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ORIGINAL ARTICLE

The Biological and Nutraceutical Properties of Methanolic Extract of *Medicago sativa* in Iran

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ABSTRACT

Background: *Medicago sativa* has been used as food by both humans and animals. However, its nutraceutical properties have yet to be clarified, and thus, the present study aimed to evaluate the biological properties of *M. sativa*.

Methods: Total phenolic and flavonoid contents of the methanolic extract of *M. sativa* were determined using Folin-Ciocalteu colorimetric and Woisky-Salatino methods. The antioxidant and antimicrobial activities of this plant were examined using *in vitro* assays. The anti-malarial and anti-platelet aggregation activities were evaluated by heme detoxification and turbidometric methods, respectively. Cytotoxicity and brine shrimp lethality assays were used for toxicological evaluations.

Results: *M. sativa* methanolic extract had 38.7±0.57 mg of gallic acid equivalents/g extract and 27.6±0.31 mg rutin equivalents/g extract concentration of phenolic and flavonoid contents. The strongest antibacterial activity was against *Staphylococcus aureus* and *Salmonella enterica* with Minimum Inhibitory Concentration (MIC) of 62.5 mg/mL, and 62.5 mg/mL, respectively. The extract showed antioxidant activity with half-maximal inhibitory concentration (IC50) of 179.1 µg/m. Regarding the anti-platelet activity, the extract with a concentration of 20 mg/mL inhibited the arachidonic acid pathway by 23.70%. The brine shrimp lethality assay results showed that LC_{50} of *M. sativa* extract was 17.62074 µg/mL; while the cytotoxicity assay revealed that the extract at concentration of 10000 µg/mL was toxic for human dermal fibroblasts and corneal epithelial cells.

Conclusion: Our study elucidated the antibacterial, antioxidant, and anti-platelet aggregation properties of the methanolic extract of *M. sativa*.

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Introduction

From ancient times to the present day, the use of herbs for medicinal and nutritional purposes has been of interest. Medicinal plants have been used in traditional forms based on ethnobotany or as modern therapeutic agents to optimize the applications of herbal medicines in respect to the efficacy and therapeutic indications for various diseases (1). Iran with its vast flora has been home to various medicinal plants with different medicinal properties for various diseases (2). One of those plants used ethnically for humans and animals is *Medicago sativa* (*M. sativa*) that new studies revealed some of its beneficial effects suggesting its is a promising therapeutic impact for some diseases (3, 4).

M. sativa (also known as Alfalfa or Lucerne) is an originally Asian herb, belonging to the Fabaceae family. It consists of diverse genotypes in which different phenotypic characteristics can be observed. This plant wildly grows in middle Asia and countries such as Iran, Afghanistan, Turkmenistan, and other parts of the world. *M. sativa* is characterized as 0.3-1 m in height and possesses a rhizome capable of penetrating as deep as 6-9 m in soil. This herb is a perennial flowering plant with a branched, sometimes, trifoliate leaves (5). Although previous studies have shown that the components and secondary metabolites of *M. sativa* such as flavonoids, phenols, and other compounds are responsible for many of the biological properties described for this plant, it is necessary to conduct more studies on species of *M. sativa* to profile the properties, thoroughly (6).

The importance of antibacterial drug resistance (ADR) is mostly observed in hospitals and community settings (7). Increased mortality and hospitalization costs due to ADR and serious side effects of some anti-bacterial agents are major reasons that highlight the need to discover new, stronger, and safer anti-bacterial agents than ever before (8). In this line, Doss *et al.* studied on anti-bacterial activity of *M. sativa* extract on bacterial strains of *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* (9). Another study surveyed *M. sativa* extract on three bacterial strains of *S. aureus*, *E. coli*, and *P. aeruginosa* (10). Chegini *et al.* evaluated the effect of *M. sativa* extract on four bacterial species in a sinusitis infection *S. aureus*, *Staphylococcus pneumoniae*, *Moraxella catarrhalis*, and *Haemophilus influenza* (11).

Malaria is an old infectious illness caused by four Plasmodium species. Malaria induces fatal periodic fevers, splenomegaly, and anemia. Based on the WHO 2020 report, from 2000 to 2019, 1.5 billion cases worldwide suffered from malaria, of which 7.6 million died. In this period, 82% of cases and 94% of deaths were reported from Africa (12). For these reasons, we decided to study the anti-malarial effects of this plant. Oxidation is a necessary process in organisms to provide cell energy (13). Reactive oxygen species (ROS) are toxic byproducts of this process which induce cell malfunction and structural disorder, immune dysfunction, and various cancers (14). Oxidative stress refers to a disproportion within anti-oxidant protection systems and ROS production (21). Anti-oxidants play a critical role in lingering and preventing oxidative stress (23, 24).

