IJMAAS International Journal of Microbiology and Applied Sciences
Volume 2 May, 2023 Research Article

In Vitro Antimicrobial Activity of Acetonic and Ethanolic Extracts of a Macrofungus *Boletus variipes*

Musa, H[*1]., Dauda, W[2] and Peter, G.W[3]

[1]Department of Botany, Ahmadu Bello University, Zaria, Nigeria.
[2]Department Agronomy, Federal University Gashua, Yobe State, Nigeria.
[3]Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria
*** Corresponding Author:** *hannatumusa23@gmail.com*

ABSTRACT

The research was carried out to determine and to compare the antimicrobial activity of different concentrations of acetonic and ethanolic extracts of a macrofungus *Boletus variipes* on three bacterial isolates: *Escherichia coli, Staphylococcus aureus,* and *Bacillus subtilis,* and two fungi: *Candida albicans* and *Aspergillus fumigatus in vitro*. The acetonic extract showed relatively strong antimicrobial activity, inhibiting both bacterial and fungal growth in the range of 12.5mg/ml-100mg/ml. Maximum antimicrobial activity of the acetonic extract was found in *E. coli* with a minimum inhibitory concentration (MIC) of 12.5mg/ml. The measured MICs for *Staphylococcus aureus, Bacillus subtilis* and *Candida albicans* were 25mg/ml, 25mg/ml and 50mg/ml respectively while *Aspergillus fumigatus* was not inhibited. The ethanolic extract did not show any antimicrobial activity and this inactivity may be due to insolubility of the active compounds of the sample in ethanol or the presence of inhibitors to the antimicrobial components. The inactivity of the ethanolic extract may also be attributed to the very low volatility of ethanol which tends to extract less of the active ingredients or compounds from the macrofungus *Boletus variipes* sample unlike the high volatility acetone.

Keywords: Antimicrobial, *Boletus viriipes*, Acetonic extracts, minimum inhibitory concentration (MIC), bacteria, fungi

Introduction

Edible and medicinal mushrooms can produce a variety of biologically active compounds and can therefore be described as a novel class of nutraceuticals which are widely used as dietary supplements (Wasser, 2002). Recent epidemiological studies from Asia demonstrated that mushroom intake protects against cancer, specifically gastrointestinal (GI) and breast cancers (Kim *et al*., 2002; Hara *et al*., 2003; Zhang *et al*., 2009). The anticancer activities of mushrooms were mainly linked to the modulation of the immune system by branched polysaccharides (glucans), glycoproteins or peptide/protein-bound polysaccharides (Borchers *et al*., 2008). Moreover, mushrooms contain minerals, vitamins (thiamin, riboflavin, ascorbic acid, and vitamin D), amino acids and other organic compounds (Mattila *et al*., 2000). Some of these natural mushroom compounds demonstrated specific activity against aberrantly activated signaling pathways in cancer cells and were able to modulate specific molecular targets in the cell function including cell proliferation, cell survival and angiogenesis (Zaidman *et al*., 2005). Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. Plants have generally served traditionally as the most important weapon against pathogens to man (Sofowora, 1980; 1992; 1993).

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The ancient man is known to have utilized plant materials as drugs against many diseases and was totally dependent on green plants for his daily need of medicament (Trease and Evans, 2002; Dionisi *et al*., 2012). The early man was able to distinguish food, medicinal and poisonous plants based on trial and error led by instinct, taste, experience and observation of animal's behaviours. For example, chimpanzees have been observed to ingest the leaves of *Vernonia amygdalina* (bitter leaf) when suffering from parasitic infections (Huffman *et al*., 1993; Nwanjo, 2005) hence man was eventually able to categorize plants into edible and non-edible.

Botany and medicine have been closely linked throughout history. Prior to this century, medical practitioners whether allopath (medical doctors), homeopaths, naturopaths, or herbalist had to know the plants in the area and how to use them since many of their drugs were derived from plants (Akujobi *et al*., 2004). Around 1900s, 80% of the drugs were derived from plants. However, in the decades that followed, the development of synthetic drugs from petroleum products caused a sharp decline in the pre-eminence of drugs from live plant sources (Ibekwe *et al*., 2000; Akujobi *et al*., 2004; Hancock *et al*., 2012). With the recent trend of high percentage resistance of microorganisms to the present-day antibiotics, efforts have been intensified by researchers towards the search for more sources of antimicrobial agents (Rogers, 2011; Dionisi *et al*., 2012). The first step towards this goal is the *in vitro* antibacterial activity assay (Tona *et al*., 1998).

