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AN EXPERIMENTAL EVALUATION OF MAHA NIMBA (Melia azadirach L.) IT'S KRIMIGHNA (ANTI FUNGAL) ACTIVITY w.s.r. to CANDIDA ALBICANS - INVITRO STUDY – A PILOT STUDY

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ABSTRACT

Many drugs of the Ayurvedic Pharmacopoeia are attributed with *Krimighna* property wherein the word *krimi* has multi-faceted meaning. *Krimi* may refer to both Macro and Micro-organisms responsible for causing diseases and it includes all microbes irrespective of whether Bacteria, Fungi or Virus. Present study is based on antifungal activity of the drug *MahaNimba*. Fungal infections or mycoses are classified as superficial, subcutaneous or systemic (deep) depending on the degree of invasion of the host. Candida albicans is a diploid fungus that grows both as yeast and filamentous cell and causal agent of opportunistic oral and genital infection in human and Candida Onychomycosis. There are abundant references in our classics about *Nimba* and its *Krimighna* activity. Hence this study is attempted to study the *Krimighna* (antifungal) activity w.s.r. to Candida Albicans. *MahaNimba* (Melia azadirach. Linn) which is also a variety of *Nimba*, also possesses the *krimighana* properties. The leaf extract of the plant was taken up for evaluation considering Candida Albicans as a type of *sleshmaja krimi* based on the symptoms. This study is carried out to see the Anti-Fungal effect of leaf extract of *MahaNimba* (Melia azadirach Linn.) against the strain of the Candida Albicans with Agar Cup-Plate Method (Well Diffusion Method). Anti fungal activity of *MahaNimba* shows less inhibitory activity compared to Standard Drug Flucanazole.

Keywords: *Mahanimbha, kaphaja krimi, krimighna,*anti-fungal, Candida albicans.

INTRODUCTION

Ayurveda is a science of life with its origin dating back to entiquity has a rich heritage. Dravya guna is a branch of Ayurveda which deals with Pharmacological properties, administration and modes of actions of drugs. In Samhitas and Nighantus the dravyas[1] and their Guna-Karmas[2] are mentioned.

Many drugs of the Ayurvedic Pharmacopoeia are attributed with *Krimighna* property wherein the word *krimi* has multi-faceted meaning. *Krimi* may

refer to both Macro and Micro-organisms responsible for causing diseases and it includes all microbes irrespective of whether Bacteria, Fungi or virus Recent Ayurvedic scholars have explained the nature of the krimi as microorganisms, which may be visible and invisible. This supports as to compare krimi to helminthes, other visible and microorganisms vizviruses, bacteria & others, which can be substantiated from the symptoms of each type of krimi. A *Krimighna* dravya acts against any of these diseases causing organism. Anti-Fungal is one such facet incorporated under this heading.[4]

Fungal infections or mycoses are classified as superficial, subcutaneous or systemic (deep) depending on the degree of invasion of the host.[5] Superficial Candidiasis is caused by Candida albicans. Candida albicans is a diploid fungus that grows both as yeast and filamentous cell and causal agent of opportunistic oral and genital infection in human and Candida Onychomycosis.

Azadirachtaindica A.juss (Neem) is one of the most useful traditional medicinal plants.[6] Every part of the tree has been used as traditional medicine for household remedy against various skin disorders.[7] There are abundant references in our classics about *MahaNimba* and its *Krimighna* activity. On reviewing the literature, it was found that the drug *Nimba* is used as *Kandugna*, *Krimigna, Kapha pitta hara, twachya* etc. In this regard, present study was taken up to evaluate the *krimigna* activity with special respect to antifungal activity. Hence this study is attempted to study the *Krimighna* (antifungal) activity w.s.r. to Candida Albicans.

MahaNimba (Melia Azadirach. Linn) which is also a variety of *Nimba*, also possesses the *krimighana* properties. This study is planned to verify as to why Melia Azadirach. Linn is named as *MahaNimba*, whether the *MahaNimba* (Melia Azadirach Linn.) is only structurally a larger version or functionally has better properties than the *Nimba* with special reference to Antifungal activity.

In this background the *Krimighna* (antifungal) activity was taken up for the study. Herein the leaf extract of both the plants are taken up for evaluation considering Candida Albicans as a type of *sleshmaja krimi* based on the symptoms.

