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# Potentials of walnut (*Tetracarpidium conophorum* Mull. Arg) leaf and onion (*Allium cepa* Linn) bulb extracts as antimicrobial agents for fish

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**The development of antibiotic-resistant pathogens due to the indiscriminate use of antibiotics has led to advocacy for the use of natural products in the treatment of fish diseases. The antimicrobial activity of methanolic and ethanolic extracts of walnut leaves and onion bulbs were evaluated against six pathogenic bacteria (*Pseudomonas aeruginosa*, *Staphyloccocus aureus*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Escherichia coli*, *Samonella typhi*) using the cup – plate method. Minimum Inhibitory Concentration (MIC) was determined using standard methods. Data were analysed using descriptive statistics. Onion bulbs and walnut leaves were also screened for secondary metabolites and this indicated the presence of saponins, tannin, alkaloids, cyanogenic glycosides and flavonoids; while anthraquinones were not detected in both plants. The zone of inhibition varied with the bacteria and type of extract. The average diameter of inhibition zones was 10 ± 0.00 and 9 ± 0.02 mm for methanolic and ethanolic extracts of walnut leaves and onion bulbs, respectively. *Staphylococcus aureus, B. subtilis* and *P. aeruginosa* were most sensitive to the extracts. However, *S. aureus* was more sensitive to the extracts of walnut leaves and *S. typhi* was the least sensitive. *Bacillus subtilis* was more sensitive to the extracts of onion compared to *E. coli* which was the least sensitive. Minimum inhibitory concentration of walnut leaves and onion bulbs extracts on the bacteria tested were both 500 µg/ml. The results indicated that walnut leaves and onion bulbs had antibacterial activity on the tested organisms and showed their prospects for their use in the treatment of fish diseases.**

**Key words:** Antibacterial activity, onion bulb extract, walnut leaf extract, fish diseases.



## INTRODUCTION

With obvious protein need worldwide and resultant intensive fish production, fish are challenged with many diseases that have led to great economic losses (Kumar and Anantharaja, 2007; Abdelkhalek et al., 2008). In the control or prevention of such devastating outbreaks, conventional antimicrobials and other chemotherapeutic agents have been used with little or no success (Jadhav et al., 2006) probably due to the development of antibiotic-resistant pathogens. Considering the potential threat of these diseases to human and animal health, current disease management tend to concentrate on environmental-friendly, preventive methods such as the use of natural products that have antimicrobial and immunomodulatory properties. Since some plant extracts

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have been reported to have antimicrobial and immunomodulatory properties, there seems to be an attractive alternative to control fish diseases as well as enhance growth (Secombes, 1994; Raa, 1996). Such plants include Onion (*Allium cepa*) and walnut (*Tetracarpidium conophorum*) leaf. Although, both plants appear to have broad spectrum anti bacterial activities as well as anti-helminthic and anti-fungal properties (Abd-Elallatif and Ebraheem, 1996); their use in fish has not been investigated.

This study was therefore aimed at screening for the antimicrobial properties of *A. cepa* and *T. conophorum* against six pathogenic bacteria of fish with a possibilty of determining the minimum inhibitory concentration by which the plant extracts can be included in fish feeds/diets.

## MATERIALS AND METHODS

### Plant collection and identification

Onion bulbs were purchased from Bodija market in Ibadan, Nigeria. Walnut leaves were obtained from a farm at Oka -Akoko, Nigeria. They were authenticated at the herbarium of the Forestry Research Institute of Nigeria (FRIN), Ibadan, where a voucher specimen was deposited under FHI 107515.

### Preparation and extraction of plant materials

#### *Onion extraction*

The onions were washed with clean sterile distilled water and allowed to air dry at ambient temperature (25°C) for 1 h. The dry outer covering of the onion were manually peeled off, washed and extracted as described by Azu and Onyeagba (2007). 200 g of the fresh onion bulbs were blended into fine powder and soaked in 100 ml of 95% ethanol for 24 h. The pulp obtained was left in a clean, sterile glass container and shaken vigorously to allow for proper extraction and it was filtered using a sterile muslin cloth after which the extract was obtained, air-dried and stored (4°C) until required.