Mushrooms with other fungi are special in the living world, being neither plants nor animals. They have been placed in a kingdom of their own called Eumycota. The word mushroom may mean different things to different people and countries (Kuo, 2010). It has emerged that special studies on the economic value of mushrooms and their products had reached a point where a clear definition of the term "mushroom" was warranted (Barros *et al*., 2007). In a broad sense "Mushroom is a macro fungus with a distinctive fruiting body, which can be either epigeous or hypogenous and large enough to be seen with naked eye and to be picked by hand" (Chang and Miles, 1992; Hsu, 2011). The most common type of mushrooms is umbrella shaped with a pileus (cap), a stipe (stem) and pores or pileus (lamellae) which bear the microspores. The increased interest in exploiting the properties of mushrooms for medicinal purposes reveals the importance of natural sources of biologically active substances (Hobbs, 2000).

Boletus variipes is a species of boletus fungus (mushroom) in the family Boletaceae. The genus (Boletus) is polyphyletic and consists of about 300 species (Mau *et al*., 2004; Komolafe and Adegoke, 2008), among which are: *B. edulis, B. aureus, B. scabaretc* with widespread distribution. This fungus is native to North America and was originally described by an American mycologist Peck in (1888).

Boletus variipes like other members of the genus is believed to contain the active biological substances necessary for antimicrobial activity. Virtually all other synthesized drug alternatives have been exploited but there are yet some loopholes as to their effectiveness (due to high resistance by microorganisms), there is need for substitute of antimicrobial agents which are natural and more effective with a very narrow range of microbial resistance. Since this fungus has high content of phenol the study is to determine the antimicrobial activity of *Boletus variipes* extract.

Some of these biologically active substances generally known as secondary metabolites include phenolic compounds found in different mushroom species. These compounds are well known for their antioxidant properties (Kim *et al*., 2008; Hong *et al*., 2009), but they also revealed antimicrobial activity emerging with potential against multi resistant microorganisms (Ozen *et al*., 2011). The increased prevalence of microorganisms which are resistant to the available antibiotics is one of the major challenges for the healthcare systems worldwide. Antibiotic-resistant infections are associated with one to two-fold increases in mortality compared to antibiotic-susceptible infections (Cosgrove and Carmeli, 2003).

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In general, it can be observed that the treatment of virus, bacteria, fungi and protozoa with the existent drugs is increasingly difficult due to rapid mutation of these organisms into new genetic variants which result in their being resistant to the antibiotics (Khalafi-Nezhad *et al*., 2005; Kuete *et al*., 2009; 2011). Moreover, antibiotic resistance imposes enormous health expenditure due to the higher treatment costs and longer hospital stays. Phenolic acids including benzoic and cinnamic acid derivatives have been pointed out as the most common in mushroom. Among benzoic acid derivatives, p-hydroxybenzoic, protocatechuic, gallic, vanillic and syringic acids were identified in different mushroom species and tannins like ellagic acid (Puttaraju *et al*., 2006; Vaz *et al*., 2011b). *In vitro* and epidemiologic studies suggest that consumption of foods rich in phenolic compounds might significantly decrease the risk of some health problems due to their antioxidant, anti-mutagenic, anti-inflammatory and antibacterial properties (Heleno *et al*., 2011, 2012). Antimicrobial activities of the aqueous and ethanolic extracts of macrofungi potentials were evaluated both *in vitro* and *in vivo* against *Aspergillus niger* and *Escherichia coli*. The percentage yields of aqueous extracts were greater than that of ethanolic extract. Both extracts showed a potentially good antimicrobial activity, however aqueous extract had more activity than ethanolic activity. The activities increased with increasing concentration. Maximum antifungal activity was shown by aqueous extract of *A. conyzoides* against *A. niger* and *A. ustus* with the average inhibition of 20 mm each while the least activity was recorded against *A. fumigatus* at the concentration of 800 mg/mL with 7 mm zones of inhibition (Barros *et al*., 2007; Ozen *et al*., 2011; Alves *et al*., 2012; Wuyep *et al*., 2017).

