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RESEARCH ARTICLE

Antibacterial Activity of Aqueous and Methanolic Extract of *Mentha piperita* against Pervasive Bacteria Isolated from Urial the *Ovis vignei*

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A B S T R A C T

The emergence of antimicrobial resistance and the emergence of novel pathogens pose a serious threat to public health and animals around the world. Peppermint (*Mentha piperita*) has a variety of therapeutic features, one of which is an antibacterial property that can be helpful in preventing infections in wildlife. This property can be found in the extracts of both the stem and the leaf of the plant. In the current investigation, an extract of *Mentha piperita* (*M. piperita*) or clocally named as peppermint stem and leaf was produced in methanol at a concentration of 70 percent to test its effectiveness as an antibacterial agent against three Gram-negative bacteria. i.e. *Escherichia coli (E. coli), Salmonella* Typhimurium *(S.* Typhimurium) and *Pseudomonas aeruginosa (P. aeruginosa)* and against one Gram-positive Bacteria *Staphylococcus aureus* (*S. aureus*). Fresh feces and skin swab samples were taken from wild sheep in the Wildlife Park Gatwala, Faisalabad, during February 2022. The isolated strains of bacteria were identified and confirmed microbiologically. Peppermint (stem and leaf) extracts prepared with methanol were examined for their efficacy against the identified pathogens. The extracts demonstrated higher inhibitory zones (leaf: 17.5mm, stem:16mm) against *S. aureus*. *S.* Typhimurium, *P. aeruginosa* and *E. coli.* The leaf extract showed zones of inhibition of 16.5, 16, 15.5 and 13mm while stem extract produced zones of inhibition of 15.5, 14, 16.5, 15mm against *S. aureus*. *S.* Typhimurium, *P. aeruginosa* and *E. coli*, respectively. Furthermore, the broth dilution method revealed that the MIC of peppermint (stem and leaf) was 0.4mg/ml against all tested bacterial strains. Altogether, these findings indicate that peppermint extracts may be useful in combating common bacterial infections of wild sheep.

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INTRODUCTION

A wide variety of bacteria can be found not only on the outside of the sheep's body but also inside of it. Bacteria are very small organisms that can be found in virtually every environment in the world. Colonizing bacteria are bacteria that grow on an animal's body without causing an infection. Pathogenic bacteria are those causing health problems and enter the body through food, water, saliva, air, and other body fluids (Jaishankar *et al.*, 2014). Bacteria have become resistant as a result of prolonged use of traditional antibiotics, which have a wide range of effectiveness by having toxic or growth-inhibitory effects on their target organisms, making the traditional antibiotics essentially ineffective (Shalayel *et al.*, 2017). The most common commensal dweller of the gastrointestinal tracts of people and warm-blooded animals is the *E. coli*, which is one of the most significant diseases causing pathogen too (Kaper *et al.*, 2004). Opportunistic bacteria such as *P. aeruginosa*, on the other hand, are only able to infect individuals with a compromised immune system and can cause severe diseases but rarely cause a disease in patients with strong immune system. Bacterial pathogenesis is typically very complex and multifactorial, with a few

notable exceptions like *Corynebacterium diphtheriae* and *Clostridium tetani*, which cause disease by means of single determinants (Casadevall and Pirofski, 2009).

In worldwide there are approximately 2 to 4 million *S.* Typhimurium cases that are reported annually (Galanis *et al.*, 2006). These bacteria produce colonies that are opaque, round, translucent, and may be smooth when grown on nutrient agar medium but with black center when cultured in SS agar. It is Gram negative bacilli under staining (Sarker *et al.*, 2021). One of the biggest issues faced by human civilization and the healthcare system is the emergence of antibiotic resistant bacteria (Ahameethunisa and Hopper, 2012). Antimicrobial resistance (AMR) is a growing concern in both domestic and wild animals. While much of the focus on AMR has been on the livestock and pets. It is also important to consider the impact of AMR on wildlife populations (Woolhouse *et al*., 2015). One of the examples is in wild sheep populations facing AMR issues. Wild sheep are found in a variety of habitats around the world, including mountain ranges, deserts, and grasslands. They are important ecologically, serving as prey for predators and contributing to the health of ecosystems. In addition, wild sheep are of significant cultural and economic importance, with some species being hunted for their meat and hides (Brown *et al.*, 2022).

