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Evaluation of Antimicrobial activity of Siddha herbo-mineral drug Neelanjana Mai against some Human Eye Pathogens

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Abstract:

Introduction: Antimicrobial agents are essentially important in reducing the global burden of infectious diseases. Neelanjana mai is a classical siddha herbo-mineral combination which is indicated for eye disease in the sastric siddha text 'Siddha Vaidhya Thirattu".**Methods**: In this study, we evaluated the antimicrobial potency of this drug on 3 bacterial species like *Staphylococcus aureus,Pseudomonas aeruginosa, Chlamydia trachomatis* with Ciprofloxacin and vancomycine as standard controls ;and 3 fungal species like *Candida albicans Aspergillus niger, Fusarium oxysporum* with Fluconazole as standard controls, using Agar disk-diffusion method. **Results:** The observed results showed potential antibacterial potency of Neelanjana Mai against the bacterial strains tested with ZOI 17±2 against P.aeruginosa and 14.67±1.53 and 15±1.55 against S.aureus and C.trachomatis respectively. Also, anti-fungal activity results revealed that against the 3 fungal strains. C.albicans, A.niger and F.oxysporum showed anti-fungal effect with ZOI 12.67±1.15, 18.67±1.53 and16.67±2.08 respectively. **Conclusion:** In summary, overall the observed results concluded that Neelanjana mai extract was effective on P.aeruginosa and A.niger with highest ZOI and confirmed the anti-microbial effect.

Keywords: Siddha, Neelanjana mai, Antifungal, Antibacterial, Eye pathogens, Agar disc diffusion method.

INTRODUCTION:

Siddha system of medicine is a paragon of knowledge for both curative and preventive health care. According to this system there are 4448 diseases mentioned in *Agathiyar Ratna Surukkam* and it gives detailed information of types, etiology and regimen of these diseases.(1) Siddha ophthalmology is a lesser explored section in which it is mentioned that there are 96 types of eye disease which further divides based on the region of pathology.(2) Neelanjana Mai is a classical siddha medicine mentioned in sasthric book *Siddha Vaidhya Thirattu* which is indicated for eye diseases of both infectious and non – infectious origin like poo (a film in eye due to excessive heat in body, or accumulation of water in eye or by small pox), padalam (cataract), Muthakasam (type of cataract), pillam (a disease of eyelid).(3) The ingredients of this drug are *anjanakkal(Plumbi sulphuratum sulphaide of lead), seenakarkandu, peetharoghini (Coptis teeta), kunkumapoo (Crocus sativus), padigara parpam(Alum), pachaikarpooram(Borneo camphor).*

In modern medicine eye disease can be caused by bacteria, virus or fungus. Analysing infectious keratitis, the most common root of this pathology is bacterial infection caused by bacterias like include *Staphylococcus aureus*, coagulase-negative *staphylococci*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*.(4,5) Among these, *P. aeruginosa* is the most common microorganism that cause bacterial keratitis among contact lens wearers. The

common mode of treatment in bacterial ophthalmic infection are topical antibiotics and monotherapy with fluoroquinolones. These regimens have almost similar effectiveness but fluoroquinolones lessen the risk of chemical conjunctivitis as well as ocular discomfort. Also use of ofloxacin, ciprofloxacin increases the risk of white corneal precipitates compared to other ophthalmic bacterial medications.(6,7) To exterminate the organism or restore damage, corneal grafting is desirable in some cases.

Ophthalmic infection caused by fungal species leads to ophthalmic mycoses and there seems to be in a firm increase in this number.(8,10) Filamentous fungal infections like, *Fusarium* and *Aspergillus* species, followed by other hyaline fungi, *Curvularia* species and other dematiaceous fungi are the most prevalent infectious fungal species causing ophthalmic pathology. Also considering infections caused by yeasts and related fungi, *Candida* species are the most familier.(9,10) Among azole antifungals, Fluconazole is considered an economical and harmless antifungal agent with a minimal toxicity profile.(11) However, the employment of fluconazole may lead to prompt progress to resistance and cross-resistance to the fungal species on extended exposure or due to a partial therapeutic regimen, which is a major challenge.

Agar disk-diffusion testing developed in 1940(12), is the authorized method used in several clinical microbiology laboratories for routine antimicrobial susceptibility testing. At the present time, many accepted and appropriate standards are published by the Clinical and Laboratory Standards Institute (CLSI) for bacteria and fungus testing.(13,14) This study was done to determine the antimicrobial activity of *Neelanjana Mai* against some human eye pathogens using the agar-disk-diffusion method.

