates, alkaline reagent, Shinoda test for flavonoids, Millon's test for proteins/amino acids, Salkowski test for steroids and terpenoids, Braymer test for tannins, the NaOH test for volatile oils, and Concentrated HCl and Sulfuric acid tests for quinones were all performed using the procedures described by Shaikh and Patil (2020). All these phytochemical tests were done on methanol, hexane, ethanol, and aqueous extracts of *N. sativa*.

2.2.2 Quantitative analysis of phytochemicals

2.2.2.1 Detection of protein content using the Bradford method

The total protein content of the plant extracts was determined by using the Bradford method based on the methodology reported by Bradford (1976). BSA (0-1 mg/ml) was used as a positive control, and absorbance was measured at 595 nm.

2.2.2.2 Detection of reducing sugar using the DNSA method

The reducing sugar was calculated using a modified version of the method described by Gusakov *et al.* (2011). With D-glucose (0-1 mg/ml) as a standard reducing sugar, the total reducing sugar in the plant extracts was computed.

2.2.2.3 Determination of total phenolic content (TPC) using the Folin-Ciocalteu (FC) method

The total phenolic content of *N. sativa* seed extracts was measured using the FC method (Lu *et al.*, 2011). Briefly, 500 µl of the extract (1 mg/ml) was mixed with 2.5 ml 10% FC reagent (v/v). After 5 min, 2.5 ml of 7.5 % sodium carbonate (w/v) was added, stirred, and left to sit for 45 min at room temperature. Finally, the absorbance was taken using a spectrophotometer (Shimadzu UV-1800) at 765 nm against a blank without extract. Gallic acid (5-500 mg/l) was used to create a calibration curve, and the results were represented in mg gallic acid equivalent per gram of dry weight (mg GAE/g DW).

2.2.2.4 Determination of total flavonoid content (TFC) using the aluminium chloride method

The total flavonoid content of *N. sativa* seed extracts was determined by using the aluminium chloride colorimetric method based on the methodology reported by Shraim *et al.* (2021). Briefly, 0.5 ml of plant extract (1 mg/ml) was mixed with 1 ml of 10% aluminium chloride, 1ml of potassium acetate (1 M), and 2.5 ml of distilled water. The absorbance of the mixtures was measured at 415 nm by using UV-spectrophotometer. Quercetin (0-50 µg/ml) was used to create a calibration curve and the results were expressed as quercetin equivalent per mg of dry weight (mg QE/g DW).

2.3 Enzyme inhibition assays

2.3.1 Inhibition of α-amylase

The method of inhibition of α-amylase by *N. sativa* seed extracts was investigated using the modified methodology disclosed by Daoudi *et al.* (2020). 500 µl of plant extract and 500 µl enzyme solution (500 µg/ml) prepared in sodium phosphate buffer (0.02 M, pH 6.9) were incubated for 10 min. To the mixture, 500 µl of 1% starch solution was added and incubated at 25°C for 10 min. After that, DNSA color reagent (1 ml) was added to stop the reaction, and the mixtures were placed in a boiling water bath for 5 min. Finally, using a Shimadzu UV-spectrophotometer, the absorbance was measured at 540 nm. The standard used was acarbose (0-1000 µg/ml). The inhibitory action of α-amylase was determined as follows:

$(A_C - A_S / A_C) \times 100\%$, where A_C is defined as the absorbance of the control (without extract) and A_S is the absorbance of the test sample (with extract).

2.3.2 Inhibition of α-glucosidase

The effect of *N. sativa* seed extracts on α-glucosidase activity was investigated using the methodology of Ouassou *et al.* (2018). 200 µl of α-glucosidase (100 µg/ml) were pre-incubated for 10 min with 200 µl of various doses of seed extracts (200-1000 µg/ml). The reaction was then initiated with 300 µl of 10 mM pNPG prepared in a phosphate buffer (50 mM, pH 6.8) as a substrate and incubated for 40 min at 37°C before being stopped with 3 ml of 100 mM Na_2CO_3. The yellow-colored p-nitrophenol produced from pNPG was measured at 405 nm to evaluate the α-glucosidase activity. The standard was acarbose (0-1000 µg/ml). The α-glucosidase inhibitory activity was calculated as follows: $(A_C - A_S / A_C) \times 100\%$, where A_C is defined as the absorbance of the control (without extract) and A_S is the absorbance of the sample (with extract).

2.4 Antioxidant assays

2.4.1 Radical scavenging activity using the DPPH method

The radical scavenging activity using the DPPH method of each sample was determined according to the methodology described by (Baliyan *et al.*, 2022). 500 µl of 0.3 mM DPPH (dissolved in methanol) and 500 µl of various concentrations of extract (200-1000 µg/ml) were used to make the reaction mixture, incubated for 30 min in the dark. The absorbance was then measured at 517 nm against methanol as a blank, with DPPH and methanol used as a control. The positive control was ascorbic acid. The formula used to compute the percentage of inhibition is as follows:

$(A_C - A_S / A_C) \times 100\%$, where A_C is defined as the absorbance of the control (without extract) and A_S is the absorbance of the sample (with extract).

2.4.2 Free radical scavenging ability using ABTS assay

The free radical scavenging ability using the ABTS assay was investigated using a methodology described by Lalhminghlui and Jagetia (2018). Briefly, 250 µl of the different concentrations of plant extracts (200-1000 µg/ml) was added to 750 µl of the $ABTS^{+}$ solution. After 10 min, the absorbance was measured at 734 nm. As a positive control, Trolox was used. The formula used to compute the percentage of inhibition is as follows:

$(A_C - A_S / A_C) \times 100\%$, where A_C is defined as the absorbance of the control (without extract) and A_S is the absorbance of the sample (with extract).

