Original Research Article

Evaluation of Bactericidal and Fungicidal efficacy of *Strychno spotatorum* Linn.(Nirmali) seeds.

ABSTRACT

Aim: Diseases due to pathogenic microbes pose a great burden on human health and they have been correlated with socioeconomic, environmental, ecological factors. The threat due to infectious diseases is further intensified by the continued emergence of new and multidrug resistant microorganisms. This scenario warrants a continuous search for antimicrobial agents preferably of plant origin due to their availability, accessibility and affordability. The present study was aimed to evaluate the antibacterial and antifungal properties of *Strychnos potatorum linn* (Nirmali) seeds using common pathogenic bacterial and fungal strains.

Methodology

Fresh and matured *S. potatorum* seeds were used for the present study. The powdered seeds were delipidated with petroleum ether (60-80°C) overnight and the extract was filtered. Soxhalation was performed with 95% ethanol to extract the phyto-ingredients from the seeds. Four gram positive, four gram negative and eight fungal strains were used. The antimicrobial activity was evaluated by disc diffusion and well diffusion methods. The Minimum inhibitory concentration (MIC) Minimum bactericidal concentrations (MBC), Minimum fungicidal concentration (MFC) were assayed.

Results

The data obtained through the disc diffusion, well diffusion, the minimum bactericidal concentration and minimum fungicidal concentrations revealed that the ethanolic extract of the seeds possesses significant antibacterial and antifungal activities. The results obtained were comparable with standard drugs widely prescribed for antimicrobial therapy.

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Conclusion

The present study provides thescientific rationale for the use of *Strychnos potatorum* seeds in traditional medicine and a rich source of phytochemicals having significant antimicrobial activities.

Key Words: *Strychnos potatorum,* antimicrobial activity, Minimum inhibitory concentration, Minimum bactericidal concentrations, Minimum fungicidal concentration.

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1. INTRODUCTION:

From the plaques of biblical times to the COVID 19 pandemic of today, infectious diseases have played an unquestionably major role in human life. A disease that occurs through the invasion of a host by a foreign agent whose behavior harms or impairs the normal functioning of the host's system is termed as infectious diseases [1,2 &3].Several factors have been implicated in the etiology of such diseases, including increasing population, the prevalence of immunosuppressive diseases, poverty, malnutrition, social practices, unplanned urbanization, lack of awareness, increased domestic and global connectivity and illiteracy [4]. Genetic alterations in pathogens have also been found to be responsible for such outbreaks to a significant extent [5].The pathogens that can infect humans and other animals may be broadly classified on epidemiological grounds into micro and macroparasites[6]. Micro parasites include viruses, bacteria, protozoa and fungi; they have the ability to reproduce directly within the individual host, their small size, relatively short duration of infectious and theyprovoke an immune response in infected and recovered individuals. However, the macroparasites lean to produce a limited immune response in infected hosts, relatively long-lived and often visible to the naked eye.

All infectious diseases either newly emerging or remerging represent a continued threat to humanity. Some pathogens, after a period of quiescent, are capable of acquiring features that enable them to reemerge their original or new hosts, usually in increasingly alarming proportions [7,8&9]. Hence the re-emerging diseases are often more pathogenic and may cause immeasurable harm in new geographic locations after apparent control [10,11].Above all, there are some pathogens whose emergence is as a result of deliberate human action. These are those engaged as biological weapons for the destruction and so their materialization is "deliberate"[12,13]. The emerging problem of multidrug resistance in pathogens to the existing drugs has made it essential to search for novel antimicrobial agents with maximum efficacy

at a low dose and without toxicity.Plants contain numerous biologically active compounds many of which form the basis for novel antimicrobial agents. Earlier, we have reported the antimicrobial properties of several medicinal plants [14-16].*Strychnos potatorum* Linn, commonly known as Nirmali is an important medicinal plantthat belongs to the family *Loganiaceae [17]*.Sanskrit writings mentioned in Sushruta Samhita from India reported that the seeds of *Strychnospotatorum* were used to clarify turbid surface water over 4000 years ago [18]. It is a moderate sized glabrous deciduous tree widely distributed in the forests of India, tropical African countries, Sri Lanka and Burma. In folklore medicine, the seeds were used for the treatment of various ailments including infectious diseases.However, the seeds lack scientific scrutiny for their antimicrobial properties. The present study is aimed to evaluate the antimicrobial properties of Nirmali seeds using common pathogenic bacterial and fungal strains.