Like other animals, wild sheep can be exposed to antibiotics through a variety of routes, including direct treatment, indirect exposure through the environment, and ingestion of contaminated feed or water. While the use of antibiotics in wild sheep is less common than in livestock, there have been instances where antibiotics have been used to treat or prevent infections in wild sheep populations (Silva *et al.*, 2020). The use of antibiotics in wild sheep populations is generally considered to be a last resort, as it can have negative impacts on the health of the animals and the environment. In addition, the use of antibiotics in wild populations can contribute to the development of AMR, which can have significant implications for both animal and human health (Espunyes *et al.*, 2021).

The development of AMR in wild sheep populations can lead to the emergence of antibiotic-resistant strains of bacteria, which can be transmitted to humans and other animals. This can result in serious illnesses that are difficult to treat with antibiotics, as well as increased morbidity and mortality in wild sheep populations (García *et al.*, 2020). To prevent the development of AMR in wild sheep populations, there is a need to promote good environmental and animal health practices. This includes promoting the use of non-antibiotic treatments for infections and promoting good animal husbandry practices to prevent the spread of infections (Arip *et al.*, 2022). Flavonoids, tannins, isoflavonoids, alkaloids, phenolic compounds, and glycosides are just some of the plant-derived chemicals that have antibacterial effects. One of the earliest human achievements across civilizations was the discovery and widespread use of medicinal plants. As an alternative, several plants' secondary metabolites have been shown to be antibacterial (Sagdic *et al.*, 2003).

Medium-sized wild sheep, the Urial (*Ovis vignei*) is the ancestor of domestic sheep (*Ovis aries*). Throughout Pakistan, the Punjab Urial can be found between the Jhelum and the Indus rivers at elevations of less than 1500 meters (Arshad and Hussain, 2018). It is considered endangered by the International Union for the Conservation of Nature (IUCN) red list from 2003. There is an abundance of data on the antibacterial characteristics of different plant species because to the long history of use of the traditional herbal medicine system (Pramila *et al.*, 2012). Extracts from plants like *M. piperita* and bay leaf that contain antimicrobial, antifungal, and antioxidant properties have been used for centuries for a wide range of applications (Irkin and Korukluoglu, 2009). Antimicrobial properties of higher and aromatic plants have long been exploited in traditional medicine, and these plants have also been used to extend the shelf life of food products (Sujana *et al.*, 2013).

M. piperita, a type of perennial plant, is widely distributed around the world. *M. piperita* is used externally as a rub or liniment and orally as a tincture, tea, extract, or oil (Brahmi *et al*., 2022). Botanists have claimed that it may have anti-aging, antiseptic, antipyretic, antispasmodic, anti-catarrhal, antimicrobial, and anti-catarrhal properties (Noureen *et al*., 2019). According to our knowledge, there have been no significant efforts to investigate the antibacterial properties of *M. piperita* against bacterial infections isolated from wild sheep. *M. piperita*, which is a member of the Lamiaceae family, is the most popular herbal treatment in the world (Tahira *et al.*, 2007). The peppermint oil and its derivatives have a potent growth-inhibiting effect on a variety of bacteria, including *E. coli*, *Streptococcus faecalis*, *Salmonella* Pullorum, *Acinetobacter* species, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Streptococcus pyogenes* and *Staphylococcus aureus* (Bohnert *et al.*, 2016; Parham *et al*., 2020).

MATERIALS AND METHODS

Collection of Peppermint: Peppermint (*M. piperita*) was purchased from a local market in Faisalabad, Pakistan, and washed three times in running water before use. Plant taxonomist identified the peppermint at the Department of Botany, Government College University Faisalabad, Pakistan. The leaves and stems of the peppermint plant were separated after being washed. Six to eight days were spent drying the stem and leaves in the shade. These were each dried, then processed to a powder and stored in two separate, sterile polyethylene bags.

Extract preparation: The stems and leaves of *M. piperita* were dried, then ground into powder in an electric grinder. The peppermint extract was prepared using 70% methanol. This methanol extract was made using both the standard and Soxhlet apparatus techniques.

Conventional method: In conventional method, powder was balanced and then dipped in the methanol (solvent), measured with measuring cylinder for the overall concentration of 100ml in beaker for 10-15 days and mixed slowly. After about 15 days, the conventional method solution was filtered by funnel using filter paper.

