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Evaluation of in vitro antibacterial activity and phytochemical screening of nigella sativa leaves extracts against human pathogenic bacteria

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***Abstract*---**Objective: The current study objective was to explore the antimicrobial activity and phytochemical screening (Alkaloids,

Flavonoid, Saponins, Phenolic compounds, terpenoid) of leaves extract of *Nigella sativa*. Study Design: Experimental study Place and duration of study: The current study was performed in Department Medical Laboratory Technology (MLT) at the University of Haripur. Haripur, Khyber Pakhtunkhwa, Pakistan from March 2021 to February 2022. Methods: Antimicrobial susceptibility test was done on Muller Hinton agar (MHA) using the disk diffusion method. Screening of *N. sativa* leaves extracts against Gram-positive bacteria Methicillin-resistant *Staphylococcus aureus (*MRSA) and Gram-negative bacteria *Pseudomonas aeruginosa*, *Salmonella species*, *Escherichia coli* and *Klebsiella pneumonia* were carried out using standard methods. Results: The results showed that ethanol, chloroform, hexane extract of nigella sativa had the best antimicrobial activity against *P. aeruginosa,* Methicillin-resistant *S. aureus*, *Salmonella species*, *E. coli* and *K. pneumonia.* Leaves extract of *N. sativa* were found to contain terpenoid, flavonoid, alkaloids, phenolic compounds except for saponins. *Nigella sativa* leaves showed good antibacterial activity against clinical multi-drug resistant bacteria. Conclusions: The results of this study provide useful bioactive compounds that show strong antibacterial activity against different multi drug resistant (MDR) including gram negative and gram positive bacteria.

***Keywords*---**Antibacterial activity*, K. pneumonia,* multi-drug resistant, *N. sativa, P. aeruginosa*.

Introduction

Medicinal plants above and beyond therapeutic agent are a huge source of evidence for a range of chemical components which may be established as effective drugs with accurate selectivity. These are the reservoir of potentially helpful chemical compound which could provide new clues and leads for current drug plan.(1) In developing countries, about 60-90% of the population use medicine derived from plants, by tradition crude extracts of plants are helpful as an herbal medicine for the treatment of most of the infectious diseases of Humans.[2] Now a day's investigators are focusing on medicinal flora since just a few plant genera have been comprehensively investigating their medicinal potential their properties, mode of action, toxicological studies, and safety considerations. *N. sativa* is among various medicinal plants since many scientists exposed its broad spectrum of pharmacological potential. It is usually well-known as black seed (Kalonji). *N. sativa* is indigenous such as; North Africa, Southwest Asia, and also in region of Southern Europe. Globally it is cultivated in various continents such as Syria, Turkey, India, Saudi Arabia, South Europe, Pakistan, Middle Eastern Mediterranean region.[3] Medicinal plants are the good source for pharmaceutical intermediates, conventional medicine, food supplements, advanced and folk medicines chemical, and usage in synthetic drugs. Plant yield derived from seeds, fruits, flowers, leaves, barks, and roots, are the component of phytomedicines.[4]

In prophetic hadith, *N. sativa* plant is considering one of the best forms of healing remedy available it has been mentioned that their black seeds are the best medication for the treatment of all diseases except death among Muslims. Due to its tremendous effects, it is also suggested for its use in our daily routine in Tibbe-Nabwi (S.A.W).[5]

N. sativa plant is flowering throughout the year grows up to 20-90 centimeter (cm) tall, had finely separated leaves, their leaf segment showing thread-like to barely linear structure. Their delicate flowers usually are of different colors like pink, purple, white, pale or pale blue, yellow, etc along with 5-10 petals. Fruit bears are inflated and large capsules along with the composition of 3-7 united follicles each contain various seeds.[6]

N. sativa has great medicinal significance and has been reported to show evidence of many pharmacological effects that include antibacterial, antiviral, antifungal, anti-parasitic ants-inflammatory, and anti-oxidant activities. The new anti-microbial compound shows great development aligned with different micro-organisms becoming significantly important, as contagious diseases which is considered as key mortality cause. Currently, the world interest in anti-microbial agents has been increased from the plant source due to the resistance against routinely used antibiotics developed by a variety of micro-organisms.[7]