The MIC values of extracts ranged from 50 mg/mL to 794 mg/mL (Albayrak *et al*., 2010). However, not many reports are available on the exploitation of antifungal or antibacterial property of plants and macrofungi for developing commercial formulations for application in crop protection. Although the medicinal uses of extract from several mushroom species have been reported, quite a number of other species are yet to be discovered. Historically, mushroom extracts have been used as a safe, effective and natural remedy for ailment and diseases in traditional medicine. They have also played significant role in providing active ingredients in controlling and reducing diseases in humans when edible species are eating. Traditionally, the screening of bioactive compounds involves, a brute force approach that demands huge investment of significant time and resources to identify a single promising lead compound from chemical libraries consisting of up to several million entities, finding an efficacious drug to bring to market have little or no guarantee. Therefore, this study was aimed at evaluating the antimicrobial efficacy acetonic and ethanolic extracts of *Boletus viriipes in vitro* against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and two fungi: *Candida albicans* and *Aspergillus fumigates*.

Materials and Methods

Collection and Processing of Mushroom Material

The mushroom growing wild on a field in Ahmadu Bello University, Samaru main campus, Zaria was collected fresh and identified as *Boletus variipes* in the Department of Biological Sciences using standard identification keys (Kuo, 2010) and assigned voucher number V/N: ABU15252013. The fresh sample was sun dried to a constant weight, finely grounded using mortar and pestle and extracted using acetone and ethanol. The test organisms used were *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Candida albicans* and *Aspergillus fumigatus* and were obtained from the Department of Pharmacognosy, Ahmadu Bello University, Zaria, Nigeria.

Extraction of Mushroom sample

From the finely powdered/ grounded sample, 100g was weighed into a beaker and 250 ml of acetone added, tightly covered, and filtered after 24hrs with a filter paper. The filtrate was then transferred to a water bath at 45^{o}C for 12hrs where it was evaporated to obtain a semi solid pure extract. The extract was stored at 4^{o}C until it was used in the experiment. All media Potato Dextrose Agar (PDA) were prepared according to the manufacturers' prescription and sterilized at 121^{o}C for 15minutes in the autoclave.

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The organisms were stored on nutrient agar slant kept in the refrigerator.

From the slant, overnight culture was prepared by inoculating a loop full of the organism from the slant into the nutrient broth and incubated at 37ºC overnight. The culture in the nutrient broth was diluted in a ratio of 1:5000 for gram negative organism (i.e., *E. coli*) and 1:1000 for Gram positive (i.e., *Staphylococcus aureus*), for each culture to contain about 10^6 colony forming unit per ml. A susceptible test was carried out using 20ml of the prepared agar medium melted and allowed to cool to 45^0C and then poured aseptically into sterile plates and allowed to solidify (Feng *et al*., 2002).

The plates were inoculated by introducing 2ml of the standardized culture, after which the excess was poured off the plates into a container containing a standard antibiotic and left to dry for 15minutes at room temperature. Using a cup borer six holes were made in the media plates 12mm to 16mm to the edge of the plate. To each well, 0.1ml of *Boletus variipes* extract of acetonic and ethanolic were poured while 0.1m of Streptomycin was used as a standard drug for the test microorganisms (bactericidal) for three replicates. Again, to each well, 0.1ml of *Boletus variipes* extract of acetonic and ethanolic were poured while 0.1m of Itranacole was used as a standard drug for the test microorganisms (fungicidal) for three replicates. The plates were left for an hour at room temperature to allow diffusion of the extract into the medium. Testing for the Minimum Inhibitory Concentration (MIC) was carried out by used of the Mueller Hinton nutrient broth agar as the growth media of the organisms for bacteria culture, while Saboud dextrose broth medium was used for fungi.