Soxhlet apparatus method: The peppermint powder was poured into the thimbles and closed with pins. Then thimbles and methanol were put into the Soxhlet apparatus. After about 7 to 8 hours, extract was prepared of cyclic system of the apparatus. Solvent was converted to colorless

from colored when cycles were repeated in the Soxhlet apparatus continuously. When the extract was separated from the apparatus then it was dried into the rotary evaporator. It was stored at 4°C in the falcon tube in the refrigerator (Abou Youssef *et al.*, 2018).

Collection of sheep fecal samples: The fresh fecal and skin samples of wild sheep were collected from the Gatwala Wildlife Park, Faisalabad, Pakistan and almost 80-100g fecal samples were stored in the polyethylene bags safely. The cotton swabs were used to collect the bacterial samples from the skin by moving it gently on the body of sheep.

Isolation and Identification of bacteria: Selective media MacConkey's agar, Cetrimide agar, Mannitol salt agar, Salmonella Shigella agar were used for the isolation of *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella* Typhimurium, respectively. Microscopic and biochemical properties were used to confirm the isolated bacterial species. The antibacterial activity of peppermint stem and leave extract was examined against three Gram's negative bacteria *E. coli*, *S.* Typhimurium and *P. aeruginosa* and one Gram's positive bacteria *S. aureus*.

Antibacterial activity: On Mueller Hinton Agar, the antibacterial activity of methanol extracts of *M. piperita* was deciphered against selected different bacterial pathogens. McFarland standard solutions were prepared in the Eppendorf's tube to examine the turbidity of bacterial culture suspension by comparing it with the McFarland standard tube. The antimicrobial susceptibility testing was performed five times with alternating agar well diffusion and disc diffusion to determine the average values. All the samples were accompanied with positive controls comprising the equal concentrations of commercially available Neomycin sulphate.

RESULTS

Colony morphology of different bacterium on MacConkey agar medium: *Salmonella* Typhimurium colonies on SS agar (selective medium for isolation of the *S. Typhimurium* species) emerged as black color (Fig. 1A). This bacterium (*S.* Typhimurium) cannot ferment the lactose and creates a hydrogen sulfide gas, thus appearing as pale with black centers. *P. aeruginosa* is commonly an encapsulated, gram negative and rod like bacterium (Fig. 1B). *E. coli* is rod shape, dark pink in color, and similarly, lactose fermenting colonies are surrounded by dark pink area (Fig. 1C). *S. aureus* is a Gram-positive bacterium that appeared in cluster-cocci form and stained purple with Gram staining. On Staph 110 agar medium its colonies emerged as yellow to golden in color and have a round shape (Fig. 1D).

Zones of inhibitions of leaf and stem extract of *M. piperita against S.* Typhimurium: *S.* Typhimurium growth was inhibited by the *M. piperita* leaf extract. In case of conventional method and Soxhlet method, inhibition zones of *S.* Typhimurium by agar well diffusion method were more prominent and measured as 16 and 17mm diameter for *M. piperita* leaf extract, respectively.

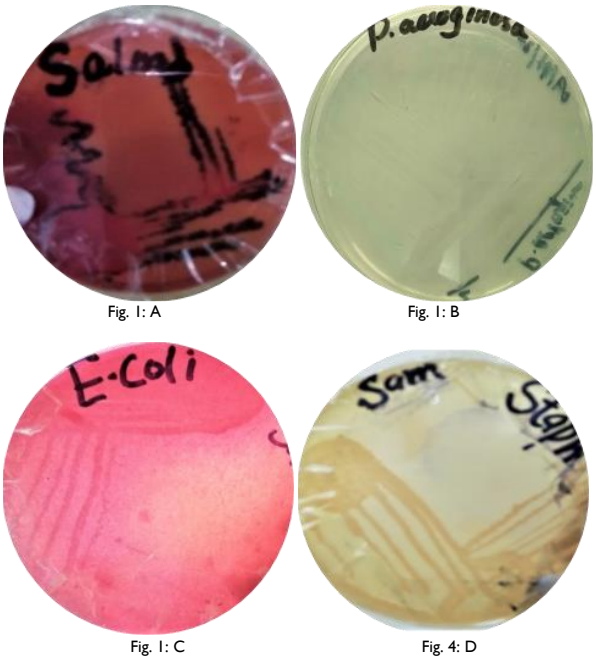

Fig. 1: A Fig. 1: B Fig. 1: C Fig. 4: D

Fig. 1: A *S.* Typhimurium growth on SS agar; B. *P. aeruginosa* growth on Cetrimide agar; C. *E. coli* growth on MacConkey agar; D. *S. aureus* growth on 110 agar media.