2.4.3 Hydroxyl radical scavenging activity (HRSA)

Hydroxyl radical scavenging activity (HRSA) was investigated using a methodology described by Jayasundara *et al.* (2018). 33 µl of the plant extracts (200-1000 µg/ml), 33 µl of 17 mM 2-deoxy2-ribose, 33 µl of 1.2 mM EDTA, 33 µl of 34 mM hydrogen peroxide (H_2O_2), 67 µl of 0.3 mM $FeCl_3$, and 67 µl of 0.6 mM ascorbic acid were used to make the reaction mixture. The reaction was carried out for 1 h at 37°C. Then, 333 µl of 1% (w/v) thiobarbituric acid (TBA) and 333 µl of 2.8% (w/v) trichloroacetic acid (TCA) was added to the mixture and incubated at 100°C for 15 min. The absorbance was measured at 532 nm after cooling and compared to a blank containing deoxyribose and buffer. As a positive control, quercetin was utilized.

2.4.4 Nitric oxide scavenging assay (NOS)

The nitric oxide scavenging assay of each sample was determined according to the methodology described by Das *et al.* (2012). Different quantities of extract in corresponding solvents were combined with sodium nitroprusside (10 mM) in PBS (0.02 M, pH 7.4) and incubated at 25°C for 1 h. As a control, the identical reaction mixture is used but with an equivalent amount of ethanol. Following the incubation period, 1.5 ml of the solution was withdrawn and diluted with 1.5 ml of Griess reagent (1% sulfanilamide in 20% glacial acetic acid, 2% phosphoric acid, and 0.1% naphthyl ethylenediamine dihydrochloride). The chromophore generated when the nitrite was diazotized with sulfanilamide and then coupled with naphthyl ethylenediamine had a 546 nm absorbance.

2.5 Glycoxidation of DNA

The H_2O_2-mediated DNA damage was studied as per the method of Ali *et al.* (2021). pBR322 plasmid DNA (0.25 µg), H_2O_2 (60 mM), were incubated with or without plant extracts in 100 mM phosphate buffer (pH 7.4). All the samples, *i.e.*, pBR322, pBR322+H_2O_2, pBR322 + extract, and pBR322 + H_2O_2 + extract, were incubated at 37°C. Following incubation, samples were separated on a 1% agarose gel electrophoresis and visualized using Gel-Doc.

2.6 Anti-inflammatory activity (Protein denaturation inhibition assay)

A protein denaturation inhibition assay was carried out using the methodology of Osman and his colleagues (2016) with certain modifications. Aspirin was used as a reference drug at a concentration of 1 mg/ml. 5 ml of reaction mixture contained 0.2 ml of bovine serum albumin (1%), 0.02 ml of plant extract (1 mg/ml), and 4.78 ml of phosphate-buffered saline (PBS, pH 6.4), which were combined and incubated in a water bath at 37°C for 15 min. The mixture was heated for 5 min at 70°C and the turbidity was measured at 660 nm after cooling. The negative control used was the phosphate buffer solution. Using the following formula, the percentage inhibition of protein denaturation was calculated as follows:

$(A_C - A_S / A_C) \times 100\%$, where A_C is defined as the absorbance of the control (without extract) and A_S is the absorbance of the sample (with extract).

2.7 Statistical analysis

Graph Pad Prism version 8 was used to conduct the analysis. The significance of the data was determined using a two-way analysis of variance (ANOVA). The data were expressed as mean ± SD (n = 3) and Tukey's multiple comparisons tests were used to evaluate differences between treatments. $^{a}p < 0.05$ when compared to positive control.

3. Results

3.1 Extraction yield

The selection of proper extraction techniques and solvents is crucial in the screening of bioactive compounds and the characterization of biological activities of plant samples (Sasidharan *et al.*, 2011). The yields for methanol, hexane, ethanol, and aqueous extracts of *N. sativa* seeds were 17.13%, 25.06%, 12.47%, and 16.8%, respectively, as shown in Table 2. The hexane extract had the maximum extraction yield, while the ethanolic extract had the lowest. The extraction yield is dependent on the solvents used and the sample's chemical makeup (Truong *et al.*, 2019).

3.2 Phytochemical screening

3.2.1 Qualitative analysis of phytochemicals

The qualitative analysis of bioactive components for four extracts may be seen in Table 1. Bioactive substances found in aqueous extract included alkaloids, carbohydrates, reducing sugar, phenol, flavonoids, steroid, terpenoid, glycosides, tannin, and quinones. Methanol extract contains a variety of bioactive components, including alkaloids, carbohydrates, steroids, terpenoids, glycosides, quinones, tannins, as well as volatile oils. Because hexane is

nonpolar, it can only extract a small number of bioactive chemicals such as steroids, terpenoids, volatile oils, and quinones. In addition, alkaloids and carbohydrates were detected in the ethanolic extract, which was absent in hexane extract.