Bora and Sharma surveyed *M. sativa* anti-oxidant activity using four methods (15). Kowalska *et al.* 2013 perused the anti-oxidant activity of some varieties of *M. sativa* with the Thin Layer Chromatography (TLC)- 2,2-diphenyl-1-picrylhydrazyl (DPPH)* method (16). Another study by Liu *et al.* demonstrated *M. sativa* polysaccharides' anti-oxidant effects (17). The study published on the effect of *M. sativa* on platelet aggregation process showed that this plant has an anti-platelet aggregation activity through the inhibition of adenosine diphosphate (ADP) which is a platelet aggregation agonist (18). It is speculated that this activity may be associated with antagonizing warfarin, an anticoagulation drug, due to the presence of vitamin K in *M. sativa* (19). So the aim of this study was to examine the biological properties of *M. sativa* indigenous to Iran.

Materials and Methods

The aerial parts of *M. sativa* belonging to the Fabaceae (Papilionaceae) were collected from Shahin-Villa area (public land in Alborz province, northern Iran, coordination of 35°50'50.6"N 50°43'56.5"E in Feb 2022). Plant material was identified by Dr. Azad Rastegar from Kurdistan Agriculture and Natural Resources Research and Education Center, Sanandaj, Iran and a Voucher specimen (48407) was deposited in the herbarium of the University of Tehran. All Experimental protocols in the present study complied with the international guidelines and legislation. The study protocols and experiments were approved by the Baqiyatallah University of Medical Sciences Ethics Committee of Research, Tehran, Iran. Collected parts of the plant were freed from debris and placed in a dry, dark place for two weeks to be dried. The dried parts were ground into powder. All experimental protocols in the present study complied with international rules and legislation. *M. sativa* powder (100 g) was extracted (3 times) with 80% methanol (Chem-Lab, Belgium), and vortexed by a shaker (GFL, Germany) at room temperature for 24 hours. After filtration (Whatman, UK),

the extract was concentrated using a rotary evaporator (Heidolph, Germany) and kept in amber vials at 4°C for subsequent tests. The extraction yield of the *M. sativa* was 17.57%.

Total phenol content was determined by Folin-Ciocalteu colorimetric method with a slight modification (20)ItemsAsXml. Folin-Ciocalteu phenol reagent (1.5 mL) was mixed with an aliquot amount of extract (0.2 mL) and then stood for 5 min. A total of 6% sodium carbonate (1.5 mL) was added to the melange and stayed away for 90 minutes at room temperature. The mixture absorbance was measured with an ultraviolet (UV)-visible spectrophotometer (Shimadzu, Japan) at 725 nm. The calibration curve was obtained in a range from 200 to 800 µg/mL of gallic acid (Merck, Germany). Results were calculated as a percentage of gallic acid equivalents in dry matter of extract. The experiment was done in triplicate.

Total flavonoid content was determined using Woisky and Salatino and AlCl3 colorimetric methods with some modifications (21). Standard solutions were 10 mg rutin solved in 80% ethanol and diluted up to 25-100 µg/mL. Each of the standard solution concentrations (0.5 mL) was mixed with 10% AlCl3 (0.1 mL), 1 M potassium acetate (0.1 mL), 95% ethanol (1.5 mL), and distilled water (DW) (2.8 mL). To determine flavonoid content, methanol extract (0.5 mL) was added to the intermediate solution and then incubated for 30 min at room temperature. To make the blank, 10% aluminum chloride (0.1 mL) was replaced by the same volume of DW's. Eventually, the absorbance of the mixture was measured by a UV-visible spectrophotometer (Shimadzu, Japan) at 415 nm. A standard curve was drawn based on standard solution concentrations and results were calculated as a percentage of $AlCl_3$ equivalents in dry extract matter. The experiment was performed in triplicate.

Five Gram-positive bacteria including *S. aureus* ATCC 6538, *Staphylococcus. epidermidis* ATCC 12228, *Kocuria rhizophila* ATCC 9341, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 1247 and five Gram-negative bacteria *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *Salmonella enterica* ATCC 19430, *Klebsiella pneumoniae* ATCC 10031, and *Salmonella typhimurium* ATCC 14028 were purchased from Iranian Research Organization for Science and Technology (IROST), Tehran, Iran and were cultured in Mueller-Hinton Agar (MHA) medium (Merck-Germany) and incubated at 37°C for 18-24 h and repeated for three times to start further tests.

Initial evaluation of the anti-bacterial effect of *M. sativa* was investigated by the cup-plate method (22). Briefly, a suspension of bacteria with turbidity equal to 0.5 McFarland standards (1×108 Colony-forming unit (CFU)/mL) was prepared. Mueller Hinton agar (MHA) plates were grooved applying this suspension. The wells of diameters of 8 mm were filled with 100 µL of concentrations of 500, 250, 125, and 62.5 mg/mL of plant extract. Also, one well was considered as a negative control by filling with solvent. The plates underwent incubation at 37°C for 18 to 24 h and were repeated 3 times. The average diameters of inhibition zones were indicated for each concentration.

