Original Article

ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF THE ETHANOL LEAF EXTRACT FROM MEDICAGO SATIVA

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Abstract

Context: *Medicago sativa* is a valuable plant for oxidative and biological research because of its ethno-medical benefits. This study aimed to assess the antimicrobial and antioxidant properties of the plant

Materials and Methods: Cefoxitin was compared to the antibacterial properties of the leaves against clinical strains of *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes, Staphylococcus aureus, and Proteus mirabilis.* M. sativa was tested for its ability to scavenge free radicals using the DPPH Spectrophotometric technique. After testing various extract concentrations, the percentage of inhibition was computed. The potential to scavenge nitric oxide was ascertained by exposing sodium nitroprusside to varying amounts of *M. sativa* extract. Additionally, the ethanol extract's phytochemical analysis was tested.

Results: When tested against harmful bacteria, the ethanol leaf extract of *M. sativa* showed antibacterial action. The outcomes showed that different concentrations had variable free radical scavenging activity levels. The qualitative

phytochemical analysis showed the presence of alkaloids, glycosides, flavonoids, saponins, reducing sugars, tannins, steroids, and terpenoids.

Conclusion

Results indicate that *M. sativa* leaf extract in ethanol has strong antibacterial properties, supporting the plant's traditional uses throughout Africa. The leaf's capacity to scavenge free radicals in DPPH and nitric oxide tests demonstrated its strong antioxidant capability.

Keywords: *Medicago sativa*; Leaf extract; antimicrobial activity; Antioxidant potential. Phytoconstituents'

Introduction

The majority of people on the planet rely on traditional medicine, which uses herbs to cure a variety of illnesses. Herbal traditions served as a source for modern medicine [1]. The use of plants or other natural substances to treat a wide range of illnesses has been made possible by herbal medicine, a respectable kind of alternative medicine that has become increasingly popular in both wealthy and developing countries [2,3]. Because the secondary metabolites of plant extracts arise as complexes of naturally occurring, structurally related analogues, their use is less harmful to human health and the environment [4]. Years of fighting diseases with crippling effects have led to an awareness of the medicinal potential of plants. This has prompted the search for drugs from unusual sources, particularly natural products from plant parts like bark, seeds, fruits, roots, leaves, and wood [5]. Furthermore, utilizing herbal medicines in folk medicine offers an intriguing and mainly unexplored avenue for developing novel potential pharmacological effects [6]. Modern science has recognized their potency and included a variety of medications derived from plants in contemporary pharmacology. Numerous investigations have been conducted to substantiate the application of these herbs against pathogenic microorganisms and illnesses associated with oxidative stress.

It is believed that phytoconstituents are good alternatives to conventional drugs used in the treatment of diseases because they are inexpensive and may have less adverse effects [6] Despite the growing concern about these plant products due to their lack of standardization, they are in high demand by traditional care givers for the treatment of various diseases including cancer. Thus, there are growing research interests geared towards natural products for finding new drug entities from among

the myriads of plant species in African forests that are largely untapped. These efforts have often been successful since many plant materials have been found to have numerous and diverse pharmacological activities. *Medicago sativa* is one such plant that may be a source of antimicrobial and as well antioxidant agents.

Medicago sativa, or alfalfa, has drawn the most interest because of its rich phytochemical profile, historical usage in traditional medicine, and possible therapeutic benefits. *Medicago sativa* is a widely grown forage crop that has been used traditionally in folk medicine to treat a variety of illnesses and provide cattle with an excellent source of nutrients.

The Fabaceae family includes the perennial flowering plant *Medicago sativa*. It has long been used for its therapeutic qualities to cure a wide range of illnesses. It has been used to treat anxiolytic disorders [7] and some liver disorders [8]. Additionally, medicinal sativa has been utilized as an antihyperlipidemic [9], an antidiarrheal drug [10], a treatment for neurological illnesses [11], and as a remedy for diabetes mellitus [12].

Thus, the aim of the present study was to investigate the antimicrobial and antioxidant activities of *Medicago sativa* leaf extract. It is expected that the results established from this study will provide valuable insights into the pharmacological potential of *M. sativa* therefore, validating and valorizing the traditional uses of this tropical plant.

Materials and methods

Ethical considerations

The Animal Care and Use Research Ethics Committee (ACUREC) of Bayero University Kano, granted approval for the study (BUK/ACUREC/CAP/PG23, dated 10th of April 2023).