Table 1: Qualitative phytochemical analysis of *N. sativa* seed extracts

	Methanolic extract	Hexane extract	Ethanolic extract	Aqueous extract
Alkaloids	+	-	+	++
Carbohydrates	+	-	-	++
Phenol	+	-	-	++
Flavonoids	+	-	-	++
Glycosides	+	-	-	+
Steroids	++	++	+	+++
Terpenoids	+	++	+	++
Volatile oils	+	+	++	-
Quinones	+	++	+	+++
Tannins	+	-	-	+++

+++: more abundant; ++: moderate; +: less abundant; -: absent

3.2.2 Quantitative analysis of phytochemicals

3.2.2.1 Detection of protein content

In an acidic matrix, the Bradford assay uses interactions between basic amino acid residues like lysine, histidine, arginine and the Coomassie brilliant blue G-250. Dye's spectral shift to the blue form of the dye occurs when it binds to proteins (Redmile-Gordon *et al.*, 2013). The protein content was determined using the standard bovine serum albumin (BSA) calibration curve (y = 0.7651x, R^2 = 0.9997). The protein of the plant extracts investigated ranged from 187.77 to 216.09 mg/ml of BSA (Table 2). Protein content was found to be 209.99 ± 0.002, 187.77 ± 0.005, 205.20 ± 0.004, and 216.09 ± 0.007 mg/ml in methanolic, hexane, ethanolic, and aqueous extract, respectively.

3.2.2.2 Detection of reducing sugar using the DNSA method

DNSA test detects the presence of reducing sugars with free carbonyl groups (C=O). This involves the oxidation of the aldehyde (in glucose) and ketone (in fructose) functional groups. During this process, dinitro salicylic acid is reduced to 3-amino-5-nitrosalicylic acid, which is transformed into an orange-colored complex with a maximum absorbance of 540 nm under alkaline circumstances (Deshavath *et al.*, 2020). Using the calibration curve of standard D-glucose (y = 0.5409x, R^2 = 0.9938), the reducing sugar content was estimated. The reducing sugar content of the plant extracts ranged from 323.81 to 370.92 mg/ml (Table 2). Reducing sugar determination in plants aids in quantifying the degree of acrylamide generation in plant-derived foods at high temperatures, which aids in measuring food quality as well as in enhancing individual health (Halford *et al.*, 2011).

Table 2: Extraction yield, total protein content, and total reducing sugar content in different extracts of *N. sativa* seeds

	Extraction yield (%)	Total protein content (mg BSA/g of dry extract)	Total reducing sugar content (mg G/g of dry extract)
Methanolic extract	17.13 ± 0.3409	209.99 ± 0.002	349.18 ± 0.005
Hexane extract	26.06 ± 0.6108	187.77 ± 0.005	323.81 ± 0.006
Ethanolic extract	12.47 ± 0.2153	205.20 ± 0.004	343.48 ± 0.003
Aqueous extract	16.82 ± 0.8458	216.09 ± 0.007	370.92 ± 0.004

Table 3: Total phenolic content (TPC) and total flavonoid content of *N. sativa* seed extracts

	TPC mg GAE/g DW	TFC (mg QE/G DW)
Methanolic extract	170.98 ± 0.00034	142.92 ± 0.0177
Hexane extract	146.36 ± 0.00231	128.519 ± 0.0202
Ethanolic extract	171.24 ± 0.0046	147.06 ± 0.0360
Aqueous extract	228.18 ± 0.0134	191.64 ± 0.0317

3.2.2.3 Determination of total phenolic content (TPC)

Using the standard curve equation of gallic acid (y = 0.003x, R^2 = 0.9987), total phenols were estimated, where y represents absorbance and x represents TPC in *N. sativa* extracts expressed in GAE/g DW. The TPC of the methanol, hexane, ethanolic, and water extracts were 170.98 ± 0.003, 146.46 ± 0.002, 171.24 ± 0.004, and 228.18 ± 0.13 mg of GAE/g DW, respectively, as shown in Table 3.

3.2.2.4 Determination of total flavonoid content (TFC)

An aluminium chloride reagent was used to assess the quantity of total flavonoids. The total flavonoid was expressed as µg of quercetin equivalents per milligram of extract using the standard curve equation: y =0.184x, R^2 = 0.9999, where y represents absorbance and x represents TFC in *N. sativa* extracts expressed in µg of quercetin equivalents per gram of dry weight of extract (µg QE/g DW). Flavonoids were found in all four extracts, but the aqueous extract had the highest level of flavonoids, with a value of 191.64 ± 0.031 µg QE/g DW. The TFC content of the other extracts varied between 128.51 ± 0.020 and 149.06 ± 0.036 µg QE/mg DW as shown in Table 2.

3.3 Enzyme inhibition assays

3.3.1 Inhibition of α-amylase

Inhibition of carbohydrate digesting enzymes is being evaluated as a treatment option for type 2 diabetes. The most significant digestive enzyme in humans is pancreatic α-amylase, a calcium metalloenzyme that catalyzes the hydrolysis of the α -1,4 glycosidic bonds of amylose, amylopectin, glycogen, starch, and other maltodextrins (Etxeberria *et al*., 2012). The results shown in Table 4 show the influence of various *N. sativa* extracts on α-amylase activity inhibition. When compared to acarbose (45.87%), the aqueous extract had a significantly good α-amylase inhibitory action of 38.21% (p<0.05). The methanolic extract's inhibitory action at 1 mg/ml resulted in a 25.96% reduction in enzyme activity, followed by the ethanol (20.87%) and hexane extract (17.31%), respectively as shown in Table 4. The IC_{50} of the standard acarbose, methanol, hexane, ethanol, and aqueous extracts was 1.24 ± 0.00705, 1.96 ± 0.1013, 2.97 ± 0.0466, 2.37 ± 0.1023, and 1.38 ± 0.0016 mg/ml, respectively (Figure 1(A)).

