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*Full Length Research Paper*

# Antibacterial properties of traditional Sudanese medicinal materials against selected enteric bacterial strains

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Received 19 August, 2020; Accepted 28 September, 2020

**Folklore medicine in Sudan used medicinal materials to treat inestinal infections caused by bacterial infections or contamination of food. Methanolic and aqueous extracts of different parts of *Acacia nilotica* (L.) Delile gum, *Haplophyllum tuberculatum* Juss.aerial parts, *Hydnora abyssinica* A. Br.fruits, *Nigella sativa L seeds, Rhynchosia minima* (L.) DC. roots,and *Usnea molliuscula lichen* were tested for antibacterial properties at a concentration of 100 mg/mL against 20 intestinal isolates including *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Salmonella typhi*, *Salmonella para typhi B,Staphylococcus aureus*, and standard bacterial strains (*Bacillus subtilis* (NCTC 8236), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 35657), *Salmonella typhi* (ATCC 1319106) and *Staphylococcus aureus* (ATCC 25923), using the Agar Diffusion Method. Standard antibiotics were used as standards drug for antibacterial effect. The highest enhancing properties were observed in methanol extracts and the lowest in aqueuos extract. *U. molliuscula* methanolic extract was the most active among all tested plant extracts, while, the aqueous extract of *H. tuberculatum* had a promising level of** efficacy **among the aqueous extracts tested. Most responsive Gram-negative clinical isolates bacteria were *S. para typhi B* and *P. aeruginosa.* Most susceptible standard bacteria were *B. subtilis* (NCTC 8236). Obtained results from investigated plants confirm their antibacterial potential and usefulness in the treatment of intestinal infections.**

**Key words:** Phytomedicine, traditional uses, antibactrial activity.

## INTRODUCTION

The increasing incidence of antibiotic resistance has been increasing throughout the world in the last few decades (Abdallah, 2011).This has led to higher mortality in humans. Consequently, one of the most intensive researched fields is the search for material with high antibacterial potency, with folklore medicine being a prime area.In this regard Sudan has a long history of traditional use of plants to treat primry health issues.However, very little research is directed towards understanding their potential for curing gastrointestinal

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tract infections,caused by various bacteria. Spread and prevelence of microbial resistance is geting more frequent worldwide (WHO, 2001). Search for new antimicrobial substances is the major weapon to combat the microbial resistance through developing new antibacterial materials to substitute with inefficient ones.

Combination of native cultures and different traditions are factors formed by Sudanese traditonal medicine.The extremely large diversity of plants in the area, different cultures due to the variation of climatic zones, and the distinctive geography created Sudanese herbal medicine. 11% of the population has access to prescribed health care.Therefore, research on the best pharmacological influence and possible undesirable side effects or toxicity is essential to enhance potency and protection of Sudanese folklore medicine (Khalid et al., 2012).

Ethnobotanical survey in the Blue Nile State, South-eastern Sudan of medicinal materials used by folklore healers was carried out. Fifty three plant species within 31 families and 47 genera were detected as being used to treat one or more diseases. The most commonly mentioned manifestations were gastro intestinal tract disorders, diseases/infestations, stiffness, and respiratory tract disorders (Musa et al., 2011).

Traditional medicine in Sudan still has the most rational source of therapy of several ailments and bacterial infections.Traditional medicine is distinguished by a special fusion of Islamic, Arabic, and African tradition. Sudanese folk plants have been reported to be characterized with a wide range of folk medicinal uses including different bacterial infections and digestive system disorders (Karar and Kuhnert*,* 2017). Different extracts of *Usnea* revealed a variable effect of antibacterial properties against *S. aureus, E. coli*, *V. cholerae*, *S. dysenteriae* and *S. flexneris.*The methanol extracts were the most active against tested bacteria; all tested organisms showed no antibacterial susceptibility against aqueous extracts of tested lichens (Sinha and Biswas, 2011). *U.molliuscula* extract showed high antimicrobial potential against all tested Gram (+ve) bacteria inclusive penicillin-resistant *S aureus* and methicillin-resistant *S.aureus* (Weckesser et al*.,* 2007). Studies revealed the effects of *N.sativa* seed extracts have dose dependent antibacterial activities on the tested organisms (Hosseinzadeh et al., 2007; Chaudhry and Tariq, 2008; Yoruk et al., 2010; Mishra, 2011; Pichette et al., 2011; Haloci et al., 2012; Monika et al., 2013).

*H. tuberculatum* showed antimicrobial evaluation against a variety of strains, revealed moderate effect against *B. subtilis*, *S. choleraesuis* and *E. coli,* gentamycin sulphate; it has 75% potency as antibacterial agent on *S.aureus* and *E.coli*. It is the most potent inhibitor against plant pathogenic bacteria and fungi (Al-Burtamani et al., 2005; Sabry et al., 2016; Abdelgaleil et al., 2020). Available literature shows that no earlier study has been performed on the antibacterial characters of *A.nilotica* gum until 2013.Study of methanolic extract of *A. nilotica* revealed moderate antibacterial effect (14-18 mm) against *E. coli* (ATCC 25922);aqueous extracts of *A. nilotica* had no effects of antibacterial characters against all tested bacteria (Mahjoub, 2013). Studies of antimicrobial evaluation of different extracts of *A.nilotica* revealed high (>18 mm) to modrate (14-18 mm) activity for different extracts which consider a good source of natural antibiotic for the therapy of different transmittable diseases (Sravani et al.,2014; Banjar et al., 2017; Al Alawi et al., 2018; Shehu et al., 2018; Ali et al., 2020). Ethanolic extracts of 8 species of *R.minima* were investigated for their antibacterial activity and phytochemical screening against clinical isolates (*B.subtilis,E.coli, P.aeruginosa* and *S.aureus*); they showed equal or nearly equal antibacterial characters against all tested bacteria (El-Kamali and El-Amir, 2010).The essential oils of *R.minima* exhibited antioxidant and antimicrobial activities against *E. coli*, *S aureus* and *C. albicans*, but not active against *C. perfringens* and *K. pneomoniae* (Gundidza et al., 2009). Methanolic extract of *H.abyssinica* showed moderate antimicrobial potential. Analysis there are tanins and phenols in the plant root extracts (Saadabi and Ayoub, 2009).