A positive control showed inhibition zone of 25mm in this case (Table 1). Inhibition zones of *S.* Typhimurium were more prominent and recorded as 16 and 15mm in diameter for *M. piperita* stem extract prepared by the Soxhlet method and conventional method respectively. A positive control shows inhibition zone of 25mm in this case (Table 2). *S.* Typhimurium prepared by Soxhlet method shows the greater inhibition zone, and becomes the sensitive bacteria towards the peppermint stem extract.

Zones of inhibition of leaf and stem extract of *M. piperita* against *P. aeruginosa:* In the case of Soxhlet method, inhibition was measured 16mm in diameter while it was 15mm for conventional method and positive control showed inhibition control 24mm (Table 1). The inhibition zone was measured 16mm for Soxhlet method while 17mm for conventional method in diameter and positive control showed inhibition zone of 24mm (Table 2).

Zones of inhibitions of leaf and stem extract of *M. piperita* against *E. coli:* In the case of conventional method, inhibition zones of *E. coli* by agar well diffusion method were more prominent and measured as 14mm diameter for peppermint leaf extract as compared to Soxhlet method that was 12mm**.** Positive control for conventional method was 26mm (Table 1). Inhibition zones of *E. coli* by agar well diffusion method, was measured as 14mm diameter for extract prepared by conventional method and 16mm for Soxhlet method. A positive control showed inhibition zone of 26mm for both (Table 2).

Zones of inhibitions of leaf and stem extract of *M. piperita* against *S. aureus:* Inhibition zones of *S. aureus* were more prominent and recorded as 18mm in diameter for peppermint leaf extract prepared by the Soxhlet method and 16mm recorded by conventional method. A positive control showed inhibition zone of 24mm for Soxhlet method and 25mm for conventional method (Table 1).

Table 1: Inhibition Zone with SD of the tested bacterial strains towards the *M. piperita* leaf extract prepared by conventional and Soxhlet method

Bacterial cultures	Leaf extract		Positive control (IZ)		Negative control
	A	B	A	B	
S. Typhimurium	17mm ±0.7	16mm ±1.41	25mm ±1.0	25mm ±2.5	0
P. aeruginosa	16mm ±1.6	15mm ±1.2	25mm ±1.7	24mm ±2.1	0
E. coli	14mm ±1.0	12mm ±1.9	25mm ±1.6	26mm ±1.5	0
S. aureus	18mm ±0.6	17mm ±2.35	25mm ±1.6	24mm ±1.9	0

Table 2: Inhibition Zone of the tested bacterial strains towards the *M. piperita* stem extract prepared by conventional and Soxhlet method

Bacterial cultures	Stem extract		Positive control (IZ)		Negative control
	A	B	A	B	
S. Typhimurium	16mm ±1.0	15mm ±2.0	25mm ±1.9	25mm ±2.5	0
P. aeruginosa	16mm ±2.2	17mm ±2.1	26mm ±1.0	24mm ±2.7	0
E. coli	16mm ±1.9	14mm ±1.6	26mm ±1.6	26mm ±1.6	0
S. aureus	17mm ±1.2	16mm ±2.5	25mm ±2.2	24mm ±2.7	0

Table 3: Minimum Inhibitory Concentration by well diffusion method of *M. piperita* stem extract

Bacterial strains	Different concentration of *M. piperita* stem extract mg/ml					
	0.05	0.1	0.2	0.4	0.6	0.1
S. aureus	-	-	14mm	15mm	17mm	17mm
E. coli	-	-	-	12mm	9mm	12mm
P. aeruginosa	-	-	-	12mm	11mm	12mm
S. Typhimurium	-	-	12mm	14mm	17mm	20mm

Table 4: Minimum Inhibitory Concentration by well diffusion method of *M. piperita* leaf extract