Objectives of the Study

- To evaluate the Antifungal properties of the leaf extract of *MahaNimba* (Melia Azadirach Linn.) against the strain of Candida albicans – In vitro study.

Material and Methodology

Materials-

Collection of drug- *Mahanimba* (Melia azadirach) collected from FRLHT, Yelahanka, Bengaluru.

Drug Authentication-

The genuinity of the drug was confirmed by Dr. Ganesh Babu, Ph.D Botanist, institute of Ayurveda and integrative medicine, FRLHT, Yelahanka, Bengaluru.

STUDY DESIGN

- Pharmacognostical study of *MahaNimba* (Melia Azadirach Linn.)
- Analytical study of M*ahaNimba* (Meliaazadirachta Linn.).

Experimental Study

- Determination of moisture content:
- Determination of ph:
- Determination of total ash content
- Determination of water and alcohol soluble extractive:
- Thin layer chromatography (TLC)
- Hplc condition for quercetin and gallic acid:
- Evaluation of antifungal activity by Well Diffusion Method with Water Extracts
  A. Screening of test extracts for antifungal activity by Well diffusion technique
- Evaluation of antimicrobial activity by Well Diffusion Method with Methanol Extracts

Antimicrobial (Anti Fungal) Activity:

There may be wide variations in the susceptibility of different strains of the same bacterial species to antibiotic. Several technologies are now available for

determination of microbial sensitivity to antimicrobial agent namely Cupplate Method, Serial Dilution Methods, Solid Dilution Method, Ditch Plate Technique, Gradient Plate Technique, Well Diffusion Method.

In the present study, the antimicrobial activity of *Maha Nimba* is performed by following Well Diffusion Method with all necessary equipments and precautions.

STAGE I

Preparation of Inoculum for Fungi:

STAGE II

Funguses are used in antifungal activity:

1. *Candida albicans* MTCC 35

Table 1: Showing Potato Dextrose Agar composition [HIMEDIA MO96]:		
Sl No	**Ingredients**	**Quantity**
1	Peptone	200 gms.
2	Dextrose	20 gms
3	Agar	15 gms
4	Distilled water	1000 ml
5	pH	5.6 ± 0.2

Preparation of Inoculum:

- ✓ **For Fungal culture:** Sterile Nutrient agar medium was cooled to 45^0C and mixed with 20% of respective fungal culture individually (That is 80 ml media and 20 ml culture).

Applications of solutions: The inoculation prepared has to be transferred to petriplates immediately of its preparation.

Incubation: - After introduction of Test and Standard drugs, the plates were placed in a refrigerator at 8^0C - 10^0C for diffusion of drugs into the media. After two hours of cold incubation, petriplates are transferred to incubator and maintained at 37^0C ± 2^0C for 24 hrs. The fungal petriplates were incubated at 27^0C ± 2^0C for 48 hrs. After the incubation period, the petriplates were observed for zone of inhibition and measured using the Vernier caliper.

Observations:

- ➢ Zone of inhibition was measured by Vernier caliper

Table 2: Nutrient Agar media composition [HIMEDIA MOO1]:		
Sl No	**Ingredients**	**Quantity**
1	Peptone	5 gms.
2	Beef extract	3 gms
3	NaCl	5 gms
4	Agar	15 gms
5	Distilled water	1000 ml
6	pH	7.4 ± 0.2

- ➢ While mixing fungal cultures, the temperature of Agar was maintained above 45^0C
- ➢ Inoculated media poured into the petriplates to a depth of 3 to 4mm & place and platform to get uniform spreading of the media.
- ➢ The growth of the fungal cultures were indicated by the turbidity of the media

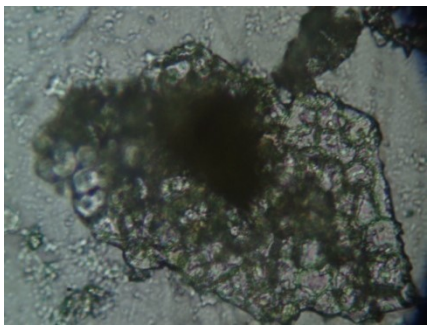
Precautions:

- ➢ pH of all the media was accurately maintained for normal ions uptake by microorganisms.
- ➢ Petridish, conical flask etc were properly sterilized by autoclaving at 15lbs/sq inch for 15 minutes.

