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"ANTIFUNGAL POTENTIAL OF *MURRAYA KOENIGII (L.)* BARK ALCOHOLIC EXTRACT AGAINST PATHOGENIC FUNGAL STRAINS: AN IN VITRO STUDY" SUB TITLE: "ANTIFUNGAL ACTIVITIES OF BARK EXTRACT *MURRAYA KOENIGII (L.)"*

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Abstract

Objective: *Murraya koenigii (M. Koenigii)* is a common indigenous plant in Tamil Nadu, India, known for its various medicinal properties. The aim of this study was to investigate the potential antifungal activity of an ethanolic extract of bark of *M. koenigii* (EEBMK) against five fungal species.

Methods: EEBMK extract was evaluated in vitro for its antifungal activity against *Aspergillus niger, Trichoderma viride, Fusarium oxysporum, Candida albicans,* and *Cryptococcus neoformans* using the agar well diffusion method.

Results: EEBMK significantly inhibited the growth of *C. neoformans, C. albicans* and suppressed the growth of *F. oxysporum* and *A. niger*. However, EEBMK showed no activity against *T. viride* strains. The antifungal activity of EEBMK was sequenced as *C. neoformans* (15.6 ± 1.15 mm) > *C. albicans* (14.3 ±1.5 mm) > *F. oxysporum* (8.3 ± 0.57 mm) > *A. niger* (8 ±1 mm).

Conclusion: The findings of this study suggest that EEBMK could be used as a natural antifungal agent to treat infections caused by certain types of fungi.

Keywords: *Murraya koenigii* ; Bark; ethanolic extract ; Antifungal activities ; Zone of inhibition

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1. Introduction

It has become evident that over 200 species of plant microbes have developed resistance to chemical pesticides, which often have negative side effects. As a result, the search for organic-based antifungal and antimicrobial agents has become increasingly important for plant protection and food preservation, particularly as there is growing interest in organic food products [1]. *M. Koenigii,* commonly called curry leaf, is an aromatic shrub or small tree native to India, Sri Lanka, Nepal, and other South Asian countries. It belongs to the family 'Rutaceae' and is one of 14 global species in the genus *Murraya,* with only two species, *M. Koenigii* (L.) Spreng., and *Murraya paniculate* (L.) Jack., available in India and Nepal[2]. *M. koenigii* is known for its antioxidant, antibacterial, antiprotozoal, antimutagenic, hepatoprotective, antiviral, antitumor, anti-inflammatory, antileukemia, antifungal, cardioprotective, antidiarrheal, neuroprotective, nephroprotective, antiulcer properties and is used to treat itching, vomiting, dysentery, leukoderma, kidney pain, and hypercholesterolemia [3].

Gabriel (2014) and Prathyusha (2016) reported that *M.koenigii* leaf exhibited antifungal activity against different fungal strains. However, there is a lack of scientific reports that indicate the antifungal activities of Bark extract of *M.koenigii* [4, 5]. Therefore, this study aimed to examine the in vitro antifungal activity of EEBMK against the five fungal species, and the growth inhibitory response of each species was evaluated to elicit the effectiveness of the extract. Health hazard induced by all fungal strains is as follows: *A. niger*, a type of black mold and a filamentous ascomycete, causes decay in most organic substances such as fruits, nuts, herbs, vegetables, wood, and beans, [6,7], although *A. niger* can cause lung and ear infections in people with weaker immune systems and may also cause some plant diseases [8]. *C. albicans*, a fungal pathogen commonly found in human GIT and female lower genital tracts, is one of the unique fungi that colonize, infect, and persist on mucosal surfaces, stimulating the immune response of mucous [9]. *F. oxysporum*, a non-dermatophyte filamentous fungus that tends to cause infections in humans, causes onychomycosis, a nail fungal infection that makes the nail brittle,[10] and strain is known to cause acute lymphocytic leukemia, and the fungus generally enters the body through nostrils or ruptured tissues and mucous membranes [11,12]. *T. viride*, a free-living fungus found in the root, soil, and leafy environments, is an asexually reproducing fungus usually isolated from tropical soil. It is used to enhance plant growth and prevent plant disease [13]. *C. neoformans* is a fungal pathogen that can cause infectious illnesses in both immune-compromised and immune-competent hosts. It can cause diseases such as meningoencephalitis and pulmonary *cryptococcosis*, which typically affect immune-compromised hosts [14].