A research was done to determine the antibacterial potential of six medicinal materials *U. molliuscula* lichen, *N .sativa* seeds, *H. tuberculatum* aerial parts, *A. nilotica* gum, *R. minima* roots and *H.abyssinica* fruits. They were obtained from Omdurman market on the origin of antimicrobial potentials with documented details for antibacterial assay against bacterial strains associated with intestinal infections. These investigations determine antibacterial potential efficacy of selected medicinal plants.This selection was guided in the first place by ethnobotanical claim in traditional medicine suggestive of their antibacterial activity and secondly by defficiency or insufficiency of information in published works on antibacterial and antioxidants potency of their extracts. This will provide baseline data for developing new antibacterial compounds in the treatment of intestinal infections, based on their folklore use.

## MATERIALS AND METHODS

Plants' taxonomy and authentication were established by Prof.Hatil H.Elkamali via differentiation with herbs specimens of Botany Department, Faculty of Science and Technology, Omdurman Islamic University during the spring season of 2012. The plants wew dried in the shade for three weeks.The air-dried plants' parts were crushed and turned into coarse powder. It was further reduced to powder using a mechanical grinder.

Two hundred grams of all air-dried plants were soaked for 24 h with 50% methanol (MeOH) in a round bottomed flask. Liquor (crude extract) was filtered with whatman grade1qualitative filter papers and then rotor-evaporated.The filtrate was dried at room temperature.Then the dried extracts were sterilized and kept in air-tight containers at room temperature until used for further tests. At the time of testing for antibacterial activities extracts were prepared at a concentration of 100 mg/mL in methanol. One hundred g of

dried plant material was crushed coarsely into powder, which was further reduced to powder using a mechanical grinder.Then it was dissolved with purified water (1L), and left for 24 h at room temperature.With whatman grade 1 qualitative filter papers the mother liquor was filtered.Thus, the aqueous extract (10%) was obtained.

The antibacterial testing was investigated by well-agar diffusion technque (Cheesbrough, 1984). Two hundard and 50 millilitres of decontaminate nutrient agar (Oxoid) were used for antibacterial testing.Suspension of 106 cells was monitored in the inoculum size of each test bacteria.Two millilitres of the inoculum suspention obtained from 24 h cultures of bacteria were supplemented with 250 ml of nutrient agar (Oxoid) and then mixed; they were inoculated on soft agar (20 mL) and flowed on 10 cm diameter sterilized petri dishes and agar plates and allowed to solidify. After solidifying, a sterile cooled-flamed cork borer using four wells (10 mm in diameter) was bored in the agar and the agar discs were removed. One hundred microliters (100 µl) of each extract solution to each plant extract was added to each well with a pipette and the plate was held for 2 h at room temperature for diffusion of extract into agar. The plates were incubated at 37°C for 24 h. Result of tested plants were evaluated to test their antibacterial activities and expressed as the diameters of the inhibition zones which were calculated as the adjacent mm. Methanol serves as negative control. Axiom laboratories, New Delhi 1100055 Multidisc was used for antimicrobial susceptibility testing of tested clinical isolates (4 *E. coli*, 5 *P. aeruginosa*, 5 *P. mirabilis*, *3 S. aureus*, 2 *S. typhi*, and 1 *S. para typhi B* ).

## RESULTS

Methanolic extracts of *U.molliuscula* revealed high (>18 mm) antibacterial properties (1Z = 20 mm) against *P. aeruginosa* no. (20)*, S.aureus, P.mirabilis* no. (11) and *S. para typhi, B* no.(17)*. B. subtilis* (NCTC 8236) was (>18 mm) (1Z = 24mm) to methanolic extracts of *U.molliuscula* (Table 1). *E.coli* (ATCC 25922), *S. aureus* (ATCC 25923), *K. pneumoniae* (ATCC 35657), and *S.typhi* (ATCC1319106) were found to be not sensitive to the tested plants (<14 mm) (Table 1). All clinical isolates were found to be resistant (< 14 mm) to methanolic extracts of *N.sativa* except *S.para typhi B* no.(17), that showed moderate (14-18 mm) antibacterial potency (1Z = 16 mm). All standard bacteria were resistant (1Z < 14 mm) (Table 1).

Methanolic extracts of *H.tuberculatum*, *A.nilotica* and *H.abyssinica* were not effective against all tested bacteria. *K.pneumoniae* (ATCC 35657), *B.subtilis* (NCTC 8236), *S. aureus* (ATCC 25923) and *S.typhi* (ATCC1319106) were resistant to the tested plant extracts (< 14 mm), except *E. coli* (ATCC 25922) that moderately (1Z = 14-18 mm) resisted (1Z = 16 mm) the methanolic extract of *A*. *nilotica* (Table 1).

All tested bacteria were less susceptible to aqueous extracts of all plants except *H,tuberculatum*. Aqueous extract of *H. tuberculatum* showed promising result against *P.aeruginosa* isolate no.(7) (1Z=18 mm), but moderate (1Z=14-18 mm) efficacy was observed against *E.coli* no.(6) (1Z=16mm)(Table 1).Aqueous extract of *U.molliuscula*, *Nigella sativa, Acacia nilotica.R.minimas, and H. abyssinica* were ineffective against all tested bacteria. Their methanolic extracts exhibited strong antioxidant properties as compared to other plants.Further work should be on antioxidant and anti-inflammatory activities of isolated compounds from active extracts (Elkamali and Mahjoub, 2015).