- Activity was conducted by using gloves & mask and it was carried in the laminar airflow.
- Zone of inhibition was recorded by placing the petriplates on colony counter.

Microscopical Study *Mahanimba*- Melia azadirach

- Outer and inner side of the endosperm filled with aleurone grains and mucilage



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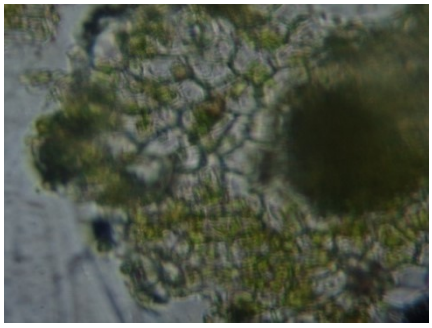

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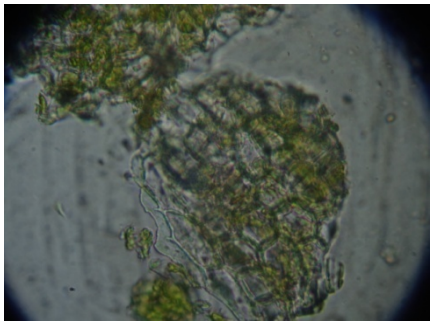

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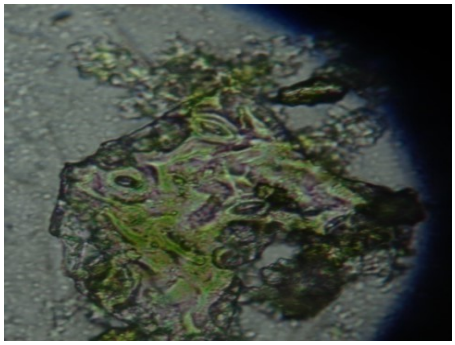

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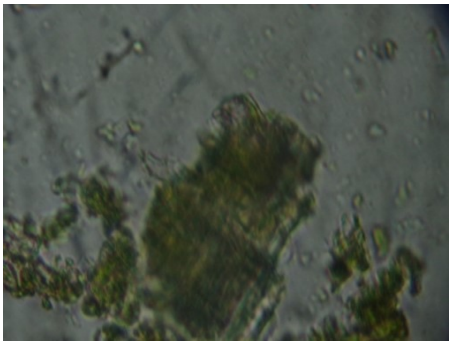


5.

Epidermis and column cell in sectional view

Table 3. MOISTURE CONTENT RESULT				
Sl. No.	Sample code	Moisture (%)	Mean±SD	Method
1	F	3.26	3.94±0.96	Loss on drying (USP 40)

Table 4. pH STUDY RESULT		
Sample code	**PH at 10%**	**PH at 1%**
F-	6.56	6.8
Mean±SD	6.135±0.60	6.4±0.57

Table 5. TOTAL ASH STUDY RESULT		
Sample code	**Total Ash (%)**	**Mean±SD**
F-2	8.89	8.73±0.23

DETERMINATION OF WATER AND ALCOHOL SOLUBLE EXTRACTIVE:

Table 6: EXTRACT STUDY RESULT

Solvent	Sample	Wt. taken for extraction (gm)	Wt. obtained (%)	Mean±SD
Alcohol	2	10	9.34	8.54±1.13
Water	2	10	27	25±2.83

Table 7: Phytochemical screening for Methanol Extract

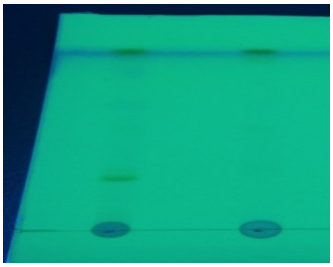
Sl.no.	Phytochemicals	Type of test		Result
1	Saponins	Foam test		-
2	Phenol	FeCl3 test		+
3	Steroids	Lieberman Burchardt test		-
4	Tannins	Braymer's test		-
5	Flavonoids	Alkaline reagent test		+
6	Terpenoids	Salkowki's test		-
7	Cardiac glycosides	Keller-Killani test		-
8	Alkaloids	Dragendoff's test		+
9	Carbohydrates	Molisch's test		-
10	Glycosides	Glycosides test		-

