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IN VITRO ANTI-FUNGAL PROPERTIES OF AQUEOUS EXTRACT OF WILD *GANODERMA SP* FROM NIGERIA

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ABSTRACT Investigation into the *in vitro* anti-fungal activities of aqueous extract of wild *Ganoderma sp.* from Lafia, Nassarawa State, Nigeria was conducted using disc diffusion technique. 100 mg/ml, 200 mg/ml, and 300 mg/ml reconstituted aqueous extract of the wild *Ganoderma* specie was tested against mixed fungal isolates from infected goats. These fungi are *Dermatophilos congolensis, Rhyzopus oryzae, Aspergillus niger* and *Penicilium sp.* Results showed that the anti-fungal activities were concentration dependent of 8 mm (100 mg/ml), 9 mm (200 mg/ml) and 11 mm (300 mg/ml) zone of inhibition of the mixed infection respectively. Although all preparations showed anti-fungal activity, only 300 mg/ml concentration have high anti-fungal activity with the zone of inhibition being 11 mm, 1 mm higher than the standard ≥ 10 mm required for an agent to be considered as possessing antimicrobial activity. This finding is relevant in discussing treatment of dermatological disease condition in goats caused by mixed fungal origin.

KEYWORDS : Fungi, *Ganoderma sp, in vitro*, pharmacology, Sensitivity

INTRODUCTION

The search for alternative antibiotics, especially against resistant strains becomes important, because microbes are rapidly developing resistance against the existing antibiotics. This antibiotic resistance is the ability of the microbe to resist the effect of medication previously use to treat them (www.amr-review.org , 2016).

In veterinary practice, the World health Organization concluded that inappropriate use of antibiotics in animal husbandry for treatment and as growth promoters are key factors in promoting antimicrobial resistance (WHO, 2017). This prompted the World Organization of Animal Health to provide series of guidelines for its members for the creation and harmonization national antimicrobial resistance surveillance and monitoring programme. (Srinivasan *et al.,* 2014)

After the discovery of antibiotics, efforts of research and development have provided new drugs to treat microbes that become resistant (Nordrum, 2015), however, this method have tremendous side effects and this potential crisis lead to a marked reduction in research and development industry, poor financial investment in antibiotic drug research (Gever, 2011, Walsh, 2013).

The most commonly used drug in the treatment of *Dermatophilosis* is Penicillin G, However, this drug may undergo enzymatic degradation and deactivation through the the production of β-lactamases by penicillin resistant strains (Criswell, 2004) or its decrease accumulation at taget sites may reduce, thus, decreasing drug permeability or increasing active efflux (Aminov and Mekie, 2007, Li and Nikaido, 2009). Infections by fungal organism cause high morbidity and mortality in both man and animals (Xie *et al.,* 2014), especially *Candida albicans* and *Aspergillus fumigatus* that are reported to cause most fungal infections and are resistant to treatment (Srinivasan, *et al.,* 2014).

The increase spread of multidrug resistant fungi is reported to be due to wide spread and indiscriminate use of anti-fungal agents (Costa *et al.,* 2014). Despite hordes of research material reporting the use of *Ganoderma sp* against some human diseases such as cancer, (Wasser, 2017, Ge *et al.,* 2014) and against *Candida* in humans (Aarati *et al.*, 2010, Alencar and Clemete, 2013, among others, there is no single report of the use of any *Ganoderma sp* extract in the treatment or management of dermatological condition in animals.

These myriads of problems associated with prolonged, and sometimes, unsuccessful treatment of dermatological condition in animals, especially *Dermatophilosis,* therefore provide the basis for research into cheaper, effective, readily available and affordable drugs from traditional sources. *Ganoderma* species have been reported to have antibacterial activities in animals and antifungal properties in humans, hence the need for search of its possible use in management of animal dermatological conditions. .

MATERIALS AND METHOD

Sample collection

Study area

The fruiting bodies of the wild *Ganoderma* specie were collected in 2016 from Lafia, Nassarawa State, during the rainy season. It was dried under room temperature, grinded to coarse powder using Lister (China) grinding machine. This powder weighing 500g was carefully wrapped in a transparent polythene bag, inserted carefully in a plastic container, and transported to Maiduguri and kept at room temperature in the Pharmacology and Toxicology laboratory, Faculty of Veterinary Medicine, University of Maiduguri, until required for use.

