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In-vitro Anti-microbial Activities of Acacia nilotica Pod extract against Saprolegniasis

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ABSTRACT

In-vitro anti-microbial activities of *Acacia nilotica* pod extract against *saprolegniasis* was carried out at University of Maiduguri. The aim was to determine the antimicrobial susceptibility of malachite green and *Acacia nilotica* on *saprolegnia* isolates. Total samples collected were 91 and sterilized swap sticks was used for the sample collection and were inoculated into prepared media of SDA (Sabouraud Dextrose Agar), the plates was incubated at 30C for 24-48 hours. The extraction of the pods was done using Plant Tissue Homogenization methods. 30g of extract powder was used in Methanol, chloroform and Ethanol. Modified Kirby Bauer disc diffusion technique was employed to determine the antimicrobial resistance pattern of the saprolegniasis isolates and data was analyzed using SPSS. The results shows malachite green as positive control indicated the zone of inhibitions ranging from 27 to 29.00mm, ethanol extract of *A. nilotica* pod extract at 100% indicated susceptibility of *saprolegniasis* with the inhibition zones ranging from 20-23mm, Methanol extract at 100% shows intermediate with the inhibitory zones ranging from l1-20mmn and Chloroform extract shows no inhibitory zone at 50, 75 and 100%, the study was concluded that ethanol and methanol of *A. nilotica* pod extract possess antimicrobial activity against the fish fungus (*saprolegnia*) which shows antifungal activities but cannot substitute malachite green in this study.

KEYWORDS

In-vitro anti-microbial, *Acacia nilotica*, *saprolegniasis*

Introduction

The *saprolegniaceae* family comprises closely related water moulds ubiquitous in freshwater and usually associated with dead tissues and fish eggs (Hart and Reynolds, 2002). This family belongs to the oomycetes, a group of fungus-like protists sharing similarities with fungi and brown algae. Other members of this group are well known plant and mammal pathogens (Kamoun, 2003). Phylogenetically very different from eumycotan fungi, oomycetes likely evolved different mechanisms for interaction with plants and animals (Kamoun, 2003; Torto-Alalibo *et al.,* 2005). Furthermore, some plant pathogenic oomycetes suppress host defence (Kamoun, 2003), a property that has been suggested to be shared by *Saprolegnia* (Alvarez *et al*., 1995). *Saprolegnia* may occur anywhere on the body of fish, but normally appear as a conspicuous, circular or crescent-shaped, white, cotton-like mycelium, particularly around the head and the caudal, adipose and anal fins (Hussein *et al*., 2001). The infection may spread over the body by radial extension until adjacent lesions merge. *Saprolegniasis* is frequently a superficial and chronic infection, with the appearance of cotton-wool-like tufts on eggs or the integument and gills of fish (Hussein *et al.,* 2001), which may extend over the entire body surface (Richards and Pickering, 1979). In severe cases, 80% of the body may be covered. In early infections, skin lesions are grey or white in colour, with a characteristic circular or crescent shape (Willoughby, 1989), which can develop rapidly and cause destruction of the epidermis. As infection develops lethargy and loss of equilibrium follow making the fish more susceptible to predation. The actual cause of death is likely to ble associated with impaired osmoregulation (Gardner, 1974; Hargens and Perez, 1975). Respiratory difficulties may also feature when infection is associated with the gills (Bruno and Stamps, 1987). Pathogenic members of the *Saprolegniaceae* may penetrate major organs (Hatai and Egusa, 1977), and the terms progressive dermatomycosis or mycotic dermatomycosis have been proposed (Wada *et al.,* 1993). Members of the genus Saprolegnia can be considered opportunistic facultative parasites, which are both saprotrophic and necrotrophic (Cooke, 1977). *Saprolegniaceae* act as primary pathogens (Noga *et al*., 1988).Fish are continually exposed to potentially pathogenic oomycetes and it therefore follows that a change in some predisposing factor or factors is