#### *Walnut leaves extraction*

The extraction was done as described by Ajaiyeoba and Fadare (2006). The air – dried walnut leaves were grounded with a hammer mill and 200 g of fine powder of walnut leaves were soaked in 100 ml of 80% methanol for 72 h. Walnut leaves were properly mixed with methanol, filtered using a sterile muslin cloth after which the extract was obtained, air – dried and stored at (25°C) until required.

#### *Media preparation*

All media used were prepared according to manufacturer's instruction as follows:

**MacConkey agar:** This agar was prepared by suspending 52 g in 1 L of distilled water. It was brought to boil to dissolved completely then, sterilized by autoclaving at 121°C for 15 min.

**Nutrient agar:** This agar was prepared by suspending 28 g in 1 L of distilled water and then sterilized by autoclaving at 121°C for 15 min.

**Mueller Hinton agar:** This agar was prepared by suspending 36 g in 1 L of distilled water and then sterilized by autoclaving at 121°C for 15 min.

**Nutrient broth:** This broth was prepared by suspending 25 g in 1 L of distilled water and then sterilized by autoclaving at 121°C for 15 min.

**Peptone water:** This was prepared by suspending 15 g in 1 L of distilled water and then sterilized by autoclaving at 121°C for 15 min.

All these media were allowed to cool after sterilization to about 45°C before pouring into Petri dishes.

### Isolation of microorganism/counts

The gills, skin, intestine and liver samples of the African catfish, *Clarias gariepinus* were separately macerated and put into sterile capped test tube containing sterilized peptone water and homogenized (Shalaby et al., 2006). Serial dilution was carried out and 1 ml each from $10^{-1}$ to $10^{-6}$ dilution factors were dispensed into Petri dishes that were appropriately labelled and molten sterile medium was poured aseptically into each Petri dish. The plates were swirled gently for even distribution of inocula and allowed to set /gel and then incubated at 37°C for 24 to 48 h. The organisms grew into visible different colonies after 24 h. Total viable count and enterobacteriacea were determined, the result were expressed in CFU/g.

### Antimicrobial assay

*Pseudomonas aeruginosa*, *Escherichia coli*, *Pseudomonas fluorescens*, *Samonella typhi*, *Staphyloccocus aereus*, *Bacillus subtilis* and *Aspergillus niger* were collected from the Laboratory stock of the Department of Microbiology, University of Ibadan, Nigeria. The pure cultures were sub cultured on Nutrient Agar slants and preserved in the refrigerator at 4°C until it is required for the study.

### Detection of antagonistic activity

A well diffusion assay as described by Schillinger and Lucke (1989) was used. Pre- poured indicator [pathogen (4 mm depth)] was overlaid with a 10 ml soft agar (0.7%) lawn of indicator culture (thus generating a potential mat for the indicator bacteria). The indicator lawn was prepared by adding 250 µl of a $10^{-1}$ dilution from an overnight culture to 10 ml of indicator organism soft agar. Wells of 5 mm diameter were cut into these agar plates by using cork borer and 100 µl of walnut leaf or onion bulb extract was placed into each well (Takahiro et al., 1991). The plates were incubated aerobically at 37°C for 24 h. The plates were examined for zones of inhibition which was scored positive, if the width of the clear zone was 5 mm or longer. The diameter of the inhibition zones were taken to be proportional to the logarithm of the antimicrobial compounds in walnut leaves and onion bulbs (Maria et al., 1994).

### Minimum inhibitory concentration of walnut leaves and onion bulbs

Doubling dilution of 2000 µg/ml of walnut leaves and onion bulbs extract were made in 5 ml volume of broth to 3.912 µg/ml. One row of the test was inoculated with 0.02 ml of 1 in 100 dilution of the overnight broth culture of the test organism (Stokes and Ridgeway, 1980). The test was incubated at 37°C for 24 h aerobically. The

**Table 1.** Microbial load of skin, gills, intestine and liver of *C. gariepinus*.

| Fish site | Organism | Microbial load ($log_{10}$cfu/g) |
|---|---|---|
| Liver | Enterobacteriacea count | 2.04±0.03 |
| | Total viable counts | 2.61±0.05 |
| Intestine | Enterobacteriacea | 2.60±0.01 |
| | Total viable counts | 3.00±0.10 |
| Skin | Enterobacteriacea | 6.48± 1.50 |
| | Total viable counts | 6.93± 1.90 |
| Gills | Enterobacteriacea | 2.78±0.08 |
| | Total viable counts | 3.11±0.02 |
| Control | Enterobacteriacea | - |
| | Total viable counts | - |