EXTRACT PREPARATION

The aqueous extract of the wild *Ganoderma* specie was prepared by weighing 10 g, 20 g and 30 g of the wild *Ganoderma* specie powder using digital electronic weighing balance (LabTech, model B1, 20001, METRA, China). They were kept in a clean 250 ml beaker, and each preparation was dissolved in 10 ml of distilled water and allowed to soak for 3 hours. Tthey were then filtered using Whitman's No. 1 filter paper. The filtrate yielded concentrations of 100 mg/ml, 200 mg/ml and 300 mg/ml solutions respectively. The filtrate was then used for the *in vitro* antimicrobial studies.

ISOLATION OF FUNGI FROM INFECTED GOATS

Isolates used in the study was obtained from the skin of a naturally infected goats, by scrapping the crust/scab from the skin of the animals, this was cultured in the laboratory for identification, the fungal isolates were sub-cultured and stored in nutrient agar in the

laboratory in the Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Maiduguri. The nutrient agar medium was obtained in dehydrated powder form (Oxoids limited, England) and reconstituted according to the manufacturer's specification; it was then maintained at refrigerated temperature and further sub-cultured in nutrient broth at 37^{0}C for 8 hours prior to antibacterial testing. Using a loop ring, 1.0 ml of the nutrient broth culture was then used to make streaks on the agar plates.

IDENTIFICATION OF THE ISOLATES

The isolates were identified by carrying out some biochemical tests, such as Gram staining, sugar fermentation test, catalase and other conventional methods as described by Gordon (1976)

The isolates were continuously sub cultured on 9% bovine blood agar to maintain a pure culture of the organisms. These isolates were comparatively studied based on cultural characteristics such as hemolyzing activity on different blood agar media, growth on different solid and liquid media (Simon citrate agar, brain-heart infusion agar, McConkey agar, deoxycolate medium, trypton phosphate broth supplemented with 10% ovine serum in trypton phosphate broth). The appearance of cultures were examined and recorded after incubation at 37^{0}C for 72 hrs.

The isolates were streaked on bovine and ovine blood agar. All plates and broth were incubated under anaerobic condition at 37^{0}C for 24 hrs. The plates and broth were examined after incubation and the results recorded.

Other test such as motility test were carried out by streaking in a semi-solid medium composed of 2.5 % w/v brain-heart infusion broth, 5.3 % w/v gelatin, 0.3 % w/v agar in distilled water, the bottles were incubated in anaerobic jar at 37^{0}C for 24 hrs. After the incubation, the degree of radial extension of haze or growth from the stab-like was examined and wet mount preparation was also used to determine the motility of the organism by putting drops of the broth culture on a clean glass slide and examined under microscope (x 100).

ANTI-FUNGAL SENSITIVITY TESTING

In vitro anti-fungal activities of the aqueous preparation of the wild *Ganoderma* specie extract was determined against the mixed fungal organisms identified using disc diffusion technique as described by the National Committee of Clinical Laboratory Standards (1993). Disc of 6 mm in diameter were prepared using Whitman's No. 1 filter paper, each disc was impregnated with the prepared concentrations of 100 mg/ml, 200 mg/ml and 300 mg/ml respectively and were dried at 50^{0}C.

TURBIDITY STANDARDIZATION

The turbidity standard was equivalent to McFarland's. This is done by preparing solutions A and B initially, Solution A was prepared by adding and mixing 1 ml of concentrated sulphuric acid in 99 ml of distilled water (100 ml), while solution B was made by dissolving 0.5 g of dehydrate borium chloride in 50 ml of distilled water. Mixture of measured quantity of solutions A and B was made to produce solution C. Briefly: Aspirate 0.6 ml of the borium chloride solution (B) and add to 99.4 ml of the sulphuric acid solution (A) and mix. Small portion (1 ml) of this mixture (C) was collected into a clean test tube and was used as control.