2. Materials and methods

2.1. Instruments & Chemicals

Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) Mueller-Hinton Agar (MHA), Amphotericin B (Himedia), DMSO, chemicals used in the study was purchased from HI media laboratories Pvt, ltd. (A-516, Swastik Disha Business Park, via Ind. Est, LBS Marg, Mumbai- 400086, India). Wire loop (Merck),Weighing balance (Conteck) = 0.001g to

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300g, Autoclave (ProGen), Laminar air flow chamber (SP Bio Engineering), UV Spectrophotometer - Shimadzu 1800, Incubator (Servo Scientific).

2.2. Identification of plant

Stem barks of *M. koenigii* were obtained from Namakkal district, Tamilnadu state, India, in December 2022. The stem barks were air-dried in the shade at room temperature. Taxonomic identification of the plant material was carried out by Prof. Senthil Kumar of the Department of Botany, Viekanandha Arts and Science College, Tiruchengodu, Tamilnadu, India. The voucher specimen (VICAS-23461) was kept in the herbarium department of Vivekanandha College of Pharmacy for Women, Sankari, Tamilnadu.

2.4. Preparation of EBEMK

The fresh bark of the curry tree was collected and dried in the shade for about two weeks. Then it was ground and made into coarse powder. The powdered bark was subjected to hot percolation extraction using a Soxhlet apparatus. Dried bark powder of *M. Koenigii* (50 g) was extracted with 250 ml ethanol for 16 hours. Then the concentrated liquid extract was allowed to stand until the residue settled, and further evaporated in a China dish using heating almonds (at 35-40° C) under reduced pressure. The resulting semi-solid residue was used for further antifungal studies. Prior to use, the dried extract was diffused with 1% DMSO followed by a 2-fold diffusion with distilled water.

2.4. Test micro-organism

The fungal cultures of *Cryptococcus neoformans* MTCC1431, *Fusarium oxysporum* MTCC284, *Aspergillus niger* MTCC282, *Candida albicans* MTCC183, *Trichoderma viride* MTCC167 used in this study were purchased from Microbial type culture and collection (MTCC), Chandigarh, India.

2.5. Antifungal susceptibility and Determination of Minimum inhibitory concentration (MIC)

The dilution method is a widely used technique for determining the MIC of antimicrobial agents and is considered the reference method for antimicrobial susceptibility testing. The MIC is the lowest concentration of the samples at which no growth can be visually detected. To determine the MIC of Amphotericin B, the drug was diluted at various concentrations (5, 10, 20, 40, 80, 100, 150, 200, 250 µg/ml) in sterile Yeast Peptone Dextrose (YPD) broth in test tubes. A standard wire loop (Merck) was then used to inoculate a scoop (10 µl) of the fungal culture into the test tubes containing 1 ml of the various concentrations of amphotericin B in YPD broth. The tubes were then incubated at 37°C for 48 to 72 hours and observed for growth or turbidity. After 48 hours, cell growth was measured using a UV spectrophotometer to determine optical absorbance at 550 nm. The percentage of inhibition of fungal growth was calculated using the following formula: [(Ac-At)/Ac] × 100, where Ac is the OD value of the negative controls and at is the OD value of the test samples. The same procedure was used for the EEBMK sample against the fungal strain *Cryptococcus neoformans*. The mean inhibitory concentration (IC50) was determined based on the percentage of inhibition of fungal growth [15, 16, 17, 18].

The antifungal activity of EEBMK was evaluated using the disk diffusion method of the Kirby-Bauer susceptibility test. *Candida albicans* and *Cryptococcus neoformans* were cultured on Mueller-Hinton agar enriched with 2% glucose [19, 20], while the other fungal strains were cultured on potato dextrose agar (PDA) medium. The test sample and standard

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were dissolved in 10% v/v DMSO. Six-millimeter sterile wells were added to the plates, and 25, 50, 75, and 100 µl of the test sample were added to separate wells. A positive control containing amphotericin B at a concentration of 10 mg/ml and a fill volume of 25 µl was added to a separate well, while 10% v/v DMSO was used as a negative control. All plates were incubated overnight at 27°C. The susceptibility of each strain was determined by measuring the diameter of the growth inhibitory zone around the plates. The experiment was repeated three times in triplicate to ensure reproducibility of observations [15,21,22].The statistical analysis was done using the Prism 9, Graph Pad Instat software system, USA. Statistical analyses were performed using one-way ANOVA, followed by Turkey's test (Post hoc test); the significance level was determined at $p < 0.05$.

