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Evaluation of Nimbooka (Citrus limon Linn.) Patra and Nimbooka phala twak for Antifungal activity – A comparative in vitro study.

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Abstract: Aim-Present study is aimed at comparative evaluation of the efficacy of *Nimbooka* (*Citrus limon* Linn.) *phala twak* and *Nimbooka patra* in fungal infections and finding out an effective therapeutic agent which is of the prime concern here. **Objectives-** To evaluate *krimighna* activity of *Nimbooka phala twak Swarasa* and *patra Swarasa* by Serial Tube Dilution method. To compare the results of *krimighna* activity of *Swarasa* of *Nimbooka phala twak* and *patra* in selected fungal strain. **Methods-**Agar Diffusion/Cup Plate method to determine the antimicrobial activities of the different samples of *Nimbooka Patra* and *Phala twak*. **Results**-It is observed from the in-vitro study that the drug *Nimbooka Phala twak* and *patra* has shown comparative antifungal activity with that of standard drug Fluconazole. Among both the drugs, *Nimbooka Patra* has shown better activity than *Nimbooka Phala twak* against *Candida albicans* where as *Nimbooka Phala twak* has shown better activity than *Nimbooka Patra* against Aspergillus niger. **Conclusion**- At a lower dose of *Nimbooka Patra* and *phala twak* is having statistically significant antifungal activity when compared with standard drug.

Keywords: *Nimbooka Phala twak, Nimbooka Patra, Antifungal Activity*

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Introduction:

In the past few decades, a worldwide increase in the incidence of fungal infections has been observed & antifungal drugs have led to the emergence of resistant strains. The challenge has been to develop effective strategies for the treatment of candidiasis and other fungal diseases considering the increase in opportunistic fungal infections in humans.[1] Statistically fungal diseases have caused over 1.6 million deaths annually and over one billion people suffer from severe fungal diseases.[2]

In present conditions effective, complete and timely management of these microbes is a must. Though the available antifungal drugs have been tried in most of the conditions, their reoccurrence is common, thus making it necessitate the need of an alternative and effective therapy. The drug *Nimbooka* (*Citrus limon* Linn.), which is easily available everywhere and is known to have both *krimighna* & *kandughna* activity.[3]

Dravyaguna shastra, a part and partial of *Ayurveda*, describes in detail the pharmacological properties, administration and modes of actions of drugs. Among the various actions explained, *krimighna* is one. The word *'krimi'* has multifaceted meanings referring to macro and micro organisms responsible for causing diseases and includes all microbes irrespective of whether bacteria, fungi or virus. *Krimighna* drugs act as antimicrobials as well as antimacrobials.[4]

Nimbooka (*Citrus limon* Linn.) belongs to Rutaceae family. In *Nighantus, Nimbooka* has been explained under *Amradiphala varga* of *Bhavaprakasha Nighantu*[5], *Aushadhi varga* of *Kaiyadeva Nighantu*[6], *Phala varga* of *Shaligrama Nighantu*.[7] It is described as '*Jantumari*' in *Raja Nighantu*,[8] as '*krimi samuha nashanam'* in *Bhavaprakasha Nighantu*. This drug is widely used in many *krimihara yogas*. Hence, the trial drugs selected for the study viz. *Nimbooka Patra* and *Phala twak* are known to have *krimighna* activity.

The two different strains of fungi namely *Aspergillus niger* and *Candida albicans*[9] selected for the study as representative microorganisms, owing to greater susceptance of Candida albicans, which is a opportunistic pathogen in the oral cavity & vagina and Aspergillus niger, an opportunistic pathogen of the uro-genital tract and of the blood stream. In the present study Agar well diffusion/Cup plate method has been followed to detect the Antifungal property of the drugs in different dosage forms and concentrations by serial tube dilution method.

Objective of the study:

- To evaluate *krimighna* activity of *Nimbooka phala twak Swarasa* by Serial Tube Dilution method.
- To evaluate *krimighna* activity of *Nimbooka patra Swarasa* by Serial Tube Dilution method.
- To compare the results of *krimighna* activity of *Swarasa* of *Nimbooka phala twak* and *patra* in selected fungal strain.

Material and Methods:

The drug was identified and authenticated by the botanist and HOD of Dravyaguna, S.J.G.A.M.C- Koppal. Phytochemical studies and In Vitro Antifungal activity were carried out at **'Drug Testing lab', BLDEA's College of Pharmacy, BLDE campus, Vijayapur.** Preparation of different dosage forms were done at **'Drug Testing lab', BLDEA's College of Pharmacy, Vijayapur.**

Microscopic studies of *Nimbooka Patra* and *Phala twak* were carried out at Laboratory, Department of Botany, KCP Science College- Vijayapur.

A. Source of the drugs selected for the study:
Bright yellow, botanically identified fresh *Nimbooka (Citrus limon Linn.) phala* was collected of average size and with thick rind. The leaves collected were kept for drying under shade for 10 days and later made in to coarse powder.

B. Preparation of the dosage forms[10]:

a. Preparation of *Swarasa* of *Nimbooka patra* and *Nimbooka phala twak*: 100 gm of each trial drug were kept in clean plastic jar and 400 ml of water was added and kept for 48 hours and macerated then filtered. Filtrate was evaporated on Magnetic stirrer evaporator; yield obtained was 9.59 gm of extract from *Nimbooka phala twak* and 11.750 gm of extract from *Nimbooka patra*. Due to its hygroscopic nature it was stored in the desiccators.

b. Preparation of methanol extract of *Nimbooka patra and phala twak*: Extraction was carried out by Maceration method. 100 gm of *Nimbooka Patra* powder yielded 7.52 gm and *Nimbooka phala twak* yielded 11.65 gm of the extract which was stored in an air tight container and then stored in desiccators.