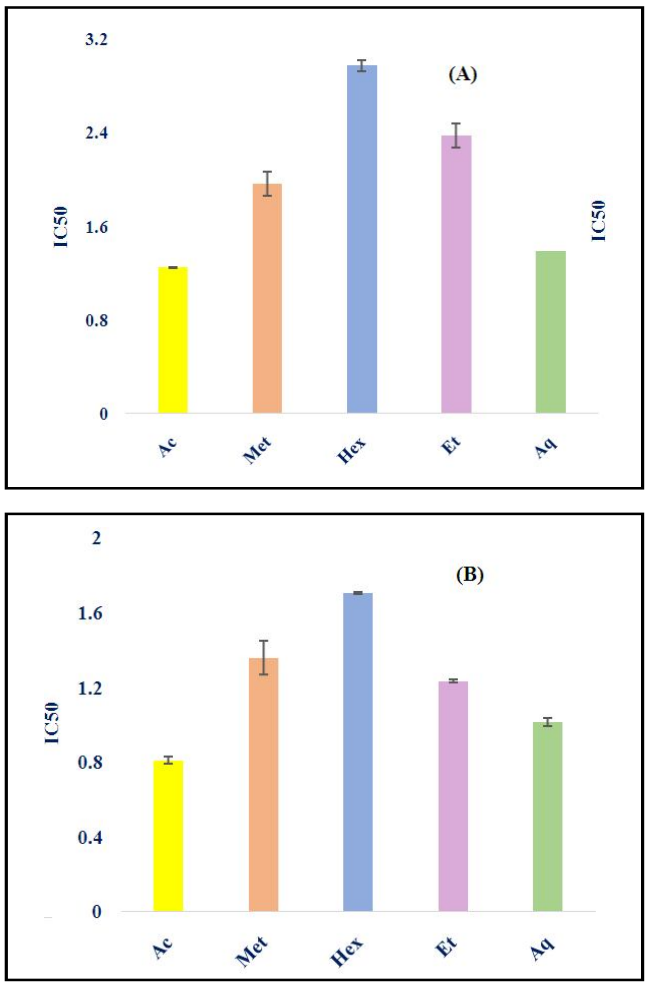

Figure 1: IC_{50} of (A) Inhibition of α-amylase, (B) Inhibition of α-glucosidase. Met: methanolic, Hex: hexane, Et: ethanolic, Aq: aqueous extract. Values are expressed as the mean ± SD (n = 3), p<0.05 when compared to acarbose.

3.3.2 Inhibition of α-glucosidase

The final phase of carbohydrate digestion is catalyzed by a second enzyme, α-glucosidase, or maltase, acting on 1,4-α bonds to produce glucose (Tundis *et al*., 2010). The inhibitory actions of *N. sativa* seed extracts against α-glucosidase *in vitro* were studied and compared to acarbose, a conventional medication. In Table 4, hexane had a weak α-glucosidase activity of 29.70% at a concentration of 1 mg/ml, whereas the aqueous extract had a stronger α-glucosidase activity of 58.05% at the same concentration (p<0.05). The enzyme inhibitory activity of the methanolic and ethanolic extracts was approximately 35.80% and 37.88%, respectively. As shown in Figure 1(B), the IC_{50} of the standard acarbose, methanol, hexane, ethanol, and aqueous extracts was 0.80 ± 0.199, 1.35 ± 0.0897, 1.70 ± 0.0052, 1.23 ± 0.0091 and 1.01 ± 0.0224 mg/ml, respectively.

Table 4: Percent inhibition of α-amylase and α-glucosidase using *N. sativa* seed extracts

	α-amylase inhibition (%)	α-glucosidase inhibition (%)
Acarbose	45.87 ± 0.0052	58.05 ± 0.0082
Methanolic extract	25.96 ± 0.0245	35.80 ± 0.0152
Hexane extract	17.31 ± 0.0015	29.70 ± 0.0022
Ethanolic extract	20.87 ± 0.0027	37.88 ± 0.0025
Aqueous extract	38.21 ± 0.0035	47.33 ± 0.0042

3.4 Antioxidant assays

3.4.1 Radical scavenging activity using DPPH assay

The stable radical DPPH has been widely used to assess the antioxidant activity of plant extracts (Kedare and Singh, 2011). Figure 2(A) demonstrates the results of DPPH radical scavenging activity in comparison to the well-known antioxidant ascorbic acid. The findings revealed that among four extracts of the tested plants, the aqueous extract had the highest activity to scavenge DPPH (74.62%), followed by methanolic (59.40%), ethanolic (40.81%), and hexane extract (27.52%), respectively at a concentration of 1 mg/ml.

3.4.2 Free radical scavenging activity using ABTS assay

The ABTS scavenging activity of various *N. sativa* extracts ranged from 8.48% to 49.45% (200-1000 µg/ml) as shown in Figure 2(B). The existence of varied amounts of bioactive chemicals such as phenolics, flavonoids, saponins, alkaloids, and tannins could explain the variance in scavenging power among different medicinal plant extracts (Yu *et al*., 2021). The ability of ABTS to scavenge radical cations also reflects its ability to donate hydrogen. High molecular weight phenolics have a better ability to quench free radicals ($ABTS^{\bullet+}$), according to Hagerman *et al*. (1998). The order of ABTS radical scavenging activity found in the present study was trolox (67.71%) > aqueous (49.45%) > methanol (44.84%) > ethanol (40.97%) > hexane (33.04%) at 1 mg/ml.