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2. MATERIALS AND METHODS

Plant Material

Fresh and matured *S. potatorum* seeds were procured from an authorized traditional medical shop in Chennai and authenticated by a qualified taxonomist in the Centre for Advanced Studies in Botany, University of Madras, and the voucher specimen was deposited in the departmental herbarium (No.BC-CS-SP-1). The seeds were shadow dried and coarsely powdered to obtain a 40 mesh range and were stored in an airtight brown container at 5°C until further use. The powdered seeds were delipidated with petroleum ether (60-80°C) overnight and the extract was filtered. Soxhalation was performed with 95% ethanol to extract the phyto-ingredients from the seeds. The extract was separated by filtration and concentrated on a rotary evaporator at 40-50°C under reduced pressure and the brownish-yellow coloured semi-solid mass obtained was dried under vacuum. The yield was around 21% of dry weight.

Bacterial and Fungal strains and growth medium

The bacterial and fungal strains used for the present study were all standard laboratory strains obtained from the stock cultures of the Division of Microbiology, University of Madras and maintained on slopes of Muller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) at 28°C.Four Gram positive bacteria (*Staphylococcus aureus, Lactobacilus bulgaricus, Candida lambica* and *Bacillus cereus* and four Gram negative bacteria (*Escherichia coli, Vibrio cholera, Enterobacter aerogenes* and *Shigella dysenteriae* were used in the present study. The fungal cultures used for the present study include *Candida albicans,*

Saccharomyces cerevisiae, Microsporumcanis, Aspergillus flavus, Rhizopus nodousus, Chrysosporium tropicum, Penicillium chrysogenum and *Penicillium notatum.*

Determination of antibacterial and antifungal activity

Preparation of inoculum

The suspension for inoculation was prepared from the broth culture. Few colonies of similar morphology of the respective bacteria were transferred with the help of a sterile inoculating loop to a Muller-Hinton broth and were incubated until adequate growth of turbidity equivalent to McFarland 0.5 turbidity standard (10^8 Colony Forming Unit (CFU)/ml) were obtained.

The fungal strains were sub cultured on slants of Sabouraud Dextrose Agar at 28°C for 7 days and the colonies were suspended in 1 ml of sterile normal saline. The resulting mixture of conidia and hyphal fragments was vortexed and the turbidity of each homogenous suspension was adjusted to match that of a 0.5 McFarland standard, as read at 530 nm. At this turbidity, the fungi density was 3×10^6 to 5×10^6 CFU $ml^{-1.}$

Preparation of the McFarland standard

0.5 ml of 0.048M $BaCl_2$ was added to 99.5 ml of 0.18M H_2SO_4 with constant stirring. The standard was distributed into screw- cap tubes of the same size and with the same volume as those used in growing the broth culture. The tubes were sealed to prevent loss by evaporation. The tubes were protected from light and stored at room temperature. The turbidity standard was agitated vigorously on a vortex mixture before use.

Antibacterial activity of the ethanolic extract of *S.potatorum* seeds was determined by the agar well diffusion method[19]. The inocula with respective test bacteria were homogenously seeded onto the 90mm Petri dishes containing 20 ml of cooled molten MH agar medium using a sterile swab in such a way as to ensure thorough coverage of the Petri dishes and a uniform thick lawn of growth following incubation [20]. Wells were dug in the medium with the help of a sterile cork borer. The stock solution of the seeds extract (2.5 mg/ml) was prepared in sterile distilled water. Dilutions of the stock solution containing 50, 100, 150, 200 and 250 µg were also prepared in sterile distilled water. 100 µl of each dilution was added to their respective wells with a sterile pipette. Control wells received only 100 µl of sterile distilled water. The plates were kept for 1 h at room temperature for the diffusion of the extract into the agar. Subsequently, all the plates were incubated at 37°C for 18-24 h. Following incubation, the plates were examined for signs of microbial growth. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the wells. Chloramphenicol (30 µg/ml) was used as a positive control and each experiment was performed in triplicates.