Table 8: TLC Characteristics Mahanimba

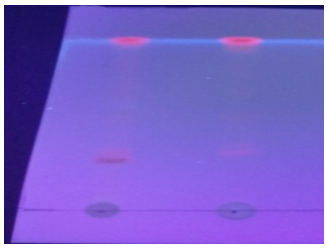
Sample code	TLC Band	Retention Factor	TLC Profile characteristics		
			366 nm	254nm	Visible light
F	2	1.00	Reddish pink	Dark green	Dark green
		0.00	Blue	Blue	Light brown

TLC characteristics

Visible light Shortwave



UV 254 nm



Long wave UV 366 nm

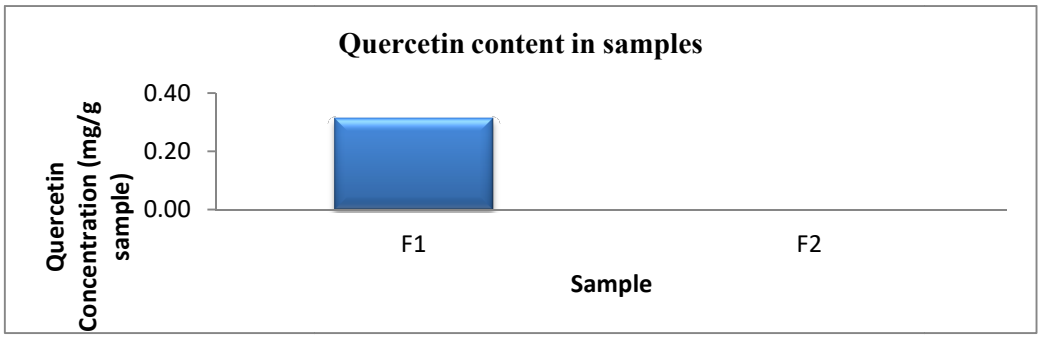
Fig. 1 TLC chromatogram of sample at Visible light, Shortwave UV 254 nm and Long wave UV 366 nm

High performance liquid chromatography

Estimation of Quercetin

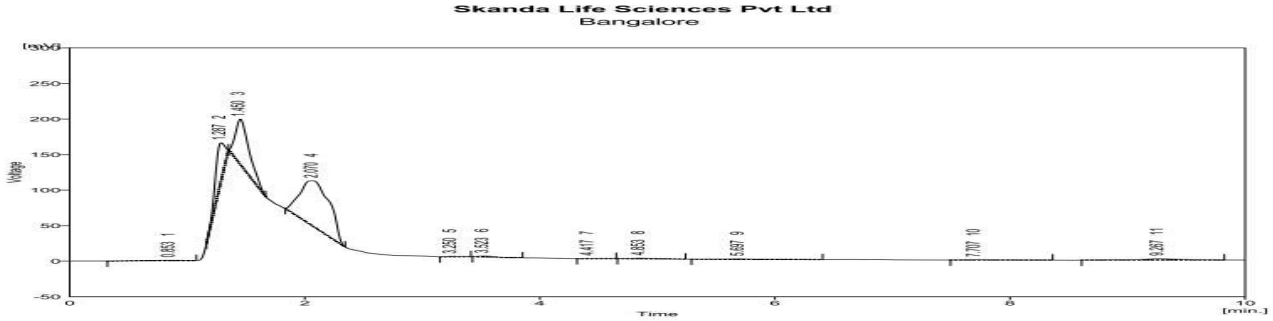
Table 9: HPLC summary report of Standard (Quercetin)

Concentration of sample injected	Compound	RT	Area	Concentration (µg/10mg)	Concentration (mg/g sample)	Chromatogrm No.
100ug/ml	Quercetin	3.41	363.26			Fig. 2
10mg/ml	*F2*	-	-	0.00	0.00	Fig. 3
						Fig. 4



Quercetin content in samples.

