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IN-VITRO ASSESSMENT OF ANTIBACTERIAL, ANTIOXIDANT, ANTIFUNGAL, AND ANTI-INFLAMMATORY ACTIVITIES OF CRUDE FRUIT EXTRACT OF *NIGELLA SATIVA*

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Abstract

While previous studies have demonstrated individual bioactivities of Nigella sativa extracts, this is the first comprehensive evaluation of antibacterial, antioxidant, antifungal, and anti-inflammatory properties using crude seed extract under standardized conditions. Using the agar disc diffusion and microdilution techniques, the extract was examined against one Gram-positive (Staphylococcus aureus) and four Gram-negative (Pseudomonas aeruginosa, Enterobacter aerogenes, Klebsiella pneumoniae, and Acinetobacter lwoffii) bacteria. K. pneumoniae had the highest Minimum Inhibitory Concentration MIC value, and P. aeruginosa was the most inhibited on the highest scale. By using the paired diene technique, it was shown that black seed has antioxidant activity (0.02 mg/mL) that was equivalent to that of ascorbic acid (1.00 mg/mL), butylated hydroxyanisole (0.06 mg/mL), and vitamin E (0.04 mg/mL). Aspergillus niger and Candida albicans were among the fungi against which the fruit extract of N. sativa was evaluated for antifungal activity; the most effective concentration was 300 µg/mL. At 100, 200, and 500 µg/mL, respectively, Nigella sativa extract exhibited anti-inflammatory action with percentages of 36.12, 59.89, and 88.33%. Due to its antibacterial, antioxidant, antifungal, and anti-inflammatory qualities, Nigella sativa can be used as an antibacterial and antifungal to treat illnesses and as a preventative measure against conditions linked to inflammation and oxidative stress.

Keywords: *Antibacterial, Antioxidant, Anti-fungal, Anti-inflammatory, Black seed, Nigella sativa*

INTRODUCTION

Plants play a vital role in sustaining human life by providing essential food sources and a wide range of bioactive compounds. For centuries, they have also served as therapeutic agents, making medicinal and aromatic plants particularly valuable both culturally and economically (1). With growing concerns over the limitations and adverse side effects of synthetic pharmaceuticals, coupled with the environmental burden of chemical production, there has been a noticeable global shift toward natural medicines and herbal remedies as safer and more sustainable alternatives (2). In diverse experimental models and bioassay systems, plant-derived compounds have shown remarkable biological activities, including antibacterial, antioxidant, antifungal, anticancer, and anti-inflammatory effects, which are highly relevant to the prevention and treatment of human diseases (3).

Nigella sativa L. (commonly known as black cumin or black seed) is a well-recognized medicinal plant belonging to the Ranunculaceae family. Traditionally, its seeds, fruits, and leaves have been utilized in folk medicine across the Middle East, South Asia, and parts of Africa for the treatment of a variety of ailments such as asthma, gastrointestinal disorders, infections, and inflammation (4). The therapeutic potential of *N. sativa* is largely attributed to its diverse phytochemical profile, which includes sterols, fatty

acids, alkaloids, essential oils, flavonoid derivatives, and thymoquinone—the latter being the most extensively studied bioactive constituent due to its strong antioxidant and anti-inflammatory effects (5, 6).

In recent decades, scientific interest in N. sativa has intensified, with numerous studies confirming its broad pharmacological properties. Extracts of this plant have been reported to exert significant antimicrobial activity against both Gram-positive and Gram-negative bacteria, thereby offering potential solutions to the rising problem of multidrug resistance (7). Similarly, the antioxidant potential of *N. sativa* has been linked to its ability to scavenge free radicals and reduce oxidative stress, processes that underlie many chronic conditions including cardiovascular and neurodegenerative diseases (8). Its antifungal activity has also been demonstrated against pathogenic fungi such as *Candida albicans* and *Aspergillus niger*, organisms commonly associated with opportunistic infections in immunocompromised individuals (9). Furthermore, the anti-inflammatory actions of *N. sativa* extracts have been validated in both in vitro and in vivo models, suggesting potential utility in managing inflammatory disorders and related pathologies (10).