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Antifungal activity of the ethanolic extract of *S. potatorum* seeds extract was evaluated by disc diffusion method. The inocula with respective fungi were homogenously seeded onto the 90mm Petri dishes containing 20 ml cooled molten SDA medium using sterile swab in such a way as to ensure thorough coverage of the plates and a uniform lawn of growth following incubation. These inoculated plates were left to dry for at least 15 min. The extract was dissolved in sterile distilled water to obtain the different concentrations of 300, 150, 75, 37.5 and 18.75 mg ml^{-1}. Amphotericin B at concentration 10 µg/disc was used as positive control and was dissolved in dimethyl sulphoxide (DMSO).DMSO was used as a negative control. Sterile filter paper disc (6mm in diameter) were impregnated with 10 µl of each different concentration of seeds extract. The discs were allowed to dry and then placed on the agar surface of each petri dish. Zone of inhibitions (in mm) was measured after 48-72 h at 28ºC. The antifungal analysis was carried out under strict aseptic conditions and each assay was repeated three times.

Minimum inhibitory concentration (MIC) Minimum bactericidal concentration (MBC), Minimum fungicidal concentration (MFC) assays

A serial of 2-fold macro-broth dilution method was performed to determine the MICs and MBCs of *S. potatorum* seeds extract for the respective tested bacterial suspensions (concentration) as recommended by the Clinical and Laboratory Standards Institute (CLSI) [21]. The minimum inhibitory concentration (MIC) of *S. potatorum* seeds extract against fungal strains was determined using the broth microdilution method as described by the National Committee for clinical laboratory standards for fungi (M27-A2). The stock solutions of *S.potatorum* seeds extract were diluted suitably as required from the stock solution. The ranges should be prepared one step higher than the final dilution range required that if a final dilution range of 0.5, 1, 2, 4, 8, and 16 mg/ml is required then a range of 1, 2, 4, 8, 16 and 32 mg/ml should be prepared to compensate for the addition of an equal volume of inoculums.

Two rows of 12 capped test tubes were arranged in the rack. In a sterile 30 ml (universal) screw capped bottle, 8 ml of MH broth (bacteria), 8ml SD broth (fungi) containing the required concentration of *S.potatorum* seeds extract for the first tube in each row was prepared from the appropriate stock solution already made. The contents of the universal bottle were mixed using a sterile pipette and transferred 2 ml to the first tube in each row. Using a fresh sterile pipette, 4 ml of broth was added to the remaining 4 ml in the universal bottle, mixed well and transferred 2 ml to the second tube in each row. Dilutions were continued in this way to as many as 10 tubes. 2 ml of broth free from *S. potatorum* seeds extract was added to the last tube in each row. The density of the bacterial suspension was adjusted (10^8 CFU/ml) to equal that of the 0.5 McFarland standard by adding sterile distilled water as detailed above. The bacterial

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suspension was suitably diluted (10^6 CFU/ml) and added to the tubes containing MH broth. The density of the fungal suspension was adjusted (3×10^6 to 5×10^6 CFU ml-1) to equal that of the 0.5 McFarland standard by adding sterile distilled water as detailed above. Chloramphenicol (30 µg) was used as a positive control for bacteria. After incubation at 37ºC for 24 h, the turbidity of the tubes was assessed visually by comparison to uninoculated control. Amphotericin B was included in the assays as positive control 10 µg/disc for fungi. After incubation at 28ºC for 42-78 h, turbidity of the tubes was assessed visually by comparison to uninoculated control.

The MIC is expressed as the lowest concentration of the seeds extract where bacterial or fungal growth with no visible growth after incubation. All assays were carried out in triplicates. The MBC was derived by sub-culturing 100 µl from each tube from the MIC assay onto substance free MH agar plates. The plates were incubated at 37ºC for 24 h and the MBC was defined as the lowest concentration of the substance that allows no visible growth on the agar plate. The MFC was determined by plating a 100 µl volume on SDA from the tubes showing no visible growth. The plates were incubated as described above in MIC. The MFC was defined as the lowest concentration of the substance that did not allow any visible growth on the agar plate.