Bacterial strains	Different concentration of *M. piperita* stem extract mg/ml					
	0.05	0.1	0.2	0.4	0.6	0.1
S. aureus	-	-	12mm	14mm	16mm	17mm
E. coli	-	-	-	8mm	8mm	9mm
P. aeruginosa	-	-	-	10mm	11mm	11mm
S. Typhimurium	-	-	8mm	12mm	16mm	17mm

In the case of Soxhlet method, inhibition zones of *S. aureus* by agar well diffusion method were more prominent and measured as 17mm diameter than conventional method inhibition zone that was measured 15mm, while a positive control showed inhibition zone of 24 and 26mm diameter zones of inhibition (Table 2).

Comparison of zones of inhibition of *M. piperita* leaf extract: When we compare the results of *M. piperita* leaf extract, which was prepared by both conventional and soxhlet method then *S.* Typhimurium, *S. aureus* showed highest zone of inhibition about 16mm in diameter towards peppermint leaf extract prepared by the Conventional method as shown in Table 1. This means these bacteria are more susceptible as compared to the other tested bacterial strains. While in case of leaf extract prepared by soxhlet method *S.* Typhimurium showed 18mm in diameter zone of inhibition among all the bacterial strains.

Comparison of zones of inhibition of *M. piperita* stem extract: When we compare the results of peppermint stem extract which was prepared by both conventional and soxhlet method. *P. aeruginosa* showed highest zone of inhibition about 17mm in diameter towards peppermint stem extract prepared by the conventional method as shown in Table 2. *P. aeruginosa* remained more susceptible as compared to the other tested bacterial strains while in case of stem extract prepared by soxhlet method, *Staph. aureus* showed highest zone of inhibition (17mm in diameter) among all the bacterial strains.

MIC determination of peppermint stem and leave extract by agar dilution method

MIC of peppermint stem extract by agar dilution method: Different concentrations of peppermint stem extract were used to determine the minimum inhibitory concentration of stem extract against tested bacterial strains. At first two dilutions, growth of all tested bacterial strains was inhibited completely on agar media of plant stem extract. This result showed that at the concentrations of 0.05 and 0.1mg/ml of peppermint stem extract, all the tested bacterial strains did not show any zone of inhibition and were resistant towards these concentrations of stem extract.

At 0.2 mg/ml concentration of stem extract there were 14 and 12mm diameter zones of inhibition against *S. aureus* and *S.* Typhimurium, respectively. However, *E. coli* and *P. aeruginosa* remained resistant to this concentration. There were 12mm zones of inhibition for both *E. coli* and *P. aeruginosa* when the concentration was increased to 0.4 mg/ml.

Further increase in concentration of stem extract to 0.6mg/ml showed 17mm zones of inhibition against *S. aureus* and *S.* Typhimurium. Finally, when the concentration was increased at a level of 1mg/ml then *S.* Typhimurium becomes much more susceptible to the stem extract and showed highest inhibition zone of 20mm and there was a zone of inhibition of about 12mm in diameter against *E. coli* (Table 3).

MIC determination of peppermint leaf extract by agar dilution method: Different concentrations of peppermint leaf extract were used to determine the minimum inhibitory concentration of leaf extract against tested bacterial strains. This result showed that at the concentrations of 0.05 and 0.1mg/ml of peppermint leaf extract, all the tested bacterial strains did not show any zone of inhibition and were resistant towards these concentrations of leaf extract.

The concentration leaf extract was increased to a level of 0.2 mg/ml, which produced zones of inhibition of 12 and 8mm against *S. aureus* and *S.* Typhimurium, respectively, while *E. coli* and *P. aeruginosa* were resistant at this concentration. When the concentration of leaf extract was 0.4mg/ml, there were zones of inhibition of 8 and 10mm against *E. coli* and *P. aeruginosa*, respectively. The concentration of seed extract was increased to 0.6mg/ml, which produced highest zones of inhibition of 16mm diameter against both *S. aureus* and *S.* Typhimurium.

The concentration of fruit extract at a level of 1mg/ml showed zones of inhibition of 17mm diameter against *S. aureus* and *S.* Typhimurium (Table 4).