MATERIALS AND METHODS:

The microorganisms used for antimicrobial analysis were purchased from Microbial Type Culture Collection and Gene Bank (***MTCC***), Chandigarh. The Bacterial microorganisms which are used in this study are *Staphylococcus aureus* (737, *MTCC*), *Pseudomonas aeruginosa (39327,ATCC), Chlamydia trachomatis* (573,*ATCC*) and the fungal microorganisms are *Candida albicans(183, MTCC), Aspergillus niger(281, MTCC), Fusarium oxysporum (1755, ATCC)* and the study drug *Neelanjana mai* was procured from GMP certified pharma.

The other materials used in the study is Nutrient Agar (Cat No:M001, Himedia), Muller Hinton Agar (Cat No: M173, Himedia), SDA agar (Cat No: M063, Himedia), Sterile discs (Cat No:

SD067, Himedia), Fluconazole 150ug disc (Cat No:SD232, Himedia), Vancomycin-30ug discs (Himedia), Ciprofloxacin-5ug discs (Himedia), DMSO (Fisher Scientific).

The bacterial strains were maintained on **Nutrient Agar** (NA) and The Fungal strains were maintained on **SDA Agar** (SDA).

Aerobic bacteria growth conditions:

Pure cultures from the plate were inoculated into Nutrient Agar plate and sub cultured at 37ºC for 24 h. Inoculum was prepared by aseptically adding the fresh culture into 2 ml of sterile 0.145 mol/L saline tube and the cell density was adjusted to 0.5 McFarland turbidity standard to yield a bacterial suspension of 1.5×10^{8}cfu/ml. Standardized inoculum was used for antimicrobial test.

Antimicrobial Test by disc diffusion method:

The medium was prepared by dissolving 38 g of Muller Hinton Agar Medium (Hi Media) in 1000 ml of distilled water. The dissolved medium was autoclaved at 15 Lbs pressure at 121^{0}C for 15 min (pH 7.3). The autoclaved medium was cooled, mixed well and poured on petriplates (25 ml/plate). The plates were swabbed with Pathogenic Bacteria and incubated for 24hours. Three wells of 6 mm were bored in the inoculated media with the help of sterile cork-borer (6 mm). Finally, The Sample loaded discs with 100ul/ml were then placed on the surface of Muller-Hinton medium and the plates were kept for incubation at 37°C for 24 hours. At the end of incubation, inhibition zones were examined around the disc and measured with transparent ruler in millimetres. The size of the zone of inhibition (ZOI) was measured in millimetres. The absence of zone inhibition was interpreted as the absence of activity (Kohner *et al*., 1994; Mathabe *et al*., 2006). The activities are expressed as resistant, if the zone of inhibition (ZOI) was less than 7 mm, intermediate (8-10 mm), and sensitive if more than 11 mm (Assam *et al*., 2010). Vancomycin with 30ug disc was used as a std control against the S.aureus and C.trachomatis respectively. Ciprofloxacin with 5ug was used as a std control for the P.aeruginosa respectively.

Fungi growth conditions:

Pure cultures from the plate were inoculated into SDA agar plate and subcultured at 37ºC for 24 h. Inoculum was prepared by aseptically adding the fresh culture into 2 ml of sterile 0.145

mol/L saline tube and the cell density was adjusted to 0.5 McFarland turbidity standard to yield a fungal suspension of 1.5×10^8cfu/ml. The standardized inoculum was used for the Antimicrobial test.

Anti-fungal Test by disc diffusion method:

Antibiotic susceptibility tests were determined by agar disc diffusion (Kirby-Bauer) method. Fungal strains viz., *Candida albicans, Aspergillus niger and Fusarium oxysporum* were swabbed using sterile cotton swabs on SDA agar plate. Up to 80µl of 1 mg/ml concentration of the extract was introduced in the sterile discs (10 mm) using sterile pipettes. The standard drug Fluconazole with 150 mcg concentration disc was used Pure cultures from the plate were inoculated into SDA agar plate and sub cultured at 37ºC for 24 h. Inoculum was prepared by aseptically adding the fresh culture into 2 ml of sterile 0.145 mol/L saline tube and the cell density was adjusted to 0.5 McFarland turbidity standard to yield a fungal suspension of 1.5×10^8cfu/ml. The standard drug Fluconazole with 150 mcg concentration disc was used (10 mm) for positive control and empty sterile disc was used for negative control. The disc was then placed on the surface of SDA medium and the compound was allowed to diffuse for 5 minutes and the plates were kept for incubation at 22°C for 48 hours. At the end of incubation, inhibition zones were examined around the disc and measured with transparent ruler in millimeters.