Determination of Minimum Inhibitory Concentration (MIC)

A plot of the square of radius diameter of the zones of inhibition against log concentration of the dilutions was done and a suitable curve drawn from the plots of each extract. Extrapolation of the curves was done to determine the log of MIC. From this log, the MIC was calculated as the antilog (Tona *et al*., 1998; Feng *et al*., 2002). The MIC is defined as the lowest concentration that will prevent the growth of the test organisms.

Determination of Minimum Fungicidal Concentration (MFC)

The MFC was determined for each of the extracts by sub-culturing the medium from each tube or well showing no visible growth in media plates. The plates were incubated at 29°C until growth was seen in the control plates. The MFC is defined as the concentrations required killing 99.9% of the cells (Elumalai *et al*., 2009).

Data collection and statistical analysis

Data obtained were subjected to Analysis of variance (ANOVA) and Duncan Multiple Range Test (DMRT) using statistical package for social science SPSS to know the significance in the zone of inhibition, effectiveness of each extract and the susceptibility of the test organism. Least significant difference (LSD) of p=0.05 was used to compare means. This was applicable to acetonic and ethanolic extracts.

Results

The results of the effect of five different concentrations (100, 50, 25, 12.5 and 6.25mg/ml) of acetonic extract of *Boletus variipes* as the mean zones of inhibition of *Boletus variipes* are shown in Table 1. Maximum antimicrobial activity of the acetonic extract was found in *E. coli* (MIC of 12.5mg/ml), which implies that the extract has the highest inhibitory effect against *E. coli*.

The minimum inhibitory concentration (MIC) of *Boletus variipes* acetonic (A) and ethanolic (B) extract (in mg/ml) is as shown in Table 2. The result showed *E. coli* as the least MIC value of 12.5mg/ml (Table 2).

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Table 1: Inhibitory effects of varying concentrations of acetonic extract of *Boletus variipes* extracts against some Bacteria and Fungi

Extract Concentration	Inhibitory zone(unit) on:				
	E. coli	***S. aureus***	***B. subtilis***	***C. albicans***	***A. fumigatus***
100	24.75±0.14^{ab}	23.50±0.29^{ab}	19.25±0.43^{ab}	16.00±0.58^{ab}	0.00±0.00^{b}
50	22.00±0.58^{ab}	9.00±0.58^{ab}	7.00±0.58^{ab}	13.75±0.14^{ab}	0.00±0.00^{b}
25	18.50±0.29^{ab}	5.50±0.87^{ab}	14.47±0.29^{ab}	0.00±0.00^{b}	0.00±0.00^{b}
12.5	17.25±0.14^{ab}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
6.25	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
0.00	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}

In each column, means followed by the same alphabet are not significantly difrent at 5% level of significance using LSD, SSPS and DMRT

The superscripts ab represents significant difference 0mg/ml and 1000mg/ml respectively at 5% level of significance ($p<0.05$). *E. coli* has the highest mean zone of inhibition value at 100mg/ml of 24.75±.14mm (Table 1).

Table 2: Minimum Inhibitory Concentration of *Boletus variipes* Acetonic (A) and Ethanolic (B) extracts

Test Organism	Concentration of Acetonic (A) and Ethanolic (B) extracts									
	100	50	25	12.5	6.25	3.125	1.563	0.781	0.391	0.2
***E. coli* A**	-	-	-	-	+	+	+	+	+	+
***E. coli* B**	-	-	-	-	+	+	+	+	+	+
***S. aureus* A**	-	-	-	+	+	+	+	+	+	+
***S. aureus* B**	-	-	-	+	+	+	+	+	+	+
***B. subtilis* A**	-	-	-	+	+	+	+	+	+	+
***B. subtilis* B**	-	-	-	+	+	+	+	+	+	+
***C. albicans* A**	-	-	+	+	+	+	+	+	+	+
***C. albicans* B**	-	-	+	+	+	+	+	+	+	+
***A. fumigatus* A**	+	+	+	+	+	+	+	+	+	+
***A. fumigatus* B**	+	+	+	+	+	+	+	+	+	+

Key: *Concentration; - = No growth; + = growth; A= Acetonic extract; B= Ethanolic extract

The minimum inhibitory concentration (MIC) of standard antimicrobial drug of acetonic (A) and ethanolic (B) extracts is shown in Table 3 while the minimum bactericidal concentration and the minimum fungicidal concentration of *Boletus variipes* acetonic (A) and ethanolic (B) extracts is shown in Table 4.