Results

In the study, the efficacy of EEBMK against five different fungal species was investigated using the agar diffusion method. The minimum inhibitory concentration (MIC) of EEBMK and the standard drug amphotericin B was determined for *Cryptococcus neoformans* by the dilution method (Tables 1 and 2). The results showed that as the concentration of the extract increased, the turbidity of fungal growth decreased significantly, indicating the ability of the extract to inhibit fungal growth. The agar diffusion method was used to compare the antifungal activity of EEBMK at different concentrations (25µl, 50µl, 75µl, and 100µl) with that of the standard drug amphotericin B (Figure 1). The results showed that the extract inhibited fungal growth in a concentration-dependent manner, with larger zones of inhibition observed with increasing extract concentration. The results showed that the extract positively affected four of the fungi tested, including *Aspergillus niger*, *Fusarium oxysporum*, *Candida albicans*, and *Cryptococcus neoformans*, but had no effect on *Trichoderma viride* (Table 3).

The study found that as the concentration of EEBMK increased, the turbidity decreased sharply from 40 µl, with an OD value of 0.41. Conversely, the OD values for Amphotericin B were 12.5, 15.63, and 65.63 for 5 µl, 10 µl, and 15 µl, respectively (Figure 2). Interestingly, at higher concentrations of 200 µl and 250 µl, no turbidity (OD=0) was observed in the EEBMK samples, indicating that fungal growth was completely inhibited at higher concentrations (Figure 3). Similarly, the standard drug Amphotericin B also exhibited no turbidity at high concentrations (OD=0) of 100 µl, 150 µl, 200 µl, and 250 µl (Table 2). However, a sudden decline in turbidity was observed at 20 µl for the standard drug, with an OD value of 0.22 compared to the OD value of 0.54 for 10 µl. Based on the study's IC 50, the MIC of EEBMK was found to be 40.17µl/ml against *Cryptococcus neoformans* (Table 2). A graph was plotted based on their % growth inhibition to visualize the antifungal activity of the EEBMK and the standard drug (Figure 2). A comparative study of anti-fungal activity of EEBMK against Amphotericin B was performed (Figure 3). Overall, the results of this study suggest that EEBMK exhibits significant antifungal activity against *Cryptococcus neoformans*, and further research may be warranted to explore its potential as a natural alternative to synthetic antifungal drugs.

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4. Discussion

M. Koenigii, commonly known as curry leaf, has been used for centuries for its medicinal properties, including its antifungal activity. Numerous studies have demonstrated its efficacy against various fungal strains. Vats et al (2011) evaluated the antifungal potential of *M. Koenigii* root extracts against *C. albicans* and *A. niger*, [23] while Afzal (2014) studied its activity against *F. moniliforme*, *F. oxysporum*, *M. phaseolina*, and *R. solanii* [24]. Doddanna (2013) found that alcoholic curry leaf extract was highly effective against *C. albicans* [21]. Gabriel (2014) reported that both aqueous and ethanolic extracts of *M. Koenigii* had antifungal properties against *C. albicans*, *P. funiculosum, P. camemberti*, and *A. niger*. Our study confirms these results and EEBMK has shown significant antifungal activity against *Cryptococcus neoformans* and suppresses the growth of *F. oxysporum, C. albicans and A. niger*.

C. neoformans and *F. oxysporum*, two pathogenic fungi, are the causative agents of Cryptococcosis and Fusariosis, respectively, the first fungus affecting immune compromised hosts and the second being a causative agent of acute lymphoblastic leukemia [14]. Disinfectants such as phenolic compounds, formaldehyde, glutaraldehyde, iodophores, and sodium hypochlorite have been shown to be effective in killing *C. neoformans* [25]. It is interesting to note that curry leaf bark powder may be a natural alternative to destroy this harmful fungal strain. *F. oxysporum* can enter the human body through the nostrils and cause lumps on the chest.[11&12] The current study found that EEBMK effectively controlled the growth of *F. oxysporum* in a concentration-dependent manner.

EEBMK and its phytomolecules are potential candidates for exploring alternative treatment of cryptococcosis and fusariosis, which could be an exciting development in the field of antifungal research. Although the results are promising, it is essential to identify the active components responsible for the antifungal properties of the extract and to conduct clinical trials to determine the safety and efficacy of the extract. Overall, the results of the study open a new avenue of research for antifungal treatments, and with continued investigation, EEBMK could become a valuable tool for the development of novel drugs for the treatment of fungal infections, and our study further contributes to this area of research by exploring the efficacy of the bark extract.