DETERMINATION OF ANTIMICROBIAL ACTIVITY

The antibacterial activity of ethanolic extract of *S.potatorum* seeds was tested against four Gram positive and four Gram negative bacteria. The inhibitory effect was assessed by the well diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also determined by the serial dilution method.The antifungal properties of ethanolic extract of *S.potatorum* seeds were tested against common pathogenic fungal strains. The inhibitory effect was assessed by the disc diffusion method. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were also determined by the serial dilution method.

3.RESULTS AND DISCUSSION

The antimicrobial agents have distinctive modes of action against various microbes. The mechanism action of antimicrobial agents generally falls under the following: Inhibition of cell wall or nucleic acid synthesis, impairment of cell membrane or ribosome function and inhibition of folate metabolism. Though antimicrobials are capable of causing the lysis of microbes, their efficacy is often compromised by a growing number of antibiotic resistant pathogens either by intrinsic or acquired resistant mechanisms. Additionally, the existence of periplasm, the space between the outer membrane and the cytoplasmic membrane in gram negative bacteria provided an additional protection from the antimicrobial agents. The

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efficacy of an antimicrobial agent is determined by their structure and affinity towards the target sites in the host cells.

The increase in the multidrug resistance of pathogenic microorganisms to time-honored antibiotics necessitates a continuous search for alternative strategies preferably from the plant origin due to their availability, accessibility, affordability efficacy, stability and safety. They are claimed to display synergistic, efficacious and agonistic/antagonistic actions at a relatively less concentration.Plants synthesize a variety of secondary metabolites such as flavonoids, alkaloids, steroids, pectins, anthroquinones and tannins to protect them against the environmental stress such as UV radiation, pollution, high temperature, extreme cold, drought, flood, tissue damage and microbial attacks [22]. However, these phytochemicals are known to elicit significant pharmacological and beneficial effects which have long been of interest to mankind [23].

Most of the currently available drugs for the treatment of various human ailments were originally derived from medicinal plants. Most of our marketed medicines are distillations, combinations, reproductions or variations of substances found in medicinal plants. Our forefathers recommended some of these medicinal plants long before their medicinal value was demonstrated and understood by scientific method. One way to prevent antibiotic resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents [24]. However, only a small percentage has been systematically studied for their antimicrobial activities [25]. Although screening of Indian medicinal plants has revealed varying degrees of antimicrobial activity against pathogenic and opportunistic microorganisms, there is still a lack of experimental scientific studies confirming the possible antimicrobial properties of a great number of these remedies [26,27].

Antimicrobial resistance is a natural biological phenomenon elicited by microbes to the selective pressure of an antimicrobial drug [28]. The ultimate goal is to offer appropriate and efficient antimicrobial drugs to the patient [29]. The use of *S.potatorum* seeds in traditional medicine in one form or the other necessitates a systematic evaluation of its antibacterial as well as antifungal activities.

Table1: Antibacterial activity of Strychnos potatorum seeds extract- Zone of inhibition in diameter (mm).

S. No.	Bacterial species	Control	50 µg	100 µg	150 µg	200 µg	250 µg	Streptomycin (10mg/ml)
	Gram Positive							
1.	*Staphylococcus aureus*	-	2.8	7.0	10.0	16.0	21.0	23

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2	*Lactobacilus bulgaricus*	-	3.2	10.0	15.5	18.0	20.0	24
3	*Candida lambica*	-	3.4	7.5	15.0	17.0	20.5	23
4	*Bacillus cereus*	-	3.8	9.0	16.5	23.0	25.0	26
Gram Negative								
5	*Escherichia coli*	-	2.5	7.0	13.0	20.5	21.5	22
6	*Vibrio cholerae*	-	3.8	9.5	15.0	22.5	25.0	25
7	*Enterobacter aerogenes*	-	1.5	10.2	17.0	24.5	22.0	26
8	*Shigellady senteriae*	-	1.0	6.0	10.0	12.0	17.0	20

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Table 1 shows the antibacterial activity of ethanolic extract of *S.potatorum* against four different Gram positive and Gram negative bacterial strains. The antibacterial potency of *S.potatorum* extract was evaluated by the presence or absence of inhibition zones and zone diameters (mm).The results of the present study suggested that the ethanolic extract of *S.potatorum* seeds have shown a maximum inhibitory zone in a dose dependant manner. However, there was no significant difference between the levels of zone of inhibition at the concentration of 200 µg and 250 µg. Among the Gram positive bacteria,*B. subtilis* showed a larger diameter of clearance than that of other Gram positive bacteria used in the present study. Among the Gram negative bacteria, *Vibrio cholera showed a larger* than that of other Gram negative bacteria. The zone of clearance achieved by *S.potatorum*seeds extract is comparable to that of standard antibiotic, chloramphenicol.

