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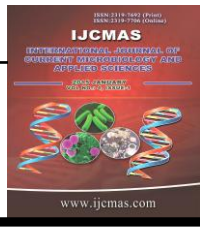

Original Research Article

Effect of Neem extract against opportunistic bacterial and fungal pathogens associated with AIDS

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Keywords

AIDS, Opportunistic infection, Neem extract, Disc diffusion method, Antimicrobial activity, Zone of inhibition

A B S T R A C T

Having HIV is not the same thing as having AIDS. With the right treatment, of AIDS patient's life quality and life expectancy could be raised. This study aims to examine the effect of Neem plant parts extract against HIV related opportunistic bacterial and fungal pathogens. Opportunistic infections are most common in immunocompromised patients which are the leading cause of death in HIV-infected patients. Antimicrobial activity of the acetone and chloroform extract of Neem leaf, fruit and bark respectively was investigated by Agar well-diffusion methods against 8 bacterial and 2 fungal pathogens. Neem bark and leaf with acetone and chloroform extract showed highest inhibitory activity as compared with all treatments; most of the pathogens were inhibited by 30mg extract concentration on disc. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were found to be sensitive bacteria against most of the plant extract as compared to the other bacteria. *Candida albicans* was inhibited more often by plant extract of Neem leaf, bark and fruit as compared to *Cryptococcus neoformans,* which was inhibited by Neem bark extract only. The side effects of the synthetic antibiotic drugs would be eliminated by replacing it with the use of medicinal bioactive compounds which could be taken throughout lifetime.

Introduction

AIDS is a condition characterized by the development of life-threatening opportunistic infection or malignancies in a patient with severe depression of the T-cell mediated immune system caused by infection with human immune deficiency virus (HIV). There are currently over 34 million people worldwide infected with human immune deficiency virus (HIV) with 15,000 new patients infected each day (Saini, 2011). India has an estimated 5.2 million HIV-infected people. The threat to their life is not from the virus alone. Opportunistic infections (OIs) and associated complications account for a considerable proportion of such mortality (Anonymous, 2007). The breakdown of body immune system is the hallmark of HIV infection. Infections which are rarely seen in those with normal immune systems are deadly to those with HIV. The effect of HIV on the immune system is monitored by

measuring the CD4 (T-helper) lymphocyte count in the blood. Depletion of CD4 cell count is a hallmark of disease progression in AIDS (AydinCiledag and Demet Karnak, 2011). OIs are caused by various pathogenic microorganisms such as bacteria, fungi, virus and parasites (Hirschtick *et al.*, 1995). Many of the antibiotics used in management of bacterial infections are experiencing increased resistance posing enormous public health concern.

Medicinal plants are part and parcel of human science from the dawn of civilization. In India they form the backbone of several indigenous traditional medicines (Nayak *et al.,* 2011). Medicinal plants are various plants used in herbalism and thought by some to have medicinal properties. Plants have a great potential for producing new drugs for human benefit. Neem is used in traditional medicine as a source of many therapeutic agents in the Indian culture and grows well in the tropical countries. Its twigs provide a chewing stick and are widely used in the Indian sub-continent (Almas, K., and Ansallafi, T.R., 1995). The chemical constituents contain many biologically active compounds that can be extracted from Neem, including alkaloids, lavonoids, triterpenoids, phenolic compounds, carotenoids, steroids and ketones, biologically most active compound is azadirachtin, it is actually a mixture of seven isomeric compounds labelled as azadirachtin A-G and azadirachtin E is more effective (Verkerk and Wright, 1993). Other compounds that have a biological activity are salannin, volatile oils, meliantriol and nimbin (Anonymous, 1992).

There is a continuous development of resistant strains which pose the need for search and development of new drug to cure diseases. Systematic screening of them may result in the discovery of novel effective antimicrobial compounds. According to a report of WHO, more than 80% of world's populations depend on traditional medicine for their primary health care needs. Extraction (as the term is pharmaceutically used) is the separation of medicinally active portions of plant (and animal) tissues using selective solvents through standard procedures. Such extraction techniques separate the soluble plant metabolites and leave behind the insoluble cellular marc. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form, and are intended for oral or external use (Handa *et al.,* 2008).

Traditional systems of medicine like Ayurveda, Unani, Homeopathy and Siddha solely rely on phyto-pharmaceuticals that are obtained from selected medicinal plants (herbs) based on traditional knowledge gained over a period of time and expertise by the because of the wide spread belief that 'herbal medicine' is safer than costly synthetic drugs which possesses side effects.