OBSERVAT ON AND RESULTS:

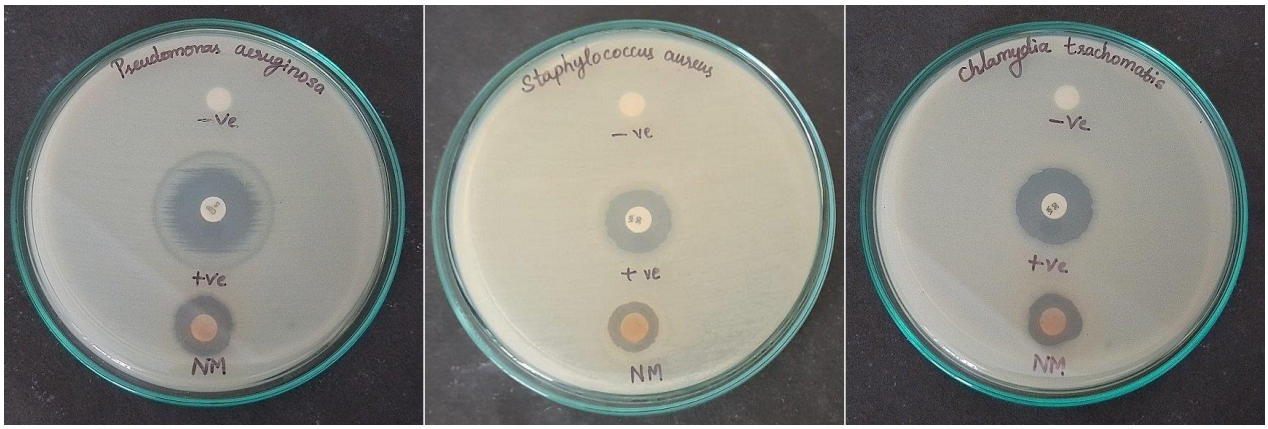


Fig 1: Anti-bacterial activity of given compound with 1 dilution (1mg/ml) against the 3 bacterial strains viz., *P.aeruginosa, S.aureus and C.trachomatis* in comparison to Positive control (Ciprofloxacin-5ug or Vancomycin-30ug) and Negative control (Distilled water) and found that the Neelanjana mai may have moderate anti-bacterial activity.

DISC DIFFUSION METHOD-P.aeruginosa							
	Zone of inhibition (mm)						
Culture condition	**Exp-1**	**Exp-2**	**Exp-3**	**Average**	**SD**	**SE**	**ZOI±SD**
Control	0	0	0	0	0	0	0
Ciprofloxacin-5ug	24	21	23	22.66667	1.527525	0.881917	22.67±1.53
NM-1000ug	15	17	19	17	2	1.154701	17±2

Table 1: Zone of inhibition of P.aeruginosa

DISC DIFFUSION METHOD-S.aureus							
	Zone of inhibition (mm)						
Culture condition	**Exp-1**	**Exp-2**	**Exp-3**	**Average**	**SD**	**SE**	**ZOI±SD**
Control	0	0	0	0	0	0	0
Vancomycin-30ug	17	19	20	18.66667	1.527525	0.881917	18.67±1.53
NM-1000ug	13	15	16	14.66667	1.527525	0.881917	14.67±1.53

Table 2: Zone of inhibition of S.aureus

DISC DIFFUSION METHOD-C.trachomatis							
	Zone of inhibition (mm)						
Culture condition	**Exp-1**	**Exp-2**	**Exp-3**	**Average**	**SD**	**SE**	**ZOI±SD**
Control	0	0	0	0	0	0	0
Vancomycin-30ug	20	18	19	19	1	0.57735	19±1
NM-1000ug	14	16	16	15.33333	1.154701	0.666667	15±1.55