Given these promising findings, the current study was designed to provide a comprehensive in vitro assessment of the antibacterial, antioxidant, antifungal, and anti-inflammatory activities of crude fruit extract of *Nigella sativa*. Specifically, the extract was evaluated against four Gram-negative and one Gram-positive bacterial species, two clinically relevant fungi, and in anti-inflammatory assays at varying concentrations. By elucidating the multifaceted bioactivities of *N. sativa* fruit extract, this study aims to contribute to the growing body of evidence supporting its therapeutic relevance and potential applications in modern medicine.

MATERIALS AND METHODS

PREPARATION OF *NIGELLA SATIVA* PLANT EXTRACTS

Fruits of the *N. sativa* plant (Fig. 1) were fresh collected from local Apothecary in Bannu and were recognized by a botanist using taxonomy. After being cleaned three times with distilled water, the fruits were roasted for 48 hours at 75 °C. 20 g of powdered fruit was dissolved after the mechanically ground dried fruits were combined with 85% methanol in a shaking water bath (50 rpm) for 24 times under 200 mL of 85% methanol for 24 hours at room temperature. Following filtering using Whatman No. 1 filter paper to remove solvent from the extract, the filtrate was concentrated using a rotary evaporator (Laborota 4000, Heidolph, Germany) at 45 °C for 35 minutes. The extract, which precipitated as a solid, was stored at 4 °C for further analysis.

Fig. 1. *Nigella Sativa* plant used for extract analyses in this study

MICROORGANISMS

The Microbiology Laboratory in University of Science and Technology, Bannu, provided the microorganisms culture utilized in this investigation. With their written consent, Clinical patients provided these microorganisms. One Gram positive *Staphylococcus aureus* bacterium and four Gram negative *Pseudomonas aeruginosa, Enterobacter aerogenes, Klebsiella pneumoniae,* and *Acinetobacter lwoffii* bacteria were cultured on nutrient agar slants at 37 °C and maintained at -80 °C.

ANTIBIOTIC DISCS AND MICRODILUTION ASSAY

Using a cell culture for each microbe, the disc diffusion technique (5) was used to assess antimicrobial activity. 50 µL of each suspension was spread out on Mueller-Hinton agar plates after its concentration was measured using a 0.5 McFarland standard. Additionally, sterile blank discs (6 mm dia) were pipetted with 50 µL of diluted fruit extract (1 g /5 mL distilled water) and left to air dry in a laminar flow hood under sterile conditions. Tiny plaques formed on the inoculation plates, indicating the bacterial development. With the exception of the disc diameter, inhibition zone diameters (in mm) were noted (6). University of Science and Technology, Bannu, graciously supplied positive controls (Cephalosporin discs). As an inhibitor of cell wall formation, cephalosporins have also been shown to cause resistance in bacteria such as klebsiella species, proteus species, and pseudomonas species (7).

According to the Clinical and Laboratory Standards Institute's methodology, the plant extract's minimum inhibitory concentration (MIC) against each microbial strain was determined using a microdilution assay in 96-well microtiter plates (CLSI, 2021). Every test was conducted in Mueller-Hinton Broth (MHB). To get a final concentration of 10 mg/ml, the plant extract was extracted into 5% dimethyl sulfoxide (DMSO). Each strain was tested using serial dilutions of the extract ranging from 512.0 to 0.06 µg/mL. Standardized microbial concentrations of 10^6 CFU/mL/well were utilized, and overnight cultures were utilized. At 37°C, incubation lasted for 24 hours. The following MIC values were used to categorize antimicrobial activity: MIC > 1,000 µg/mL = no activity; 512 to 1,000 µg/mL = mild; 128 to 512 µg/mL = moderate; 32 to 128 µg/mL = excellent; 10–32 µg/mL = strong; MIC < 10 µg/mL = extremely strong (CLSI, 2021). The trials were duplicated, and average data were reported.