Source of microorganisms: Microorganisms were procured Department of microbiology, BLDEA's College of Pharmacy, Vijayapur.

Preparation of the media: Sabouraud Dextrose Agar Media (HIMEDIA –M096)

Preservation of the cultures: Cultures from Department of microbiology, BLDEA's College of pharmacy, Vijayapur were supplied on Agar media in cotton plugged test tubes.

Agar Diffusion/Cup Plate method: To determine the antimicrobial activities of the different samples of *Nimbooka Patra* and *Phala twak*.

Preparation of the samples[11]: Required amount of 100 mg of the extract was diluted in 10 ml sterile distilled water to get concentration of 10 mg/ml. 1ml of concentration of 10 mg/ml was taken in separate test tube and to that 1 ml of sterile distilled water was added to achieve the concentration of 5 mg/ml. 1 ml of concentration of 10 mg/ml was taken in separate test tube and to that 4 ml of sterile distilled water was added to achieve the concentration of 2 mg/ml. 1 ml of concentration of 10 mg/ml was taken in separate test tube and to that 9 ml of sterile distilled water was added to achieve the concentration of 1 mg/ml. Control – distilled water was taken as negative control. Standard drug – 100 mg of Fluconazole tablet was dissolved in 100 ml of Phosphate buffer saline of pH 7.4. The Phosphate buffer saline is prepared by adding Disodium hydrogen phosphate= 10.9 gm, Sodium di-hydrogen phosphate=3-2 gm, Sodium chloride= 90 gm in 1000 ml water.

Addition of the samples:

A sterile cork borer of 6 mm diameter was used to make bores in the medium which was already solidified. 4 bores were made in the plate in order to accommodate 2 different concentrations of 1 sample – for eg. 1 mg/ml and 2 mg/ml concentration of aqueous extract of *Nimbooka patra,* Standard-Fluconazole and negative control-distilled water. Same procedure is done for different concentrations of other extracts i.e., aqueous extract of *Nimbooka phala twak* and alcohol

extract of *Nimbooka patra* and *phala twak*. Sterile 1ml pipette used to fill 0.2 ml of samples, Standard and Negative control into the boxes. Plates were covered with sterile lid immediately. Proper markings were done to identify the respective bores pertaining to different samples. All the plates were allowed for the diffusion of the solution at room temperature for an hour. After an hour of pre-incubation, the plates were transferred to the incubator. It was incubated at 37° C for 24 hours. After 24 hrs of incubation, the zone of inhibition produced by different dosage forms of *Nimbooka Patra* and *Phala twak*, Standard drug were measured in mm and recorded.

Table: 1 Showing Different groups to evaluate Antifungal Activity of Nimbooka Patra and Phala twak.

Sl.No.	*Aspergillus niger*	*Candida albicans*
	Test Group I	Test Group II
1	*Nimbooka Patra* Water extract	*Nimbooka Patra* Water extract
2	*Nimbooka Patra* Methanol extract	*Nimbooka Patra* Methanol extract
3	*Nimbooka Phala twak* Water extract	*Nimbooka Phala twak* Water extract
4	*Nimbooka Phala twak* Methanol Extract	*Nimbooka Phala twak* Methanol Extract
5	Std-Fluconazole	Std- Fluconazole
6	Negative Control	Negative Control

OBSERVATIONS AND RESULTS

Table: 2 Observation during the collection of the drugs:

Sl.no	Observations	*Nimbooka patra*	*Nimbooka phala twak*
1.	Collected drug	2 kg	200 gm
2.	Yield after drying	500 gm	-
3.	Yield of the coarse powder	450 gm	90 gm
Organoleptic observations			
1.	Rupa	Blackish green	yellow
2.	Rasa	Slightly Bitter	Bitter
3.	Gandha	Pleasantly aromatic	Pleasantly aromatic