CONCLUSION

The study demonstrated the antifungal activity of EEBMK against *C. neoformans, F. oxysporum, A. niger, and C. albicans* in concentration dependent manner. However, it was found to be ineffective against *T. viride*. Based on our findings, we suggest that the plant extract can be used as a natural fungicide, opening new possibilities for creating natural antifungal formulations for the treatment of many fungal-related disorders.

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Conflict of interest: None.

Abbreviations: EEBMK: Ethanolic extract of bark of *M. koenigii*, *A. niger*:*Aspergillus niger*, *T. viride : Trichoderma viride*, *F. oxysporum : Fusarium oxysporum*, *Candida albicans: C. albicans*, *C. neoformans : Cryptococcus neoformans.* GIT: gastro intestinal tract, SDA: Sabouraud Dextrose Agar, PDA: Potato Dextrose Agar, MHA: Mueller-Hinton Agar, YPD: Yeast Peptone Dextrose, DMSO: Dimethyl sulfoxide, MIC: Minimum inhibitory concentrations. OD: Optical density

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Table 1. Optical density values of different concentration *Cryptococcus neoformans* in plant extract

S. No	Sample Concentration µl/ml	OD Value	% of Inhibition	IC 50
1	5	0.62	3.13	40.17
2	10	0.61	4.09	
3	20	0.61	4.09	
4	40	0.41	35.94	
5	80	0.25	60.94	
6	100	0.1	84.38	
7	150	0.02	96.88	
8	200	0	100	
9	250	0	100	

Table 2. Optical density values of different concentration of Amphotericin B against the *Cryptococcus neoformans*.

S. No	Sample Concentration µl/ml	OD Value	% of Inhibition	IC 50
1	5	0.56	12.5	15.35
2	10	0.54	15.63	
3	20	0.22	65.63	
4	40	0.1	84.38	
5	80	0.04	93.75	
6	100	0	100	
7	150	0	100	
8	200	0	100	
9	250	0	100	

Table 3. Illustrated antifungal activities of EEBMK and Amphotericin B Standard drug against five different strains fungal species in agar diffusion method.

S.No.	Test organism	Zone of inhibition in (mm)	
		Amphotericin B (10 mg/ml)	EEBMK
1	*Aspergillus niger*	20 ±2	8 ±1
2	*Trichoderma viride*	16 ±1	NA
3	*Fusarium oxysporum*	15 ±1	8.3 ± 0.57
4	*Candida albicans*	35 ± 2	14.3 ±1.5
5	*Cryptococcus neoformans*	30.6 ±1.5	15.6 ± 1.15

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Each value is mean of 3 batches (n=3) with standard deviation
NA: No activity; + control Amphotericin B = 10 mg/ml was used with loaded volume of 25µl.

Figures legends

Figure1. Anti-fungal study of ethanolic extract of bark of *M. koenigii (EEBMK).* (A) Activity against *Aspergillus niger* (B) Activity against *Candida albicans* (C) Activity against *Cryptococcus neoformans* (D) Activity against *Trichoderma viride* (E) Activity against *Fusarium oxysporum*

Figure2. A comparative study on minimum inhibitory concentration. Standard: Amphotericin B; EEBMK: Ethanolic extract of bark of *M. koenigii*

Figure3. Comparative anti-fungal studies (A) Activity against *Aspergillus niger* (B) Activity against *Fusarium oxysporum* (C) Activity against *Candida albicans* (D) Activity against *Cryptococcus neoformans.* The statistical analyses were done using the Prism 9, Graph Pad Instat software system, USA. Each value is mean of 3 batches (n=3) with standard deviation. *** Extremely significant at $p < 0.05$.