Materials and Methods

Collection of plant materials

The samples for antibacterial and antifungal activity testing were collected from local places. Neem plant parts i.e. bark, leaves and fruit were collected in cotton bags by cutting it in to small pieces. Authentication of plant material was done at Medicinal Plant Nursery, Mahatma Phule Krishi Vidhyapeeth Rahuri. Tal – Rahuri, Dist. Ahmednagar, Maharashtra, India.

Preparation of plant extract

After collection of the plant parts were cleaned (running tap water for 10 min and

then with distilled water for 5 min), shade dried (10 to 15 days) and powdered by using mixer grinder. The plant extracts were obtained by using Soxhlet apparatusas depicted in Fig. 1. Two types of solvents were used namely acetone and chloroform. The excess solvent present in the recovered extracts were allowed to evaporate by keeping it at room temperature. The dry weights of extract were measured. 1 mg/ml stock solutions of the extracts were prepared by dissolving the 1mg of dry extract in to 1ml of respective solvent and stored at room temperature until further antimicrobial assay.

Collection of clinical samples

CD4 count was done for the HIV patients as shown Table 1. The blood for CD4 detection had been collected within one week of collection of clinical specimens for culture of bacteria and fungus. The CD4 count is the number of CD4 cells per microliter (μL) of blood. It is used to stage the patient's disease, determine the risk of opportunistic illnesses, assess prognosis, and guide decisions about when to start antiretroviral therapy (ART). Clinical samples namely blood, urine, sputum, pus and wound, nail and head scrapings were collected from the suspected symptomatic HIV positive patients, under the watchful eye of experienced medical practitioner. All unsuitable specimens were discarded and a repeat specimen was collected.

Isolation and Identification of pathogens

Bacterial and fungal pathogens tested in this study were isolated from clinical samples of suspected symptomatic HIV patients. Eight bacterial and two fungal pathogens were isolated and confirmed by staining, morphological and biochemical characteristics.

Preparation & Maintenance of stock culture

The clinical isolates of bacterial and fungal pathogens were inoculated on nutrient agar slants and potato dextrose agar respectively and incubated overnight at 37^{o}C. These cultures were stored in a refrigerator at 4^{o}C. Fresh slopes cultures were prepared every 3-4 weeks until subjected to further antimicrobial study.

Preparation of inoculum culture

The inoculums for most of the microorganisms was prepared by transferring a loop full culture of microorganisms from the agar slant to a tube containing 5 ml of liquid media with the help of nicrome wire loop and incubated for 24 h at 37 ^{0}C. The tubes were shaken occasionally to aerate for promoting growth. Fresh 24 hr culture of bacteria was suspended in sterile distilled water to obtain a turbidity of 0.5 McFarland standard. The final inoculum size was adjusted to 5×10^{5} CFU/ml.

Inoculating the agar plates

Approximately 10ml of sterilized medium was poured in autoclaved petri-plate for base agar under sterilized condition. The overnight grown culture of turbidity approximately $5x10^{5}$ bacterial and fungal was used to inoculate agar assay plates. Petri dishes were poured with 1ml of bacterial and fungal suspension which had been growing overnight for 24 h at 37°C and was swabbed with a sterile cotton swab by rotating the assay plates**.**

Antimicrobial assay

The antimicrobial assay of acetone and chloroform extracts of Neem bark, leaves and fruit was performed by disc diffusion

method. From the 1 mg/ml extract stock solution, sterilized paper disc of 6mm diameter had prepared from card sheet paper, and were impregnated with plant extract with help of micropipette in such way that each disc will receive the extract concentration as 5mg, 10mg, 20mg, 30mg and kept for drying in laminar air flow for 2-4 hrs for evaporating of the solvents. Dried discs containing extracts of plant material and control discs were then aseptically placed on the culture media.

Plates were then incubated upside down for 18-24 h at 37 ^{0}C. Negative control discs were prepared by imprinting the discs with acetone and chloroform respectively. Similarly, standard positive control discs (containing antibiotics) of HiMedia were used. The diameter of inhibition zones was measured with help of Hi antibiotic zone scale (HiMedia).

Results and Discussion

Preparation of plant extract

Standard procedure for extraction of antibacterial and antifungal compounds by using Soxhlet apparatus was followed (Mukherjee and Pulok, 2010). Acetone and chloroform extracts of Neem (Leaf, Bark and fruit) were collected; stock solutions were prepared and stored at room temperature for further studies shown in Table 3.