ANTIOXIDANT ACTIVITY OF FRUITS

The paired diene approach, which is based on assessing an extract's capacity to prevent linoleic acid peroxidation by stabilizing its double bond into a conjugated diene, was employed to ascertain the antioxidant activity of *N. sativa* fruit extracts (8). To create an emulsion, 100 µL of each extract sample (0.01–30 mg/mL in methanol) was combined with 3 mL of 10 mM linoleic acid (Sigma Chemical Co., St. Louis, MO, USA) in 0.2 mM sodium phosphate buffer (pH 6.6). The tubes were then incubated in the dark at 37 °C for 15 hours to cause oxidation. Following incubation, 7 mL of 65% methanol in deionized water was added, and a Hitachi U-2001 spectrophotometer (Tokyo, Japan) was used to measure each mixture's absorbance at 234 nm. The following formula was used to evaluate antioxidant activity:
Antioxidant activity (%) = [(DA234 of control – DA234 of sample/ DA234 of control] × 100.

All measurements were done 3 times. As the standard antioxidant control, α-tocopherol, butylated hydroxyanisole (BHA) and ascorbic acid (Sigma) were used. Results were presented using EC_{50} values (mg/mL), which are the concentrations required to produce 50% antioxidant activity, once determined by linear regression analysis (9).

ANTIFUNGAL ACTIVITY

Nigella sativa plant extracts were investigated for their antifungal qualities against *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 9142). Using absorbance at 530 nm, fungal strains were cultivated at 37 °C for 14–24 hours, and cell densities were normalized to 0.5 McFarland (≈10^8 CFU/mL). The disc diffusion technique was used to test for antifungals (10). To cut to the chase, nutrient agar plates (Merck, Germany) were infected with 100 µL of each fungal solution, which constituted 10^8 CFU/mL. Sterile discs measuring six millimeters in diameter were immersed in 10 µL of extract at several concentrations (50, 100, 150, 200, 250, and 300 µg/mL) and then cut in half before being applied to the infected plates. After 24 hours of culture at 37 °C, the zones of inhibition (in mm) were measured on the plates. The negative control was 2 µL of 5% DMSO (v/v), while the positive control contained ketoconazole (50 µg/disc).

Using extract dilutions ranging from 512 to 0.05 µg/mL, minimum inhibitory concentration (MIC) values were determined using a broth microdilution technique in accordance with Clinical and

Laboratory Standards Institute recommendations (CLSI, 2023). Fungal suspensions were prepared and standardized to 0.5 Mcfarland (570 nm; 10^8CFU/mL) in Luria–Bertani medium. A 96-well microtiter plate was aspirated with 100 µL of fungal suspension and extract, and it was then incubated for 24 hours at 37°C. The growth controls were fungal cultures without extract, while the negative control was a media devoid of fungus. MICs were defined as the lowest concentration of an extracted sample that, when compared to the control, visibly reduced fungal growth (11).

ANTI-INFLAMMATORY ACTIVITY

The protein denaturation method, which evaluates the extract's ability to prevent heat-induced denaturation of proteins—a process linked to the inflammatory response—was used to ascertain the anti-inflammatory efficacy of *Nigella sativa* plant extract (12). Three milliliters of phosphate-buffered saline (pH 6.5), two milliliters of egg albumin, and one milliliter of N. sativa extract at varying concentrations—100, 200, and 500 µg/mL in distilled water—were all included in the reaction mixture. Protein denaturation was caused by incubating the mixture for 15 minutes at 25°C and then for 12 minutes in a water bath at 65°C. After cooling, absorbance was measured using a Shimadzu A160 spectrofluorometer (Shimadzu, Japan) at 660 nm. The blank was double-distilled water. The following formula was used to determine the percentage of protein denaturation inhibition:

Inhibition (%) = [(Absorbance of control – Absorbance of sample) / Absorbance of control] x 100.

[As – Ac/Ac]×100 is the percentage inhibition, where "As" and "Ac" stand for sample absorption and control absorption, respectively (13).