**Table 2.** Antibacterial activities (diameter of inhibition zone, mm) of walnut leaves and onion bulbs.

| Pathogen | Diameter of zone of inhibition (mm) | | |
|---|---|---|---|
| | Onion | Walnut leaves | Control |
| *Pseudomonas aeruginosa* | 10±0.01 | 12±0.00 | - |
| *Bacillus subtilis* | - | 12±0.02 | - |
| *Pseudomonas fluorescens* | 11±0.00 | 11±0.01 | - |
| *Staphyloccocus aureus* | 11±0.01 | 13.5±0.03 | - |
| *Escherichia coli* | 9±0.02 | - | - |
| *Salmonella typhi* | 10±0.00 | 10±0.01 | - |
| *Aspergillus niger* | 16.5±0.02 | 10±0.00 | - |

minimum inhibitory concentration was the lowest concentration that prevented the growth of bacterial after 24 h incubation (Osoba, 1979).

#### Statistical analysis

The microbial load of fish tissue (skin, gills, intestine and liver) and antibacterial and antifungal activities (diameter of inhibition zone, mm) of walnut leaves and onion bulbs against the tested pathogens resulting from the experiment were subjected to one-way analysis of variance (ANOVA) using SPSS (Statistical Package for Social Sciences Version 15.0).

## RESULTS

### Determination of microbial load in *Clarias gariepinus*

The microbial load of fish tissue (skin, gills, intestine and liver) were determined and the results show that the highest enterobacteriacea was recorded in skin and least in liver while no enterobacteriacea was recorded in control. Also, the highest total viable counts was recorded in skin and least in liver while no total viable counts was recorded in the control as shown in Table 1.

### Detection of antagonistic activities of onion bulbs and walnut leaves

Ethanolic and methanolic extracts of onion bulbs and walnut leaves respectively shows antibacterial and antifungal properties in the present study. The walnut leaves extracts exhibited the highest activities with all the pathogens investigated. Although, the onion bulbs extract had highest antifungal property, while no antibacterial activity were recorded for *B. subtilis* and *E. coli* in onion bulbs and walnut leaves respectively (Table 2).

### Determination of minimum inhibitory concentration (MIC) of onion bulbs and walnut leaves

The minimum inhibitory concentration of the ethanolic and methanolic extracts of onion bulbs and walnut leaves against 6 pathogenic bacteria isolated from aquatic animals were examined in the present study and their potency were assessed by the MIC and it was recorded that both plants shows 500 µg/ml against all the tested pathogens (Table 3).

**Table 3.** Minimum inhibitory concentration of onion bulbs and walnut leaves.

| | | Minimum inhibitory concentration in µg/ml | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Isolates±** | **2000** | **1000** | **500** | **250** | **125** | **62.5** | **31.3** | **15.65** | **7.825** | **3.912** |
| Onion | *Pseudomonas aeruginosa* | - | - | - | + | + | + | + | + | + | + |
| | *Bacillus subtilis* | - | - | - | + | + | + | + | + | + | + |
| | *Pseudomonas fluorescens* | - | - | + | + | + | + | + | + | + | + |
| | *Staphyloccocus aureus* | - | - | - | - | - | + | + | + | + | + |
| | *Escherichia coli* | - | - | - | - | - | - | - | + | + | + |
| | *Salmonella typhi* | - | - | - | + | + | + | + | + | + | + |
| | Control (without isolates) | - | - | - | - | - | - | - | - | - | - |
| Walnut leaves | *Pseudomonas aeruginosa* | - | - | - | + | + | + | + | + | + | + |
| | *Bacillus subtilis* | - | - | - | + | + | + | + | + | + | + |
| | *Pseudomonas fluorescens* | - | - | - | + | + | + | + | + | + | + |
| | *Staphyloccocus aureus* | - | - | - | - | + | + | + | + | + | + |
| | *Salmonella typhi* | - | - | - | + | + | + | + | + | + | + |
| | Control (without isolates) | - | - | - | - | - | - | - | - | - | - |

Key:
+ = indicating growth showed by turbidity of the broth.
- = no growth.