necessary for infection to occur. *Salmonids* are susceptible to *saprolegniasis* throughout the freshwater stage of their life cycle, particularly leading up to and during smoltification (Pickering, 1994). Environmental stress factors, including poor water quality, adverse water temperatures and, in aquaculture, handling or overcrowding, can all result in increased occurrences of oomycete infections (Bailey, 1984). *saprolegniaceae* species are known to cause *saprolegniasis*, a fish tegumentary mycosis characterized by visible patches of filamentous mycelium covering the epidermal tissues (Beakes *et al*., 1994). If untreated, saprolegniasis leads to death, presumably by heamodilution (Hatai and Hoshia, 1994) reported teratogenicity and toxicity (Meyer and Jorgenson, 1983; Alderman and Polglase, 1984). Pathogenic *saprolegniacea* can act either as secondary or as primary pathogens (Whisler, 1996).

Saprolegniasis is one of the most important freshwater fish diseases, both in nature and aquaculture, where it causes economically important losses worldwide. This problem has achieved new heights since the discovery of malachite green, a triarylmethane dye efficiently used to control *saprolegniasis* since the mid-1930s was prohibited in most producer countries because of its suspected teratogenicity and toxicity.

Most frequently used chemicals for the treatment of *Saprolegniasis* in aquaculture are Malachite Green, Formalin and Hydrogen peroxide. These chemical compounds have shown to be adversely affecting the aquatic life forms as well as human health. Also chemical methods pose various problems resulting in immunosuppressive, teratogenic, carcinogenic and mutagenic effects on repeatedly treated fish. The major reason behind the restriction imposed on the use of such chemicals is their possible carcinogenic and mutagenic effects due to bioaccumulation, bio magnifications inhuman populations, there is need for tentacle spread to hardness, natural products that can replace those chemicals completely.

Materials and Methods

Study Area

The study was conducted at the University of Maiduguri. Maiduguri is the capital city of Borno State, Nigeria, located in Northeastern Nigeria shares borders with neighboring countries, such as the Niger Republic, Chad, and Cameroon. Within Nigeria, Maiduguri shares borders with Adamawa, Yobe, and Gombe and has Sahel savannah vegetation. The annual average

Temperature of Maiduguri ranges from 19.1°C to 34.7°C, and the average annual precipitation is 562 mm (NPC, 2006).

Sample Size

The minimum sample size desired was calculated to be 60 using the sample size formula for cross sectional studies of population ≤10,000 described by Araoye (2004) as follow;

$$n_t = \frac{Nn}{N+n}$$

But can only be calculated from population ≥10,000; given by

$n = \frac{z^2pq}{d^2}$ $n = \frac{z^2pq}{d^2}$ **(**Desired sample size when the population is greater than 10,000)

z = standard normal deviate at 80%confidence level usually set at 1.26

p= prevalence of *Saprolegniasis* reported in Asaba(17.4 %) (Nwabueze *et al*., 2013)

Where q= 1-p = 0.5

d= degree of precision or acceptable error margin (5% or 0.05 for this study).

This gives

$$n = \frac{1.26^2(0.174)(0.826)}{(0.05)^2}$$

$$n = \frac{1.26^2(0.174)(0.826)}{(0.05)^2}$$

= 91.27

= 91

Sample Collection and Preparation

Swab Stick was appropriately labeled, study number was used for the collection of the samples. The samples was collected from the hatchery and immediately transported to the Microbiology laboratory and inoculated onto PDA, Sabouraud Dextrose Agar (SDA) (Hi-Media Laboratories, Mumbai). These plates was incubated at 30^0C for 24-48 h. Plates was observed for growth, and a Gram smear will be performed for different types of colonies. The cultural characteristics cotton-like colonies of saprolegniasis on PDA, Sabouraud Dextrose Agar (SDA) was observed. Characteristics of saprolegniasis colonies was identified by colony morphology, pigment formation; was performed on the isolates (Dauner et al., 2010). A suspension of each confirmed isolates was prepared in peptone water to match 0.5 McFarland turbidity standards.