Using the same quantities of *N. sativa* plant methanol extracts (100, 200, and 500 lg/mL) diluted in distilled water, the assay's reference drug was the potent non-steroidal anti-inflammatory medication diclofenac sodium (14).

STATISTICAL ANALYSIS

The extract's antibacterial, antioxidant, antifungal, and anti-inflammatory properties were prepared for many testing. first accessible at Assigncode. Using SPSS v. 11.5 (IBM SPSS, New York, USA), analysis of variance was applied to the data using a totally randomized design in order to determine the least significant difference (LSD) at $P < 0.05$.

RESULTS AND DISCUSSION

ANTIBACTERIAL ACTIVITY

It was observed that the fruits of *N. sativa* showed varying degrees of inhibition against each of the tested microorganisms (Table I). The inhibitory zones of *A. lwoffii, E. aerogenes, K. pneumonia, S. aureus, and P. aeruginosa* had respective radius of 7, 18, 14, 16, and 25 mm, indicating that the *N. sativa* plant extract had the greatest impact on *P. aeruginosa* and the least effect on *A. lwoffii.*

Cephalosporin, the control medication, did not affect *P. aeruginosa or A. lwoffii*, whereas *N. sativa plant* extract affects (Table I). The corresponding minimum inhibitory concentrations (MIC) for *A. lwoffii, E. aerogenes, K. pneumonia, S. aureus*, and *P. aeruginosa* were 138, 40, 180, 95, and 25 lg/mL. Because the minimum and highest MIC values matched those of *P. aeruginosa and A. lwoffii*, respectively, this finding validated the disc diffusion data (Fig. 2). Values are mean of 350 ±SE of three replicates. Means with different letters within a column are significantly different ($P < 0.05$; LSD).

The health of humans is seriously threatened by microorganisms that are resistant to antibiotics. *Staphylococcus aureus, Acinetobacter lwoffii, Enterobacter aerogenes, Klebsiella pneumoniae, and Pseudomonas aeruginosa* are some of the more concerning species of these antibiotic-resistant bacteria (15) Gram-positive and very versatile, *Staphylococcus aureus* is a serious concern in hospital and community-acquired infectiousness. Skin infections, food poisoning, toxic-shock syndrome, endocarditis, pneumonia, bacteremia, and osteomyelitis are only a few of the illnesses it causes (16). Because it may remain latent in a host for prolonged periods of time without endangering healthy people, *A. lwoffii* is linked to hospital environments. In immunocompromised individuals, *Enterobacter aerogenes* mostly causes opportunistic

infections; nevertheless, new research has raised concerns about its community infections (17). One of the more frequent causes of hospital-acquired pneumonia, particularly in patients with weakened immune systems, is *Klebsiella pneumoniae*, which is also linked to a disproportionately high rate of hospital-acquired infections, including pneumonia, urinary tract infections, surgical wound infections, and biliary tract infections (18). One of the most significant Gram-negative pathogens that infects immunocompromised individuals, especially those who stay in hospitals for an extended period of time, is *P. aeruginosa* (19).

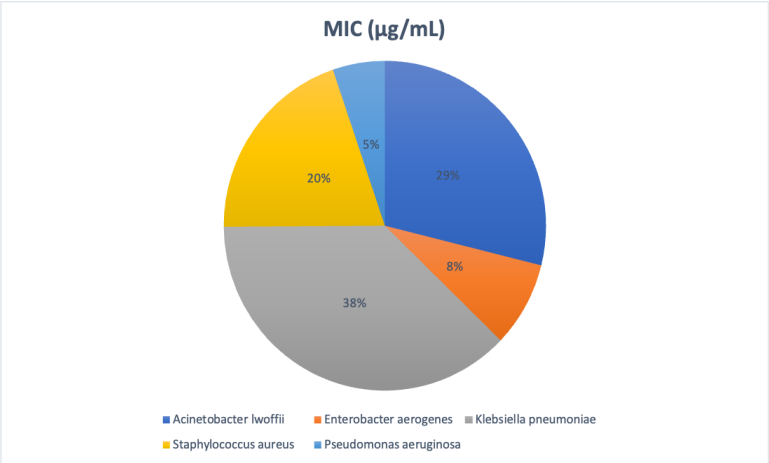

Fig. 2. MIC (Minimum Inhibitory Concentration) values of *Nigella sativa* methanolic plant extract against five bacteria