3.4.3 Hydroxyl radical scavenging activity (HRSA)

The most reactive and prevalent radicals generated endogenously during aerobic metabolism to induce cell damage *in vivo* are hydroxyl radicals (Mates and Sanchez-Jimenez, 2000). We looked at how well the samples inhibited deoxyribose breakdown, which indicates

that they are hydroxyl radical scavengers. Figure 2(C) shows the hydroxyl radical scavenging capabilities of different extracts of *N. sativa* (200-1000 µg/ml) in comparison to quercetin. The decreasing order of hydroxyl radical scavenging activity depicted was quercetin (68.82%) > aqueous (63.60%) > methanol (60.25%) > hexane (50.73%) and ethanol (48.85%) with $p<0.05$. The extracts scavenged the hydroxyl radical generated by Fe^{2+} ascorbic acid and EDTA-H_2O_2 system in a dose-dependent manner. This investigation suggests that *N. sativa* seeds could be used instead of synthetic antioxidants to combat hydroxyl radical oxidation.

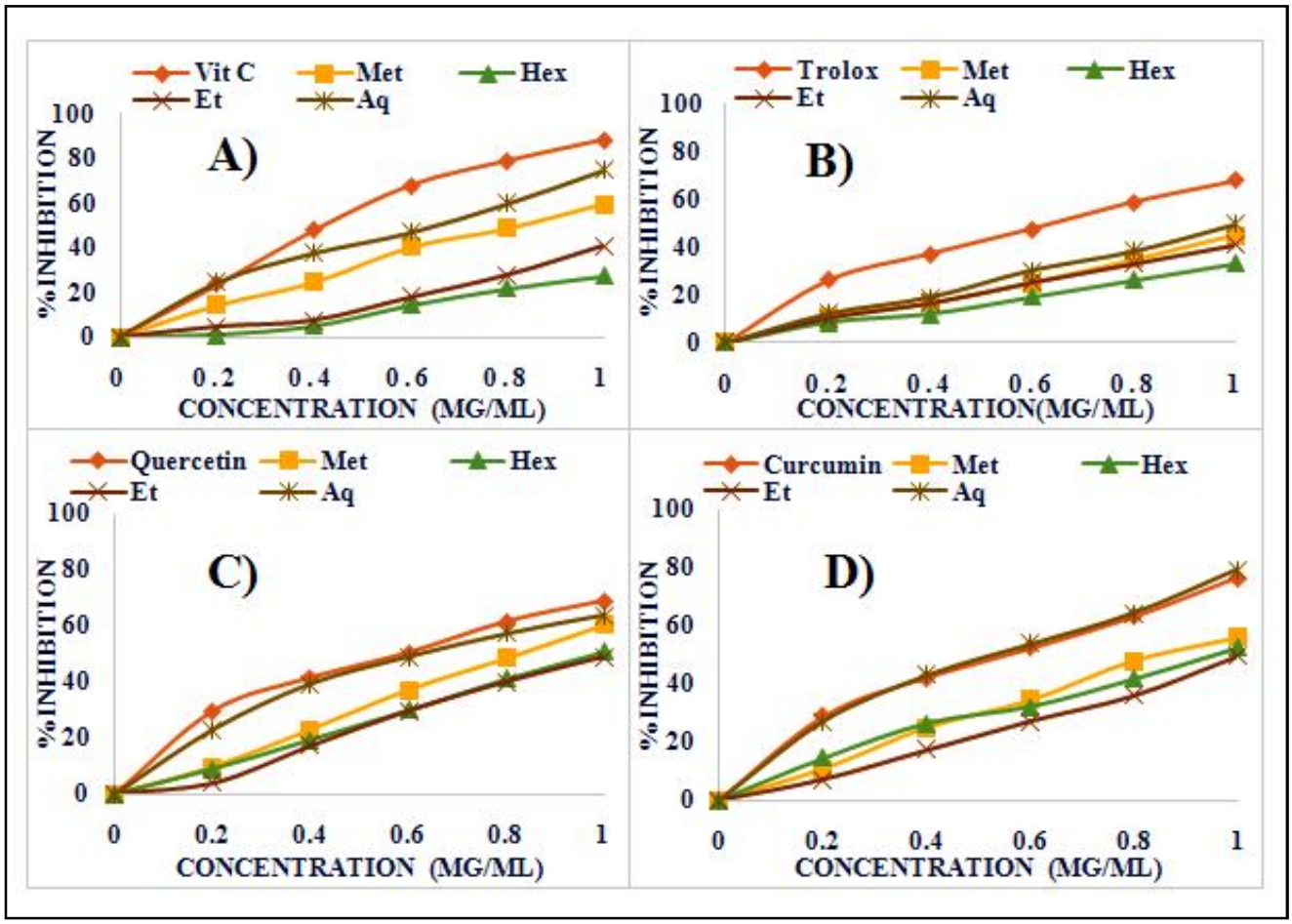

Figure 2: (A) Radical scavenging activity using the DPPH method; (B) Free radical scavenging ability using a stable ABTS radical cation; (C) Hydroxyl radical scavenging activity (HRSA); (D) Nitric Oxide scavenging (NOS) activity. $p<0.05$ when compared to positive control.

3.4.4 Nitric oxide scavenging assay (NOS)

The toxicity of nitric oxide is increased when it reacts with the superoxide radical to generate the peroxynitrite anion ($ONOO^-$). Nitric oxide, produced by sodium nitroprusside, interacts with oxygen to make nitrite. The extract prevents the creation of nitrite by directly fighting with oxygen in the nitric oxide process (Hazra *et al.*, 2008). The aqueous extract exhibits similar effective nitric oxide scavenging activity as conventional curcumin, according to the findings. As shown in Figure 2(D), *N. sativa* extracts significantly inhibited nitric oxide in a dose-dependent manner. Curcumin was utilized as a control agent, which showed 76.59% inhibition at a concentration of 1 mg/ml. The decreasing order of hydroxyl radical scavenging activity depicted was aqueous (79.39%) > methanol (56.30%) > hexane (52.75%) and ethanol (49.61%), respectively.