Plant Collection and Identification

Freshly picked alfalfa (Medicago sativa) leaves were gathered from farms in the Imo State, Nigeria's Owerri West Local Government Area. A sample specimen MOUAU/ZEB/21/009 was placed in the university herbarium for reference after the plant was recognized and verified by Mr. Ibe Ndukwe, a taxonomist at the Department of Forestry, College of Environmental Sciences, Michael Okpara University of Agriculture, Umudike, Abia State.

Taking the leaves off

After being washed, the recently collected leaves were allowed to air dry for 14 days at room temperature. Afterwards, a Warring commercial blender was used to powder the leaves. The coarse powder of *M. sativa* leaves, weighing eight hundred grams (800 g, was measured using a sensitive digital weighing scale). For 48 hours, the powder was soaked in a flask filled with 2.5 L w/v of 80% ethanol and shaken occasionally. Whatman (No.1) filter paper was used to filter the resulting mixture, and the filtrate was then concentrated using a rotary evaporator and dried on a water bath. Before being used, the greenish semi-solid was placed in a sterile McCartney bottle and refrigerated at 40 ^{o} ^{C}. The lead extract was made by reconstituting it in distilled water until it reached the required concentration.

Screening for phytochemistry

The phytochemical screening of the extract of *M. sativa* leaf was carried out to identify different phytoconstituents such as alkaloids (Hager's and Wagner's tests), tannins (ferric chloride test), saponins (Froth test), terpenoids (Salkowski test), steroids (Liebermann- Burchard test), flavonoids (ammonia and sulfuric acid test), cardiac glycosides (Keller-Killani test), anthraquinones (Borntrager's test), and polyphenol (Folin-Ciocalteu reagent) [13].

Origin of the clinical isolates

Six strains of bacteria were employed: *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes, Staphylococcus aureus, and Proteus mirabilis*. All clinical strains were acquired from the Alex Ekwueme Federal Teaching Hospital's Microbiology Department in Abakaliki, Nigeria. They were grown on a new, suitable agar plate 24 hours before any antimicrobial tests and kept on an agar slant at 8 °C.

Culture medium and susceptibility to antibiotics

Before conducting an antibiotic susceptibility test, discrete colonies of fresh cultures from several bacterial isolates were carefully combined in 5 ml of nutrient broth and cultured for 24 hours at 37 °C. The agar well diffusion method developed by Essiet et al. (2019) was utilized to measure the antibacterial activity of the ethanol leaf extract of M. sativa [14]. A sterile petri plate was filled with 0.5 ml of the isolate broth

cultures, which contained 105 cfu/ml of organism. Then, 15 ml of Muller Hinton agar was added. After being well combined, the substance was given time to solidify. Holes were bored in the plates with a standard sterile cork borer of 8 mm in diameter and the leaf extract was reconstituted in distilled water at different concentrations of 6.25, 12.5, 25, 50 and 100 µg/ml, solvent blank and standard antibiotic (cefoxitin) were applied in each of the wells in the culture plates. The studies were performed in duplicate, and plates were allowed to stand for 2 h for pre-diffusion of the extract and incubated at 37 °C for 24 h

Thereafter, the diameters of the zones of inhibition were measured against the test organisms. Cefoxitin disc (20 µg/ml) was used as a reference standard, while distilled water was used as a control. The growth was compared with the reference and the control.

Determination of minimal inhibitory concentration (MIC).

The ethanol leaf extract of M. sativa was found to have a minimal inhibitory concentration in accordance with the methodology Essien et al. (2017) described [15]. A test tube rack holding six sterile tubes was filled with 0.5 cc of sterile nutritional broth per test tube. To achieve concentrations of 100, 50, 25, 12.5, and 6.25 µg/ml, the extract was first serially diluted. Cefoxitin was used as a positive control at a concentration of 20 µg/ml, while 96 % ethanol was used as negative control.