To determine Minimum Inhibitory Concentration (MIC), the micro-dilution method was used conforming to Clinical and Laboratory Standards Institute (CLSI) recommendations (23). The extract was mixed with Muller-Hinton Broth (MHB) medium (Merck-Germany) to prepare homogeneous concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.95, 0.97, and 0.48 mg/mL (one row was considered for evaluation of the extract sterility) and the solvent was used as a negative control. The bacterial suspensions corresponding to 0.5 McFarland were prepared and then diluted 20 times with sterile normal saline. From every bacterial suspension, 10 µL was added to each well of micro-plate (SPL-Korea). Also, one row was regarded as a growth control without plant extract. The micro-plates were incubated at 37°C for 24 h and the procedure was repeated three times. The lowest concentrations with no visible growth were recorded as MICs. To determine Minimum Bactericidal Concentration (MBC), 20 µL were picked up from the wells of MIC experiments that showed no growth and cultured on MHA plates. The number of colonies was counted after 18-24 h of incubation at 37°C. Each experiment was performed three times and data were recorded.

The anti-malarial activity was surveyed using the inhibition test of heme detoxification (ITHD) method (24). The procedure for the ITHD was preparing 200 µg/mL sample solution by solving extract in dimethyl sulfoxide (DMSO). To achieve 60 µg/mL hemin solution, fresh 120 µg/mL hemin solution in DMSO was diluted with 1 M acetate buffer (pH=4.8). Tween 20 was later diluted with DW up to 12 µg/mL. These solutions were distributed respectively in a 96-well microplate with a 2:9:9 ratios. Controls were prepared without hemin at the same condition to omit residual intervention absorbance of the sample matrix. All microplates were incubated at 60ºC for 24 h for reaction completion. The absorbance of mixtures was measured with an ELISA reader at 405 nm.

The performance of ITHD was evaluated by calculating the negative predictive value (NPV) and

the positive predictive value (PPV) using the following equation; while a positive control of Chloroquine diphosphate and a negative control of DMSO were used in these assays and the experiments were done in triplicate:

$$NPV = \frac{True\ Negative\ number}{True\ and\ False\ Negative\ number}$$

$$PPV = \frac{True\ Positive\ number}{True\ and\ False\ Positive\ number}$$

To assess antioxidant activity, the free radical scavenging ability of *M. sativa* extract in diverse concentrations from 6.25-400 µg/mL was measured with stable diphenyl-1-picrylhydrazyl (DPPH*) radicals. Each of the extract concentrations (2.5 mL) was mixed with 0.3 mM DPPH* solution (1 mL) and incubated at room temperature for 30 min. Absorbance reduction was measured at 517 nm. To calculate DPPH* percentage inhibition (IDPPH* %), a defined equation was used (25) as

$$I_{DPPH}(\%) = \left[\frac{A_C-(A_S-A_B)}{A_C}\right] \times 100.$$

AC, AS, and AB were related to the absorbance of the control, sample, and the blank. Half-maximal inhibitory concentration (IC50), which is the concentration (µg/mL) of the sample that inhibits 50% scavenging of DPPH* was calculated with GraphPad Prism 8.4.2. Positive controls of gallic acid and rutin were used in these assays and all experiments were repeated in triplicate.

To evaluate anti-platelet aggregation, the methanol extract was dissolved in DMSO at the concentration of 20 mg/mL and this stock was passed through a 0.22 µm syringe filter (Jet-China). Anti-platelet aggregation was done with a turbidometric method and a decrease in light transmission was recorded (26). The levels of transmission were calibrated by platelet-poor plasma (PPP) and platelet-rich plasma (PRP). The amount of 200 µL of PRP was divided in the test cuvettes and incubated in an aggregometer chamber at 37ºC temperature. Platelet aggregation was measured by using PRP after activating and adding arachidonic acid (AA), ADP, and collagen (Col). Totally, 1 µL of prepared extract solution in DMSO/PVA was added into PRP and it took 5 minutes to be active with AA or ADP or Col. The anti-platelet aggregation activity was reported as percent of inhibition compared with the measured vehicle alone and the percent of inhibition was calculated (27) with the formula of Percent of inhibition=100×((1-(D/S)), while D=Sample aggregation, and S=Aggregation in the presence of solvent (DMSO/PVA1%).

Regarding toxicological investigation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to evaluate the viability of human dermal fibroblasts (HDF) and human corneal epithelial (HCE) cells in the vicinity of *M. sativa*, 5×10^3 cells per well were seeded into 96-well plates (Biologix, China). Following seeding and adhesion of HDF and HCE cells, the experiment was conducted the following day by adding *M. sativa* extract to the wells at the concentrations of 200, 400, 600, 800, 1000, and 10000 µg/mL (n=3 in each group). The negative controls included the cells which not treated with the extract. After 48 and 72h of incubation in 5% CO_2 at 37ºC, the medium was replaced with a culture medium containing 100 µL of serum-free medium and 15 µL of MTT in 5 mg/mL concentration. After 4h incubation in 5% CO_2 at 37ºC and removing the culture medium, formazan crystals that were produced by the consumption of MTT by living cells were dissolved in dimethyl sulfoxide (DMSO). After 15 min incubation at 37ºC, by measuring the formazan absorbance at 570, optical density (OD) units were used to represent numerical values. Brine shrimp lethality assay was conducted according to the previously described methodology since it is one of the most relevant toxicological tests for the evaluation of biologically active compounds from medicinal plant extracts (28).