**Table 4.** Determination of important phytochemical of ethanolic and methanolic extracts of onion bulbs and walnut leaves respectively.

| Sample | Parameter | Concentration |
|---|---|---|
| Onion bulb | Alkaloids | + |
| | Cyanogenic glycosides | + |
| | Anthraquinones | - |
| | Saponins | + |
| | Tannins | + |
| | Flavonoids | + |
| Walnut leaves | Alkaloids | + |
| | Cyanogenic glycosides | + |
| | Anthraquinones | - |
| | Saponins | + |
| | Tannins | + |
| | Flavonoids | - |

+ = present and available in moderate quantity/ concentration.
- = not present.



### Determination of phytochemicals in onion bulbs and walnut leaves

Preliminary phytochemical screening of onion bulbs and walnut leaves for secondary metabolites showed the presence of saponnins, tannins, alkaloids, cyanogenic glycosides while anthraquinones was not detected in both plants; flavonoids was present in onion bulbs while flavonoids was not detected in walnut leaves. The concentration of these metabolites was available in moderate quantity (+) in both plants as shown in Table 4.

## DISCUSSION

In fish health management, the most useful index of success is prevention of disease and systematic physico-chemical analysis of the water and monitoring of the micro organism in the aquaculture system (Krishna et al., 2012). The epithelial surfaces of fish such as those of skin, gill or gastrointestinal tract are the first contact areas for potential pathogens (Narvaez et al., 2010). The result of this work revealed that the microbial counts in the liver, intestine, skin and gill of *C. gariepinus* varies with the skin

and gills having the highest values of enterobacteriacea and total viable counts. This agrees with Shalaby et al. (2006) that bacterial load is greater on the skin and gills than any part of fish as these parts are ones constantly exposed to challenges. However, higher bacterial loads were observed in the guts of fish than surrounding water. This finding was similar to Ampofo and Clerk (2010). The use of herbal extracts is widely expected to become an alternative therapy in aquaculture as a prophylactic and to control fish diseases. Studies concerning antimicrobial properties of herbal extracts against bacteria with fish culture importance *in vitro* and *in vivo* are still limited; hence, this study was carried out and the result revealed that the extracts of walnut leaves and onion bulb (methanol and ethanol) respectively had antimicrobial activities against the tested pathogenic bacteria (*P. aeruginosa*, *B. subtilis*, *P. fluorescens*, *S. aureus*, *E. coli*, *S. typhi*).

For walnut leaves, the widest zone of inhibition was obtained with *S. aureus* (13.5 mm), followed by *P. aeruginosa* (12 mm) and *B. subtilis* (12 mm); while the least zone of inhibition was obtained in *S. typhi* (10 mm) and *E. coli* did not show zone of inhibition. With onions, *B. subtilis* (12 mm) had the widest zone of inhibition, followed by *P. fluorescens* (11 mm), *S. aureus* (11 mm), while the least was obtained from *E. coli* (9 mm). These corroborate earlier reports that plant extracts are usually more active against gram positive bacteria than gram negative bacteria (Abu Shanab et al., 2004; Basri and Fan, 2005). The walnut leaves had better antibacterial activity with higher diameter of inhibition zone than the onion bulbs while onion bulbs had a better anti –fungal activity against *A. niger* than walnut leaves. The factors responsible for high value of antimicrobial activity in walnut leaves are not fully elucidated but may be due to the presence of two antibacterial constituents – walnut essential oil and juglore – that directly initiate steps against contagious micro organism and large concentration of vitamins C and presence of secondary plant metabolites (astringent tannins); the fact that the methanolic extracts of walnut leaves and onion bulbs inhibited the growth of the tested bacteria.

Similar observations were made by Abu Shanab et al. (2005); where the methanolic extract of the dried ripe berries of *Rhus coriaria* inhibited the bacteria studied. Similar studies using extracts of plants against pathogens include Ajaiyeoba and Fadare (2006), Azu and Onyeagba (2007), Selvamohan et al. (2012) and Panghal et al. (2011). The minimum inhibitory concentration of walnut leaves and onion bulbs revealed that 500 µg/ml was the least concentration that prevented the growth of bacteria after 24 h incubation. *S. aureus* and *E. coli* had 125 and 31.3 µg/ml, respectively for onion while *S. aureus* had 250 µg/ml for walnut leaves. However, over use of antibiotics for bacterial disease in fish can lead to the emergence of drug – resistant strains and can create serious health problems (Aly et al., 2008; Das et al., 2009; Harikrishnan et al., 2011). Muniruzzaman and Chowdhury (2004) also reported that the extracts obtained from bulb of onion (*A. cepa*) had inhibitory effect on *P. fluorescens* at minimum inhibitory concentration (MIC) 1.2 mg/ml. This report agreed with the present study in which *A. cepa* showed inhibitory activity against *P. fluorescens*.