The culture of each concentration was diluted using sterile normal sterile saline to give an inoculums size of 10^6 cfu/ml (Cheesbrough, 2004). A swab of the inoculums using cotton wool was spread on the surface of the dried nutrient agar plates. The plates were then incubated at 37^{0}C for 30 minutes, thereafter, the impregnated disc were applied aseptically. The plates were incubated at 37^{0}C for 24 hours. Similarly, discs that were impregnated with Charmil^R (200 mg/ml) were applied to a separate agar plates and served as positive control. Inoculated agar plates devoid of antifungal or extract were prepared and served as negative control. After the inoculation period, a meter rule was use to measure the diameter of zone of inhibition produced by the extract or anti-fungal agent on the agar plates. A fungal isolate is considered susceptible to the effect of the extract if the diameter of the zone of inhibition produced by the extract is ≥ 10 mm (Cheesbrough, 2004**).**

FUNGAL ORGANISM IDENTIFICATION

The fungal organisms were identified using cultural microscopy of plates cultured on Sabouraud dextrose agar. This was stained with lactophenol cotton blue and examined under the microscope. Some structures used for identification of fungi include; chains of conidia, sterigmata, conidiophores and vesicle for *Aspergillus fumigatous*. Structures of mycelia rhizoids

and sporangiophores, sporangia and sporangiopores were used to identify *Rhyzopus oryzae* and *penicilium sp* was identified by chaining of a single cell conidia, branches and metulae

RESULTS

Analysis of scab/crust content from the skin of the infected goats revealed the presence of *Dermatophilos congolensis (100%), Rhyzopus oryzae (25%), Aspergillus niger* (33.3%) and *Penicilium sp (8.3%)*, (Table 1), while biochemical test (Table 2) revealed strong presence of *Dermatophilos congolensis* organism. An *in vitro* microbial sensitivity test showed concentration dependent activities of the extract with concentration of 300 mg/ml having the highest zone of inhibition of 11 mm (Table 3, Plate 1).

Table 1: Isolated Fungal Organisms From The Skin Scrapings Of Infected Goats

Goat No.	*Dermatophilus congolensis*	*Rhyzopus oryzae*	*Aspergilus niger*	*Penicilium sp*
1.	1	1	1	
2.	1			
3.	1		1	
4.	1			
5.	1	1	1	
6.	1			
7.	1			
8.	1			
9.	1		1	
10.	1			
11.	1	1		
12.	1			1
Total	**12 (100 %)**	**3 (25.0 %)**	**4 (33.3 %)**	**1 (8.3 %)**

Indicating mixed infection

Table 2: Results of biochemical tests to identify *Dermatophilos congolensis* organisms

Microorganism	Tests									
	Cat.	Mot.	Glu.	Fruc.	Man.	Xyl.	Suc.	Mal.	Lact.	Sorb.
Dermatophilos congolensis	+	+	+	±	±	±	±	±	-	±

Key: Cat-catalase, Mot-motility, Glu-glucose, Fruc-fructose, Man-mannitol, Xyl-xylose, Suc-sucrose, Mal-maltose, Lact-lactose and Sorb-sorbitol.

Table 3: *In vitro* anti-fungal activities of different concentrations of aqueous extract of wild *Ganoderma sp*. against the mixed infection in goats

S/No.	Anti-fungal activities (mm) of three concentrations (mg/ml) of aqueous extract of wild Ganoderma sp.			
	100 mg/ml	200 mg/ml	300 mg/ml charmil^R	
1.	8 mm	9 mm	11 mm	0 mm

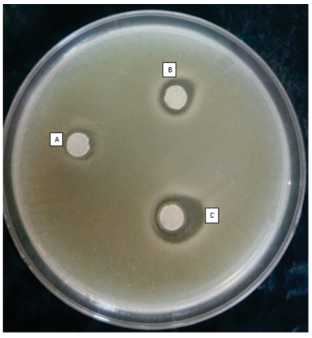

Plate 1: *in vitro* anti-fungal activity of aqueous extract of wild *Ganoderma* specie against mixed fungal infection at different concentrations. (A) 100 mg/ml, (B) 200 mg/ml and (C) 300 mg/ml.

DISCUSSION

The *in vitro* anti-fungal activities of the aqueous extract of *Ganoderma lucidum* have been reported by many authors (Ofodile *et al.,* 2005, Sivaprakasam *et al.,* 2011, Pushpa *et al.,* 2013). However, in their reports, susceptibility to a single isolate of an identified microbe was tested. In this study, there was mixed fungal infection involving *Dermatophilos congolensis, Aspergillus niger, Rhyzopus oryzae* and *Penicilium sp.* These organisms, showed a concentration dependent susceptibility to aqueous extract of the wild *Ganoderma sp.* This finding agree with that of Sivaprakasam *et al* (2011) and Pushpa *et al.,* (2013) who reported antibacterial and antimicrobial activity of extract of *Ganoderma lucidum* against various microbes. The antifungal activity observed in this study is similar to other reports by Hexiang and Ng (2006) Aarati *et al* (2012), Sivaprakasam *et al* (2011) with, Alencar and Clemente (2013), Pushpa *et al* (2013), and Uma Gowrie *et al* (2014), who reported activities of different solvents of *Ganoderma* extract against *Aspergillus species, Candida albicans, Mucour sp. Curbilaria sp. and Botrytis cinerea,* all are reported to be resistant strains of fungus (Lloyd and Sellers, 1976). There is no report of activity of *Ganoderma* extract against either *Dermatophilos congolensis* or *Rhyzopus oryzae.*