Briefly, cysts of *Artemia franciscana* were purchased from an Iranian local supplier (Iran Artemia Company, Tehran, Iran), and they were hatched in the laboratory using conical containers with a volume of 1000 mL; while an environment temperature of 26±2°C was maintained within the container along with oxygenation. Water salinity of 35 ppt and pH of 8.5 were provided for hatching of larvae (nauplii). For toxicity tests, plant extracts with concentrations of 1000, 100, and 10 µg/mL were prepared and added to the test tubes adding up to 5 mL with artificial seawater. A total of 0.5 mL of DMSO was used for the positive control group and artificial seawater was applied for the negative control. After 24 hours, *A. franciscana* movements were examined under a light microscope and the percentage of 50% lethal concentration (LC_{50}) value was analyzed using the probit method (29). The values were expressed as mean±S.D (n=3). Statistical analysis was performed using SPSS (SPSS Inc., version 18, Chicago, Ill. USA). Analysis of variance (ANOVA) was employed to compare differences between groups. Moreover, GraphPad Prism (Version 8.4.2) was used for IC50 calculations. The statistically significant level was set as p value<0.05.

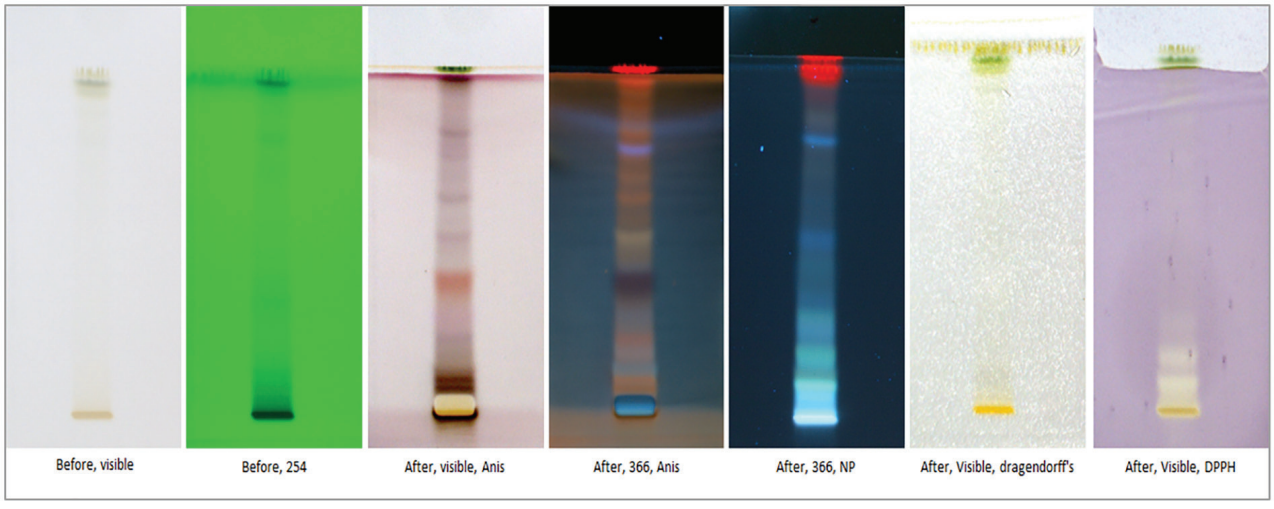

Figure 1: Preliminary phytochemical investigation of *M. sativ*a methanolic extract by HPTLC.

Results

Regarding total phenolic and flavonoid content, *M. sativa* hydromethanolic extract had 38.7±0.57 (mg of gallic acid equivalents/g extract) and 27.6±0.31 (mg rutin equivalents/g extract) concentration of phenolic and flavonoid contents, respectively. In phytochemical investigation of *M. sativa*, the methanolic extract by using High Performance Thin Layer Chromatography (HPTLC) and F254 TLC plates, and chloroform-ethyl-acetate-acetone-formic acid (4:3:2:1) revealed several minor and major-colored spots indicating the presence of various compounds in the extract. The color increased by using UV at 254 and 366 wavelengths and by anisaldehyde-sulfuric acid and Dragendorff's spraying reagents as shown in Figure 1.