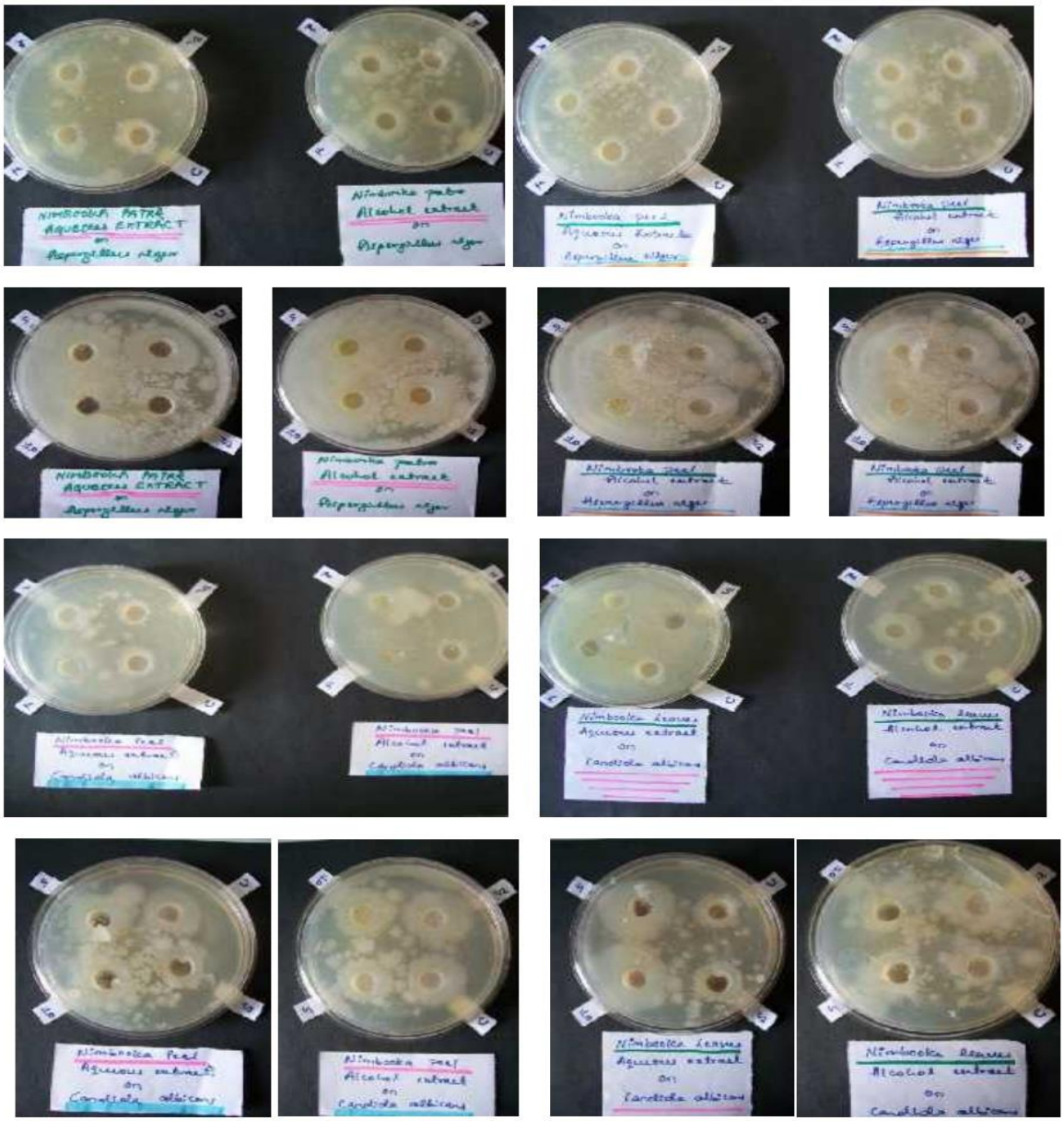
Table: 3 Observations during preparation of Methonolic extracts

Sl.no	Observations	*Nimbooka patra*	*Nimbooka phala twak*
1.	Coarse powder taken	100 gm	100 gm
2.	Extract obtained	7.52 gm	11.65 gm
3.	Yield	7.5 %	11.6 %
4.	Colour	Blackish Green	Brownish yellow
5.	Consistency	Semi Solid	Semi Solid
6.	Nature	Sticky	Sticky
7.	Odour	Pleasant	Pleasant
8.	Taste	Bitter	Pungent
9.	Solubility in water	Easily soluble	Easily soluble
10.	Colour of prepared solution	Greenish Black	Brownish yellow

Table: 4 Observations during preparation of Water extract

Sl.no	Observations	*Nimbooka patra*	*Nimbooka phala twak*
1.	Coarse powder of the drug taken	100 gm	100 gm
2.	Water taken	400 ml	400 ml
3.	Colour	Blackish Green	Dark brown
4.	Consistency	Thin watery	Thin watery
5	Odour	Pleasant	Pleasant
6	Taste	Bitter	Pungent
7	Extract Obtained	11.75 gm	9.59 gm
8	Yield	9.59 %	

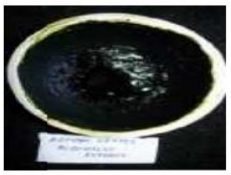
Antifungal Activity of Nimbooka extracts at different concentrations

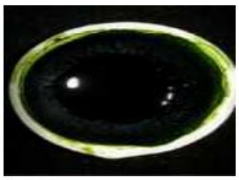





Fresh Nimbooka fruit and leaves

Nimbooka peel and dried leave

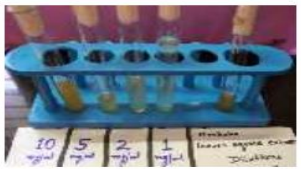


Nimbooka Peel & leaves Methanol extract

Nimbooka Peel & Leaves Water extract




Inoculation of Fungal strains: Candida Albicans and Aspergillus Niger




Preparation of different concentrations of the extracts



Preparation of Petriplates

Observations during pilot study:

- **Solution of Water Extracts**: Water extracts of *Nimbooka patra* was Blackish Green and that of *Nimbooka phala twak* was Brownish yellow in color.
- **Solution of Methanol Extracts**: Methanol Extracts of *Nimbooka patra* and *Nimbooka phala twak* dissolve in water easily and was Blackish Green and Brownish yellow in color respectively.

During the pilot study of the antimicrobial assay it was found that

- Drugs exhibited the zone of inhibition by 24 hrs.
- Zone of inhibition was maintained till 48 hrs.
- However by 72 hrs there was complete fungal growth.