3.5 Glycoxidation of DNA

The preventive effects of plant extracts on Fenton-induced damage of pBR322 supercoiled DNA were studied further to toe if they could protect against oxidative DNA damage. As shown in Figure 3, two bands of control DNA were visible, one of open circular DNA and the other of supercoiled DNA (Lane 1). In the absence of plant extract (Lane 2), combined treatment of pBR322 DNA with H_2O_2, resulted in the production of open circular DNA *via* strand scission of the supercoiled DNA, but plant extracts (Lane 3-10) prevented this strand scission. It has been found that the aqueous extract (Lane 10) prevented the most DNA damage when compared to methanolic (Lane 4), hexane (Lane 6), and ethanolic (Lane 8) extracts, respectively. The inclusion of flavonoids and phenolic substances, which can limit the creation of ROS by complexing cations (iron, copper) that assist in the formation of hydroxyl radicals, may be responsible for the extract's DNA protection (Banan and Ali, 2016; Jha *et al.*, 2018).

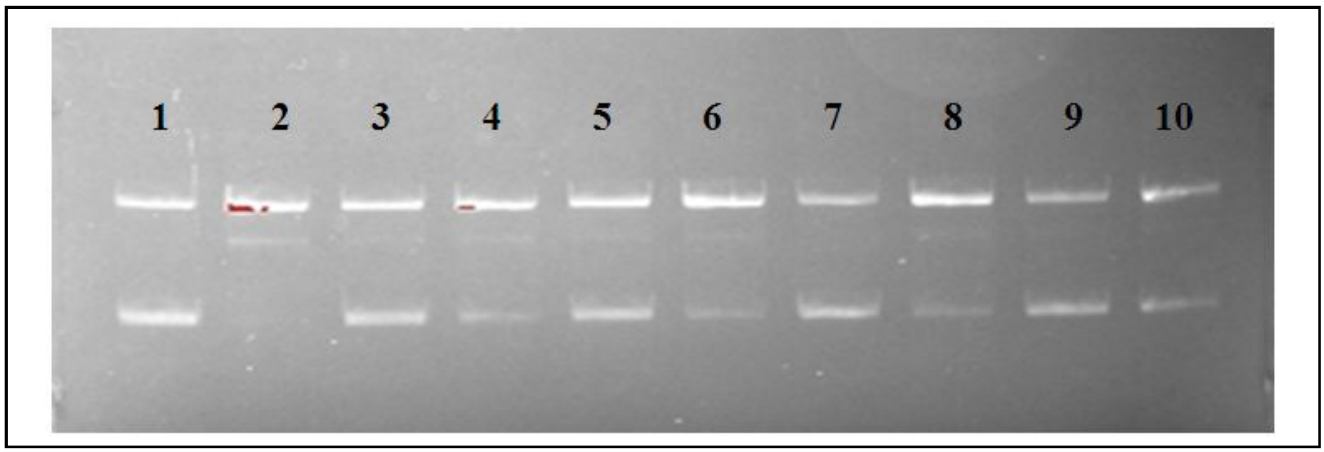

Figure 3: Electrophoresis patterns of pBR322 DNA breaks by ·OH generated from the Fenton reaction and prevented by different extracts of *N. sativa* seeds. (a) Lane 1: control DNA; lane 2: + DNA + H_2O_2; lane 3: DNA + Met; lane 4: DNA H_2O_2 + Met; lane 5: DNA + Hex; lane 6: DNA+ H_2O_2 + Hex; lane 7: DNA+ Et; lane 8: DNA+ H_2O_2 +Et; lane 9: DNA+ Aq; Lane 10: DNA+ H_2O_2 +Aq.

3.6 Anti-inflammatory activity (Protein denaturation assay)

The current study was based on a comparison of the *in vitro* anti-inflammatory activities of several *N. sativa* seed extracts. The greatest activity for inhibiting albumin denaturation was determined to be 68.74 ± 0.014% in aspirin, followed by aqueous extract (57.56 ± 0.018%), methanolic (43.30 ± 0.009%), ethanolic (35.98 ± 0.011%) and hexane extracts (28.68 ± 0.010%), respectively, at 1 mg/ml (Figure 4). The two-way ANOVA showed significant results with $p<0.05$ between aspirin and all four extracts of *N. sativa*.