The results of measuring the antibacterial effect of *M. sativa* extract on Gram-positive bacteria in an agar medium showed the same effectiveness of 500, 250, and 125 mg/mL concentrations on *S. epidermidis* with a growth inhibition zone of 10.33±0.57 and with decreasing concentration. In 62.5 mg/mL, the diameter of the growth inhibition zone decreased and reached 9.66±0.73 mm. This extract did not affect other Gram-positive bacteria. Regarding the effect of *M. sativa* extract on control of gram-negative bacteria, it was shown that the effect of this extract on *P. aeruginosa* bacteria was such that with decreasing concentration, the area of non-growth was also reduced. The maximum and minimum growth inhibitions for this bacterium for concentrations of 500 and 62.5 mg/mL were 19.33±0.57 mm and 13.33±1.15 mm, respectively. The highest growth inhibition area for Gram-negative bacteria for the concentrations used in this study was correlated to the concentration. The antibacterial effect of 500 and 250 mg/mL of *E. coli* was 10.00 and 9.00 mm, respectively, and lower concentrations were shown to be ineffective. None of the concentrations used in this study were effective on *K. pneumonia* and only a concentration of 500 mg/mL affected *S. enterica*. The lowest antibacterial effect recorded by this method was related to the antibacterial concentrations of 500 and 250 mg/mL of *S. typhimurium* with an area of 2.60±0.63 and 1.70±0.73 mm, respectively. It should be noted that other concentrations had no effect.

The trend of the bacteriostatic effect of different concentrations of hydroalcoholic extract of *M. sativa* indicated that the lowest and best effect was related to the concentration of 62.5 mg/mL of *S. aureus* and *S. enterica* (Table 1). The extract with a concentration of 125 mg/mL was able to stop the growth of *S. epidermidis*, *K. rhizophila*, *B. cereus*, and all Gram-negative tested bacteria. The highest inhibitory concentration was related to the concentration of 250 mg/mL of *B. subtilis*. In respect to the bactericidal properties of *M. sativa* extract, the most effective bactericidal concentration was 125 mg/mL against *S. enterica*. Also, this extract was able to kill *P. aeruginosa* bacteria at a concentration of 250 mg/mL and a concentration of 500 mg/mL had a bactericidal effect on the following bacteria *S. epidermidis*, *B. subtilis*, *E. coli*, *K. pneumonia*, and *S. typhimurium*. Bactericidal effects on *S. aureus*, *K. rhizophila*, and *B. cereus* were not observed at the concentrations measured in our study.

M. sativa methanolic extract did not have any antimalarial activity as the last concentration of this extract (200 µg/mL) in the wells could inhibit only 34%; while the positive result should be above 90% inhibition. *M. sativa* methanolic extract showed a concentration dependent antioxidant activity by an increase in the DPPH free radical scavenging percentage shown in Figure 2. The IC_{50} which is the concentration required to scavenge DPPH by 50% was equal to 179.1 µg/mL.

Table 1: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of *M. sativa* methanolic extract (mg/mL) (n=3).

Type	Bacteria	MIC	MBC
Gram-positive	*Staphylococcus aureus* (ATCC 6538)	62.5	-
	Staphylococcus epidermidis (ATCC 12228)	125	500
	Kocuria rhizophila (ATCC 9341)	125	-
	Bacillus subtilis (ATCC 6633)	250	500
	Bacillus cereus (ATCC 1247)	125	-
Gram-negative	*Escherichia coli* (ATCC 8739)	125	500
	Pseudomonas aeruginosa (ATCC 9027)	125	250
	Salmonella enterica (ATCC 19430)	62.5	125
	Klebsiella pneumonia (ATCC 10031)	125	500
	Salmonella typhimurium (ATCC 14028)	125	500

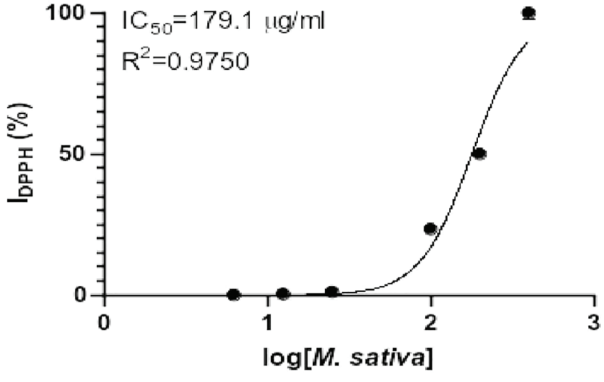

Figure 2: DPPH radical scavenging activity of *M. sativa* methanolic extract.

Anti-platelet aggregation analysis showed that the inhibition of platelet aggregation occurred via three different mechanisms, which indicates that the *M. sativa* extract with a concentration of 20 mg/mL was able to inhibit the arachidonic acid, ADP, and collagen pathways with 23.70, 8.40 and 2.11%, respectively. The highest level of inhibition was related to the arachidonic acid pathway.