The diameter of the zones of inhibition was used to calculate the antibacterial activity of all samples

Antioxidant activity

Evaluation of DPPH's Scavenging Activity

After Onoja and Anaga's DPPH Spectrophotometric technique experiment, the free radical activity of *M. sativa* was examined [16]. At varying doses (50, 100, 200, 300, and 400 µg/mL), the test extracts (0.05 ml) and the DPPH methanol solution (200 µm) were added. Ascorbic acid served as the reference standard, and methanol as a control (negative). At 517 nm, the absorbance was measured following a 30-minute incubation. The capability to scavenge the DPPH radical was calculated using the following equation:

DPPH Scavenged (%) = $((A_B - A_A)/A_B) \times 100$;

Where A_B is absorbance of blank at t= 0 min; A_A is absorbance of the antioxidant at t= 30 min

Nitric Oxide Scavenging Radical Determination

With a few minor adjustments, this was carried out using the methodology outlined by Pacher et al.[17]. Using the Griess reaction, nitric oxide was extracted from sodium nitroprusside and quantified. A standard phosphate buffer solution containing 5 mM sodium nitropruside was treated with varying concentrations (50-400 µg/mL) of ethanol extract dissolved in phosphate buffer (0.05 m, PH 7.4), and all tubes were incubated for 5 hours at 25 ºC. Additionally, the buffer used as a control was incubated. After that, 0.5 mL was taken out and diluted using 0.5 mL of Griess reagent, which contains 0.1% napthylethylene diamine dihydrochloride, 1% sulphanilamide, and 2% 0-phosphoric acid. At 546 nm, the absorbance of the chromophore created when nitrite was diazotized with sulphanilamide and then coupled with napthylethylene diamine was measured. Three duplicates of the experiment were conducted.

Examining statistics

The findings were presented as the average ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Tukey's post hoc test were used to assess the significance of differences between the means of the control and treatment groups. When $P < 0.05$, the results were considered significant.

Results

Analysis of phytochemistry

According to the phytochemical investigation results, alkaloids and glycosides were the most common secondary metabolites in the ethanol extract of M. sativa. Flavonoids, saponins, and reducing sugars came next. Trace amounts of terpenoids, steroids, tannins, phlorotannins, and resins were also present. The outcomes also showed that the *M. sativa* ethanol extract lacked anthraquinone.

Antimicrobial activity

The antibacterial activity of *M. sativa* ethanol leaf extract was assessed in order to determine how efficient it was against dangerous bacterial species. The extract showed strong suppression against all tested strains of bacteria. The leaf extract was most efficient against *E. coli, P. aeruginosa, S. aureus, K. pneumonia, E. aerogenes, and P. mirabilis.* At a dosage of 20 µg/ml, cefoxitin totally inhibited all bacterial strains, with the exception of *E. coli*, which only displayed very little inhibition (Table 1). Table 2 presents the

findings of the reported minimal inhibitory concentration (MIC).

DPPH scavenging activity of the extract

Ascorbic acid was employed as a standard antioxidant control, and the leaf extract of *Medicago sativa* demonstrated a significant antioxidant action in a dose-dependent manner.

Nitric Oxide radical scavenging activity

Ethanol leaf extract of *M. sativa* has potent nitric oxide scavenging activity as compared to the standard, vitamin C

Table 1. Antibacterial activity of the ethanol leaf extract of *M. sativa* and Cefoxitin

Organisms	Inhibition zones (mm)	
	Extract	Cefoxitin
Escherichia coli	17	30
Pseudomonas aeruginosa	22	26
Staphylococcus aureus	20	26
Klebsiella pneumonia	20	30
Enterobacter aerogenes	22	28
Proteus mirabilis	20	32

Table 2. Minimum inhibitory concentration (MIC) of *M. sativa* ethanol leaf extract

Organisms'	**Minimum inhibitory concentration (µg/ml)**
Escherichia coli	45
Pseudomonas aeruginosa	25
Staphylococcus aureus	40
Klebsiella pneumonia	30
Enterobacter aerogenes,	35
Proteus mirabilis	36

Table 3: DPPH scavenging effect of ethanol leaf extracts of *M. sativa* at different Concentrations

Concentrations of *M. sativa* and vitamin C (mg/ml)	**% inhibition of DPPH by *M. sativa***	**% inhibition of DPPH *by* Vitamin C**
3	74	75
4	72	74
5	68	70
10	57	62
15	41	57
20	31	54
25	14	45

Results are expressed as mean ± SEM, n= 3 replicate.

Table 4: Nitric oxide scavenging effect of ethanol leaf extracts of *M. sativa* at different Concentrations

Concentrations of *M. sativa* and vitamin C (mg/ml)	% inhibition of NO by *M. sativa*	% inhibition of NO *by* Vitamin C
3	72	83
4	87	91
5	91	95
10	76	82
15	54	62
20	43	58
25	33	55

Results are expressed as mean ± SEM, n= 3 replicate.