In different countries, plants are a potential source of the anti-microbial agent. One of the major public health dilemmas in the global predominance of infectious disease caused by bacterial agents is emerging.[8] The most common bacterial agent that causes several severe Human infections including *E. coli, K. pneumonia, MRSA*, and *P. aeruginosa.*[9]

Medicinal plants, roots, leaves, and different vegetables have a powerful defense mechanism against different microbial agents that gives protection against various diseases due to the presence of naturally occurring phytochemicals in them. Phytochemicals contain common sugars, proteins, and chlorophyll as primary constituents while secondary components include alkaloids, phenolic compounds, and terpenoids.[10] The study aimed to investigate the antibacterial activity of *N. sative* leaves against different Gram-negative and positive bacterial pathogens and screening of important Phytochemicals in the leaves of *N. sativa*.

Materials and Method

Inclusion and Exclusion Criteria

We collected Plant material from “Agriculture Research Form” University of Haripur. A total of 54 plants were obtained randomly from the field and all are included in this study.

The current study was performed in Department of Medical Laboratory Technology (MLT) at the University of Haripur. Haripur, Khyber Pakhtunkhwa, Pakistan from March 2021 to February 2022. The study was approved by institutional ethical committee (UoH/MLT/2021/2025)

Plant material was provided by the “Agriculture Research Form” University of Haripur. A total of 54 plants were obtained randomly from the field. The leaves of each plant were separated. Each sample was washed with tap water and left to dry. Pestle and mortar was used for mashing and 3.13-gram powder of each sample was added into 125 mL of chloroform, hexane, and ethanol (solvents) respectively. These diluted samples were incubated in a rotary shaking incubator for 21 days. Samples were filtered with sterile filter paper and this filtered extract was subjected to the rotary evaporator for evaporation on 125 rpm at 44 ℃ and then transferred into sterile falcon tubes. After that 250 mg of each extract was added into 100 mL of dimethyl sulphoxide (DMSO) achieving a concentration of 25 mg/100 mL and the rotary shaker was used at 125 rpm for 1 hour to attain a homogeneous mixture.

Mueller-Hinton agar (MHA) was prepared according to manufacturer guidelines and performs sterilization was performed at 121 ℃ for 15 minutes in an autoclave. For antibacterial testing MDR, clinical isolates were used in this study, which includes (Gram-positive bacteria MRSA, and Gram-negative bacteria *P. aeruginosa, Salmonella* spp. *E. coli*, and *K. pneumonia*. All the isolates were identified using standard microbiological procedures. MDR was defined as no susceptibility towards one agent in three or more than three antimicrobial classes.[11]

An antimicrobial susceptibility assay was done to check the ability of the antimicrobial compound to inhibit bacterial growth in vitro. Antimicrobial susceptibility test was done using the Muller Hinton agar with the help of the disk diffusion method. After 24-hour incubation, inhibition zone was noticed and properly measured. After pouring of 30 mL of sterile molten agar medium into disposable Petri plates, allowed them for solidification. With the help of a pipette about 300-400 µL, bacterial strains suspension was placed on the media, and the suspension was spread by a glass spreader throughout the plate. Sterile borer was used to prepare the bores on the medium and 0.1 mL of the extract was introduced to the respective bore. The plates were placed in the refrigerator at 4 ℃ containing organisms to facilitate the diffusion of the extract and incubated at 37 ℃ for 24 hours using standard methodology by the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2012). The zone of inhibition was observed on the next day. Screenings of the leaves of *N. sativa* for various phytochemical constituents were carried out using standard methods.

Phytochemical screenings of crude extracts were performed to find out the existence or absent of bioactive molecule like alkaloids, saponins, terpenoids, flavonoids, etc. The extract was dissolved in 2N HCL solution for the presence of Alkaloids. Further added 3 to 4 drops of Mayer’s reagent to this mixture. Mayer’s reagent was prepared by mixing 3 mL of potassium iodide solution and 2 mL mercuric chloride solution. The presence of creamish precipitate was an indicator of the alkaloid’s presence. Five mL distilled water along with the extracts was added to a test tube. Then added 3-4 drops solution of neutral 5% ferric chloride. Dark green was the indicator of phenolic compounds. Saponin's presence was confirmed with the help of the frothing test. In this test, the leave extract was shaken vigorously in distilled water and leaves for 10 min. The saponin presence was detected by the formation of stable emulsion. Extract of about 1 mL was

added with a few drops of 20% NaOH solution in the test tube. The yellow color was observed on the addition of an acid colorless solution depicting the flavonoids availability. One mL extract was mixed thoroughly with chloroform of 2 mL, followed by the addition of 3 mL concentrated H_2SO_4. A precipitate of reddish-brown in color at the interface shows the terpenoids presence.