The discovery of plant-based natural medicines to combat germs has drawn increased interest. 40 of the 45 Indian medicinal plants that were studied by (20) in the form of ethanolic extracts had antibacterial efficacy against Candida albicans and one or more drug-resistant pathogens. Remarkably, eleven of the plants including Terminalia belerica, Punica granatum, and Eucalyptus sp showed more extensive antibacterial activity (21). The antimicrobial activity of medicinal plants from Southern Africa against diseases were found relevant to dermatology (22). They found that plants like Eucalyptus camaldulensis and Diospyros mespiliformis had high antibacterial activity. Another study found that the essential oils of Sinapis arvensis have antibacterial efficacy against *P. aeruginosa*, *S. aureus*, and MDR bacteria (23). Recent research on the plant *Nigella sativa* revealed the antibacterial properties of secondary metabolites, including fatty acids, sterols, alkaloids, and flavonoids (24).

Table I. Mean Diameters of Inhibition zone of *Nigella Sativa* Methanol Pant Extract and two positive controls

Bacteria	Fruit Extract	Cephalosporin
Acinetobacter lwoffii	7 ± 0.05	0 ± 0.00
Enterobacter aerogenes	18 ± 0.17	5 ± 0.57
Klebsiella pneumoniae	14 ± 0.08	0 ± 0.00
Staphylococcus aureus	16 ± 1	4 ± 0.21
Pseudomonas aeruginosa	25 ± 0.57	0 ± 0.00

*Values are mean of ± SE of three replicates.
Means with *different letters* within a column are significantly different ($P<0.05$; LSD)

ANTIOXIDANT ACTIVITY

A summary of the extract's antioxidant activity test findings is provided in Table II. The extract's EC50 values are inversely correlated with the effectiveness of its antioxidant activity. According to Table III, the antioxidant activity, or EC50 values, for a-tocopherol, butylated hydroxyanisole (BHA), ascorbic acid, and *N. sativa* plants were 0.04, 0.06, 1.00, and 0.02 mg/mL, respectively. The fruit extract only differed substantially from ascorbic acid ($P < 0.05$) but not from a-tocopherol ($P < 0.05$) or BHA ($P < 0.05$)

The antioxidant and free radical-scavenging properties of different portions of *Nigella Sativa* plants collected in Zabul from the Iranian provinces of Sistan and Balochistan were assessed in a study (18). When compared to aqueous and chloroform extracts, they found that the methanolic extracts had the strongest antioxidant and free radical scavenging properties. Fruits were the first to exhibit these processes, followed by leaves and roots. The methanolic, aqueous, and chloroform extracts had scavenging potencies of 86%, 70%, and 57%, respectively. In a related study, antioxidant activity of *N.*

sativa fruits were measured that were collected from Gonabad, Razavi Khorasan Province, Iran. It was found that the antioxidant activity was unaffected by the fruits' geographic location, suggesting that *N. sativa* can be a great source of bioactive phytochemicals and antioxidants (20).

Several conventional assays have been employed using dichloromethane, ethanol, and methanol extracts of root bark and leaves from various Korean medicinal plants to assess their free radical scavenging potential and antioxidant activity. (22). Using catechin, morin, naringenin, quercetin, and rutin as standards, the study demonstrated the antioxidant power of these flavonoids. The plant extracts that showed the greatest antioxidant activity were the leaves of Saururus chinensis and the root bark of *Morus alba* L. A 2007 study compared various plant parts from the Hamadan region of western Iran that were grown under similar natural conditions, including the leaves of *Lavandula officinalis* L., roots of *Verbena officinalis* L., flowers of *Calylophus lavandulifolius*, leaves of *Melissa officinalis* L., and flowers of *Althea kurdica*. The results showed significant variation in antioxidant activity, with the leaves of *M. officinalis* exhibiting higher antioxidant content than those of *L. officinalis*.