Antifungal Activity:

Group I: *Aspergillus niger*

- All the 6 test samples showed antifungal activity against *Aspergillus niger*
- *Nimbooka peel* Water extract at a concentration of 10mg/ml showed highest Zone of inhibition of 52 mm against *Aspergillus niger*.
- *Nimbooka peel* Methanol extract at a concentration of 5 mg/ml and 10

mg/ml showed highest Zone of inhibition of 49 mm against *Aspergillus niger*.

- *Nimbooka leaf* Water extract at a concentration of 10mg/ml showed highest Zone of inhibition of 43 mm against *Aspergillus niger*.
- Standard drug Fluconazole showed highest zone of inhibition of 40mm against *Aspergillus niger*.
- *Nimbooka leaf* Methanol extract at a concentration of 10 mg/ml showed highest Zone of inhibition of 39.5 mm against *Aspergillus niger*.

Note: *Nimbooka peel* water extract was found to be very effective in *Aspergillus niger* when compared to other test dosage forms.

Group II: *Candida albicans*

- All the 6 test samples showed antifungal activity against *Candida albicans*
- *Nimbooka leaf* water extract at a concentration of 10 mg/ml showed a zone of inhibition of 40mm against *Candida albicans*
- *Nimbooka leaf* methanol extract at a concentration of 5 mg/ml showed a zone of inhibition of 30 mm against *Candida albicans*
- *Nimbooka peel* water extract at a concentration of 10 mg/ml showed a zone of inhibition of 30mm against *Candida albicans*
- *Nimbooka* peel methanol extract at a concentration of 5 mg/ml showed a zone of inhibition of 33 mm against *Candida albicans*
- Standard drug Fluconazole at concentration 100 mcg/ml showed zone of inhibition of 21 mm against *Candida albicans*.
- **Note: *Nimbooka leaf* water extract was found to be very effective in *Candida albicans* when compared to other test dosage forms.**

Comparison between dosage forms of each drug (the mean value of zone of inhibition against the 2 organisms) – Anti fungal Activity

Nimbooka patra

- Among the different dosage forms viz. Methanol extract and water extract, the mean difference was insignificant at the significance level at 5% between the organisms Aspergillus niger and Candida albicans

Nimbooka phala twak

- Among the different dosage forms viz. Methanol extract and water extract, the mean difference was insignificant at the significance level at 5% between the organisms Aspergillus niger and Candida albicans.

Comparison of antifungal activity of different drug delivery forms against each test organism.

1 **Standard – Fluconazole :**
   - Aspergillus niger was sensitive to Fluconazole with a zone of inhibition of 25.5 ± 8.43 mm
   - Candida albicans was sensitive to Fluconazole with a zone of inhibition of 19. ± 1.78 mm

1. ***Nimbooka patra* methanol extract :**
   - Candida albicans was sensitive to Nimbooka patra methanol extract with a zone of inhibition 24 ± 3.4mm.
   - Aspergillus niger was sensitive to Nimbooka patra methanol extract with a zone of inhibition 29.5 ± 7.95mm
2. ***Nimbooka patra* water extract :**
   - Aspergillus niger was the most sensitive to Nimbooka

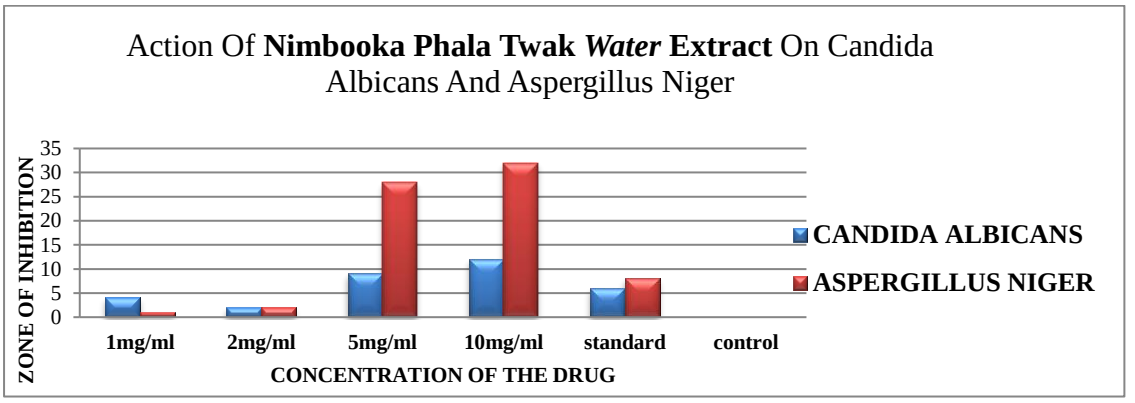
patra water extract with a zone of inhibition 27.3 ± 10.69 mm.