Viability and proliferation rate were assessed for HDF and HCE cells. The 200, 400, 600, 800, and 1000 µg/mL extract concentrations were not different between the treated and control groups in define optical density (OD) (Figure 3).

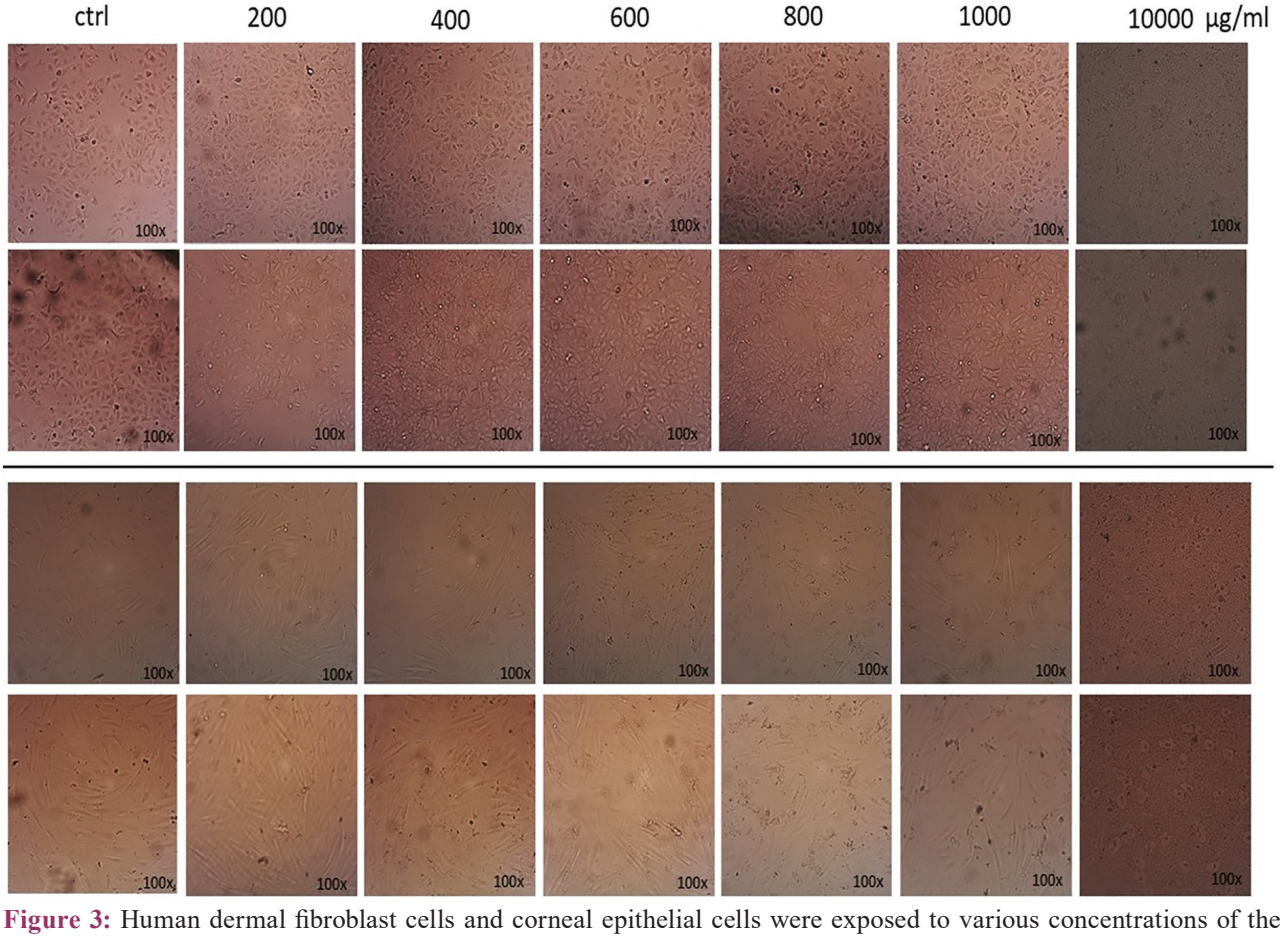

Figure 3: Human dermal fibroblast cells and corneal epithelial cells were exposed to various concentrations of the methanolic extracts of *M. sativa* (100x).