The free radical-scavenging properties of methanolic extracts of immature and mature leaves were investigated from nine coastal plants throughout Maharashtra, India's west coast (25). The young leaves of *Hibiscus tiliaceus* L. had the highest antioxidant activity (76%) followed by those of *Syzygium jambos* (71%), *Calophyllum inophyllum* L. (68%), and *Colubrina asiatica* (55%). These species exhibited significant antioxidant potential due to their high quantities of flavonoids (6.03–16.63 mg/g of dry weight) and total polyphenols (12.12–26.23 mg/g of dry weight).

These researchs' findings show that the plants have significant antioxidant capability and the ability to scavenge free radicals. An essential component of this function, phenolic chemicals found in plants may aid in shielding cells from oxidative damage. This damage, which is brought on by free radicals and other reactive oxygen species, has been linked to Parkinson's disease, dementia, arthritis, asthma, and cancer (26).

Table II. EC_{50} values (mg/mL) of the *Nigella Sativa* methanolic fruit extract in two assays

Compounds	Antioxidant activity
Fruit extract	0.02 ± 0.00 b
α-Tocopherol	0.04 ± 0.00 b
BHA	0.06 ± 0.02 b
Ascorbic acid	1.00 ± 0.00 a
Fruit extract	0.02 ± 0.00 b

Values are mean of ±SE of three replicates.
Whereas, the means with different letters in a column are highly significant ($P < 0.05$; LSD)

ANTIFUNGAL ACTIVITY

Table III displays the antifungal activity of N. sativa plant extract against two fungus strains. Inhibition zone concentrations in *A. niger* were 2.1, 5.6, 9.2, 14.5, 15.7, and 18.2 mm per 50, 100, 150, 200, 250, and 300 lg/mL of fruit extract. Following treatment with 50, 100, 150, 200, 250, and 300 lg/mL of fruit extract, the corresponding inhibition zones of Candida albicans were 1, 2.5, 6.3, 13.8, 15.1, and 17.9 mm. The inhibition zones for *A. niger* and *C. albicans* were 1.0 mm or 14.7 mm for DMSO (negative control) and 0.5 mm 21.1 mm for ketoconazole (positive control). *A. niger* and *Candida albicans* had respective MICs of 284 lg/mL and 342 lg/mL.

Antifungal agent resistance has increased significantly during the last ten years, with significant ramifications for healthcare, human morbidity, and death. In order to treat severe fungal infections, new, safer, and more effective medications must be introduced as antifungal resistance develops. In the search for new antifungal medicines, medicinal plants have emerged as promising agents. Secondary metabolites such as phenols, flavonoids, coumarins, quinones, saponins, xanthones, alkaloids, lectins, polypeptides, terpenoids, and essential oils from natural sources have been the subject of several research in recent years about their antifungal potential (27). According to certain earlier research, Zygophyllaceae plant species include bioactive substances such alkaloids and flavonoid derivatives that have been used for their antifungal properties (28).

Table III. Anti-fungal activity of *Nigella Sativa* plant extract against two fungal strains

Fruit extract (lg/mL)	*Aspergillus niger*	*Candida albicans*
50	2.1 ± 0.0	1.0 ± 0.0
100	5.6 ± 0.0	2.5 ± 0.2
150	9.2 ± 0.1	6.3 ± 0.0
200	14.5 ± 0.0	13.8 ± 0.0
250	15.7 ± 0.0	15.1 ± 0.0
300	18.2 ± 0.7	17.9 ± 0.0
DMSO (negative control)	1.0 ± 0.0	0.5 ± 0.0
Ketoconazole (positive control)	14.7 ± 0.0	21.1 ± 0.0
MIC	284.5 ± 0.3	342 ± 0.0