Table 3: Zone of inhibition of C.trachomatis

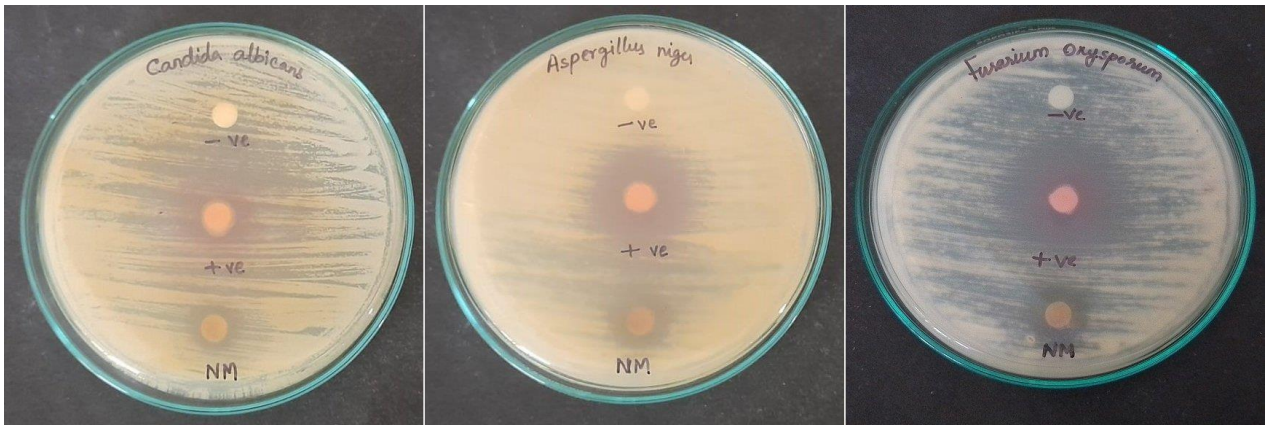


Fig 2: Anti-fungal activity of given compound with 1 dilution (1mg/ml) against the 3 fungal strains viz., *C.albicans, A.niger and F.oxysporum* in comparison to Positive control (Fluconazole with 150ug) and Negative control (Distilled water) and found that the Neelanjana mai (NM) may have satisfactory anti-fungal activity.

DISC DIFFUSION METHOD-C.albicans							
	Zone of inhibition (mm)						
Culture condition	**Exp-1**	**Exp-2**	**Exp-3**	**Average**	**SD**	**SE**	**ZOI±SD**
Control	0	0	0	0	0	0	0
Fluconazole-150ug	22	20	18	20	2	1.154701	20±2
NM-1000ug	12	14	12	12.66667	1.154701	0.666667	12.67±1.15

Table 4: Zone of inhibition of C.albicans.

DISC DIFFUSION METHOD-A.niger	
	Zone of inhibition (mm)

Culture condition	Exp-1	Exp-2	Exp-3	Average	SD	SE	ZOI±SD
Control	0	0	0	0	0	0	0
Fluconazole-150ug	24	23	24	23.66667	0.57735	0.333333	23.67±0.58
NM-1000ug	17	19	20	18.66667	1.527525	0.881917	18.67±1.53

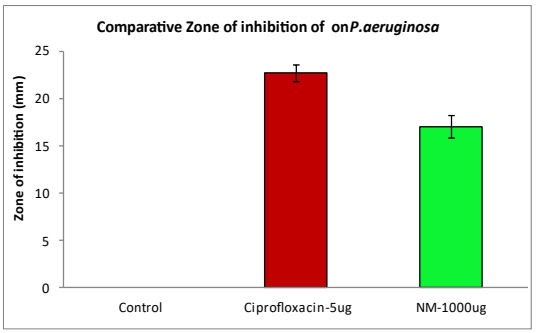
Table 5: Zone of inhibition of A.niger.

DISC DIFFUSION METHOD-F.oxysporum							
	Zone of inhibition (mm)						
Culture condition	Exp-1	Exp-2	Exp-3	Average	SD	SE	ZOI±SD
Control	0	0	0	0	0	0	0
Fluconazole-150ug	27	28	24	26.33333	2.081666	1.20185	26.33±2.08
NM-1000ug	16	19	15	16.66667	2.081666	1.20185	16.67±2.08

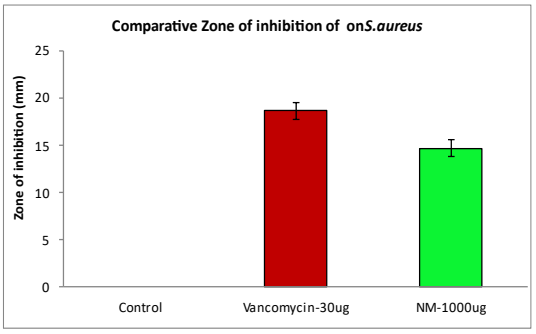
Table 6: Zone of inhibition of F. oxysporum.