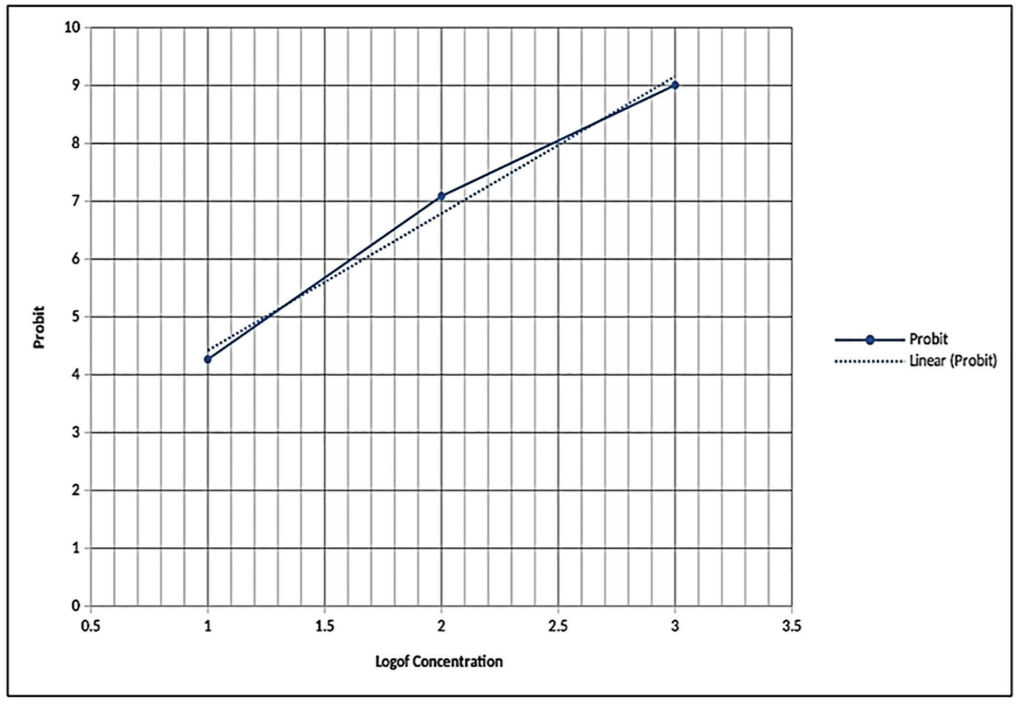

Figure 4: Probit analysis of LC_{50} estimation of the methanolic extract of *M. sativa*.

However, the extract at 10000 µg/mL concentration was toxic for these cells and the OD of treated cells was statistically different from the non-treated group. This result was confirmed by cell morphology imaging. As shown in Figure 3, the extract at a concentration lower than 1000 did not significantly affect cell culture, but with increasing concentration; cell death happened in both cell lines. According to the probit analysis, the LC_{50} value of *M. sativa* extract was 17.62074 µg/mL (Figure 4).

Discussion

In the present study, Folin-Ciocalteu colorimetric method and Woisky and Salatino technique were used to determine total phenol and flavonoid contents of methanol extract of whole-part *M. sativa*, respectively. The findings revealed 38.7 mg/dry matter (DM) gallic acid equivalent (GAE) for total phenol and 2.76 mg/DM rutin for total flavonoid content. Krakowska, Rafińska, reported the amount of total phenolic content by Folin-Ciocalteu method and total flavonoids content by Rouphael method based on the solvent type, method of extraction, and the part of the plant which was used. The maximum result was observed for 70% ethanol extract of flowers driven from Ultrasound-Assisted Extraction (UAE) method and the lowest for 96% ethanol extract of root as 48.4 and 6.9 mg GAE/g DM, respectively. The mentioned paper also suggested that the more polar solvent (i.e., 70% ethanol when compared to 96%) was, it had more content of phenolic compounds (6). The same basis of comparison for flavonoids suggested that the highest amount of flavonoids were present in the leaves extracts prepared by supercritical fluid extraction (SFE) method with supercritical CO_2 and ethanol (139 mg RE/g DM, RE is rutin equivalent) (6). Types of flavonoid and phenolic compounds found in *M. sativa* were described before in Bora *et al.*'s study (15).

HPTLC results confirmed the presence of various secondary metabolites in *M. sativa* extract such as flavonoids like quercetin, myricetin, luteolin, apigenin, chrysoeriol, tricin, medicarpin, coumestrol, sativan, vestitol, and formononetin and also presence of triterpenes and the steroids β-sitosterol, stigmasterol, and α-spinasterol. Alkaloids as trigonelline, stachydrine, and l-homostachydrine were observed too. In comparative studies, there was compelling evidence that *M. sativa* could disrupt the life cycle of so many bacterial species. Some researchers used two methods to describe the antibacterial activity of *M. sativa* methanolic extract; and their MIC for *S. pneumoniae*, *H. influenza*, and *M. catarrhalis* that was 125 mg/mL, while the disk diffusion method showed 16 mm for *M. catarrhalis*, 13 mm for *S. pneumoniae* and 10 mm for *H. influenza* (11). It should be mentioned that others did not report any anti-bacterial activity against *S. aureus* (10).

It was shown that *M. sativa* leaves methanol extract has been studies against three bacterial species via methods of disc dif., methods-turbidity, and MIC assay (10). Three bacterial species were included as *E. coli*, *P. aeruginosa*, and *S. aureus* and it was shown that 23, 22, and 23 mm were at the disc diffusion test, respectively, and 37, 12.03, and 111

Table 2: The antioxidant activity of *M. sativa* extract from different plant parts (DPPH method).