Isolation and identification of pathogens from suspected symptomatic HIV patients

As per the guidelines and procedure for biochemical test referred from *Microbiology Laboratory Manual* (Sundarraj, 2010), 8 major bacteria and 2 major fungi were isolated from the clinical samples of suspected symptomatic HIV patients. All the isolated bacterial and fungal species were confirmed through morphology, staining and biochemical tests and all the test results were compared with the standard test results for confirmation shown in Table 2. Isolated pathogen with its percentage of occurrence in respective clinical samples is mentioned in Table 1. Eight major bacterial pathogens were isolated from the samples; *Pseudomonas aeruginosa (53%), Escherichia coli (79%), Salmonella typhi (33%), Shigella (33%), Streptococcus pneumoniae (75%), Staphylococcus aureus (83%), Acinetobacter (29%), Klebsiella pneumoniae (40%).* Only two fungal spp., were identified i.e. *Candida albicans (43%), Cryptococcus neoformans (50%).*

Effects of neem extract against opportunistic pathogens

Table 4 and Table 5 represents the comparative results of the zones of the inhibition formed by the acetone and chloroform extract prepared from different Neem plant parts against bacterial and fungal opportunistic pathogens tested by disc diffusion assay.

Effect of acetone solvent extract on bacterial pathogen

Neem leaf extract 30mg, showed highest zone of inhibition (20mm) against *Pseudomonas aeruginosa* and *Staphylococcus aureus* followed by (16mm) for *E. Coli* and *Klebsiella pneumoniae* respectively.

Streptococcus pneumoniae and *Staphylococcus aureus* were inhibited by Neem bark extract with zone of inhibition (14mm) at 30mg extract concentration on disc. Earlier studies concluded that Neem leaf extract 30mg/ml was more effective than bark extract against *Staphylococcus aureus* with zone inhibition 17mm (Chaturvedi *et al.*, 2011). Neem fruit extract

does not showed promising result in terms of zone of inhibition except, *Shigella* inhibited by 30gm extract with zone of inhibition (14mm).

Effect of chloroform solvent extract on bacterial pathogen

Neem leaf extract with chloroform showed antibacterial activity against *Streptococcus pneumoniae* and zone of inhibition (18mm) was observed with 30mg extract concentration. Neem bark was found to be having antibacterial activity against *Staphylococcus aureus* with zone of inhibition 17mm. Neem fruit extract showed zone of inhibition (12mm) at 30mg concentration against *E. Coli.*

Effect of acetone and chloroform solvents extract on fungal pathogen

Candida albicans growth was inhibited by both type of solvent extracts of Neem Leaf, bark & fruit. Highest zone of inhibition (17mm) was observed against Neem leaf with chloroform extract. The 5 % aqueous leaf extract of neem caused an inhibition in growth of *Candida albicans* (Mahmoud *et al.*, 2011). *Cryptococcus neoformans* was found to be resistant against most of the types of extract except Neem bark with acetone and chloroform extract shown zone of inhibition (12mm and 13mm) respectively.

Table.1 Isolation of pathogens from clinical samples of HIV positive patients

Organisms	**Specimens**	**No. of HIV patients clinical samples**	**Positive clinical samples**	**% of Occurrence**	**CD4 count/µL**
Pseudomonas aeruginosa	Sputum, wound swab	36	19	53	300-380
Escherichia coli	Stool	14	11	79	340-360
Salmonella typhi	Stool	9	3	33	250-410
Shigella	Stool	6	2	33	280-390
Streptococcus pneumoniae	Sputum	28	21	75	410-500
Staphylococcus aureus	Sputum	30	25	83	300-390
Acinetobacter	Wounds, skin scraping	14	4	29	270-350
Klebsiella pneumoniae	Stool	5	2	40	400-490
Candida albicans	Oral swab	7	3	43	380-490
Cryptococcus neoformans	CSF	2	1	50	100-150

Table.2 Summary of the Staining, Morphology and Biochemical characteristics of isolated bacteria and fungi form clinical samples of suspected symptomatic HIV patients