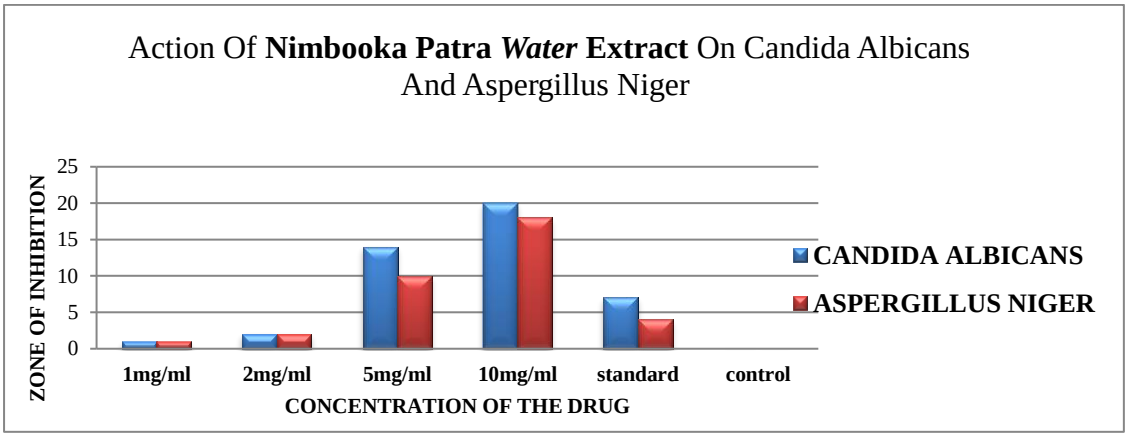
- Candida albicans was less sensitive to Nimbooka patra water extract with a zone of inhibition 22 ± 11.28 mm

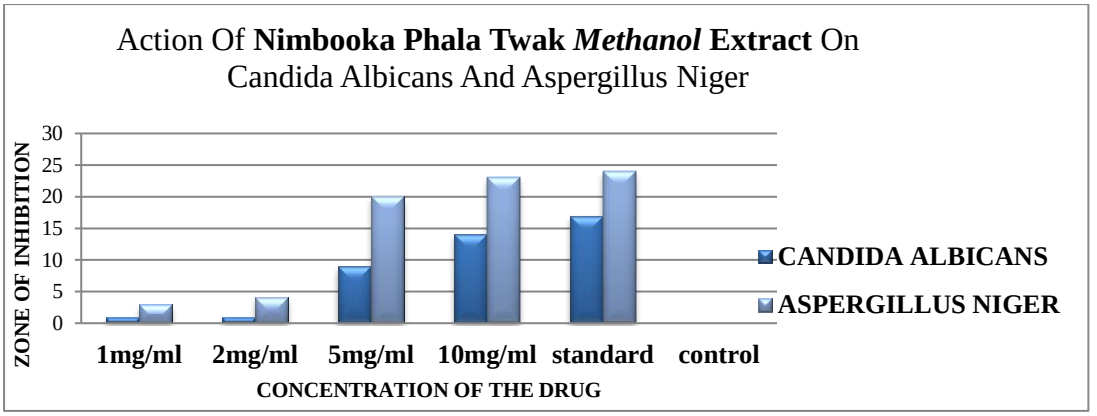
3. ***Nimbooka phala twak* methanol extract :**
   - Candida albicans was the less sensitive to Nimbooka phala twak methanol extract with a zone of inhibition 21.5 ± 8.46 mm
   - Aspergillus niger was most sensitive to Nimbooka phala twak methanol extract with a zone of inhibition 32.34 ± 11.8 mm
4. ***Nimbooka phala twak* water extract :**
   - Aspergillus niger was the most sensitive to Nimbooka phala twak water extract with a zone of inhibition 30 ± 14.6 mm
   - Candida albicans was sensitive to Nimbooka phala twak water extract with a zone of inhibition 24 ± 4.9 mm

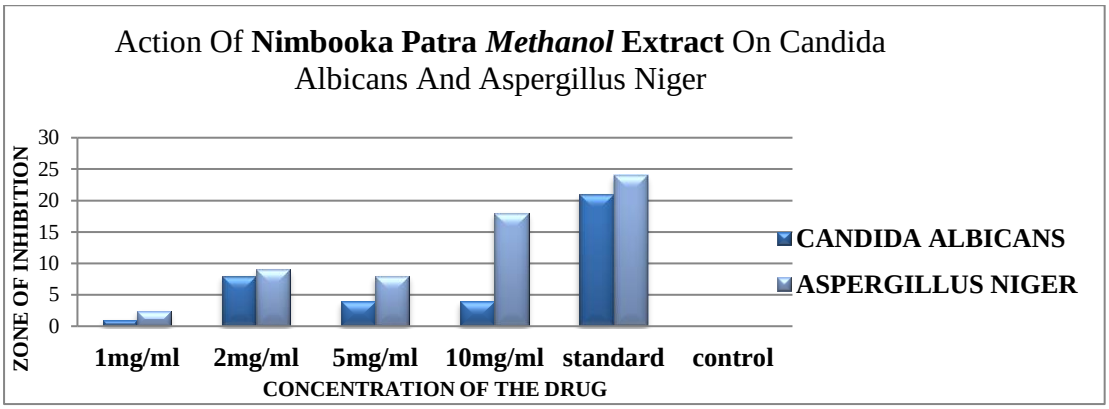
Note: Aspergillus niger was found to be more sensitive when compared to Candida albicans.