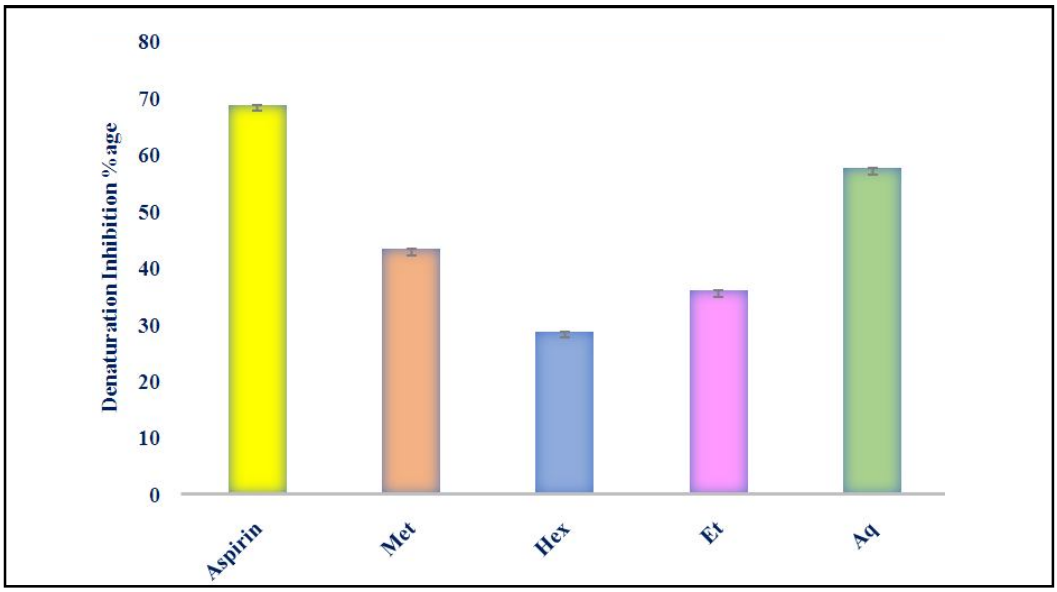

Figure 4: Denaturation inhibition % of different extracts of *N. sativa* seeds. Met: methanolic, Hex: hexane, Et: ethanolic, Aq: aqueous extract. Values are expressed as the mean ± SD (n = 3), $p<0.05$ when compared to aspirin.

3.7 Correlation between total phenolic content and antioxidant assays

The correlation between antioxidant assays and total phenolic content was investigated, and all four antioxidant assays, *i.e.*, DPPH, ABTS, HRSA, and NOS revealed a significant positive relationship, as shown in Figure 5(A-D). According to the results, the extract prepared in a polar solvent (*i.e.*, water) had a high TPC and TFC, as well as antioxidant potential, followed by extracts prepared in somewhat polar (methanol, ethanol) and nonpolar (*i.e.*, hexane). As shown in Table 5, correlation coefficients (r) were calculated to better understand the relationship between antioxidant activity and phenolic content of the plant extracts investigated. The antioxidant capabilities of total phenols were shown to have strong correlations, demonstrating that phenolic compounds contribute to the suppression of oxidative processes.

Table 6: Correlation coefficients between the total phenolic content and antioxidant assays (DPPH, ABTS, HRSA, and NOS Assays) of *N. sativa* seed extracts

	TPC vs DPPH	TPC vs ABTS	TPC vs HRSA	TPC vs NOS
Methanolic extract	0.9279	0.9708	0.9622	0.9518
Hexane extract	0.9675	0.9469	0.9599	0.9195
Ethanolic extract	0.9864	0.9424	0.9277	0.954
Aqueous extract	0.9349	0.9796	0.8025	0.9146

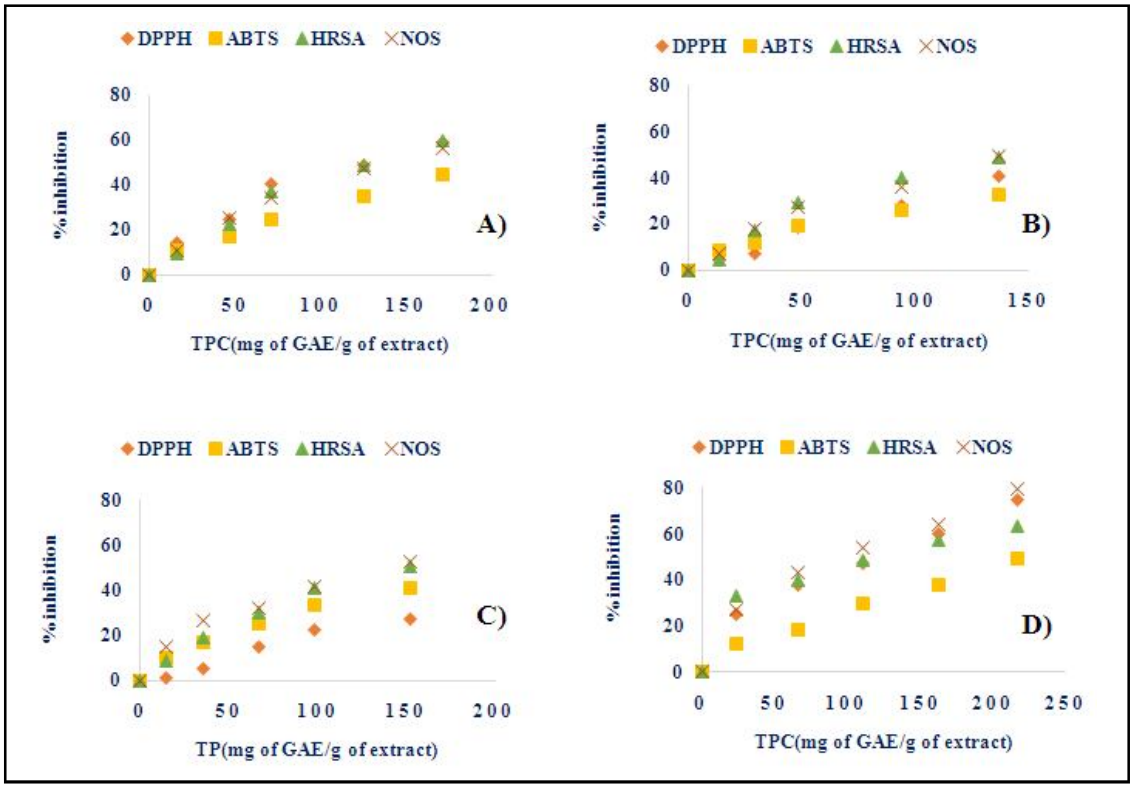

Figure 5: Correlation analysis between the total phenolic content (x-axis) and antioxidant capacities (y-axis) measured by DPPH, ABTS, HRSA and NOS of A) methanolic, B) hexane, C) ethanolic, and D) aqueous extracts.