Table 2: MICs and MBCs of Strychnos potatorum seeds extract on Gram positive and Gram negative bacteria.

	Minimum Inhibitory Concentration (MIC)	**Minimum Bactericidal Concentration (MBC)**

Bacterial species	***Strychnos potatorum seeds* extract (mg/ml)**	**Chloramphenicol (µg/ml)**	***Strychnos potatorum seeds* extract (mg/ml)**	**Chloramphenicol (µg/ml)**
Gram positive				
Staphylococcus aureus	4.5	3	4	3
Lactobacilus bulgaricus	5	2	3	5
Candida lambica	3.5	4	4	4
Bacillus cereus	5	3	2	3
Gram negative				
Escherichia coli	3	3	3	7
Vibrio cholerae	4	2	5	3
Enterobacter aerogenes	4.5	4	7	5
Shigella dysenteriae	2	3	4	2

The minimum inhibitory concentration and minimum bactericidal concentration of *S.potatorum* seeds extract as well as the standard antibiotic, chloramphenicol is shown in Table 2. The MIC value of *S.potatorum*seeds extract against both Gram positive and Gram negative bacterial strains varies from 2 mg to 5 mg and the results are comparable with the standard antibiotic, chloramphenicol. The highest MIC values were shown by *Lactobacilus bulgaricus* in Gram positive bacteria and by *Enterobacter aerogenes* in gram negative bacteria. The lowest MIC values were displayed by *Candida lambica* in Gram positive bacteria and *Shigella dysenteriae* in gram negative.

The results of the study indicated *S.potatorum*seeds extract showed siginificant inhibitory activity against Gram-positive bacteria, *Bacillus subtilis and* gram negative bacteria *Vibrio cholera. B. subtilis* showed a larger diameter of clearance than that of other Gram positive bacteria used in this study. Similarly, *S.potatorum* seeds extract showed a maximum zone of clearance in the Gram negative bacteria, *Vibrio cholera* than that of other Gram negative bacteria.

Minimum inhibitory concentrations are considered the “gold standard” for evaluating the susceptibility of microorganisms to antimicrobials and therefore used to assess the credentials of all other methods of susceptibility testing [30]. A lower MIC value indicates that less drug is required for inhibiting growth of the organism; therefore, antimicrobials with lower MIC values are considered as effective antimicrobial agents. The highest MIC and MBC values were shown by *Lactobacilus bulgaricus* in Gram positive bacteria and by *Candida lambica* in gram negative bacteria. The lowest MIC and MBC values were displayed by *Shigella dysenteriae* in Gram negative bacteria and *Bacillus cereus* in gram positive.

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S. No.	Strains	Contro l	0.175 mg/disc	0.375 mg/disc	0.75 mg/disc	1.5 mg/disc	3 mg/disc	Amphotericin B
1	*Candida albicans*	-	9.	11.0	14.5	24	26	27

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Table3: Antifungal activity of *Strychnos potatorum seeds extract* against fungal species tested by disc diffusion assay.

			2					
2	*Microsporum canis*	-	-	09.0	13.0	15.0	16	19.5
3	*Saccharomyces cerevisiae*	-	12	14	16	19.5	22	24
4	*Aspergillus flavus*	-	9	12	15.0	17	19	20.5
5	*Rhizopus nodousus*	-	9	11.0	13	16	20	23
6	*Chrysosporium tropicum*		8	10.0	13	17	19	21
7	*Penicillium chrysogenum*	-	-	13	17	19	22	24
8	*Penicillium notatum*	-	12	15	18	22	25	26

Table 3 shows the antifungal activity of ethanolic extract of *S.potatorum* against eight different fungal species. The antifungal potency of *S.potatorum* seeds extract was evaluated by the presence or absence of inhibition zones and zone diameters (mm). It is evident that the ethanolic extract of *S.potatorum* seeds showed a maximum inhibitory zone in a dose dependant manner. However, there was no significant difference between the levels of zone of inhibition at the concentration of 1.5 mg and 3 mg/disc. The antifungal potency of *S.potatorum* seeds extract on the *C. albicans* showed a larger diameter of clearance than that of other strains. Moreover, the zone of clearance achieved by *S.potatorum* seeds extract is comparable to that of standard drug, Amphotericin B.