Sr. No.	Name of Organism	Gram Staining	Morphology	Catalase	Oxidase	Motility	Coagulase	TSI	Urease	Indole	Methyl red	Voges-Proskauer	Citrate	Glocose	Lactose	Maltose	Sucrose
1.	*Pseudomonas aeruginosa*	-	*Bacilli*	+	+	+	-	- - / -+	-	-	-	-	+	***WF***	***NF***	***NF***	***NF***
2.	*Escherichia coli*	-	*Bacilli*	+	-	+	-	***AA/- -***	-	+	+	-	+	***F***	***F***	***WF***	***NF***
3.	*Salmonella typhi*	-	*Bacilli*	+	-	+	-	***KK*** / - +	-	-	+	-	+	***F***	***F***	***W F***	***NF***
4.	*Shigella*	-	*Bacilli*	-	-	-	-	***KK*** / - +	-	-	+	-	-	***F***	***F***	***W F***	***NF***
5.	*Streptococcus pneumoniae*	+	***Cocci***	-	***NP***	***NP***	+	***NP***	***NP***	***NP***	***NP***	***NP***	***NP***	***NP***	***NP***	***NP***	***NP***
6.	*Staphylococcus aureus*	+	***Cocci***	+	***NP***	***NP***	+	***NP***	***NP***	***NP***	***NP***	***NP***	***NP***	***NP***	***NP***	***NP***	***NP***
7.	*Acinetobacter*	-	***Cocci***	-	-	-	-	***AA/- -***	+	-	-	-	+	***F***	***WF***	***NF***	***NF***
8.	*Klebsiella pneumoniae*	-	*Bacilli*	+	-	+	-	***A K/+-***	+	-	-	+	+	***F***	***F***	***NF***	***NF***
9.	*Candida albicans*	On Sabouraud's dextrose agar colonies were white to cream colored, smooth, glabrous and yeast-like in appearance. Clusters of round blastoconidia were present at some septae. Thick walled chlamydospores were seen.															
10.	*Cryptococcus neoformins*	Colonies (SDA) cream-coloured smooth, mucoid yeast-like colonies. Indian Ink staining microscopic observation -Positive - Distinct, wide gelatinous capsules was present.															
NP – Not performed, F = Fermenter, WF = Weak Fermenter, NF = Non Fermenter, + = positive, - = Negative, A/A = Acid slant acid butt, K/A = Alkaline slant acid butt																	

Table.3 Summary of the concentration of the extract obtained from different plant parts g/ml

Sr. No.	Extract	Wt. of extract (g)	Wt. of empty tube (g)	Total weight of extract (g)	Stock solution g/ml
Acetone					
	Neem				**1g/ml**
1.	Fruit	**39.2**	**21.8**	**17**	
2.	Bark	**26.3**	**21.98**	**4.3**	
3.	Leaves	**25.5**	**21.48**	**4**	
Chloroform					
	Neem				**1g/ml**
4.	Fruit	**32**	**22**	**10**	
5.	Bark	**29.1**	**21.68**	**7.4**	
6.	Leaves	**33.6**	**21.52**	**12.08**	