Bacteria: Zone of inhibition±SD (mm)			
Sample code	**P.aeruginosa**	**S.aureus**	**C.trachomatis**
Negative control	0	0	0
Ciprofloxacin-5ug	22.67±1.53	--	--
Vancomycin-30ug	--	18.67±1.53	19±1
NM-1mg/ml	17±2	14.67±1.53	15±1.55

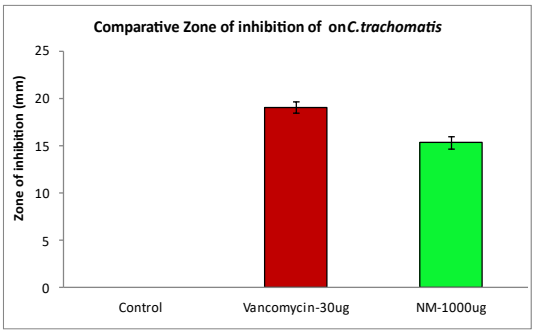
Table 7: Diameter of zone of inhibition (mm) of Test compound, Neelanjana mai (NM) against the microorganism at 1mg/ml concentration after the incubation period of 24hrs. The presented values were the average of 3 independent individual experiments (N=3). Keys: Positive Control for Bacteria: Ciprofloxacin with 5ug for P.aeruginosa and Vancomycin with 30ug for S.aureus and C.trachomatis and Negative Control-Distilled water



Graph 1 : Comparative zone of inhibition of P.aeurginosa



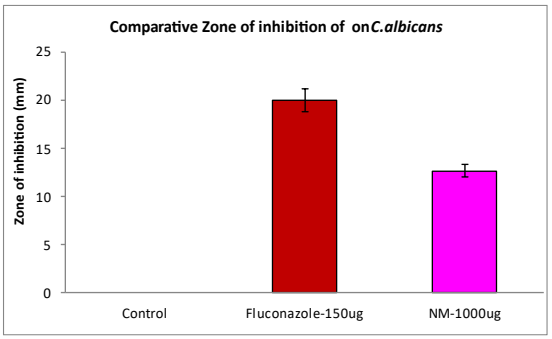
Graph 2: Comparative zone of inhibition of S.aureus



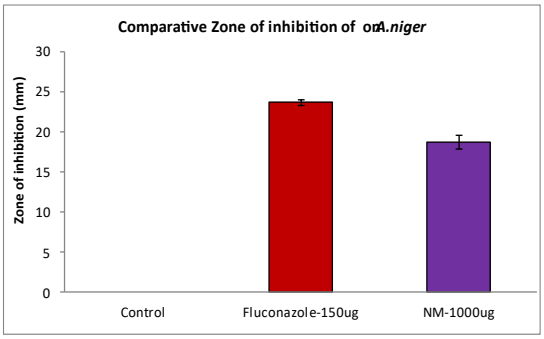
Graph 3: Comparative zone of inhibition of C. trachomatis

Fungi: Zone of inhibition±SD (mm)			
Sample code	**C.albicans**	**A.niger**	**F.oxysporum**
Negative control	0	0	0
Fluconazole-150ug	20±2	23.67±0.58	26.33±2.08
NM-1mg/ml	12.67±1.15	18.67±1.53	16.67±2.08

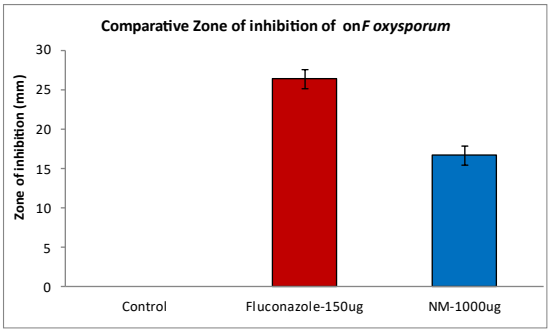
Table No.8: Diameter of zone of inhibition (mm) of Test compound, Neelanjana mai (NM) against the microorganism at 1mg/ml concentration after the incubation period of 24hrs. The presented values were the average of 3 independent individual experiments (N=3). Keys: Positive Control for Fungi: Fluconazole with 150ug and Negative Control-Distilled water



Graph 4: comparative zone of inhibition of C.albicans.