4. Discussion

Extraction is a vital first step in the study of medicinal plants since it is necessary to extract the appropriate chemical components from plant materials for subsequent examination. The results of this study are similar to the data reported by Khattak *et al.* (2008) in which the extraction yields of hexane, acetone, methanol, and water extracts were 31.9%, 24.6 %, 28.7%, and 25.4%, respectively.

The aqueous extract of the seed contained the most phytochemicals of the four extracts. Phytochemicals have been observed to have a variety of activities that could help avoid chronic diseases. Alkaloids, for example, can help to prevent chronic diseases. Saponins have antimicrobial and antihyperlipidemic properties. Analgesic effects are seen in both steroids and triterpenoids. The central nervous system's actions are controlled by steroid and saponin hormones (Matsuura and Fett-Neto, 2015).

The variations in TPC results between the current and prior research could be attributable to differences in the extraction solvent, plant component employed, analysis method, environmental stress, and climatic and geographical variables (Iloki-Assanga *et al.*, 2015). According to Dalli *et al.* (2021), the aqueous extract has the most polyphenols (51.63 ± 1.95) mg GAE/mg DW, followed by methanolic, and ethanolic extract has the lowest value (5.47 ± 0.49 mg GAE/mg DW). In another study, the total polyphenol content of the methanolic extract was 1.3714 ± 0.0315 mg GAE/g DW, which is higher than the level of the acetone extract (0.5962 ± 0.0046 mg GAE/g DW) (Mechraoui *et al.*, 2018). Flavonoids were found in all four extracts, but the aqueous extract had the highest level of flavonoids. According to a previous study, the chloroform extract of *N. sativa* seeds has the highest amount of flavonoid content, *i.e.*, 6.93 ± 0.28, followed by ethyl acetate fraction (5.96 ± 0.25), aqueous extract (3.17 ± 0.1) and hexane extract (0.49 ± 0.07) mg QE/g DW, respectively (Boudiaf *et al.*, 2010). When compared to acarbose, the aqueous extract had a significantly good α-amylase inhibitory action as compared to other extracts.

The scavenging effects of methanol, ethanol and aqueous extracts using different methods like DPPH, ABTS, HRSA, and NOS were astonishingly good; however, the antioxidant activity of hexane extract was poor. In a study by Goga and his colleagues (2012), samples' ability to decrease stable DPPH radical was 3.01 ± 0.03 mg/ml for ethanolic extract and 8.177 ± 0.11 mg/ml for hexane extract. As per the study of Fatima *et al.* (2018), the action of the oil and capsules made using *N. sativa* seeds was higher, with a radical inhibition of 16% and 17%, and was lower in methanolic and aqueous extracts of *N. sativa* seeds (12% and 7%), respectively. *N. sativa* extracts significantly inhibited nitric oxide in a dose-dependent manner. DNA damage has been noticed in NO. High levels of NO have been demonstrated to cause deamination of deoxynucleotides as well as bases in intact DNA *in vitro* and to be mutagenic *in vivo* (Madabhushi *et al.*, 2014). Hence, the high NO scavenging capacity of *N. sativa* may aid in stopping the chain of reactions that are harmful to human health. And, it has been observed that plant extracts prevented this strand scission in pBR3222 DNA.

The greatest activity for inhibiting albumin denaturation was determined to be in the order of aspirin, followed by aqueous, methanolic (43.30 ± 0.009%), ethanolic (35.98 ± 0.011%) and hexane extract (28.68 ± 0.010%), respectively. Phytochemicals obtained from herbal plants, such as flavonoids and phenolic compounds, are the primary source of antioxidative property (Li *et al.*, 2014) and hence operate as an anti-inflammatory moderator. Inflammation can be reduced by the creation of pro-inflammatory chemicals such as TNF-α, IL-6, *etc.* These anti-inflammatory chemicals react with free radicals, which may cause irreversible damage to cell membranes, resulting in tissue damage and even cell death (Zhang and An, 2007).

The correlation between antioxidant assays and total phenolic content was investigated, and all four antioxidant assays revealed a significant positive relationship. A recent study found that the presence of phenolics, carotenoids, and flavonoids in medicinal plants resulted in substantial overall antioxidant activity in some bioactive components (Dai and Mumper, 2010). Our findings are consistent with those of Rajurkar and Hande (2011), who determined a substantial relationship between total phenolic content and antioxidant assays.

5. Conclusion

The presence of various phytochemicals indicates that this herb has a wide range of biological capabilities. The aqueous fraction of *N. sativa* seeds is high in antioxidative and anti-inflammatory bioactive components, allowing it to be developed as a diabetes nutraceutical. The study demonstrated that *N. sativa* can be employed to prevent and manage free radical-induced oxidative stress. Their anti-inflammatory properties could be attributed to the antioxidants found in the plants. The correlation results show that phenolic components possess a substantial impact on the plants' antioxidant effects. For future herbal medication formulations, more research is needed to elucidate the specific antioxidants involved in these activities and their modes of action.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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