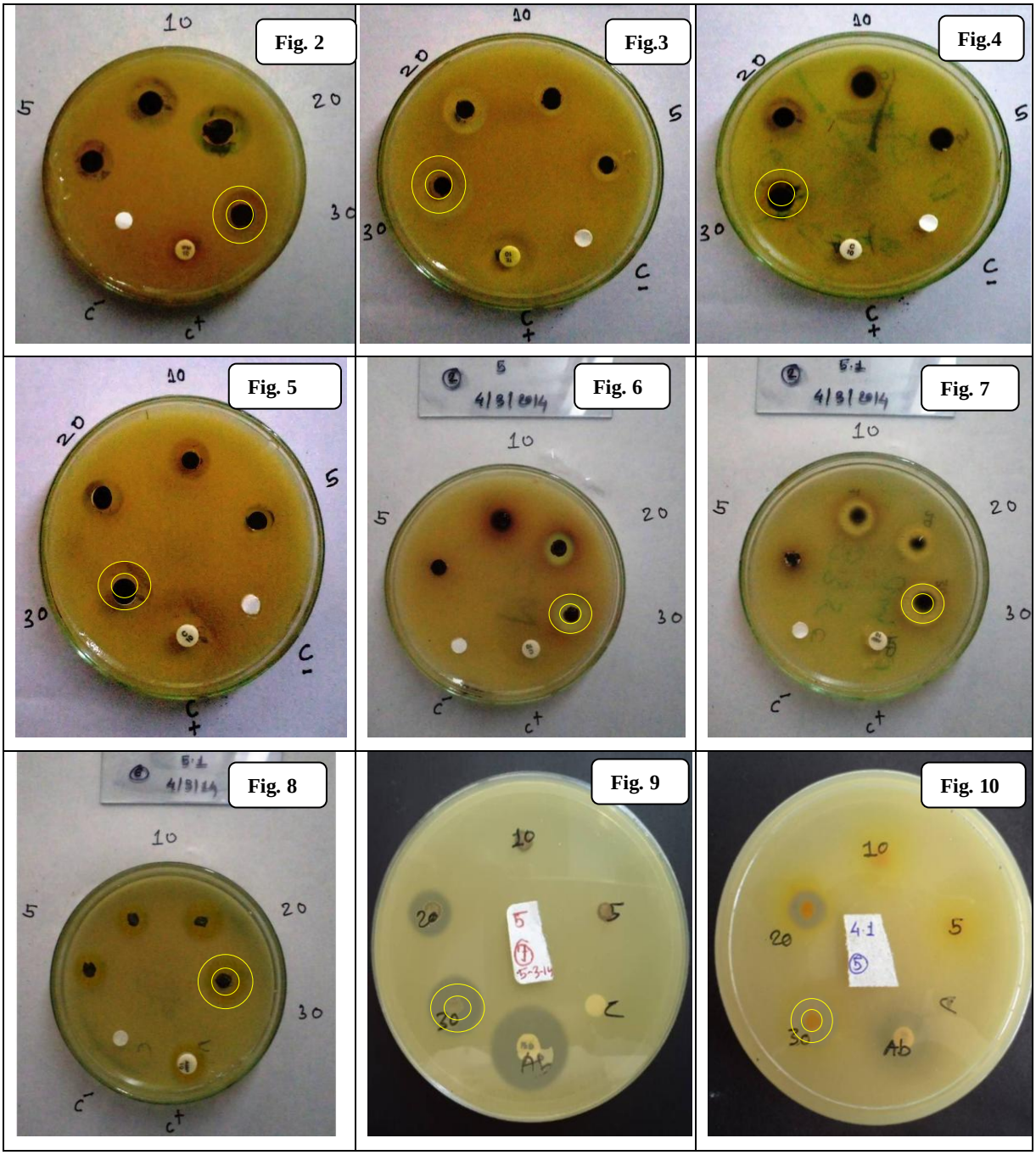
Table.4 Comparative results of the zones of the inhibition formed by the acetone extract prepared from different plant parts of Neem against bacterial and fungal opportunistic pathogens tested by disc diffusion assay

Plant Name	Plant part	Extraction solvent	Concentration of Extract mg/ml	Zone of inhibition in mm									
				Pseudomonas aeruginosa	*Escherichia coli*	*Salmonella typhi*	*Shigella*	*Streptococcus pneumoniae*	*Staphylococcus aureus*	*Acinetobacter*	*Klebsiella pneumoniae*	*Candida albicans*	*Cryptococcus neoformans*
Neem	Leaf	Acetone	**5**	10	-	-	-	-	11	-	10	-	-
			10	15	10	-	-	-	13	-	13	-	-
			20	16	12	-	14	12	18	-	15	11	-
			30	20	16	-	15	-	20	-	16	12	-
	Bark	Acetone	**5**	-	-	-	-	-	-	-	-	-	-
			10	-	-	-	-	-	10	-	-	-	-
			20	11	-	-	11	12	13	-	-	-	-
			30	12	-	-	13	14	14	-	12	13	12
	Fruit	Acetone	**5**	-	-	-	-	-	-	-	-	-	-
			10	-	-	-	-	-	-	-	-	-	-
			20	-	-	-	-	-	-	-	-	-	-
			30	-	10	-	14	-	-	-	-	12	-
Disc with Acetone			**-Ve C**	-	-	-	-	-	-	-	-	-	-
Antibiotic disc			**+Ve C**	+	+	+	+	+	+	+	+	+	+

Table.5 Comparative results of the zones of the inhibition formed by the chloroform extract prepared from different plant parts of Neem against bacterial and fungal opportunistic pathogens tested by disc diffusion assay