Microscopic

Direct wet preparation of the Saprolegniasis sample was place on a free glass slide to examine

Media preparation

SDA was used for the isolation of Saprolegniasis according to manufacturer's instruction.

Gram staining

Lactophenol cotton blue stain technique

Lactophenol Blue Stain is a mounting medium and staining agent used in the preparation of slides for microscopic examination of fungi.

Procedure

1. Place a drop of Lactophenol Blue on a clean microscope slide.
2. Using an inoculating needle, gently remove a small portion of growth midway between the colony center and edge. Place the growth in the drop Lactophenol Blue on the slide.
3. With two sterile dissecting needles, gently tease the fungus apart so that it is thinly spread out in the Lactophenol.
4. Place a coverslip edge of the Lactophenol and slowly lower it. Avoid trapping air bubbles under the coverslip.
5. If desired, seal the edges of the coverslip with nail polish or permount to preserve the mount as a reference slide.
6. Examine the slide under the microscope.

Collection and Extraction of Plant Material

The fully mature pods of *Acacia nilotica* was collected from the Botanical garden, Department of Biological Sciences, University of Maiduguri, Borno State, Nigeria. The plants were identify and authenticated by a taxonomist at herbarium unit of the Department of Biological Sciences, University of Maiduguri. The plant materials was washed with water to remove dust and dried under shade for three days and then pulverized into powder form, using sterile mortar and pestle.

Preparation of plant extract

The extraction of the pods was done using Plant Tissue Homogenization methods. 30g of extract powder will be weight, using digital weighing balance and dissolved into 80ml of three different solvents at (99%) Methanol, chloroform and Ethanol was which shaken vigorously for 10 minute using orbital Shaker. The samples will be made homogenous, and then incubated for 24-48hours as described by Banu and Cathrine (2015).

Antimicrobial Sensitivity Testing (Modified Kirby-Bauer disc diffusion technique)

Modified Kirby Bauer disc diffusion technique was employed to determine the antimicrobial resistance pattern of the saprolegniasis isolates using Malachite Green. A disc of blotting paper impregnated with Malachite Green placed on a plate of SDA uniformly inoculated with the test organism (saprolegniasis). The antifungal

diffuses from the disc into the medium, and the growth of the test organism will be inhibited. The zone of inhibition was recorded in millimeter, and the resistance pattern will be determined using the standard method.

Control

Malachite green will be used as a drug control in this experiment since it is the most effective synthetic chemical used in treating *saprolegniasis* worldwide. The pathogen will be treated in 99.99% MG solution. *Acacia nilotica* pods extract was further diluted in 50%, 75% and at 100% in solvents concentration (ethanol, methanol and chloroform).

Statistical Analysis

The data from all experiment was analyzed using SPSS version 21.0 (2009). Standard deviation between replicates sample for all experiments was calculated and an unpaired two-tailed t-test was performed to determine whether there was a statistically significant differences (degree of freedom 95%, p-value of < 0.05) in saprolegnia isolates.

Result

Table 4.1 The distribution of microbiological isolates shows that Out of 91 samples, 33 isolates was from eggs, 29 from fins and 29 also from the skin of fish which makes the total of 91 samples. Out of 33 isolates from eggs, 20 were positive isolates (microscopy) and 8 for gram stain. Of 29 isolates from fins, 12 were positive isolates (microscopy) and 4 for gram stain while out of 29 isolates from skin, 8 were positive isolates (microscopy) and 6 for gram staining respectively.