Co-Trimoxazole (BA) at concentration of 25 mcg showed promising effect (1Z=24 mm) against *S.para typhi B* no. (17), and moderate effect (14-18 mm) (1Z=16-14 mm) against *E.coli* no.(9), *P.aeruginos* no.(15) and *S. aureus* no.(14) (Table 2). *P. mirabilis* no. (3) exhibits antibiotic-resistant (<14 mm) to BA and showed a high degree (>18 mm) of sensitivity (1Z=18 mm) to the methanolic extracts of *U. molliuscula*. *P. mirabilis* has the ability to cause various human diseases; it is primarily corresponding with urinary system disorders and is a major health concern due to its complications and frequent recurrence. Usnic acid rich in phenols is accountable for the antioxidant activities of *U.molliuscula* methanolic extract; it possesses strong antioxidant activities against different antioxidant systems *in vitro*. It is considered as the source of natural antioxidants. It can be used simply as possible food complement, and in pharmaceutical implementations (Elkamali and Mahjoub, 2015). *B.subtilis* (NCTC 8236) showed promsing (>18 mm) degree of sensitivity (1Z =24mm) to methanolic extracts of *U.molliuscula,* which exhibit antibiotic-resistant to Co-Trimoxazole and Ceftizoxim; same goes to Piperacillin/Tazobactam, Chloramphenicol and Ciprofloxacin*.* Our results support that the traditional therapeutic information for *U.molliuscula*, in near future, can surely change the traditional antimicrobial agents to which there is increased occurrence of drug interactions. The study recommends that *U.molliuscula* is promising for increasing phytomedicines with antibacterial potential. More studies on the selection and distinguishing of active concepts and assessing possible synergism among extract components for their antimicrobial potentiels depending on the main results obtained might be considered enough (Ali et al., 2012).

Piperacillin/Tazobactam (TZP) at concentration of 100/10 mcg showed good (14-18 mm) efficacy (1Z=16 mm) against *E.coli* no. (6) (Table 2); it also showed promising (>18 mm) result (1Z=20 mm) against *B.subtilis* (NCTC 8236); whereas S*. typhi* (ATCC1319106), *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) were found to be resistant (< 14 mm). *E. coli* no.(6) exhibited antibiotic-resistant to most synthetic antibiotics and showed good (14-18 mm) degree of sensitivity (1Z=16 mm) to the methanolic extracts of *H.tuberculatum* in the same way as TZP (1Z = 16 mm). The phytochemical profile of leaf extracts of *H.tuberculatum* revealed the presence of alkaloid and polyphenolic compounds may be basic contributors to the antioxidant potential of these extracts (Hamdi et al., 2018).

Chloramphenicol (CH) at concentration of 30 mcg showed moderate (14-18 mm) efficacy (1Z=14 mm) against clinical isolates *P.aeruginosa* no.(15) *S.aureus* no. (14), *P. mirabilis* no. (19) and *E. coli* no. (9) showed

**Table 1.** Screening of Six Medicinal Plants for Antibacterial Activity against Gastrointestinal Tract Clinical Isolates and Standard bacteria.

| S/N | Bacteria | Extract | *U. molliuscula* | *N. sativa* | *H. tuberculatum* | *A. nilotica* | *R. minima* | *H. abyssinica* |
|---|---|---|---|---|---|---|---|---|
| | | | **Clinical isolates bacteria** | | | | | |
| 1 | *Salmonella typhi* | Me OH | 6 | 2 | - | 2 | - | - |
| | | $H_2O$ | - | - | - | - | - | - |
| 2 | *Proteus mirabilis* | Me OH | 2 | 2 | 2 | 10 | - | 4 |
| | | $H_2O$ | - | - | - | - | - | - |
| 3 | *Proteus mirabilis* | Me OH | 18 | 2 | 2 | 8 | 2 | 8 |
| | | $H_2O$ | - | - | - | - | - | - |
| 4 | *Proteus mirabilis* | Me OH | 4 | 2 | 2 | 2 | - | 2 |
| | | $H_2O$ | - | - | - | - | - | - |
| 5 | *Escherichia coli* | Me OH | 4 | - | 2 | 2 | - | - |
| | | $H_2O$ | - | - | - | - | - | - |
| 6 | *Escherichia coli* | Me OH | 2 | 8 | 2 | 2 | 2 | 10 |
| | | $H_2O$ | - | - | 16 | - | - | - |
| 7 | *Pseudomonas aeruginosa* | Me OH | 4 | - | 2 | - | - | 2 |
| | | $H_2O$ | - | - | 18 | - | - | - |
| 8 | *Pseudomonas aeruginosa* | Me OH | 4 | 2 | 2 | 11 | 2 | 2 |
| | | $H_2O$ | - | - | - | - | - | - |
| 9 | *Escherichia coli* | Me OH | 6 | - | - | 2 | - | - |
| | | $H_2O$ | - | - | - | - | - | - |
| 10 | *Escherichia coli* | Me OH | 4 | 2 | - | - | - | 2 |
| | | $H_2O$ | - | - | - | - | - | - |
| 11 | *Staphylococcus aureus* | Me OH | 20 | 8 | 2 | 4 | 2 | 4 |
| | | $H_2O$ | - | - | - | - | - | - |
| 12 | *Staphylococcus aureus* | Me OH | 2 | - | - | - | - | 2 |
| | | $H_2O$ | - | - | - | - | - | - |
| 13 | *Pseudomonas aeruginosa* | Me OH | 8 | 4 | 6 | 4 | 4 | 2 |
| | | $H_2O$ | - | - | - | - | 6 | 6 |
| 14 | *Staphylococcus aureus* | Me OH | 4 | 2 | 2 | 2 | - | 2 |
| | | $H_2O$ | - | - | - | - | - | - |
| 15 | *Pseudomonas aeruginosa* | Me OH | 4 | 2 | - | 2 | - | 2 |
| | | $H_2O$ | - | - | - | - | - | |
| 16 | *Proteus mirabilis* | Me OH | 14 | 6 | - | - | - | 2 |
| | | $H_2O$ | - | - | - | - | - | -- |
| 17 | *Salmonella para typhi B* | Me OH | 20 | 16 | 2 | 8 | 2 | 4 |