*In vitro/in viv*o	Plant part	Solvent	Result	Reference
Present study *In vitro*	Aerial parts	Methanol 80%	IC_{50}=179.1 µg/mL	Present study
In vivo	Aerial parts	Methanol	70% (concentration, 5 µg/mL)	(30)
In vitro	Leaves	Methanol	54.42% (concentration, 250 µg/mL)	(31)
In vitro	Seeds and whole part	Water-glycerin	Max of 40% (concentration,10%)	(32)
In vitro	Flower	Ethanol	Max of 70 µmol TEAC/g DM	(6)
In vitro	Root	Methanol	IC_{50}=100.38 µg/mL	(33)

at MIC assay, respectively (10). The same bacterial species were included in the present study, but the results showed differences, particularly in the case of *S. aureus*. A survey showed that the type of solvent played a definitive role in the anti-bacterial activity of *M. sativa* (9). Some extracts, particularly those containing benzene and petroleum ether solvent were reported to have no anti-bacterial effect on *S. aureus*, *K. pneumonia*, *E. coli*, and *P. aeruginosa* (9) ote>. Regarding antimalaria activity, more than 90% of heme detoxification inhibition was considered a positive response, while *M. sativa* extract did not show any antimalarial activity in this method. So, it is recommended to use other antimalarial assays.

The result of DPPH anti-oxidant test was demonstrated to vary with the method of extraction, solvent concentration, and the part of the plant which is used; as the most anti-oxidant activity was seen in the case of 96% ethanol extract from the flower part (Table 2) (6). In addition to the DPPH radical scavenging method, some researchers used ABTS radical scavenging, iron chelating activity, lipid peroxidation assay, nitric oxide scavenging assay, and alkaline DMSO assay for different concentrations of the same extract; and the result showed the IC_{50} values to be 12.33 µg/mL, 115.79 µg/mL, 49.06 µg/mL, 21.77 µg/mL, and 15.91 µg/mL, respectively (33).

It should be mentioned that according to Bora *et al.*'s study when compared to the standard radical scavengers of butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), *M. sativa* methanol extract could differently display the antioxidant activity. Although the diphenylpicrylhydrazyl (DPPH) scavenging test results for *M. sativa* extract were always weaker than BHA in the same concentration (25-75 µg/mL) that it could surpass that of BHT with the same concentration (30). Regarding the anti-platelet activity, results of the present study were in the percentage of the inhibition with the extract concentration of 20 mg/mL on three platelet aggregation agonists of ADP, arachidonic acid, and collagen. On the other hand, some researchers showed this effect based on percentage of inhibition of ADP to be 5.0 µM in optimum concentration. The result was indicated in a concentration-dependent manner and the IC_{50} for anti-platelet aggregation test was reported as 5.9±0.01% mg/mL (18). However, there is one study suggesting that due to the presence of vitamin K in *M. saliva*, it may antagonize or delay the effect of warfarin, which acts through antagonizing vitamin K activity (19).

Cytotoxic evaluation by the MTT method showed that *M. sativa* was safe at a concentration lower than 1000 µg/mL. This concentration did not induce any cell death or any change in viability. Our data is in agreement with a recent study demonstrating the beneficial effects of *M. sativa* on the viability and oxidative protection of small intestinal epithelial cells against injuries (34)**.** New studies showed the protective effect of *M. sativa* extract toward cell injuries attributed to the antioxidant activity of total phenols and flavonoid contents of the plant (35-37). Moreover, a research showed that applying *M. sativa* as an ingredient in topical cosmetic formulations did not cause ocular and dermal toxicity. These findings are consistent with our findings on the toxicological evaluations of *M. sativa* extract on HDF and HCE cells (38).

In our study, LC_{50} of *M. sativa* extract was estimated to be 17.62074 µg/mL. The estimated median lethal concentration of different species of *Medicago,* except *M. sativa,* after 48 hours of exposing *Artemia salina* was illustrated to be at a range of 4.1 to 181.3 µg/mL for *M. rigidula* and *M. doliata*, respectively (39). Other studies reported the range of LC_{50} values for *Medicago* species through the brine shrimp lethality assay (37, 40). However, their reports were different from our investigation in terms of the extraction method, plant species, and the brine shrimp lethality test, and thus different results can be expected.

Conclusion

The results obtained in this study indicated that the methanolic extract of *M. sativa* possessed antibacterial, antioxidant, and anti-platelet

aggregation properties. These effects could be related to the phenolic and flavonoid contents of this plant. Further studies are encouraged to clinically evaluate the therapeutic and nutraceutical properties of *M. sativa* in human and animal diseases.

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Authors' Contribution

ESS, MAS, and SA: Conceptualization, methodology, data curation, investigation, visualization, supervision, project administration, and original draft preparation; EKV, MK, and KR: Analysis, validation, editing, and reviewing; All authors read and approved the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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