Table 1: Distribution of Microbiological Analysis of Isolates

Sources	No. Test	No. Positive (microscopy)	No. positive (Gram Stain)
Eggs	33	20	8
Fins	29	12	4
Skin	29	8	6
Total	**91**	**40**	**18**

No= number

Table 4.2 The means and standard deviation of saprolegnia as affected by percent (%) treatment of *A. nilotica* shows there is significant ($p< 0.05$) differences as affected by percent extracts. The control (malachite green) had a constant means value of

inhibition zones at 28.20mm which of all extract is the highest followed by Ethanol and methanol. *A. nilotica* pod extract was used at 30g/80ml of ethanol further diluted to 50, 75 and at 100% showed significant effect on *saprolegniasis* at p< 0.05. It shows the mean value of inhibition zones of 10.00mm at 50%, 17.20mm at 75% and 21.20mm at 100%. Similarly Methanol extract of *A. Nilotica* at 30g/80ml further diluted in 50,75 and 100% also showed significant effect on *saprolegniasis* has the mean value of inhibitory zone of 8.40mm at 50%, 10.80mm at 75% and 16.60mm at 100% respectively. Chloroform extract of *A. nilotica* pod extract at 30g/80ml further diluted in 50,75 and 100% showed insignificant (p< 0.05) on the treatment of *saprolegniasis* with the inhibition zones of 0.00mm at both 50,75 and at 100%.

Table 2: Means and Standard Deviation of inhibition zones as Affected by the Percent (%) Treatment of A. *nilotica*

Extracts	Malachite green	50%	75%	100%	Significant
CE	28.20±0.84	0.00±0.00	0.00±0.00	0.00±0.00	ns
EE	28.20±0.84^{a}	10.00±4.64^{d}	17.20±2.05^{c}	21.20±1.30^{b}	*
ME	28.20±0.84^{a}	8.40±3.78^{c}	10.80±4.55^{c}	16.60±3.05^{b}	*
Total	**28.20±0.77**	**6.13±5.55**	**9.33±7.82**	**12.60±9.65**	*****

CE= Chloroform extract, EE = ethanol extract, ME = Methanol extract and Means within the same row having different subscripts are significantly different * (P<0.5).

Table 4.3 The means and standard deviation of inhibition zones of *saprolegniasis* as affected by extracts and replicates indicates that chloroform extract had a significant (P<0.05) effect on all replicates with the mean value of 7.00mm in replicates 1 and 2, 7.25mm in replicate 3 and 4 and 6.75mm in replicates 5 which recorded the lowest. Ethanolic extract showed significant (P<0.05) differences in all replicates. It showed the mean value of 17.00mm, 18.80mm, 19.75mm, 21.75mm and 18.75mm in replicate 1,2,3,4, and 5 with 21.75mm recorded highest in replicate 4.

Similarly methanolic extract had significant (P<0.05) differences in all replicate with the mean value of 12.80mm, 14.25mm, 15.50mm,19.50mm and 18.25mm in replicate 1,2,3,4 and 5 were as replicate 4 was recorded the highest mean value of 19.50mm respectively.

Table 3: Means and Standard Deviation of inhibition zones as Affected by Extracts and replicates

Extracts						**Significant**
	1	2	3	4	5	
CE	7.00±14.00^{b}	7.00±14.00^{b}	7.25±14.50^{a}	7.25±14.50^{a}	6.75±13.50^{c}	*
EE	17.00±9.63^{d}	18.50±9.29^{bc}	19.75±7.80^{b}	21.75±5.62^{a}	18.75±6.24^{b}	*
ME	12.80±10.66^{e}	14.25±9.95^{d}	15.50±10.34^{c}	19.50±6.81^{a}	18.25±6.75^{ab}	*
Total	**12.27±11.31**	**13.25±11.34**	**14.17±11.51**	**16.17±11.09**	**14.78±10.31**	*****

CE= Chloroform extract, EE = ethanol extract, ME = Methanol extract and Means within the same row having different subscripts are significantly different * (P<0.5).