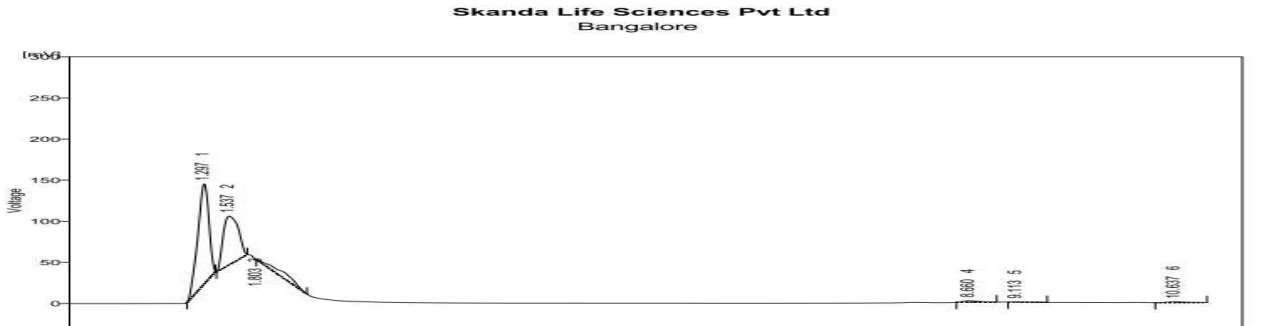
HPLC Chromatogram of Quercetin standard concentration 100µg/mL



Result Table (Uncal - C:\SPINCHROM\WORK1\DATA\2018\FEB\DR FAHIM\23022018_NEEM F1_100UG_002)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]
1	0.853	15.236	0.652	0.7	0.3
2	1.287	299.191	54.625	14.3	29.0
3	1.450	537.460	63.453	25.6	33.7
4	2.070	1142.772	63.974	54.4	33.9
5	3.250	3.688	0.478	0.2	0.3
6	3.523	11.371	1.241	0.5	0.7
7	4.417	2.716	0.271	0.1	0.1
8	4.853	11.625	0.750	0.6	0.4
9	5.697	17.136	0.716	0.8	0.4
10	7.707	10.384	0.494	0.5	0.3
11	9.267	47.501	1.915	2.3	1.0
	Total	2099.060	188.563	100.0	100.0

HPLC chromatogram of F110mg/mL



Result Table (Uncal - C:\SPINCHROM\WORK1\DATA\2018\FEB\DR FAHIM\23022018_NEEM F2_100UG_003)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]
1	1.297	898.457	121.177	54.4	65.8
2	1.537	592.540	59.299	35.9	32.2
3	1.803	122.230	0.199	7.4	0.1
4	8.660	20.442	1.857	1.2	1.0
5	9.113	3.485	0.358	0.2	0.2
6	10.637	13.802	1.131	0.8	0.6
	Total	1650.956	184.022	100.0	100.0

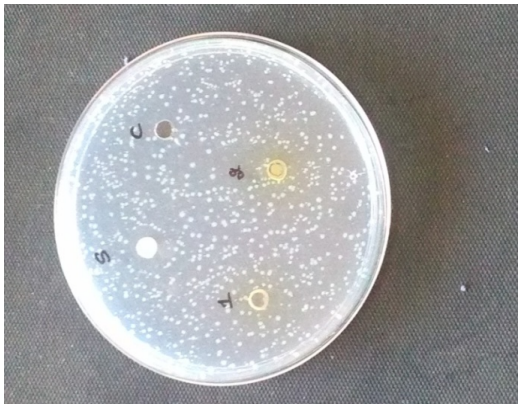
HPLC chromatogram of F2 10mg/mL

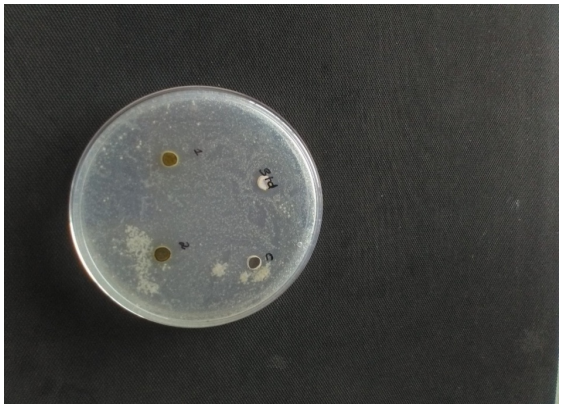
Evaluation of antifungal activity by Well Diffusion Method

Table 10: Efficacy of *MahaNimba* against *Candida Albicans*: water Extract

Test Compounds	Concentration (µg/well)	Zone of inhibition(mm) *Candida albicans*	Well refernce number
Control	Water	-	**C**
Flucanazole (standard)	100	11±0.0	S
Sample (Water extracts)		5.5+-0.0	f
		5.5±0.0	F-2

Above table shows, in 2000µgm/ml concentration the Tested drug *MahaNimba* shows less inhibitory activity as compared with the Standard drug Fluconazole (100µgm/ml).