ANTI FUNGAL ACTIVITY






Action Of Nimbooka Phala Twak Methanol Extract On Candida Albicans And Aspergillus Niger
ZONE OF INHIBITION
30
25
20
15
10
5
0
1mg/ml
2mg/ml
5mg/ml
10mg/ml
standard
control
CONCENTRATION OF THE DRUG
CANDIDA ALBICANS
ASPERGILLUS NIGER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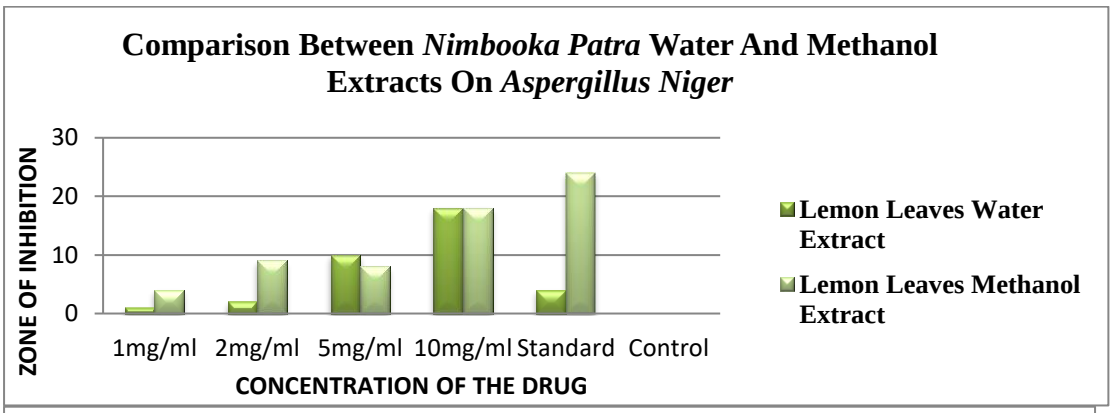
Comparison Between Nimbooka Patra Water And Methanol Extracts On Aspergillus Niger
ZONE OF INHIBITION
30
20
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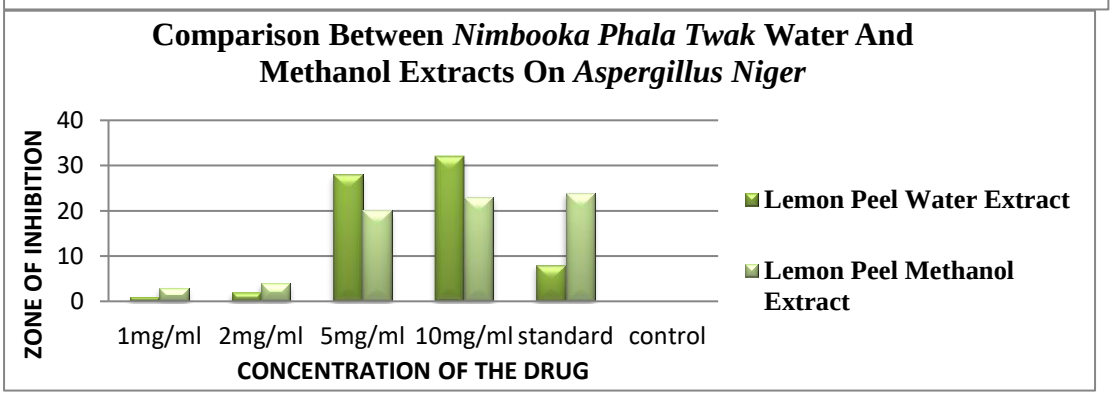
1mg/ml
2mg/ml
5mg/ml
10mg/ml
Standard
Control
CONCENTRATION OF THE DRUG
Lemon Leaves Water Extract
Lemon Leaves Methanol Extract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40
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10mg/ml
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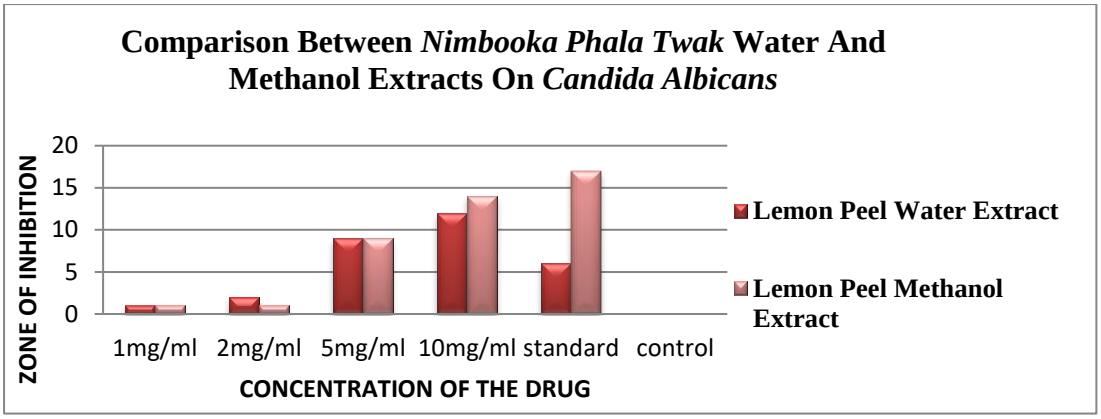
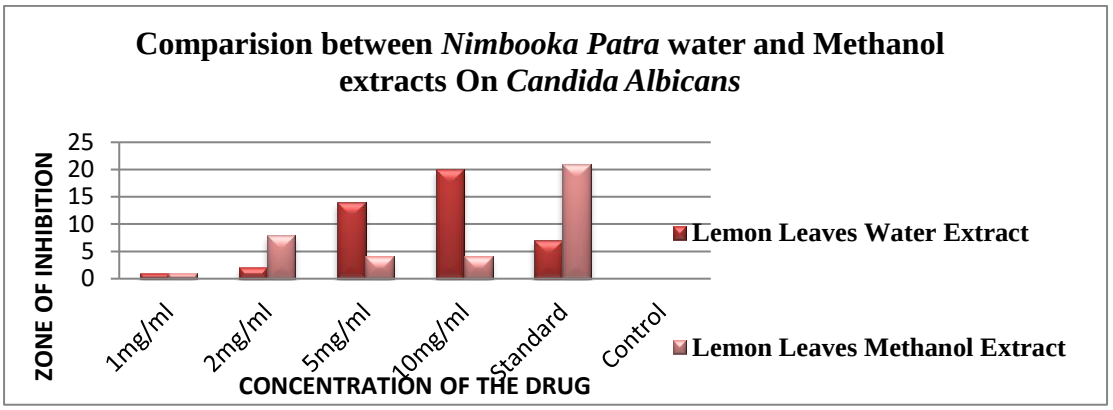
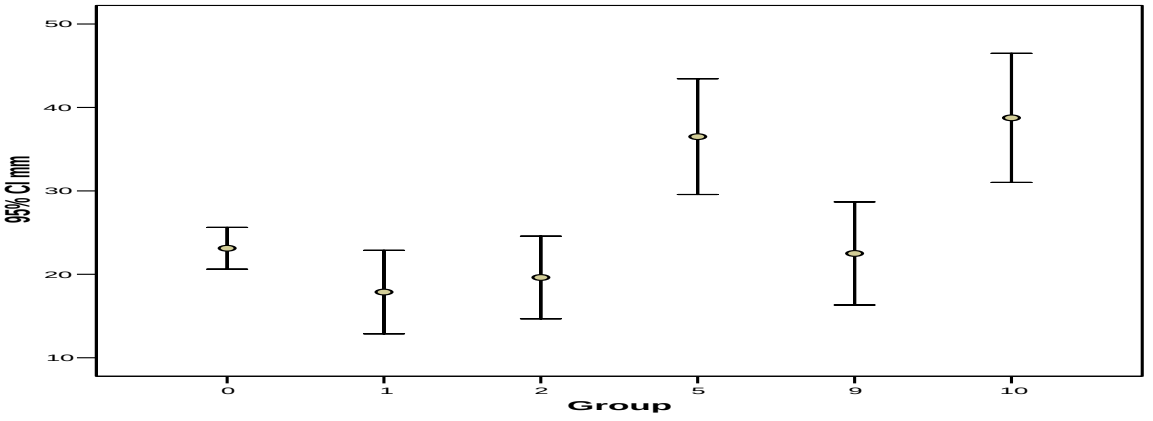
CONCENTRATION OF THE DRUG
Lemon Peel Water Extract
Lemon Peel Methanol Extract