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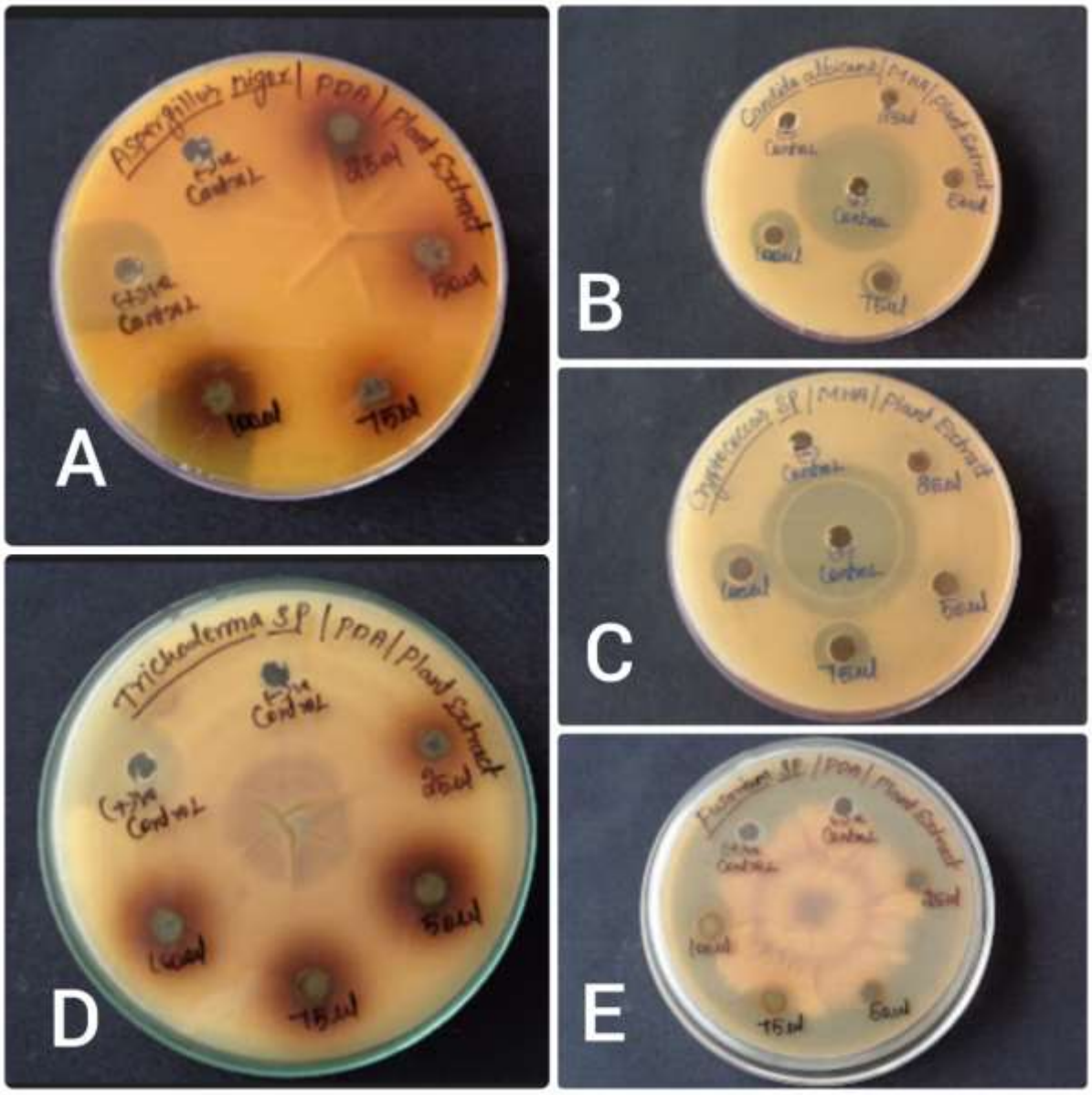

Figure1. Anti-fungal study of ethanolic extract of bark of *M. koenigii (EEBMK).* (A) Activity against *Aspergillus niger* (B) Activity against *Candida albicans* (C) Activity against *Cryptococcus neoformans* (D) Activity against *Trichoderma viride* (E) Activity against *Fusarium oxysporum*