*Data presented as mean ± SE of inhibition zone diameter (mm) for various concentration of extract, controls and minimum inhibitory concentration (MIC) (lg/mL), DMSO Dimethyl sulfoxide

ANTI-INFLAMMATORY ACTIVITY

The anti-inflammatory test values for 100, 200, and 500 lg/mL of *N. sativa* plant extract, which are 36.12, 59.89, and 88.33%, respectively has been depicted in Fig. 3. The anti-inflammatory percentage of diclofenac (control) at 100, 200, and 500 lg/mL was 90.31, 97.33, and 109.53%, respectively. The degree to which *N. sativa* plant extracts inhibited protein denaturation varied considerably across all treatments ($P < 0.05$).

Redness, fever, edema, loss of function, and discomfort are pathophysiological processes that clinically suggest inflammation (31). Flavonoids possess a wide range of biological and pharmacological properties, including antibacterial, anti-inflammatory, immunomodulatory, antiviral, anticancer, and antithrombotic effects (29). Flavonoids have been the focus of several investigations and have shown anti-inflammatory effects in a range of animal models of inflammation. These substances may have organ-specific effects on mast cells, neutrophils, lymphocytes, macrophages, inflammatory-related cells, and cellular processes. Some flavonoids, for instance, hinder the growth of T cells, while others prevent mast cells from releasing histamine (30).

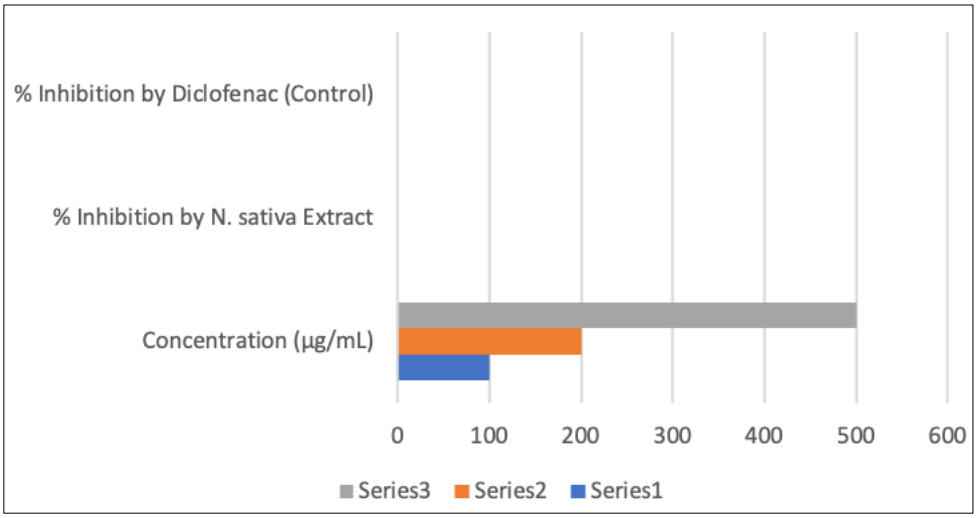

Fig. 3. Anti-inflammatory effect of *Nigella Sativa* fruit extract

CONCLUSION

With its antibacterial, antioxidant, antifungal, and anti-inflammatory properties, *Nigella Sativa* plants show promise as novel medications. However, for the plant to be used as an herbal drug, the organic compounds and active agents in the fruits must be identified. Additionally, the toxicity of the active ingredients, serum-attainable levels, pharmacokinetic characteristics, side effects, and diffusion in various body sites must be established. Food pathogen development was inhibited and lowered by *Nigella Sativa* plants. This plant may thus be used to prolong food shelf life and lessen food poisoning.

Conflict of interest:

The authors declare no conflict of interest.

Authors' contribution:

MZ, DF & AZ Conceived, prepared original draft and architected the research design; SR & RN Executed the experimental protocols, curated data and performed statistical analyses; SZ, SAK, MYA & AK Provided technical support and data analysis.

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