**Table 1.** Contd.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $H_2O$ | - | - | - | - | - | - |
| 18 | *Salmonella typhi* | Me OH | 4 | - | 2 | - | - | - |
| | | $H_2O$ | - | - | - | - | - | - |
| 19 | *Proteus mirabilis* | Me OH | 4 | 2 | 2 | - | - | 4 |
| | | $H_2O$ | - | - | - | - | - | - |
| 20 | *Pseudomonas aeruginosa* | Me OH | 20 | - | - | - | - | 2 |
| | | $H_2O$ | - | - | - | - | - | - |
| | | **Standard bacteria** | | | | | | |
| 1 | *Staphylococcus aureus*(ATCC 25923) | Me OH | 6 | 6 | 8 | 8 | 6 | 6 |
| | | $H_2O$ | - | 6 | - | - | - | - |
| 2 | *Bacillus subtilis*(NCTC 8236) | Me OH | 24 | 8 | 8 | 8 | 6 | - |
| | | $H_2O$ | 6 | - | - | - | - | - |
| 3 | *Escherichia coli* (ATCC 25922) | Me OH | 10 | 4 | 4 | 16 | 2 | 4 |
| | | $H_2O$ | - | - | - | - | - | - |
| 4 | *Klebsiella pneumonia*(ATCC 35657) | Me OH | 2 | 2 | 4 | 4 | 2 | 2 |
| | | $H_2O$ | - | - | - | - | - | - |
| 5 | *Salmonella typhi*(ATCC1319106) | Me OH | 2 | 2 | 2 | 2 | 2 | 4 |
| | | $H_2O$ | - | - | - | - | - | 2 |

Sensitive: (> 18), intermediate: (14-18mm), Resistant:(< 14 mm), - no inhibition. Tested extracts concentration: 100 mg/mL (0.1 mL /well). Values of the tests are the mean of four replicates.

promising (>18 mm) result (1Z=20 mm) against *B. subtilis* (NCTC 8236); while other standard bacteria *S. aureus* (ATCC 25923), *E.coli* (ATCC 25922),*S. typhi* (ATCC1319106) and *K. pneumoniae* (ATCC 35657) were found to be chloramphenicol resistant (<14mm). *Bacillus subtilis* (NCTC8236) showed high sensitivity (1Z=24 mm) to methanolic extract of *U.molliuscula* and antibiotics resistant, except Piperacillin/ Tazobactam, Ciprofloxacin, Chloramphenicol, and Levofloxacin .*U. molliuscula* extracts can be used in the therapy of infectious diseases caused by resistant bacteria due to their great potential as chemotherapeutic agents against microorganisms.

Ceftizoxime (CI) at concentration of 30 mcg was found effective (1Z=20 mm) against *P. aeruginosa* no. (13), *P. mirabilis* no.(2), moderate effect (1Z=16 mm) was observed against *P. aeruginosa* no.(15), it was ineffective to *S. aureus* (ATCC 25923),*B. subtilis* (NCTC 8236),*K. pneumoniae* (ATCC 35657) and *S.typhi* (ATCC 1319106) except *E.coli* (ATCC 25922).Standard bacteria *E.coli* (ATCC 25922) exhibit moderate sensitivity to most synthetic antibiotics,showed good (14-18 mm) degree of sensitivity (1Z=16 mm) to the methanolic extracts of *A.nilotica* in the same extent as to CI and AK (1Z=16 mm).The pharmacognostical study revealed that *A.nilotica* species can be characterized on the basis of its macroscopic, microscopic and phytochemical properties. It was found to contain different secondary metabolites such as alkaloids, saponins, tannins and flavanoids (Saini et al., 2008). This information represents an ample chance to establish drug based design depending on their significant role in the folk medicine, efficacy against many pathogenic microoganisms, and their significant phytochemical coumpounds.

Ofloxacin (OF) at concentration of 5 mcg showed good effective result (1Z=18 mm) against *P. aeruginos* no.(7),and *S.para typhi B* no.(17) (Table 2). *S. typhi* (ATCC1319106), *S. aureus* (ATCC 25923), *B. subtilis* (NCTC 8236), *E. coli* (ATCC 25922), and *K. pneumoniae* (ATCC 35657) exhibit antibiotic-resistant to OF (<14 mm) (Table 3). *S. aureus* no.(11), *P. mirabilis* no. (3) *P. aeruginosa* no.(20) exhibit antibiotic-resistant to OF and showed high (> 18 mm) degree of

**Table 2.** Standard antibiotics against gastrointestinal tract clinical isolates.

| Antibiotics | Concentration mcg /ml | Clinical isolates | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *S. typhi no.*(1) | *P. mirabilis no.*(2) | *P. mirabilis no.*(3) | *P. mirabilis no.*(4) | *E. coli no.*(5) | *E. coli no.*(6) | *P. aeruginosa no.*(7) | *P. aeruginosa no.*(8) | *E. coli no.*(9) | *E. coli no.*(10) | *S. aureus no.*(11) | *S. aureus no.*(12) | *P. aeruginosa no.*(13) | *S. aureus no.*(14) | *P. aeruginosa no.*(15) | *P. mirabilis no.*(16) | *S.para typhi no.*(17)*B* | *S. typhi no.*(18) | *P. mirabilis no.* (19) | *P. aeruginosa no.*(20) |
| AS | 20 | 2 | 8 | - | - | 6 | - | - | - | 4 | - | - | 2 | 10 | - | - | - | 6 | - | 2 | - |
| BA | 25 | - | 10 | - | - | - | 6 | 10 | - | 16 | - | - | - | 10 | 14 | 14 | - | 26 | - | 12 | - |
| CF | 30 | 14 | 2 | - | 20 | 12 | 8 | 4 | - | 6 | - | - | - | 10 | 2 | 4 | - | 8 | - | 2 | - |
| TZP | 100/10 | 10 | 12 | - | - | 12 | 16 | 12 | - | 12 | 12 | - | 6 | 10 | - | - | - | 12 | - | 12 | - |
| CH | 30 | 8 | 8 | - | - | 10 | - | - | - | 14 | 8 | - | - | 10 | 16 | 16 | - | 4 | - | 16 | - |
| CP | 5 | 10 | 10 | - | 10 | - | 20 | 24 | - | 6 | 14 | - | - | 12 | 8 | 10 | - | 30 | - | 4 | - |
| CI | 30 | 14 | 20 | - | - | - | - | - | - | 12 | - | - | - | 20 | 12 | 16 | - | - | 6 | 10 | - |
| TE | 30 | 2 | 2 | - | - | 2 | 20 | 4 | - | - | 10 | - | - | 6 | - | - | - | 12 | - | - | - |
| OF | 5 | 10 | 10 | - | - | 8 | 10 | 18 | - | 6 | 14 | - | 6 | 10 | 4 | 6 | - | 18 | - | 4 | - |
| GM | 10 | 10 | 10 | - | - | 14 | 12 | 10 | - | - | 18 | - | - | 8 | - | 16 | - | 14 | 10 | - | - |
| AK | 30 | 10 | 10 | - | - | 14 | 10 | 14 | - | - | 20 | - | - | 8 | - | 8 | - | 8 | 6 | - | - |
| GF | 5 | 10 | 10 | - | - | 14 | 8 | 18 | - | 12 | 14 | - | 16 | 10 | 10 | 14 | - | 16 | - | 10 | - |