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Table 4: Antifungal activity of Strychnos potatorum seeds extract against fungal species tested by MIC and MFC.

Fungal species	MIC		MFC	
	***Strychnos potatorum* seeds extract**	**Amphotericin B ($\mu g\ ml^{-1}$)**	***Strychnos potatorum* seeds extract**	**Amphotericin B ($\mu g ml^{-1}$)**

	(mgml^{-1})		(mgml^{-1})	
Candida albicans	3	1.5	3	3
Microsporumcanis	6	2.5	6	3.5
Saccharomyces cerevisiae	2.0	1.8	2	3
Aspergillus flavus	3.5	2.5	4	3
Rhizopus nodousus	6	3.5	6	6
Chrysosporium tropicum	4	2	4	1
Penicillium chrysogenum	3	2.5	3	2
Penicillium notatum	3.2	4.5	2.8	3

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The minimum inhibitory concentration and minimum fungicidal concentration of *S.potatorum* seeds extract as well as the standard antifungal drug, Amphotericin B is depicted in Table 4. The MIC value of *S.potatorum* seeds extract against fungal strains varies from 1 mg to 7 mg and the results are comparable with the standard antifungal agent, Amphotericin B. The lowest MIC was shown by *Saccharomyces cerevisiae* and the highest MIC values by *Microsporum canis.*

Fungal diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide. Human infections, particularly those involving the skin and mucosal surfaces, constitute a serious problem, especially in tropical and subtropical developing countries [31]. In humans, fungal infections range from superficial to deeply invasive or disseminated, and have increased dramatically in recent years. Although new drugs have been introduced to combat this problem, the development of resistance to antifungal drugs has become increasingly apparent, especially in patients who require long-term treatment or who are receiving antifungal prophylaxis, and there is growing awareness of shifts of flora to more-resistant species.

The fungal strains used in the present study were selected on the basis of their clinical importance. Agar disc diffusion method was performed in the present study to investigate the antifungal activity of *S.potatorum* seeds extract. The highest activity (diameter of zone of inhibition 25 mm) was demonstrated by the ethanolic extract of *S.potatorum* against *C. albicans* while the lowest activity was observed against *Microsporum canis*. The results of the *in vitro* antifungal assay revealed that the growths of fungal strains were affected by the *S.potatorum* seeds extract by forming clear inhibition zones.

The MICs and MFCs showed that *Microsporum canis* has the highest MIC (7mg/ml) and MFC (7mg/ml) while the lowest MIC of 2 mg/ml was demonstrated by *Saccharomyces cerevisiae*. The fungistatic or fungicidal effect of natural products and the mechanisms involved are cytoplasm granulation, cytoplasmic membrane rupture and inactivation and/or inhibition of intracellular and extracellular enzymes. These biological events could take place separately or concomitantly culminating with mycelium germination inhibition and it is also reported that plant lytic enzyme act in the fungal cell wall causing breakage of β-1,3 glycan, β-1,6, glycan and chitin polymer [32]. The observed antifungal effect of the extract might be due to the presence of biologically important ingredients present in the *S.potatorum* [33-36].

The presence of biologically important secondary metabolites such as alkaloids, sterols, flavonoids, tannins, glycosides, triterpenoids, saponins and phenolic compounds in the delipidated seeds extract readily accounts for its observed antibacterial and antifungal properties[37].The results obtained are corroborated with the earlier reports.

4. CONCLUSION

The significant bactericidal and fungicidal effects of *S.potatorum* extract suggest that the *S.potatorum* may be considered as a potential source for the development of novel antibacterial, antifungal agent against pathogenic bacteria and fungi.

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