Data Analysis

There is no such frequency or percentages were used in this study so simply used Microsoft Excel sheet

Results

This study results showed that ethanol, chloroform, hexane extract of *N. sativa* had the best antimicrobial activity against *P. aeruginosa,* MRSA, *Salmonella* spp., *E. coli*, and *K. pneumonia.* Dimethyl sulfoxide (DMSO) was used as a negative control to check accuracy of the experimental work. The different Zones of inhibition in millimeters (mm) were shown in Table 1 and Table 2 against MRSA, *Salmonella* spp, *E. coli*, *P. aeruginosa*, and *K. pneumonia.* The inhibition effect of leaves extract of *N. sative* against study isolates was shown in (Figure-1).

Table-1: Zone of inhibition shown against MRSA, *Salmonella* spp, *E. coli*, *P. aeruginosa*, and *K. pneumonia*

Zone of inhibition in mm Isolates No	MRSA, *Salmonella* spp, *E. coli*, *P. aeruginosa*, and *K. pneumonia.*		
S 1	Chloroform	Hexane	Ethanol
Salmonella	18 mm	16 mm	21 mm
MRSA	13 mm	14 mm	14 mm
E. coli	16 mm	13 mm	16 mm
P. aeruginosa	17 mm	14 mm	17 mm
K. pneumoniae	11 mm	10 mm	13 mm
S 2	Chloroform	Hexane	Ethanol
Salmonella	20 mm	18 mm	20 mm
MRSA	15 mm	16 mm	12 mm
E. coli	27 mm	15 mm	16 mm
P. aeruginosa	28 mm	21 mm	23 mm
K. pneumoniae	23 mm	16 mm	12 mm
S 3	Chloroform	Hexane	Ethanol
Salmonella	23 mm	19 mm	21 mm
MRSA	16 mm	14 mm	14 mm
E. coli	17 mm	15 mm	16 mm
P. aeruginosa	18 mm	21 mm	23 mm
K. pneumoniae	13 mm	16 mm	12 mm
S 4	Chloroform	Hexane	Ethanol
Salmonella	16 mm	17 mm	13 mm
MRSA	13 mm	11 mm	14 mm
E. coli	18 mm	21 mm	21 mm
P. aeruginosa	16 mm	12 mm	14 mm

K. pneumoniae	15 mm	13 mm	17 mm
S 5	Chloroform	Hexane	Ethanol
Salmonella	22 mm	21 mm	20 mm
MRSA	17 mm	17 mm	11 mm
E. coli	13 mm	17 mm	14 mm
P. aeruginosa	15 mm	19 mm	21 mm
K. pneumoniae	14 mm	15 mm	14 mm
S 6	Chloroform	Hexane	Ethanol
Salmonella	16 mm	17 mm	13 mm
MRSA	17 mm	20 mm	15 mm
E. coli	22 mm	18 mm	21 mm
P. aeruginosa	15 mm	17 mm	20 mm
K. pneumoniae	18 mm	21 mm	20 mm
S 7	Chloroform	Hexane	Ethanol
Salmonella	14 mm	16 mm	19 mm
MRSA	18 mm	13 mm	11 mm
E. coli	18 mm	18 mm	16 mm
P. aeruginosa	15 mm	14 mm	14 mm
K. pneumoniae	19 mm	21 mm	17 mm
S 8	Chloroform	Hexane	Ethanol
Salmonella	20 mm	16 mm	14 mm
MRSA	18 mm	21 mm	19 mm
E. coli	18 mm	18 mm	16 mm
P. aeruginosa	17 mm	14 mm	14 mm
K. pneumoniae	18 mm	21 mm	16 mm
S 9	Chloroform	Hexane	Ethanol
Salmonella	20 mm	11 mm	13 mm
MRSA	14 mm	17 mm	12 mm
E. coli	11 mm	12 mm	10 mm
P. aeruginosa	12 mm	16 mm	18 mm
K. pneumoniae	14 mm	13 mm	16 mm
S 10	Chloroform	Hexane	Ethanol
Salmonella	16 mm	17 mm	18 mm
MRSA	14 mm	12 mm	16 mm
E. coli	18 mm	18 mm	18 mm
P. aeruginosa	19 mm	13 mm	15 mm
K. pneumoniae	14 mm	13 mm	12 mm