Sensitive: (> 18) , intermediate: (14-18mm), Resistant:(< 14 mm), - no inhibition. Tested extracts concentration: 100 mg/mL (0.1 mL /well). Values of the tests are the mean of four replicates. Ampicillin/sulbactam (AS) 20mcg,Co-Trimoxazole( BA) 25 mcg,Cefotaxime (CF) 30 mcg,Piperacillin/Tazobactam (TZP) 100/10 mcg, Chloramphenicol (CH) 30 mcg,Ciprofloxacin (CP) 5 mcg,Ceftizoxime (CI) 30 mcg,Tetracycline (TE) 30 mcg,Ofloxacin (OF) 5 mcg,Gentamicin (GM) 10 mcg,Amikacin (AK) 30 mcg,Gatifloxacin (GF) 5 mcg.

**Table 3.** Antibacterial activity of antibiotics against different standard bacteria.

| Antibiotics | Ampicillin/ sulbactam (20 mcg) | Co-Trimoxazole (25 mcg) | Cefotaxime (30mcg) | Piperacillin/ Tazobactam 100/10 mcg | Chloramphenicol (30 mcg) | Ciprofloxacin (5 mcg) | Ceftizoxime (30 mcg) | Tetracycline (30 mcg) | Ofloxacin (5 mcg) | Gentamicin (10 mcg) | Amikacin (30 mcg) | Gatifloxacin (5 mcg) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sa.t | 4 | - | - | - | 6 | - | - | 10 | - | - | - | - |
| K.n | 4 | 10 | 4 | 10 | 10 | 16 | - | 10 | 10 | 10 | 10 | 10 |
| B.s | 6 | - | 8 | 20 | 20 | 20 | - | 10 | 10 | 10 | 10 | 10 |
| E.C | - | - | 6 | - | - | 14 | 16 | 18 | 12 | 14 | 16 | 14 |
| S.a | - | - | - | - | - | - | - | - | - | - | - | - |

Sensitive: (> 18), intermediate: (14-18 mm), Resistant:(< 14 mm), - no inhibition. Tested extracts concentration: 100 mg/mL (0.1 mL /well). Values of the tests are the mean of four replicates. Ampicillin/sulbactam(AS) 20mcg,Co-Trimoxazole BA 25 mcg,Cefotaxime (CF) 30 mcg,Piperacillin/Tazobactam (TZP) 100/10 mcg, Chloramphenicol (CH) 30 mcg,Ciprofloxacin( CP) 5 mcg,Ceftizoxime (CI) 30 mcg,Tetracycline (TE) 30 mcg,Ofloxacin (OF) 5 mcg,Gentamicin (GM) 10 mcg, Amikacin (AK) 30 mcg, Gatifloxacin (GF) 5 mcg.

sensitivity (1Z = 20-18 mm) to the methanolic extracts of *U. molliuscula*. The appearance and rapid development of antibiotic resistance by infectious bacterial isolates are critical threats to the international public health; it leads to a significant threat to public health worldwide due to the bounded therapy options and unconcerned discovery of new types of antibiotics (Trojan et al., 2016).Incorporating sensitivity data within study may be worth it in fulfilling treatment strategies for diseases.*P.mirabilis* no.(16) showed antibiotic-resistant to synthetic antibiotics and showed good (14-18 mm) degree of sensitivity (1Z=14 mm) to the methanolic extracts of *U. molliuscula*. *S. para typhi B* no.(17) showed promising degree of sensitivity (1Z=20 mm) to the methanolic extracts of *U.molliuscula* same extent as to OF. Plant chemical constituents as alkaloids, flavonoids, tannins,and phenolic compounds work as protection mechanisms contrary to predation by many microbs,insects and herbivores. Flavonoids have antibacterial potential through their capability to complex with extracellular and disolved proteins and to complex with bacterial outer cell walls (Vijayasanthi et al., 2012). *E.coli* no.(6) exhibited antibiotic-resistant to OF and showed a good (14-18 mm) degree of sensitivity (1Z=16 mm) to the methanolic extracts of *H.tuberculatum* same extent as to Piperacillin/Tazobactam. *P.aeruginosa* no.(7) showed fairly high effect (1Z = 18 mm) against *H.tuberculatum* methanolic extracts in the same extent as to OF.Phytochemical constituents of *H tuberculatum* revealed polyphenolic compound as resveratrol,myricetin and quercetin flavonol kaempferol and rutinand rosmarinic acid (Abdelkhalek et al.,2012). Evidence indicated that bioactive substances (antioxidants) possess extranutritional charateristics and advanced role in food-disease association.Radical scavenging activity under physiological conditions needs additional research to show and to determine whether there is any link between their radical scavenging properties and their antimicrobial potential (Ramadan et al., 2003).

## DISCUSSION

*U. molliuscula* methanolic extracts revealed high (>18 mm) antibacterial effect (1Z = 20 -18 mm) against *P.aeruginosa*, *S.aureus*, and *P.mirabilis*. There is need for more research on the isolation and recognition of active ingradients and to assess the probable synergism between extract constituents for their antibacterial activities based on the results obtained (Ali et al*.,* 2012). *P. aeruginosa*, *S. aureus*, and *P. mirabilis* showed antibiotic-resistant against *U.molliuscula* extracts. There is need to discover and develop new antimicrobial medications for the treatment of diarrhoea and other bacterial infections due to *P.mirabilis* and *E. coli* through support scientific base and inclusion of traditional practices in present system of medicines.