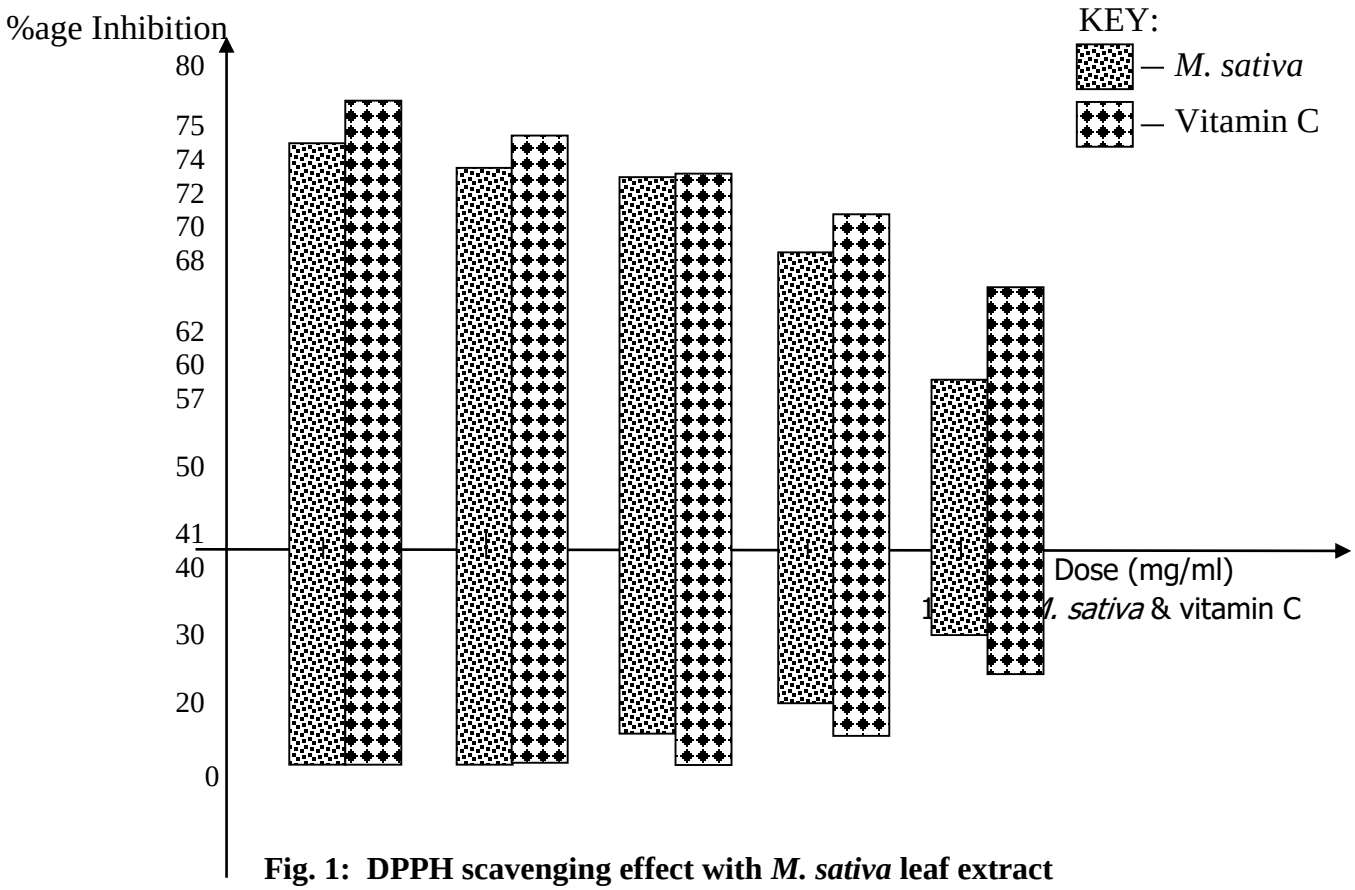

Fig. 1: DPPH scavenging effect with *M. sativa* leaf extract

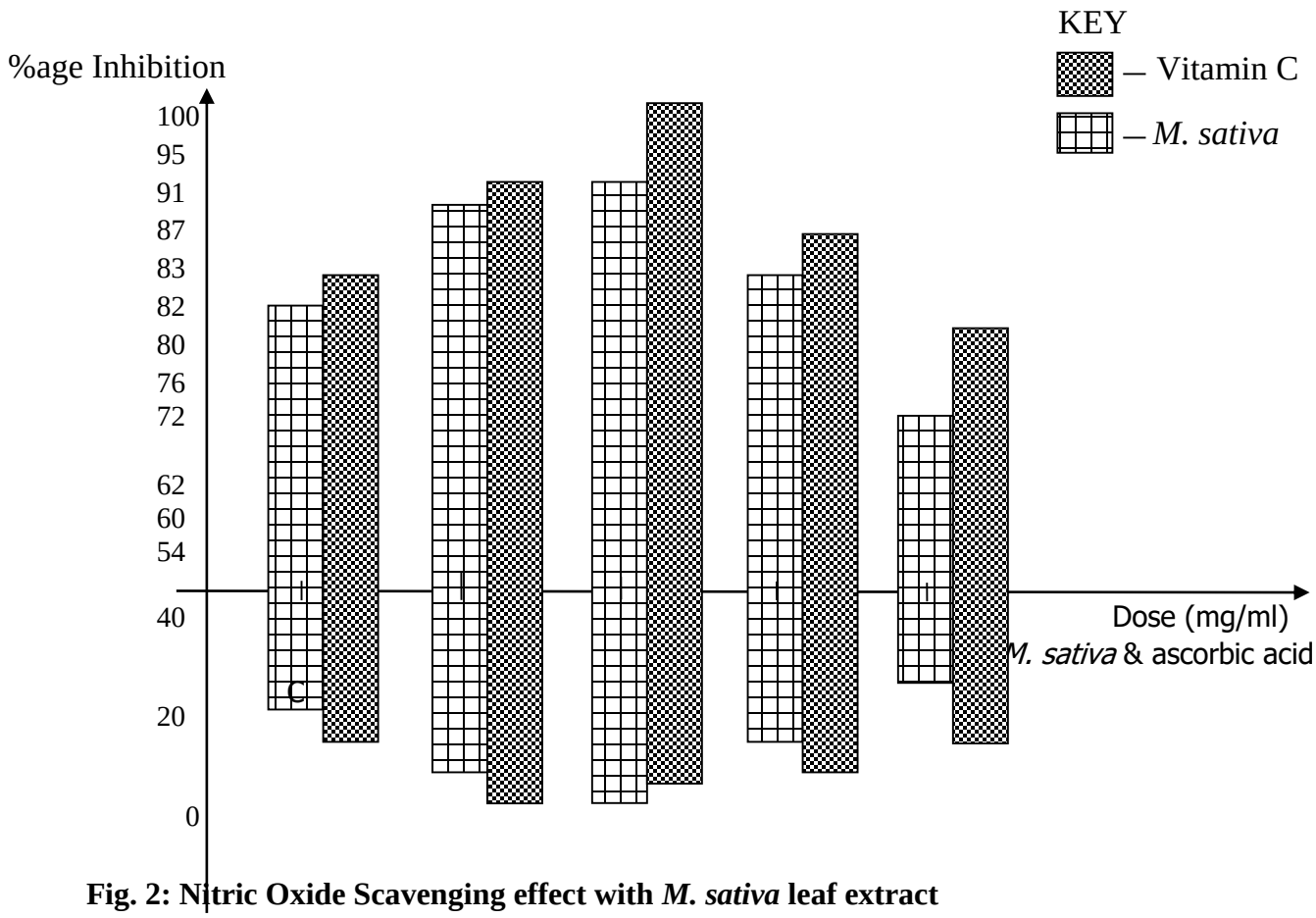

Fig. 2: Nitric Oxide Scavenging effect with *M. sativa* leaf extract

Discussion

Herbal medicines have a lot of promise to offer new therapeutic agents and are becoming increasingly popular in primary healthcare across the globe. However, concerns exist regarding the dearth of scientific data regarding the safety characteristics of most of these products [18]. The chemical components of herbal recipes are responsible for their pharmacological actions and their positive and negative effects [19]. There are several ways to extract the components of every medicinal plant. A phytochemical examination of the M. sativa ethanol leaf extract showed that alkaloids, saponins, tannins, polyphenols, flavonoids,

steroids, glycosides, and anthraquinones were present in varying amounts. First, screening medicinal plants for secondary metabolites aids in identifying bioactive molecules that can potentially lead to both beneficial and negative outcomes, including medication discovery [20]. These biochemicals could be the cause of the impacts this study saw.