S= Sample

Table-2: Phytochemical evaluation of leave extract

Phytochemicals	Observation	Results		
S 1		Ethanol	Hexane	Chloroform
Alkaloid	Cremish precipitate	Positive	Positive	Positive
Saponin	Emulsion form	Negative	Negative	Negative
Flavonoid	Yellow color	Positive	Positive	Positive
Terpenoid	Reddish-brown precipitate	Positive	Positive	Positive
Phenolic compound	Dark green color	Positive	Positive	Positive
S 2		Ethanol	Hexane	Chloroform

Alkaloid	Cremish precipitate	Positive	Positive	Positive
Saponin	Emulsion form	Negative	Negative	Negative
Flavinoid	Yellow color	Positive	Positive	Positive
Terpenoid	Reddish-brown precipitate	Positive	Positive	Positive
Phenolic compound	Dark green color	Positive	Positive	Positive
S 5		Ethanol	Hexane	Chloroform
Alkaloid	Cremish precipitate	Positive	Positive	Positive
Saponin	Emulsion form	Negative	Negative	Negative
Flavinoid	Yellow color	Positive	Positive	Positive
Terpenoid	Reddish-brown precipitate	Positive	Positive	Positive
Phenolic compound	Dark green color	Positive	Positive	Positive
S 6		Ethanol	Hexane	Chloroform
Alkaloid	Cremish precipitate	Positive	Positive	Positive
Saponin	Emulsion form	Negative	Negative	Negative
Flavinoid	Yellow color	Positive	Positive	Positive
Terpenoid	Reddish-brown precipitate	Positive	Positive	Positive
Phenolic compound	Dark green color	Positive	Positive	Positive
S 7		Ethanol	Hexane	Chloroform
Alkaloid	Cremish precipitate	Positive	Positive	Positive
Saponin	Emulsion form	Negative	Negative	Negative
Flavinoid	Yellow color	Positive	Positive	Positive
Terpenoid	Reddish-brown precipitate	Positive	Positive	Positive
Phenolic compound	Dark green color	Positive	Positive	Positive
S 8		Ethanol	Hexane	Chloroform
Alkaloid	Cremish precipitate	Positive	Positive	Positive
Saponin	Emulsion form	Negative	Negative	Negative
Flavinoid	Yellow color	Positive	Positive	Positive
Terpenoid	Reddish-brown precipitate	Positive	Positive	Positive
Phenolic compound	Dark green color	Positive	Positive	Positive
S 9		Ethanol	Hexane	Chloroform
Alkaloid	Cremish precipitate	Positive	Positive	Positive
Saponin	Emulsion form	Negative	Negative	Negative
Flavonoid	Yellow color	Positive	Positive	Positive
Terpenoid	Reddish-brown precipitate	Positive	Positive	Positive
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S 10		Ethanol	Hexane	Chloroform
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Flavonoid	Yellow color	Positive	Positive	Positive
Terpenoid	Reddish-brown precipitate	Positive	Positive	Positive
Phenolic compound	Dark green color	Positive	Positive	Positive