Only one of all *S. aureus* clinical isolates was more sensitive and effective against methanolic extract of *U.molliuscula* than Gram (-ve) antibiotics (Tables 2 and 3). Lichen metabolite usnic acid exhibits antimicrobial activity against plant and man infectious microorganism, including antimicrobial efficacy against antibiotic-resistant bacterial strains (Ingólfsdóttr, 2002). Aqueous extract of *U. molliuscula* lichen did not exhibit antibacterial properties against all tested bacteria.The obtained results may account for the reason people in Sudan refuse to use aqueous extract countiniously as a treatment of gastrointestinal tract. It is speculated that it may possess anti-inflammatory activities, where it is used in Sudan as a bitter stomachic, for cough and also by women to relive menstrual pain (Kheir, 1966).

*Nigella sativa* methanolic extract showed moderate (1Z=14-18 mm) potency (1Z=16 mm) against *S.para typhi B* (Table 1).More and more beneficial effects of *N.sativa* should be explored in order to maximize its utility in effective treatment and cure for various diseases.A single strain of *S. typhi B* clinical isolates was resistant to most of the plant extracts analyzed.This bacterial species is of importance in gastrointestinal tract infections and deserves a wider investigation, including a large number of strains.Aqueous extract did not exhibit antibacterial potentials against all tested bacteria.Thymoquinon possesses anti-inflammatory activities,protects the cell membrane integrity through inhibition of lipid peroxidationm and alters levels of leukotrienes and prostaglandins favoring cytoprotection of the gastric mucosal cells (Kanter et al., 2005).Scavenging activity of the free radical ions was increased due to effectiveness of human serum albumin (HSA) isoforms (‘N’ form at pH 7.4 and ‘B’ form at pH 9.0) in the presence of Thymoquinone (TQ), the main constituent in *N. sativa* (Ishtikhar et al., 2015).

All plant aqueous extracts except *H. tuberculatum* do not possess significant antibacterial efficacy against tested bacteria.Phytochemical investigation revealed the presence of Terpenes and β-phellandrene,limonene, β-ocimene, β-caryphyllene,myrcene, and α-phellandrene, the most rich oil components (Al-Burtamani et al., 2005).It is speculated that they may possess anti-inflammatory characteristics. The potential therapeutic assessment of the medicinal plants has been the subject of continual research for their anti-inflammatory componants, including the terpenes which have pharmacological actions (Souza et al., 2014). *H. tuberculatum* aqueous extract showed moderate (1Z=14-18 mm) potency (1Z=18-16 mm) against clinical isolates *E. coli* and *P. aeruginosa*. Antibacterial activity of the extract showed promising result against *P. aeruginosa* and *E. coli* clinical isolates, the most common one and problematic among opportunistic pathogens.Resistance to antimicrobial drugs may be a problem. The use of an appropriate combination therapy is important. This plant plays vital role in man health, possesses different pharmacological properties and bioactive materials; therefore, it might

participate in various drug productions. Its cultivation is very important.

*U. molliuscula*, *H.tuberculatum,* and *N.sativa* extracts show positive microbial potency. These plants may be considered as important sources for new antimicrobial drugs. Therefore they will contribute to the devolopment of new methods to treat infectous diseases and intestinal disorders caused by some pathogenic strains.*U molliuscula* in folk medicine is used widely for the treatment of different diseases,but scientifically few of them have been invetigated .Thus more scientific research should be harmonized to investigate unutilized activities. Thymoquinone is a phytochemical compound found in the plant *N.sativa; it* was found to be effective against Gram -positive bacteria (Kokoska et al*.,* 2008). Depending on doses the oil showed antibacterial activity against all tested bacteria (Salman et al*.,* 2008). Thymoquinone and Thymohydroquinone may be used for the treatment of infections alone or in combination with some antibiotics, especially in case of highly susceptible Gram (+ve) bacteria *S.aureus* (Halawani, 2009).More and more beneficial effects of *N. sativa* should be explored in order to maximize its utility for effective treatment and curing of various diseases (Naz, 2011).

Methanolic extract of *A. nilotica*, *R. minima, H. abyssinica* and aqueous extracts of *C. phelypaea* did not exhibit antibacterial properties against all tested bacteria except *A. nilotica* against *E. coli* (ATCC 25922) (Tables 1).It is speculated that it may possess anti-inflammatory activities. The antioxidant activity of *H. abyssinica* aqueous extract and *A. nilotica* extracts reported by Mahjoub (2013) revealed that the solvent extracts exhibited strong to modrate antioxidant activity as compared to other plants .Aqueous and methanolic extracts of *R. minima* showed the presence of alkaloids, flavonoids, tannins, trepenoids, glycoside and steroid were absent (Mali and Mahale, 2008). In recent years, the trend towards natural products that are considered as antioxidant, antimicrobial, anti-inflammatory and similar agents is rapidly increasing in the prevention and treatment of diseases. Accordingly, various speculations on natural products could arise and lead to information on pollution (Sevindik et al., 2017).

## Conclusion

The results demonstrate that the extremely active plant was *U. molliuscula*. Methanolic extracts of *U. molliuscula* of all extracts possessed significant antibacterial efficacy. All the plants' aqueous extracts did not clearly show antibacterial activities against all tested bacteria except *H. tuberculatum*. *U. molliuscula* showed high antibacterial activity against *P. aeruginosa, S. aureus, P.mirabilis* and *S.para typhi, B* clinical isolates; also *B. subtilis* (NCTC 8236) showed high sensitivity (1Z = 4mm) to methanolic extracts of *U.molliuscula* . All plants' aqueous extracts were less effective against the bacterial growth of all tested Bacteria except *H. tuberculatum; they* showed high effect against *P.aeruginosa* and moderate effect against *E.coli* clinical isolates.Aqueous extract of *U.molliuscula*, *N. sativa, A. nilotica. R. minima, and H. abyssinica* were found to be ineffective against all tested bacteria. Studied medicinal materials of *U. molliuscula* were satisfactory on the basis of their antibacterial properties. An adequate toxicological testing must be carried out to confirm the capability of using these plants to fight against infectious dieases.

## CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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## REFERENCES

Abdallah EM (2011). Plants: An Alternative Source for Antimicrobials. Journal of Applied Pharmaceutical Science 1(06):16-20.