The result indicates that ethanol extract of *A. nilotica* at 100% extract shows susceptible to *saprotegniasis* with the inhibition zones ranging from 20 to 23.00mm with the mean value of 21.00mm (p<0.05) compared to malachite green with the mean value of 28.20mm (p<0.05). 75% extract shows intermediate with the zones ranging from 15 to 19.0mm with the mean value 17.20mm (p<0.05) compared to malachite green with the mean value of 28.20mm (p<0.05) while at 50% extract, it shows resistance to *saprolegniasis* with zones ranging from 5 to 13.00mm with the mean value of 10.00mm respectively. So also, the methanol extract of *A. nilotica* at 100% extract shows intermediate with the inhibition zones ranging from 11 to 20.00mm with the mean value of 16.00mm (p<0.05), 75% extract indicate resistance with zones ranging from 6 to 17.00mm with mean value 0f 10.80mm similarly to 50% extract with the zones ranging from 5 to 12mm with the mean value of 8.40mm respectively. Chloroform extract shows no inhibitory zones at all 50, 75 and 100% extract.

Table.4. Comparative study of inhibitory zones of malachite green with ethanol, methanol and chloroform extract of A. *nilotica* effect on saprolegniasis

Replicates	Malachite Green	Zones of Inhibitions(mm)								
		Ethanol conc.			Methanol conc.			Chloroform conc.		
		50%		75%	50%		75%	50%		75%
		100%			100%			100%		
1	28.00	5.00	15.00	20.00	5.00	6.00	11.00	0	0	0
2	28.00	6.00	18.00	22.00	6.00	8.00	15.00	0	0	0
3	29.00	10.00	19.00	21.00	6.00	9.00	18.00	0	0	0
4	29.00	16.00	19.00	23.00	13.00	17.00	19.00	0	0	0
5	27.00	13.00	15.00	20.00	12.00	14.00	20.00	0	0	0
Means value ($p<0.05$)	28.00	10.00	17.20	21.20	8.40	10.80	16.00	0	0	0

Key: ± 21.20 Susceptible ±16.20 Intermediate ±10.00 Resistance

Discussion

91 samples was collected from different location and part of the fish. There samples were collected using sterilized swap stick on eggs, fins and skins of the fish. Of 91 samples, 33 isolates were from eggs which was collect at the hatchery and 29 from fins and skins of fish were collected from two different commercial farm while the microscopy which are positive isolates were 20, 12 and 8 in eggs, fins and skins respectively. The gram stain was also observed to 8,4 and 6 in eggs, fins and skin of the fish. In this study research of fungal infection, it showed that samples collected

from eggs are more susceptible to *saprolegniasis* than other part of the fish.

Malachite green (MG) was used as control in this study research which showed the highest inhibition zone of mean value 28.20mm. It indicates the effectiveness in killing or destroying the mycelia established on the media within 24 - 48 hours. MG was found to be the most effective fungicide among 49 compounds tested against an oomycete fungus (Campbell *et al.,* 2001). It has also look revenged the growth of *Haliphthorus* on rock robster (Diggles, 2001) and ful-2 on salmon (Huang *et al.,* 1996). Ethanolic extract of *A. nilotica* (pods) shows significantly ($p<0.05$) with the highest inhibition zones of 21.20mm and minimal 10.00mm. Amjad ur Rahman et al. 2014 recorded the inhibitory zones ranging from 4 to 29mm against five strains of bacteria which is in contrast to (Satish *et al.* 2008) found that aqueous extract of *A. nilotica* exhibited significant study against the test bacteria which showed the zones of inhibition from 9 to 35mm. Also Amjad ul Rahman *et al. (*2014) revealed that ethanol extract of *A. nilotica* was more effective against all test bacterial than chloroform. Banso (2009) using agar diffusion method found that ethanol stem bark extract of *A. nilotica* produced antimicrobial activity against *streptococcus virindas, B. subtilis, S. aureus, E. coli and Shigella sonnei.* Again Amjad ul Rahman et al. (2014) reported that ethanol and chloroform extract of A. nilotica was equally efficient against gram negative *E. coli and P. aeruginosa* as well as against fungi *A. Niger and A. flavus w*hich in contrast to this research, chloroform extract showed insignificant effect on saprolegniasis fungal infection this is due to the ability of ethanol to extract side range of chemical and chloroform extract might have extracted less number of the constituents (Abdul Shakoor *et al.,* 2012). Also Similarly, Khan (2009) reported the antifungal activity of ethanolic extracts against multidrug-resistant (MDR) strains of *Candida species. Acacia nilotica* bark and leaf extract showed antifungal activity against *Aspergillus flavus* (12 mm) followed by leaf extract of *Zizphus mauritian* (11 mm) (Mahesh and Satish, 2008)