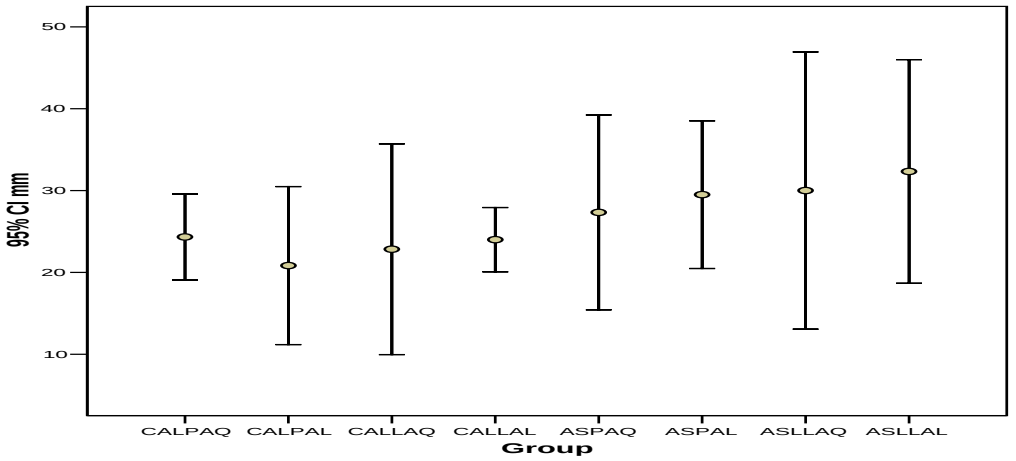


Total Antifungal Activity

Different concentration	Aspergillus niger		Candida albicans		% of Difference	± SE	t	P
	n	Mean ± S.D.	N	Mean ± S.D.				
1 mg/ml	4	18±2.94	4	17.75±8.65	1.39	4.57	0.054	>0.05
2 mg/ml	4	22.5±3.31	4	16.75±699	25.56	3.87	1.485	>0.05
5 mg/ml	4	42±8.36	4	31±3.16	26.19	4.47	2.459	>0.05
10 mg/ml	4	45.75±5.85	4	31.75±5.9	30.60	4.16	3.366	<0.05
standard	4	25.75±9.74	4	19.25±2.06	25.24	4.98	1.305	>0.05
control	4	25±2.58	4	21.25±2.21	15.00	1.70	2.203	>0.05