DISCUSSION

The main role of antimicrobial agents is to lessen the burden of infectious diseases worldwide (Bhatia and Narain, 2010; Abou Youssef *et al*., 2018). Finding novel

antimicrobial drugs is therefore of utmost relevance in view of the evidence of the rapid global spread of resistant clinical isolates. Even new families of antimicrobial drugs will, however, have a brief shelf life given the history of the rapid, widespread evolution of resistance to recently introduced antimicrobial agents (Coates *et al.*, 2002).

In our investigation, *P. aeruginosa* had the best sensitivity with an inhibitory zone measuring 18mm in diameter when the *M. piperita* extract was made using the traditional approach. Additionally, when the extract was created using the Soxhlet apparatus method, both *E. coli* and *P. aeruginosa* had the same zone of inhibition, which was 24mm in diameter. Our research is consistent with that of Hammadi and Adnan (2021), who utilized various dilutions on four different species of harmful bacteria, including *S. aureus*, *S. pneumoniae, E. coli* and *K. pneumoniae*. By measuring the diameter of growth inhibition zones and tabulating the concentration that resulted, the agar well diffusion method was used to evaluate the Mentha's antibacterial activity. Inhibition zone for *E. coli* measured at 0.25mg per liter, 0.25mg per liter, and 0.25mg per liter, respectively, had diameters of 21, 17, and 9mm. Through the LSD value, it was determined that there were significant variations between the first three dilutions.

Like peppermint leaf extract made using the traditional approach, *S. aureus* displayed the largest zone of inhibition, measuring around 17mm in diameter. The maximum zone of inhibition was similarly given by *S. aureus* when the leaf extract was made using the Soxhlet method, and it measured about 17mm in diameter. This indicates that *S. aureus* grows increasingly susceptible to the Soxhlet-prepared peppermint leaf extract. According to quantitative and qualitative tests, peppermint extract's antibacterial efficacy significantly inhibits the growth of *S. aureus* and *Bacillus cereus* bacteria. Increasing the methanolic extract concentration increased the inhibitory effect. This study demonstrated that Gram-positive bacteria were substantially more sensitive to the inhibitory effects of peppermint methanolic extract than Gram-negative bacteria.

Our MIC values are consistent with those of Mahmoudi *et al.* (2019), the lowest inhibitory concentration of peppermint stem extract against the examined bacterial strains was established using various concentrations of the extract. On agar plates containing plant stem extract, the growth of all tested bacterial strains was totally suppressed after the first two dilutions. This finding indicates that all the tested bacterial strains are resistant to this dose of peppermint stem extract, which is present at concentrations of 0.05 and 0.1 mg/ml.

Both *E. coli* and *P. aeruginosa* become sensitive to this dose at a further rise in seed extract concentration. The maximum inhibition zone against *S. aureus* and *S.* Typhimurium was found to be approximately 16mm in diameter with further increase in seed extract concentration to 0.6mg/ml.

This study is consistent with Singh *et al*. (2015), who reported that Gram-positive bacteria are more sensitive to peppermint extract than Gram-negative bacteria; the diameter of non-growth for gram-negative bacteria like *E. coli* and *K. pneumonia* is 12.4 and 5.1mm, respectively, compared to 17.2 and 13.1mm for Gram-positive bacteria like *S. pyogenes* and *S. aureus* (Singh *et al.*, 2015). The current study also shows that *P. aeruginosa* is sensitive towards peppermint stem extract and *S. aureus* and *S.* Typhimurium are sensitive towards peppermint leave extract.

Conclusions: The traditional uses for *M. piperita* span a wide range of medical benefits and have a considerable healing impact. Topical applications of *M. piperita* leaf extracts may be used to treat various microbiological infections. Additionally, the susceptibility of certain microorganisms to various extracts varies. The *M. piperita* plant's extract has antibacterial action against *S. aureus, S.* Typhimurium, and *E. coli*, and after additional research on laboratory animals and its side effects, it can be utilized as an alternative medication. Finally, it can be said that *M. piperita's* chemical constituents should undoubtedly be used to treat a variety of bacterial illnesses. The present study's results are very positive and suggest further research on this herb in order to examine its potential for treating infectious disorders as well.

Authors contribution: SSR, MG and ARH conceptualized the work, SSS and AR planned and executed the study, SS, MG, ARH and TS proofread the manuscript and WB helped in the experiments, MG and ARH helped in the writeup of the manuscript.

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