The results in Table 3 showed that Streptomycin and Itranazole had stronger activity than the tested extract which was represented by its significant difference from the mean inhibitory values obtained when the extract was used ($p<0.05$). While the results in Table 4 showed that when each culture containing a microorganism was sub-cultured in a sterile medium, their determined MBCs was 50mg/ml for *E. coli* and *Candida albicans*, and 25mg/ml for *Staphylococcus aureus* and *Bacillus subtilis*.

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Table 3: Minimum Inhibitory Concentration (MIC) of standard antimicrobial drug of Acetonic (A) and Ethanolic (B) extracts

Test Organism	Concentration of acetonic (A) and ethanolic (B) extracts									
	100	50	25	12.5	6.25	3.125	1.563	0.781	0.391	0.20
***E. coli* A**	-	-	-	-	+	+	+	+	+	+
***E. coli* B**	-	-	-	-	+	+	+	+	+	+
***S. aureus* A**	-	-	-	+	+	+	+	+	+	+
***S. aureus* B**	-	-	-	+	+	+	+	+	+	+
***B. subtilis* A**	-	-	-	+	+	+	+	+	+	+
***B. subtilis* B**	-	-	-	+	+	+	+	+	+	+
***C. albicans* A**	-	-	+	+	+	+	+	+	+	+
***C. albicans* B**	-	-	+	+	+	+	+	+	+	+
***A. fumigatus* A**	+	+	+	+	+	+	+	+	+	+
***A. fumigatus* B**	+	+	+	+	+	+	+	+	+	+

Key: *Concentration; - = No growth; + = growth; A= Acetonic extract; B= Ethanolic extract

Table 4: Minimum Bactericidal and Fungicidal Concentration (MBC) of *Boletus variipes* Acetonic (A) and Ethanolic (B) extracts

Test Organism	Concentration of acetonic (A) and ethanolic (B) extracts									
	100	50	25	12.5	6.25	3.125	1.563	0.781	0.391	0.20
***E. coli* A**	-	-	+	+	+	+	+	+	+	+
***E. coli* B**	-	-	+	+	+	+	+	+	+	+
***S. aureus* A**	-	-	-	+	+	+	+	+	+	+
***S. aureus* B**	-	-	-	+	+	+	+	+	+	+
***B. subtilis* A**	-	-	-	+	+	+	+	+	+	+
***B. subtilis* B**	-	-	-	+	+	+	+	+	+	+
***C. albicans* A**	-	-	+	+	+	+	+	+	+	+
***C. albicans* B**	-	-	+	+	+	+	+	+	+	+
***A. fumigates* A**	+	+	+	+	+	+	+	+	+	+
***A. fumigatus* B**	+	+	+	+	+	+	+	+	+	+

Key: *Concentration; - = No growth; + = growth

Discussion

The results of the effect of the five different concentrations (100, 50, 25, 12.5 and 6.25mg/ml) of acetonic extract of *Boletus variipes* on the microorganisms showed relatively strong antimicrobial activity, inhibiting both bacterial and fungal growth. The acetonic extract inhibited the growth of both bacteria and fungi in the range of 12.5mg/ml-100mg/ml. Maximum antimicrobial activity of the acetonic extract was found in *E. coli* (MIC of 12.5mg/ml), which implies that the extract has the highest inhibitory effect against *E. coli*. The zones of inhibition of the standards and extracts, though of same volume varied slightly in the replicates, this might be due to uneven distribution of test organisms on agar surfaces or slight difference in temperature and the flatness of the plates at the time it was used. Okigbo *et al*. (2005) reported that inactivity of plant extracts may be due to age, extracting solvent, method of extraction and time of harvesting of the plant materials.

The minimum inhibitory concentration (MIC) of *Boletus variipes* acetonic (A) and ethanolic (B) extract showed *E. coli* with the least MIC value of 12.5mg/ml (Table 2). This implies that the extract has the highest inhibitory effect against *E. coli*.