Abdelgaleil SA, Saad MM, Ariefta NR, Shiono Y (2020). Antimicrobial and Phytotoxic Activities of Secondary Metabolites from *Haplophyllum Tuberculatum* and *Chrysanthemum Coronarium.* South African Journal of Botany 128:35-41.

Abdelkhalek A, Salem MZ, Hafez E, Behiry SI, Qari SH (2020). The Phytochemical, Antifungal, and First Report of the Antiviral Properties of Egyptian *Haplophyllum tuberculatum* Extract, Biology 9:248-265.

Al Alawi SM, Hossain MA, Abusham AA (2018). Antimicrobial and Cytotoxic Comparative Study of Different Extracts of Omani and Sudanese Gum acacia. Beni-Suef University Journal of Basic and Applied Sciences 7(1):22-26.

Al-Burtamani SK, Fatope MO, Marwah RG, Onifade AK, Al-Saidi SH (2005). Chemical Composition, Antibacterial and Antifungal Activities of the Essential Oil of *Haplophyllum Tuberculatum* from Oman. Journal of Ethnopharmacology 96(1):107-112.

Ali A, Akhtar N, Khan BA, Khan MS, Rasul A, UZ-Zaman Sh, Khalid N, Waseem Kh, Mahmood T, Ali L (2012). *Acacia nilotica*: A Plant of Multipurpose Medicinal Uses. Journal of Medicinal Plants Research 6(9):1492-1496.

Ali KS, Salih TA, Daffalla HM (2020). In vitro Phytochemical, Larvicidal and Antimicrobial Activities of Gum Arabic Extract. Walailak Journal of Science and Technology 17(3).

Banjar MM, Khafaji AM, Maher YA (2017). Antimicrobial Activity of Hydrogen Peroxide, Sesame and Gum Arabic against *Streptococcus*. International Journal of Health Sciences and Research 7(1):97-104.

Chaudhry NM, Tariq P (2008). In Vitro Antibacterial Activities of *Kalonji, Cumin* and Poppy Seed. Pakistan Journal of Botany 40(1):461-467.

Cheesbrough M (1984). Culture Media. In: Medical Laboratory Manual for Tropical Countries. Tropical Health Technology and Butterworth-Heineman. Cambridge 3(60-9):407-428.

El-Kamali HH, El-Amir MY (2010). Antibacterial Activity and Phytochemical Screening of Ethanol Extracts Obtained from Selected Sudanese Medicinal Plants. Current research Journal of Biological Sciences 2(2):143-146.

Elkamali HH, Mahjoub SE (2015). Antioxidant potential of Some Sudanese Medicinal Plants used in Traditional Medicine.International Journal of Scientific World 3(2):192-198.

Gundidza M, Gweru N, Magwa ML, Ramalivhana NJ, Humphrey G, Samie A, Mmbengwa V (2009). Phytochemical Composition and Biological Activities of Essential Oil of *Rhynchosia minima* (L) (DC) (*Fabaceae*). African Journal of Biotechnology 8(5):721-724.

Halawani F (2009). Antibacterial Activity of Thymoquinone and Thymohydroquinone of Nigella sativa L. and Their Interaction with Some Antibiotics. Advances in Biological Research 3(5-6):148-152.

Haloci E, Manfredini S, Toska V, Ziosi SV, Topi I, Kolani H (2012).Antibacterial and Antifungal Activity Assesment of *Nigella Sativa* Essential Oils. International Journal of Medical, Health, Biomedical, Bioengineering and Pharmaceutical Engineering 6(6):270-272.

Hamdi A, Viane J, Mahjoub MA, Majouli K, Hussein M, Gad H, Kharbach M, Demeyer K, Marzouk Z, Heyden YV (2018). Polyphenolic Contents, Antioxidant Activities and UPLC–ESI–MS analysis of *Haplophyllum tuberculatum* A. Juss leaves extracts. Macromolecules106:1071-1079.

Hosseinzadeh H, Bazzaz BS, Haghi MM (2007). Antibacterial Activity of Total Extracts and Essential oil of *Nigella Sativa* L. Seeds in Mice. Pharmacolgyonline 2:429-435.

Ingólfsdóttr K (2002) .Usnic acid. Phytochemistry 61(7):729-736.

Ishtikhar M, Rabbani G, Khan Sh, Khan RH (2015). Biophysical Investigation of Thymoquinone Binding to 'N' and 'B' Isoforms of Human Serum Albumin: Exploring the Interaction Mechanism and Radical Scavenging Activity .Journal of Royal Society of chemistry 24. https://pubs.rsc.org/en/content/articlelanding/2014/ra/c4ra09892g/unauth#!divAbstract

Kanter M, Demir H, Karakaya C, Ozbek H (2005). Gastroprotective Activity of *Nigella sativa L* Oil and its Constituent, Thymoquinone against Acute Alcohol-Induced Gastric Mucosal Injury in Rats. World Journal of Gastroenterology 11(42):6662-6666.

Karar MG, Kuhnert N (2017). Herbal Drugs from Sudan: Traditional Uses and Phytoconstituents. Journal of Pharmacognosy Review 11(22):83-103.

Khalid H, Abdalla WE , Abdelgadir H, Opatz T, Efferth Th (2012). Gems from Traditional North-African Medicine: Medicinal and Aromatic Plants from Sudan.Natural Products and Bioprospecting 2:92-103.

Kheir YM (1966). Investigation of Certain Plants used in Sudanese Local Medicine. M.Pharm.Thesis. Khartoum University.

Kokoska L, Havlik J, Valterova I, Sovova H, Sajertova M, Jankovska I (2008). Comparison of Chemical Composition and Antibacterial Activity of *Nigella sativa* Seed Essential Oils Obtained by Different Extraction Methods. Journal of Food Protection 71(12):2475-2480.

Mahjoub SE (2013). Antibacterial and Antioxidant Activities of Some Medicinal Plants Used in Sudanese Traditional Medicine. PHD Thesis. Omdurman Islamic University. Omdurman, Sudan.

Mali RG, Mahale NB (2008). Evaluation of *Rhynchosia minima* (Linn.) DC Leaves for Anthelmintic Activity. International Journal of Pharmaceutical Sciences and Nanotechnology 1(2):191-194.