Medicinal plant reports are increasing; the leaf extract showed potent antibacterial activity from bioactive compounds. [21]. Plant compounds like terpenoids, alkaloids, and phenolics disrupt bacterial membranes by targeting enzymes. [21]. Numerous studies have examined the effectiveness of plant products worldwide and the reports of medicinal plants' antimicrobial qualities are growing [4, 21, 22]. The leaf extract had strong activity against the pathogenic bacteria under investigation in this work, indicating the presence of bioactive chemicals that may operate as an antibacterial agent or as a lead compound in manufacturing an antibacterial agent. Many people believe that the antibacterial components of plant products, such as terpenoids, alkaloids, and phenolic compounds, interact with the enzymes and proteins of the bacterial cell membrane to disrupt it [21, 22]. Numerous studies have examined the effectiveness of plant products and their potent compounds as antimicrobial agents to stop the growth of pathogenic microbes [21, 22]. Furthermore, the enzyme's activity releases a flow of protons into the cell's outside, which can either kill the cell or prevent enzymes needed to produce amino acids from working [23]. It has also been reported that alkaloids and flavonoids have antibacterial effects [24]. Their conclusions clarified the cause of the *M. sativa* leaf extract's antibacterial properties against the examined species.

M. sativa showed broad antimicrobial activity, with its ethanol leaf extract effective against multiple Gram-positive and Gram-negative bacteria.

Using a variety of in vitro models, this study also looked into the antioxidant capacity of *Medicago sativa* leaf extract. The study investigated the capacity of seven concentrations, ranging from 3 to 25 mg/mL, to scavenge free radicals. The DPPH radical activity and the nitric oxide (NO) scavenging impact are two important assays used to assess the antioxidant properties of the ethanol leaf extract.

Using vitamin C as a reference antioxidant, the percentage suppression of DPPH radicals was compared with the DPPH radical activity of M. sativa extract at various doses. The results showed that the response was concentration-dependent, and that M. sativa had significant DPPH radical scavenging activity. *M. sativa* showed a potent suppression of DPPH radicals at 3 mg/mL, similar to what was seen with vitamin C. The percentage inhibition steadily dropped as the concentration rose. It's interesting to note that *M. sativa* consistently showed antioxidant activity at different concentrations that was comparable to that of vitamin C, indicating a strong capacity for scavenging free radicals.

Additionally, the ethanol leaf extract of *M. sativa's* nitric oxide scavenging (NO) activity was investigated. The outcomes showed that the activity was concentration dependent. *M. sativa* showed remarkable suppression of NO at 4 mg/mL, greater than the inhibition seen by vitamin C. As the concentration rose, the trend of declining percentages of inhibition persisted. Several investigations have examined the antioxidant characteristics of several plant extracts, such as *M. sativa*, and have attributed these benefits to the existence of bioactive substances such phenolic acids and flavonoids [25].

Similar results in DPPH radical scavenging activity were reported by Johnson et al. [25] for leaf extracts of *Medicago sativa*, with inhibition rates ranging from 60% to 75% at doses comparable to those investigated in this study. Furthermore, the results of a study by White et al. [26] that showed *Medicago sativa* extracts significantly inhibited nitric oxide are consistent with the observed NO scavenging action. Our study's concentration-dependent response is in line with earlier research on plant extracts, highlighting the significance of dosage in assessing the effectiveness of antioxidants [19, 28].

Moreover, the fact that *M. sativa* has similar antioxidant activity to the well-known antioxidant ascorbic acid implies that the plant extract could be used as a natural substitute for treating illnesses linked to oxidative stress. This result is consistent with research that suggests antioxidants produced from plants should be investigated as possible therapeutic agents [29, 30].

Conclusion

The study shows M. sativa ethanol leaf extract has broad antibacterial and promising antioxidant activities, similar to ascorbic acid. Its effects suggest potential therapeutic use against microbial infections and oxidative stress-related conditions. Further in vivo studies and clinical trials are needed to confirm these properties and establish safe, effective applications.

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Conflict of interest

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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