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However, the minimum inhibitory concentration of the standard antimicrobial (streptomycin) and antifungal (Itranacole) in µg/ml gave the least MIC value of 7.813µg/ml which was found in *B. subtilis* and represents the most susceptible to the standard drug followed by *E. coli, S. aureus and C. albicans* and the least susceptibility was found in *A. fumigatus* with MIC value of 250µg/ml. The values obtained signify that the standard drugs were more effective than the extract. The higher activity of Itraconazole was expected since the extracts have various impurities as compared to the drug that is already a synthetically processed molecule and has undergone refining processes that have established it as a standard antifungal (Wuyep *et al*., 2017). These results could be expected due to the fact that numerous tests have proved that bacteria are more sensitive to the antibiotic drugs compared to fungi (Hugo, 1983).

The measured MICs for *Staphylococcus aureus* and *Bacillus subtilis* was 25mg/ml while for *Candida albicans* was 50mg/ml. *Aspergillus fumigatus* did not show any inhibition. The extract inhibited the growth of both bacteria and fungi in the range of 12.5mg/ml-100mg/ml. The intensity of the antimicrobial effect depended on the concentration and the tested organism. The *Boletus variipes* extract in the same concentration showed a stronger antibacterial effect than antifungal activity. The reason for different sensitivity between the fungi and bacteria can be found in different transparency of their cell wall (Amadioha and Obi, 1999; Ademola and Eloff, 2011). The cell wall of the gram-positive bacteria consists of peptidoglucans (mureins) and teichoic acids, while the cell wall of the gram-negative bacteria consists of lipo-polysaccharides and lipo-polyproteins, whereas, the cell wall of fungi consists of polysaccharides such ashitchin and glucan (Barros *et a*l., 2009; Agatemor, 2009). According to the work of Heleno *et al*. (2012), the minimum inhibitory concentration of acetone and methanol extracts of *Boletus aestivalis, Boletus edulis* and *Leccinum carpini* on tested bacteria and fungi were between 1.25 - 10 mg/ ml. This is quite low compared to that obtained from the extract of *Boletus variipes* which is 12.5-100mg/ml, suggesting that the extract of the former is ten times more active than the later. Generally, the acetone extracts exerted stronger antimicrobial activity than ethanol extracts (Erasto *et al.*, 2007). The measured MIC values according to the same author for *Leccinum carpini* against bacteria were 1.25-5 mg/ml for the acetone and 2.5-10 mg/ml for the ethanol extract. Both extracts of this mushroom inhibited the tested fungi in concentrations 5 mg/ml and 10 mg/ml. The acetone and ethanol extract of *Boletus aestivalis* and *Boletus edulis* had approximately equal antimicrobial activity and were inhibited in the tested bacteria and fungi in concentrations of 2.5 mg/ml, 5 mg/ml and 10 mg/ml. The antimicrobial activities of the extract of *Boletus variipes* on the test organisms were compared to those of Streptomycin (standard antibacterial drug) and Itranazole (standard antifungal drug).

The minimum inhibitory concentration (MIC) of standard antimicrobial drug of acetonic (A) and ethanolic (B) extracts (Table 3) showed that Streptomycin and Itranazole had stronger activity than the tested extract which was represented by its significant difference from the mean inhibitory values obtained when the extract was used ($p<0.05$). In a negative control, a sterile culture media inoculated, each of the microorganisms grew implying also a significant difference in mean inhibitory values when compared with the extract ($p<0.05$). The minimum fungicidal concentration of *Boletus variipes* acetonic (A) and ethanolic (B) extracts showed that, when each culture containing a microorganism was sub-cultured in a sterile medium, their determined MBCs was 50mg/ml for *E. coli* and *Candida albicans*, and 25mg/ml for *Staphylococcus aureus* and *Bacillus subtilis* (Table 4). These values are equally quite high when compared to the standards (Ben- Ami *et al*., 2010; Akinyemi *et al*, 2004).

In conclusion, the acetonic extract showed relatively strong antimicrobial activity, inhibiting both bacterial and fungal growth and the intensity of the antimicrobial effect depended on the concentration and the test organism. This implies that the standard antibiotics used are more effective against the tested bacteria and fungi. Further studies can be done on the isolation and characterization of new compounds from *B. variipes* that contained antimicrobial constituents.

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