Graph 5: comparative zone of inhibition of A.niger.



Graph 6: comparative zone of inhibition of F.oxysporum.

In this study, anti-bacterial activity of 1 test compound viz., Neelanjanamai (NM) was assessed by disc diffusion method. The observed results showed potential antibacterial potency of NM against the bacterial strains tested, *P.aeruginosa, S.aureus & C.trachomatis* similar to the std control used for the study. Graphs 1-3 shows comparative zone of inhibition of bacterial

species. Among the 3 different bacteria, NM extract was potential against the P.aeruginosa was sensitive and the other 2 species moderately showed anti-bacterial effect. Ciprofloxacin with 5ug for *P.aeruginosa* and Vancomycin with 30ug for *S.aureus and C.trachomatis* was used as a reference std control for the current study.

Graphs 4-6 shows comparative zone of inhibition of Fungal Species used in the study. Anti-fungal activity of same compound, Neelanjana mai (NM) results revealed that against the 3 fungal strains. C.albicans, A.niger and F.oxysporum showed effective anti-fungal effect with higher zone of inhibition. A.niger was more sensitive than the other species with relatively higher ZOI. Fluconazole with 150ug was used as a std control for the anti-fungal activity.

DISCUSSION:

Neelanjana Mai is a common eye medicine available in all siddha pharmacies and is mainly used in the treatment of infectious and non-infectious disease and this study was mainly done to determine its antimicrobial activity. As mentioned before, the ingredients of this drug are *anjanakkal (Plumbi sulphuratum sulphaide of lead) , seenakarkandu, peetharoghini (Coptis teeta), kunkumapoo (Crocus sativus), padigara parpam(Alum), pachaikarpooram (Borneo camphor). Anjanakkal* the main ingredient of this drug is widely used by Indian women as an eye protector against sun's heat and light and also it is mentioned in literature that it is effective against watery ophthalmic conditions.(15) Coptis teeta has active ingredient berberine which is more effective in eradicating Chlamydia trachoma as compared to sulfacetamide and in avoiding relapse of symptoms(16,17) and also Saffron increases oxygen diffusion, thus improves ocular blood flow, and increases glutathione (GSH) levels that protect against reactive oxygen species (ROS) and apoptosis.(17,18) There are also literature evidence available that *peetharoghini* soaked water can be used as eye wash and also *kukumapoo* can be used in case of cataracts.(19) So from this it can be elucidated that each ingredient in this drug has a potent action for the welfare of ophthalmic condition.

Even though many eye medicine are mentioned in Siddha literature, studies done to validate them is only a fistful. In this antimicrobial study, we can find that *Neelanjana mai* has Zone of inhibition±SD (mm) about 17±2 against P.aeruginosa and , 14.67±1.53 and 15±1.55 against

S.aureus and C.trachomatis respectively. Also, Zone of inhibition±SD (mm) against fungal infections like C.albicans, A.niger and F.oxysporum was 12.67±1.15, 18.67±1.53 and16.67±2.08 respectively.

On comparing with other studies like antimicrobial activity and effeciveness of Ophthacare brand eye drops in the treatment of various ophthalmic disorders such as acute and chronic conjunctivitis, eye strain, dacryocystitis and pterygium(20), the ZOI of S. aureus, P. aeruginosa, C. albicans are 27.60 ± 1.45, 10.33 ± 0040, 37.66 ± 0040 in this study. Here Neelanjana mai seems to have more effectiveness against P. aeruginosa.In another study, aimed to determine the in vitro assessment of different concentration of natural and artificial medicines on ocular microbes, with selected traditional medicines such as Elaneer Kuzhambu, Himalaya Ophthacare eye drops and allopathic medicines like Ciplox and Catina, it was found that ZOI for Elaneer Kuzhambu, Himalaya Ophthacare eye drops in pureform without dilution was 7.33 ± 0.5773 and 7.66 ± 0.5773 respectively. And in case of Ciplox and Catina eye drops, it was 12.66 ± 0.5773 and 8.66 ± 0.5773 respectively.(21) So, after further studies like toxicity studies, Neelanjana mai can be introduced to clinical trials.

CONCLUSION:

In summary, the overall observed results concluded that Neelanjana mai extract was effective on *Pseudomonas aeruginosa* and *Aspergillus niger* with highest ZOI and confirmed the anti-microbial effect.

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CONFLICT OF INTEREST:

It is declared that there are no conflicts of interest among authors.

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