Plant Name	Plant part	Extraction solvent	Concentration of Extract mg/ml	Zone of inhibition in mm									
				Pseudomonas aeruginosa	*Escherichia coli*	*Salmonella typhi*	*Shigella*	*Streptococcus pneumoniae*	*Staphylococcus aureus*	*Acinetobacter*	*Klebsiella pneumoniae*	*Candida albicans*	*Cryptococcus neoformans*
Neem	Fruit	Chloroform	**5**	-	-	-	-	-	-	-	-	-	-
			10	-	-	-	-	-	-	-	-	-	-
			20	-	10	-	-	-	-	-	-	-	-
			30	-	12	-	10	-	-	-	-	14	-
	Leaf	Chloroform	**5**	-	-	-	-	12	11	-	-	-	-
			10	10	-	-	-	13	12	-	-	13	-
			20	11	-	-	-	14	15	-	12	15	-
			30	12	-	12	-	18	16	-	13	17	-
	Bark	Chloroform	**5**	-	-	10	-	-	12	-	-	10	-
			10	-	-	-	-	-	13	-	-	12	-
			20	-	-	-	14	14	16	-	-	13	12
			30	14	-	-	16	16	17	-	10	15	13
Disc with Chloroform			**-Ve C**	-	-	-	-	-	-	-	-	-	-
Antibiotic disc			**+Ve C**	+	+	+	+	+	+	+	+	+	+

Fig.1 Soxhlet apparatus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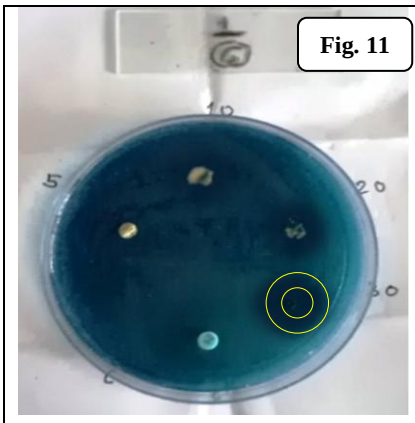

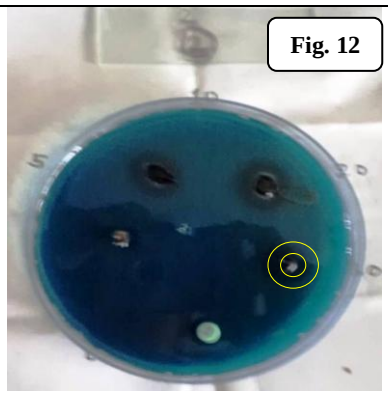

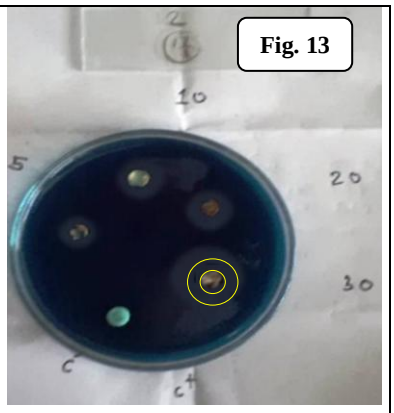

Figs 2-13: Zone of inhibitions against opportunistic bacterial and fungal pathogens formed by acetone and chloroform Neem extract. **(Fig. 2-5)** Zone of inhibition (20mm) against *Pseudomonas aeruginosa* and *Staphylococcus aureus* followed by (16mm) for *E. Coli* and *Klebsiella pneumoniae* respectively by formed by Neem leaf with acetone extract. **(Fig. 6-7)** Zone of inhibition (14mm) against *Streptococcus pneumoniae* and *Staphylococcus aureus* formed by Neem bark with acetone extract. **(Fig. 8-10)**Zone of inhibition (18, 17 and 12mm) against *Streptococcus pneumoniae, Staphylococcus aureus and E. coli* formed by Neem leaf, bark and fruit with chloroform extract respectively. **(Fig. 11-13)** Zone of inhibition (17mm) against *Candida albicans* formed by Neem leaf with chloroform extract and zone of inhibition (12mm and 13mm) against *Cryptococcus neoformans* formed by Neem bark with acetone and chloroform extract, respectively.

Conclusion

Many of the existing synthetic drugs cause various side effects. Hence, plant compounds based drug development could be useful in meeting this demand for newer drugs with minimal side effects (Srivastava *et al.*, 2000). It is possible that Neem may take a role as an adjuvant to the use of antibiotics or as a replacement of current antibiotics to treat the opportunistic infections. The present study showed the effectiveness of Neem plant part extract with chloroform and acetone as a solvent against the most common opportunistic infection associated with AIDS. Further study also needs to be conducted to determine the active compound (s) that poses antimicrobial activities and their concentration.

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