S= sample

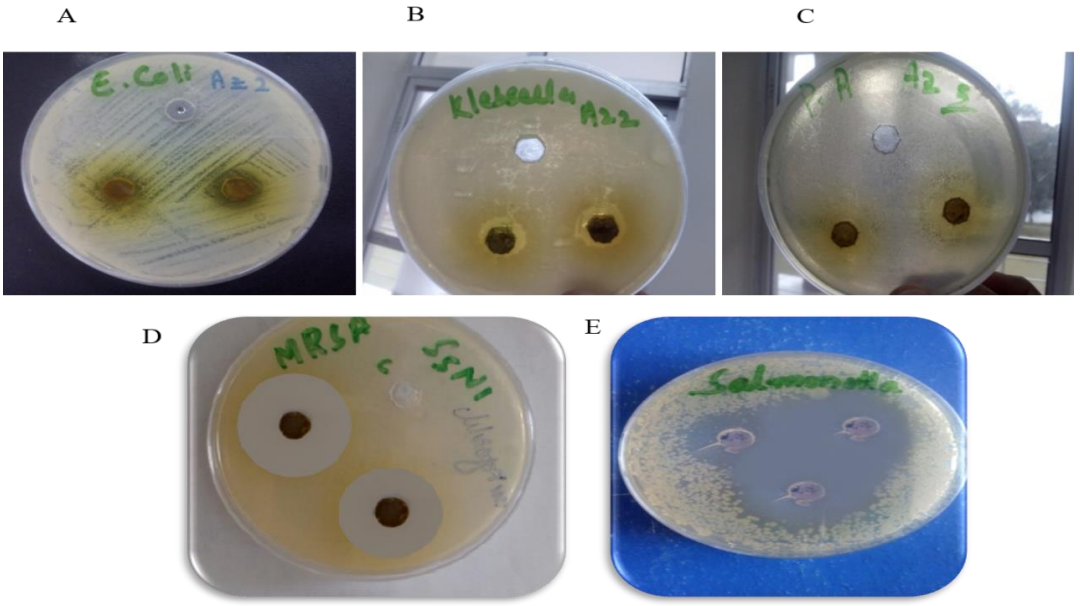

Figure-1: Zone of inhibition shown by leaves extract of *N. sativa* against (A) *E. coli,* (B) *K. pneumoniae,* (C) *P. aeruginosa,* (D) methicillin-resistant *S. aureus* (MRSA), (E) *Salmonella*

Approximately 10 samples of leaves extract of *N. sativa* were observed to detect presence of bioactive secondary metabolites. The screening of leaf extracts of *N. sativa* based on phytochemical tests. These tests indicate the presence of various bioactive compounds that might be responsible for bacterial growth inhibitors for their medical aspects. The observations made in phytochemical tests are shown in Table-2. Phytochemical evaluation of extract of *N. sativa* leaves was shown in Figure-2.

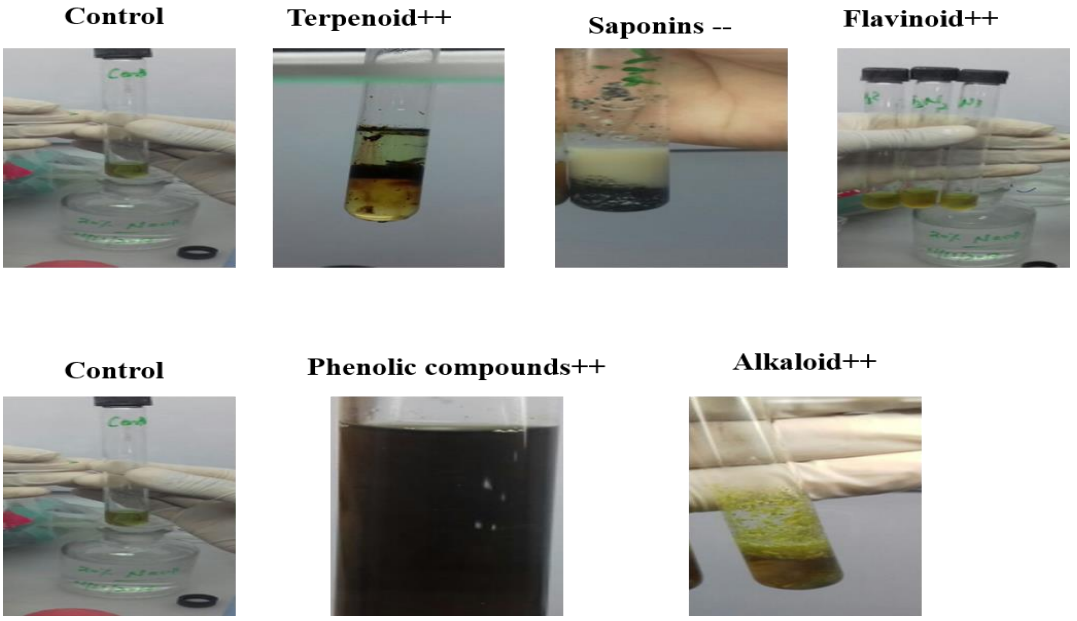

Figure-2: Phytochemical screening extract of *N. sativa* leaves

Alkaloid: Cremish precipitate was observed in leave extract of *N. sativa*, contained in a solution of organic compounds (Ethanol, Hexane, Chloroform). Indicate a positive result. **Saponins:** Saponins were observed negative in leaves extract of *N. sativa*, contain in the solution of organic compound (Ethanol, Hexane, Chloroform). There's no stable emulsion form in leaves extract. **Flavonoid:** Yellow color was observed in leaves extract of *N. sativa* contain in the solution of (Ethanol, Hexane, Chloroform). Indicate the positive result. **Phenolic compound:** Dark green color was observed in leaves extract of *N. sativa* contain in a solution of (Ethanol, Hexane, Chloroform). Indicate a positive result. **Terpenoid:** Reddish-brown precipitate was observed in leaves extract of *N. sativa* contain in a solution of (Ethanol, Hexane, Chloroform). Indicate a positive result.