In late sixties, Hart and Tiszkiewiczk (1968) reported that there is no cure for *D. congolensis* infection in animals, this is because most treatment of this condition are done with parenteral administration of penicillin G, a Gram positive antibiotic with little effect against this bacterial organism, that finds home in skin tissues, that is less perfuse with blood supply, hence less concentration of the administered drug reaching the target organism. It was later reported that treatment of *D. congolensis* in animals have always been a herculean task (Bida *et al.,* 1976, Oduye *et al.,* 1976 and Scanlan *et al.,* 1984) with longer duration of the parenteral drug and topical drugs were found to be ineffective. The antifungal activity of *G. lucidum* against plant pathogenic fungi *Calendula officialis* L was also reported by Shahid *et al* (2016).

There had always been less consideration for combination of antibacterial drug with antifungal agents, hence, reports of resistance of the disease (Lloyd and Sellers, 1976). Microbial analysis from crusts/scabs collected from the skin of the infected goats' revealed mixed infection of both bacterial and fungal organisms. Therefore, administration of specific treatment against any of the two alone, will not only result to resistance development, but will allow the untreated organism to proliferate, hence the reported elongation of the treatment of this condition in animals.

In 2004, the European Union achieved a decline in antimicrobial resistance in humans through limiting the use of antimicrobials in agriculture and food industry without jeopardizing animal health or economic cost (Angulo *et al.,* 2004).

Extracts from recognized wild *Ganoderma sp* may be an effective chemo prophylactic or therapeutic agents against resistant strains of microbes. In immunocompromised patients, anti-fungal resistance do occur (Xie *et al.,* 2014), this happens more if the patient is infected with either; *Cryptococcus neoformis* and *Aspergillus fumigatous* which are all reported to develop anti-fungal resistance (Srinivasan *et al.,* 2014). Proper exploitation of *Ganoderma sp.* in antimicrobial therapy will reduce the use of antibiotics in both man and animals. Since the *Ganoderma sp* appear to have broader antimicrobial activity against bacteria (Gram positive and Gram negative) and fungal organism from reports of various authors (Wasser, 2005, Hexiang and Ng, 2006, Ogbe *et al.,* 2008, Aarati *et al.,* 2010, Sivaprakasam *et al.,* 2011, Pushpa *et al.,* 2013, Uma Gowrie *et al.,* 2014 and Happuarachchi *et al.,* 2016. Reports revealed that aqueous extract of wild *Ganoderma sp*. from Nigeria is not toxic at 5000 mg/ml (Shamaki *et al.,* 2017), while it was reported that extract from *Ganoderma lucidum* has specific cytotoxic effect against infecting microbes (Wasser, 2017a,b).

Among the three concentrations (100 mg/ml, 200 mg/ml and 300 mg/ml) of the test extract, recorded zone of inhibition were 8 mm, 9 mm and 11 mm respectively, indicating a concentration dependent activity. A microbial isolate is considered susceptible to the effect of the test extract if the diameter of the zone of inhibition produced by the extract is ≥ 10 mm (Cheesbrough, 2004**).** Therefore, the extract can be said to possess anti-fungal activity at 300 mg/ml**.** The use of *Ganoderma* preparations can equally reduce multidrug resistant microorganisms reported as biggest threat by Center for Disease Control USA (CDC, 2016).

In conclusion, aqueous extract of a wild *Ganoderma sp*. from Nigeria have demonstrated concentration dependent, anti-fungal activity against wild strains of *Dermatophilos congolensis*, resistant *Aspergillus niger, Rhyzopus oryzae* and *Penicilium sp.* respectively, and was therefore recommended that further work is required to identify and isolate each of these identified organisms separately and test them against synthetic anti-fungal agents as controls accordingly.

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