Inhibitory activity of test sample against *C.albicans* S-standard (Fluconazole); Control (Water); Sample Water extract (2000µg/well)

Conclusion:

The zones of inhibition were observed against test compounds and standard antifungal are summarized in above table and Fig. The Sample shows less inhibitory activity against *C. albicans* than Standard Drug Fluconazole

Evaluation of antimicrobial activity by Well Diffusion Method with Methanol Extracts

Table 11. Inhibitory activity of test compounds against *Candida albicans*

Test Compounds	Concentration (µg/well)	Zone of inhibition(mm) *Candida albicans*	Well refernce number
Control	Methanol	-	**C**
Flucanazole (standard)	100	11±0.0	S
Sample (Methanol)		5.5±0.0	

Table 12: Determination of Minimum inhibitory Concentration			
No.	**Sample Code**	**MIC (µg/mL)**	**Test parameters**
		C.albicans	
1	Flucanazole	250	**Methodology** Microbroth dilution technique using Culture Medium: Potato dextrose broth for *C.albicans.* Sample test concentrations 1000, 500, 250, 125, 62.5, 31.25, 15.62 µg/mL.
2	Melia Azedarach	500	

DISCUSSION

Maha Nimba is used as *Kandughna, Krimighna, Kapha pitta hara, twachya.* In this regard present study was taken up to evaluate the *krimigjna* activity with special respect to Antifungal activity.

Analysis of Physical constants: **Total Ash**: was found to be 8.57% and 8.89% which shows the amount of inorganic matter present and values are within standard limits.

Alcohol soluble extractive value and Water soluble extractive value: was found to be 7.74% and 23% respectively of *Nimba* whereas 9.34% and 27% of *MahaNimba* indicating the presence of more water soluble principles like flavanoids, etc.

***Rasa Panchaka*:** The properties of the drug in *Ayurveda* are based on the *rasa panchaka*. Action of the drug also varies with the *rasa panchaka*.

Action of the Drug and Mode of Action of the Drug:[15] The drug possesses rasa like *Tikta* and *kashaya* which are mostly *kapha* and *pittashamaka* and also *avidahi*. *Tikta rasa* helps in reducing the *kleda* and *kashaya rasa* helps in *dravshoshan,* thus it proves beneficial in the cases of *Krimighna* activity. The drug also possesses *laghu* and *ruksha guna*. *Laghu guna* is *Kaphashaamaka*, causes *mala kshaya*, *ruksha guna* has the *shoshana shakti,* it causes *mala shoshana,* this causes *Dravam shashoshan*, thus being used in *Krimi.*

Antimicrobial Activity: Antimicrobial activity is a technique in which response of an organism to a particular antimicrobial agent can be established. Many methods are employed for evaluation of antimicrobial activity of a drug. In the present study Well Diffusion method was selected. It is simple and relatively inexpensive which makes it still the method of choice for the average laboratory.

Each kind of microorganism has specific growth requirements; most of the microbes can be grown in culture medium in the laboratory. In the present study, Potato dextrose agar media for fungi.

Growth of organism was confirmed by turbidity of the media. Agar universally used as a solidifying agent, it common for bacteria & fungi, which has not been replaced by any other agent from 100 years.

Tested drug *MahaNimba,* standard antifungal drug Fluconazole was used at 2000µgram/ml and 100 µgram/ml concentrations the Results are expressed by determining the zone of inhibition measuring in mm by using vernier caliper.

Results of Antimicrobial activity: *MahaNimba* has shown less antifungal activity than the proven drug Fluconazole. *MahaNimba* can be an alternative to Fluconazole & should be tried in clinical trial also.

CONCLUSION

The drug *MahaNimba* has multiple pharmacotherapeutic properties. Among them *Krimighna* property was studied with respect to Antifungal activity. The leaf extract of the drug was used for studying anti fungal activity in two forms that is water extract and Methanol extract. The Phytochemical study reveals the observation conducted using water extract of *Maha Nimba* showed the presence of *Carbohydrate, flavanoids*, Saponins, *terpenoids.* The antifungal activity of *MahaNimba* was compared with standard drug Flucanazole. The anti fungal activity of *MahaNimba* showed less inhibitory activity than Flucanazole.

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