Discussion

N. sativa belong to herb Family known as Ranunculaceae, considered as a marvel with an annoying religious and historical background because of its inclusive range of pharmacological potential.[3] The worldwide spread of infectious diseases caused by bacterial pathogens poses a serious threat to public health.[12] Antibacterial activity of black seed and its crude extract has been demonstrated previously.[13] The seed of black cumin comprises active compounds including tannins, terpenoids, saponins, and alkaloids. Black cumin seeds class of alkaloid is alkaloids isochinolin, pyrazol alkaloids, and active substances such as anti-microbial function.[14]

The mechanism of action of Alkaloids is interferes with DNA, establishing bacterial cellular activities and as a result lysis of bacteria cell occurred.[15] The Cytoplasmic membrane lysed by Saponin; disturb cell organelle and bacterial cell permeability.[16] Other than that, saponins also inhibit the DNA replicating

enzymes (DNA-polymerase) and result in interfering in DNA synthesis.[17]. This study demonstrated that *N. sativa* leaves extract has inhibitory and lethal effects against pathogenic bacteria, against *P. aureginosa, K. pneumoniae, E. coli, Salmonella species,* and MRSA. One study from Malaysia reported that black cumin seed extract is effective against Gram-positive and negative bacteria.[18], as this study is supporting our observation. In the current study, Ethanol, Chloroform, and hexane extract possess antibacterial activity against *P. aureginosa, K. pneumonia, E. coli, Salmonella species,* and MRSA. This antibacterial activity is due to the presence of terpenoid, flavonoid, phenolic compounds, and alkaloid. Previous studies support our current study findings[19-21] as the aqueous extract of *N. sativa* antibacterial activity.

Previous study from Pakistan[22], showed the result in disconcordance where *Nigella* extract in methanol against MRSA was ineffective. Another previous study reported the activity of medicinal plants against some infectious organisms while ineffective to some others.[23] These studies findings suggest that variation in results may be due to harvesting conditions, geographical region, chemical constituents, and various extraction techniques. In this study, the hexane portion shown a high antibacterial activity against gram negative bacteria. The inhibition zone from 12.6 mm for *K. pneumoniae* to 18.3 mm for both *Enterobacter* and *E. coli.* While ethanol fraction had more significant potential activity against MRSA which is 22.3 mm in comparing with hexane and chloroform fractions that more effect on *E. coli*, *K. pneumonia* and *Enterobactoer.* Methanol have more inhibition growth activity against *K. pneumonia* and MRSA that had inhibition zone 10 $\pm$ 2.9 and 14.3 mm respectively. A similar finding was observed previously.[22] Our study demonstrates that by using different extraction methods different phytochemicals fractionate were obtained which show diverse antimicrobial activity. These need to be confirm further by their phytochemical analysis.

Conclusion

This study noticed various secondary metabolites in the extract of *N. sativa* leaves. The leave extracts could be used for the treatment of complicated infections, and as some new potent antimicrobial agents.

Acknowledgement

Department of Medical Laboratory Technology (MLT) at the University of Haripur. Haripur, Khyber Pakhtunkhwa, Pakistan

Limitation Of Study

Our study demonstrates that by using different extraction methods different phytochemicals fractionate were obtained which show diverse antimicrobial activity. These need to be confirm further by their phytochemical analysis. Qualitative phytochemicals study need to be done.

Conflict of Interest: None

Author's Contribution

AB: Substantial contributions to the conception or design of the work; the acquisition, analysis, or interpretation of data for the work; Drafting the work. Agreement to be accountable for all aspects of the work in ensuring, **FAS; AA; GH; ER:** Substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data, **IR:** Drafting the article or revising it critically for important intellectual content. Final approval of the version to be published.

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