Methanolic extract in this study had significantly effect on the study fungi with the mean value ranging from 8.40mm to 16.60mm respectively. Antifungal activity was confirmed by using aqueous and methanol extract of *Acacia nilotica* pods, which exposed antifungal activity against *Candida albicans and Aspergillus niger* (Farzana *et al.,* 2014). Also Dabur *et al.*

(2007) analyzed water extract of A. nilotica was found to be most active against bacteria as well as fungal pathogens. Saini *et al.* (2008) found that the methanolic extract of *A. nilotica* (pod) and *Acacia catechu* (bark) were reported to be active against bacterial and fungal. Their finding showed highest activity against *E. coli, S. aureus and A. niger*.The methanol leaf extracts of *Acacia nilotica* showed significant activity against *E. coli, S. aureus and X. a. pv. malvacearum* around 15 mm. The highest antibacterial activity of 20 mm in *B. subtilis* and least activity recorded in *E. coli* measured 14 mm. Bark extract of *Acacia nilotica Linn* exhibit highest activity against *B. subtilis* and *S. aureus* (15 mm) and lowest in *P. fluorescens* (Mahesh and Satish, 2008). Using of methanol and aqueous extract of *Acacia nilotica* pods exposed antibacterial activity against *Escherichia coli, Staphylococcus aureus and Salmonella* typhi (Farzana *et al.*, 2014).

Extracts and replicates of *A. nilotic*a pod extract against saprolegniasis are significantly different ($p<0.05$). Chloroform extract had the mean value ranging from 7.00mm to 6. 75mm in replicate 1 to 5. Ethanol extract recorded the highest among all extracts had the mean value ranging from 17 to 21.20mm in replicate 1 to 5. Similarly, methanol extract recorded higher among the extract had the mean value ranging from 12.80 to 19.50mm respectively. The concentration of extract inhibit different diameter zones. This is in the opinion of Martiningsih (2017) which states that the diameter of the inhibition zone formed in each treatment is different, this is because the growth of fungal isolates in the media is influenced by several factors, including the concentration of antimicrobial substances, the number of microorganisms, the presence of organic matter, temperature, degree of acidity. (P^H) and species of organisms. Also based on this study research, the diameter reduces as concentration reduces. This is by the opinion of Fadilah (2018), which states that the smaller the concentration of the extract, the less the amount of active substance dissolved in the extract so that the ability to inhibit fungal growth is lower. Conversely, the higher the concentration, the more active substances that function as anti-fungi, so that the ability to inhibit fungal growth is greater.

Conclusion

Malachite Green most frequently used chemicals for the treatment of *Saprolegniasis* in aquaculture. This chemical compound have shown to be adversely affecting the aquatic life forms as

well as human health. Also chemical methods pose various problems resulting in immunosuppressive, teratogenic, carcinogenic and mutagenic effects on repeatedly treated fish. In this study research, Ethanol and Methanol had activity on *saprolegniasis* which can partially reduce the risk of using chemicals which shown to be adversely affecting the aquatic life as well as human health. The ethanol and methanol of *A. nilotica* pod extract possess antimicrobial activity against the fish fungus (*saprolegnia*) which shows antifungal activities but cannot substitute malachite green in this study.

Recommendation

High concentration of ethanol and methanol should be used when extracting samples as low concentration might show resistance of the study organism. It is also recommended that chloroform should not be used when extracting samples because it renders the *A. nilotica* less effective and further studies should be carried out on the chemistry of interaction between chloroform and *A. nilotica* and research should be planned to try using combination of extracts so as to prevent fish from fungal infection and it is always of advantageous to use natural herbs over harmful chemicals.

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