0= control, 1= 1mg/ml, 2=2mg/ml, 5=5mg/ml, 9= standard, 10=10mg/ml,



CA= Candida albicans, AS= Aspergillus niger

LPAQ= lemon peel aqueous extract, LLAL= lemon peel alcohol extract

LLAQ=lemon leaves aqueous extract, LLAL=lemon leaves alcohol extract

DISCUSSION

Pilot study:

- The methanol extracts of *Nimbooka Patra* and *Phala twak* dissolved in water completely, wherein both dissolved easily and uniform solution was obtained.
- Agar cup plate method- was preferred than other methods because of the requirement of the minimum sample size; several samples can be tested on one micro organism.
- Pilot study was carried out to determine the effective concentration at which the trial drugs showed the anti fungal activity.
- It was observed that during the pilot study both fungal strains showed encouraging growth in sabouraud dextrose agar media. Hence it was taken up for the study proper.
- Standard drug Fluconazole showed good activity at 100mcg/ml. Hence this concentration was selected for the study proper.
- Distilled water was selected as the negative control group, to rule out the role of methanol in antifungal activity.

Study proper:

- The parameters selected for the antifungal activity were zone of inhibition in milligram in agar cup plate method.
- The study proper was carried out in 2 dosage forms viz. methanol and water extract, because in vitro studies allow extracts as dosage because different extracts reveal the different phyto-constituents present in it.
- In agar well diffusion/cup plate method, *Nimbooka Phala twak* water extract showed good activity against Aspergillus niger whereas *Nimbooka Patra* water extract showed good activity against Candida albicans. It showed antifungal activity at 10 mg/ml.

Test group I: Aspergillus niger

Nimbooka phala twak water extract was found to be very effective in Aspergillus niger

when compared to other test dosage forms. This may be due to presence of Hesperidin, Quercetin, Volatile oil, Pectin, Calcium oxalate, Bitter substances, α-terpineol, Linailye acetate, Gerange acetate, Luteolin-7-rutinoside, Quercetin-3, 5-diglucoside.

Test group II: Candida albicans

Nimbooka leaf water extract was found to be very effective in Candida albicans when compared to other test dosage forms. This may be due to presence of Rectin, Hesperidin, Neral, Geraniol, Limonene, Citral.

Comparison between the groups:

Nimbooka Phala twak in different dosage forms was more active and sensitive against Aspergillus niger when compared with Candida albicans. This shows that *Nimbooka Phala twak* water extract is more sensitive for Aspergillus niger when compared with that of Candida albicans.

Nimbooka phala twak water extract has high significant activity with 52 mm when compared to that of methanol extract (49 mm).

Though all the forms of *Nimbooka Phala twak* are sensitive against both fungal strains, the sensitivity of Nimbooka Phala twak may be due to the presence of *Amla, Katu rasa, ushna veerya* and *Tiksna guna*. Whereas in *patra*, presence of *Amla, katu rasa, ushna veerya* and *laghu guna*.

The well known standard drug Fluconazole showed better antifungal activity against Aspergillus niger and Candida albicans.

Krimighna activity of Nimbooka Patra and Phala twak (Ayurvedic Perspective):

- For a *krimi* to manifest it requires *drava* with *kledamsha* and this is very true in fungal infection also. Thus the drug due to the *kaphahara* and *kleda achushana* ability has a direct action on the *krimi*.
- *Nimbooka Patra* as well as *Phala twak* possesses *Amla & Katu rasa*, *laghu guna* and *ushna veerya*[12, 13, 14]. The *Amla & katu rasa* is considered to be *Dhatu hrasaka* (causes the depletion of the *dhatus*)
- *Nimbooka* has its action like *Agnivardhana, Rochana, Pachana, Deepana, Varnya, Balakara, Shleshma* and *Vatahara* due to *Tikshana, Ushna guna* and hence does the *Prakruti Vighata* by reducing the *lakshanas* of *krimi* like *Chardi, Vibandha, Mandanala, Arochaka*, etc[12, 13, 14, 15, 16].
- Hence it can be inferred that *Nimbooka* due to its above properties succeeds in checking the growth of the microbe. This can be very much compared to the fungistatic activity or *prakruthi vighatha* where in an unfavorable environment for the growth of fungus is created.
- The activities of *krimighna dravyas* in In-vitro studies is limited to the antimicrobial activity alone but in-vivo they may have wide spectrum of activities such as Anti inflammatory, phagocytosis, neutralizing the circulating endotoxins produced by the micro organisms etc. thus proving that the *krimighna dravyas* have a holistic approach.

CONCLUSION

Nimbooka Phala twak has a higher yield in methanol extract when

compared to *Nimbooka Patra*. *Nimbooka Patra* has a higher yield in water extract when compared to *Nimbooka Phala twak*. Agar diffusion/cup plate method is the most convenient method to screen the herbal extracts. Among both the drugs, *Nimbooka Patra* has shown better activity than *Nimbooka Phala twak* against Candida albicans where as *Nimbooka Phala twak* has shown better activity than *Nimbooka Patra* against Aspergillus niger. Among the two extracts of *Nimbooka,* water extracts showed better activity than methanol extracts. It is observed from the in-vitro study that the drug *Nimbooka Phala twak* and *patra* has shown comparative antifungal activity with that of standard drug Fluconazole. Thus it can be concluded that at a lower dose of *Nimbooka Patra* and *phala twak* is having statistically significant antifungal activity when compared with standard drug.

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