The antibacterial activity of ethanol extract of medicinal plants screen showed antibacterial activity and inhibited the growth of bacterial strains, *P. aeruginosa, Bacillus cereus* and *E. coli* (Yadav and Khan, 2012); this report supported the present study. Ethanol extract of onion bulb and methanol extract of walnut leaves showed very high potentials as antimicrobial agents and 500 µg/ml minimum concentrations prevented the growth of bacteria. This could be further explored for improved productivity in aquaculture industry. These strong antimicrobial activities could be due to the presence of various phytoconstituents such as alkaloids, flavonoids, tannins, saponins and sterols (Ravikumar et al., 2010). Plants generally contain chemical compounds (such as saponins, tannins, oxalates, phytates, trypsin inhibitors, flavonoids and cyanogenic glycosides) known as secondary metabolites, which are biologically active (Soetan and Oyewole, 2009). Secondary metabolites may be applied in nutrition and as pharmacologically-active agents (Soetan and Oyewole, 2009). They have antibacterial and antiparasitic properties. Plants are also known to have high amounts of essential nutrients, vitamins, minerals, fatty acids and fibre (Gafar and Itodo, 2011). The results obtained in this study showed the presence of alkaloids, cyanogenic glycosides, saponins, tannins and flavonoids in onion bulbs and walnut leaves but anthraquinones were not detected in onion bulbs and walnut leaves.

The concentrations of these metabolites in the onion bulb and walnut leaves were moderately available (+) (Table 4). Although, Ajaiyeoba and Fadare (2006) described that these secondary metabolites were present in higher concentration in walnut leaves (++). Azu and Onyeagba (2007) also ascribed the antimicrobial properties to the presence of flavonoid in onion bulb. Flavonoids (quercetin) have inhibitory activity against disease - causing organisms in animals. Preliminary research indicates that flavonoids may modify allergens, viruses and carcinogens and so may be biological response modifiers. *In vitro* studies show that flavonoids also have anti allergic, anti – inflammatory, anti- microbial, anti – cancer and anti – diarrheal activities (Cushnie and Lamb, 2011). Tannins are plant polyphenols which have ability to form complexes with metal ions and with macro-molecules such as proteins and polysaccharides (Dei et al., 2007). Dietary tannins are said to reduce feed efficiency and weight gain in animal (Dei et al., 2007). Environmental factors and the method of preparation of samples may influence the concentration of tannins present. Tannin presence influences protein utilization and build defense mechanism against micro organism (Cushnie and Lamb, 2011).

Saponins are glycosides, which include steroid sapo-

nins and triterpenoid saponins. High levels of saponins in feed affect feed intake and growth rate in animal (Dei et al., 2007). Saponins (in excess), causes hypocholestrolaemia because it binds cholesterol making it unavailable for absorption (Soetan and Oyewole, 2009). Saponins also have haemolytic activity against red blood cell (RBC) (Ogbe and Affiku, 2011). Saponin-protein complex formation can reduce protein digestibility (Ogbe and Affiku, 2011). Saponins reduced cholesterol by preventing its reabsorption after it has been excreted in the bile. Proper food processing would reduce anti-nutrients (Akinyeye et al., 2011). Haniffa and Shanthi (2012) reported that the phytochemical screening of some medicinal plants revealed the presence of alkaloids, carbohydrates, flavonoids, saponnins and phenolic compounds which are associated with antimicrobial activities and curative properties against pathogen which are similar to the findings of this study. This study showed that walnut leaves and onion bulbs have antimicrobial properties which may be applicable in fish farming and that the minimum concentration of 500 µg/ml of walnut leaves and onion bulbs could be added to fish feed to prevent the growth of microorganisms.

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