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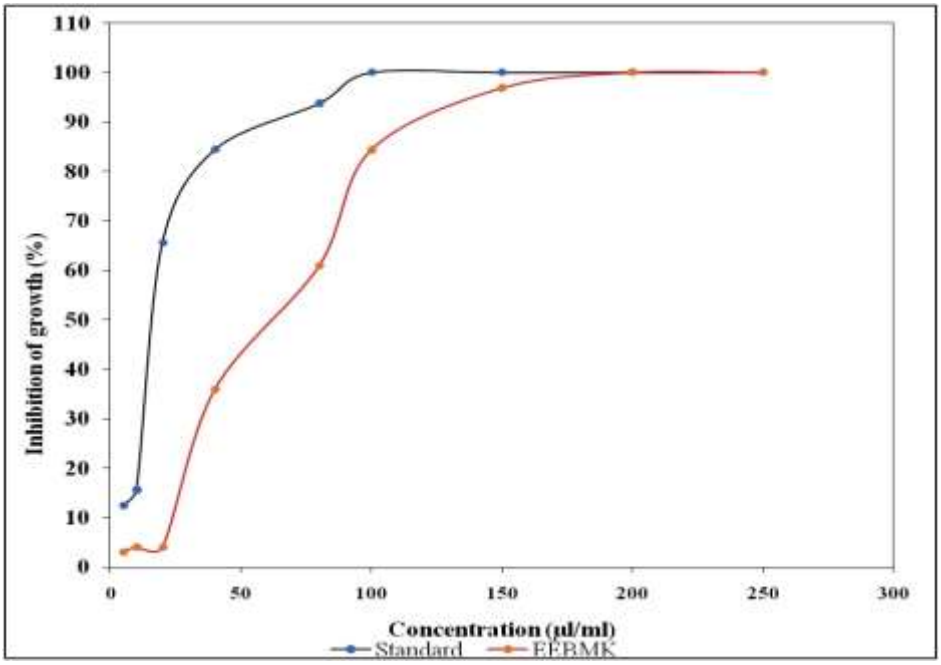

Figure2. A comparative study on minimum inhibitory concentration. Standard: Amphotericin B; EEBMK: Ethanolic extract of bark of *M. koenigii*

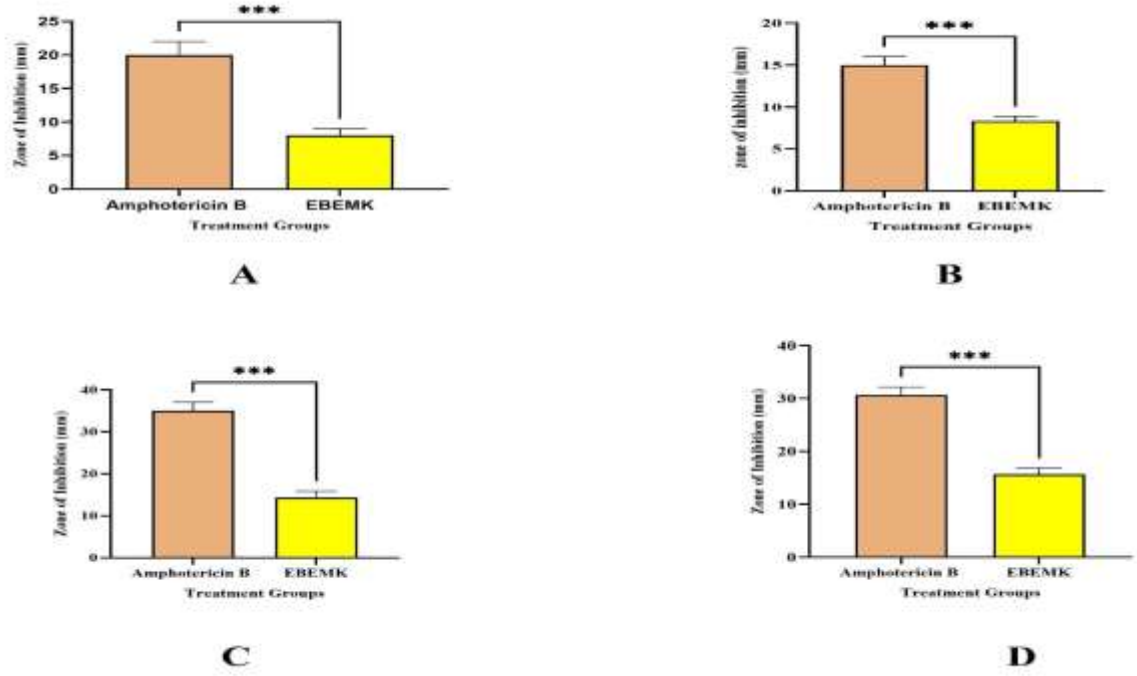

Figure3. Comparative anti-fungal studies (A) Activity against *Aspergillus niger* (B) Activity against *Fusarium oxysporum* (C) Activity against *Candida albicans* (D) Activity against *Cryptococcus neoformans.* The statistical analyses were done using the Prism 9, Graph Pad Instat software system, USA. Each value is mean of 3 batches (n=3) with standard deviation. *** Extremely significant at $p < 0.05$.