Mishra RP (2011). Effect of Metal Ions and Drugs on Antibacterial Activities of *Nigella Sativa* (L.) Seeds.Webmed Central Ayurvedic Medicine 2(8):WMC002074.

Monika T, Sasikala P, Bhaskara MV (2013). A Investigational Study of Antibacterial Activities of *Nigella sativa* on Mastits in Dairy Crossbred Cowst. International Journal of Advanced Scientific and Technical Research 4(3):277-283.

Musa MS, Abdelrasool FE, Elsheikh El A, Ahmed LA, Mahmoud AL, Yagi SM (2011). Ethnobotanical Study of Medicinal Plants in the Blue Nile State, South-eastern Sudan. MPR 17:4287-4297.

Naz H (2011). *Nigella sativa***:** the Miraculous Herb.Pak. Journal of Biochemistry and Molecular Biology 44(1):44-48.

Pichette A, Marzouk B, Legault J (2011). Antioxidant, Anti-inflammatoyy, Anticancer and Antibacterial Activities of Extracts form *Nigella sativa* (Black Cumin) Plant Parts. Journal of Food Biochemistry. https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1745-4514.2011.00567.x

Ramadan MF, Kroh LW, Mörsel JT (2003). Radical Scavenging Activity of Black Cumin (Nigella sativa L.),Coriander (Coriandrum sativum L.), and Niger (Guizotia abyssinica Cass.) Crude Seed Oils and Oil Fractions. Journal of Food Chemistry 1-9.

Saadabi AM, Ayoub SM (2009). Comparative Bioactivity of *Hydnora abyssinica* A. Braun against Different Groups of Fungi and Bacteria**.** Journal of Medicinal Plants Research 3(4):262-265.

Sabry OM, El Sayed AM, Sleem AA (2016). Potential Anti-Microbial, Anti-Inflammatory and Anti-Oxidant Activities of Haplophyllum tuberculatum Growing in Libya. Journal of Pharmacognosy 2(1):1000116.

Salman MT, Khan RA, Shukla I (2008). Antimicrobial activity of *Nigella sativa* Linn. Seed oilagainst multi-drug resistant bacteria from clinical isolates. CSIR. http://nopr.niscair.res.in/handle/123456789/5637

Saini ML, Saini R, Roy Sh, Kumar A (2008). Comparative Pharmacognostical and Antimicrobial Studies of Acacia Species (Mimosaceae) Mohan Lal. Journal of Medicinal Plants Research 2(12):378-386.

Sevindik M, Akgul H, Pehlivan M, Selamoglu Z (2017). Determination of Therapeutic Potential of Mentha Longifolia ssp. Longifolia. Fresenius Environmental Bulletin 26(7):4757-4763.

Shehu Z, Lamayi DW, Sabo MA, Shafiu MM (2018). Synthesis, Characterization and Antibacterial Activity of Kaolin/Gum Arabic Nanocomposite on *Escherichia Coli* and *Pseudomonas Aeruginosa.* Research Journal of Nanoscience and Engineering 2(2):23-29.

Sinha SN, Biswas M (2011).Evaluation of Antibacterial Activity of Some Lichen from Angla, Sikkim, India. International Journal of pharma and Bio Sciences 2(4):23-28.

Souza MT, Almeida JR, Araujo AA , Duarte MC, Gelain DP , Moreira JC , Santos MR, Quintans LJ (2014). Structure–Activity Relationship of Terpenes with Anti-Inflammatory Profile – A Systematic Review. Basic and Clinical Pharmacology and Toxicology115:244-256.

Sravani P, Kiranmayee Y, Narasimha MS, Reddy VS, Asha S, Kumar RB (2014). In vitro Experimental Sstudies on Selected Natural Gums and Resins for their Antimicrobial Activity. Research Journal of Pharmaceutical, Biological and Chemical Sciences 5(1):154-172 .

Trojan R, Razdan L, Singh N (2016). Antibiotic Susceptibility Patterns of Bacterial Isolates from Pus Samples in a Tertiary Care Hospital of Punjab, India. International Journal of Microbiology. https://www.hindawi.com/journals/ijmicro/2016/9302692/

Vijayasanthi M, Kannan V, Venkataswamy R, Doss A (2012). Evaluation of the Antibacterial Potential of various solvent extracts of Acacia nilotica linn Leaves. HYGEIA - Journal for Drugs and Medicines 4(1):91-96.

Weckesser S, Engel K, Simon-Haarhaus B, Wittmer A, Pelz K, Schempp CM (2007). Screening of Plant Extracts for Antimicrobial Activity against Bacteria and Yeasts with Dermatological Relevance. Phytomedicine1-9.www.sciencedirect.com.

WHO Publication (2001). WWW. WHO. Int / emc-documents/ antimicrobial-resistance/ docs/ EGlobal-strat. 22-4-2003.

Yoruk O, Gur FO, Uyanik H, Yasar M, Mutlu V, Altas E, Baysal E, Taysi S (2010). Antioxidant Effects of *Nigella Sativa* in the Treatment of Experimentally Induced Rhinosinusitis. Macedonian Journal of Medical Sciences 3(2):132-137.

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*Full Length Research Paper*

# Prevalence and drug-resistance profile of plasmid-borne extended spectrum beta-lactamase (ESBLs) resistance genes in multidrug resistant *Escherichia coli* from HIV-1 positive individuals in Jos, Nigeria

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**Production of extended-spectrum beta-lactamases (ESBLs) can lead to treatment failures when the antibiotics are used. This study detected ESBLs genes on Multidrug Resistant *Escherichia coli* from HIV-infected individuals attending some hospitals in Jos. Eighty (80) isolates of multidrug resistant (MDR) *E. coli* were screened for plasmids. ESBLs genes including *bla*-$_{CTX}$, *bla*-$_{TEM}$ and *bla*-$_{SHV}$ were detected on the plasmids using polymerase chain reaction (PCR) with 100 bp DNA ladder as DNA molecular weight marker. Out of the total 38 plasmids tested, ESBLs genes occurred in 13 (34.21%), with the *bla*-$_{TEM}$ dominating [7 (53.85%)] over the *bla*-$_{CTX}$ [4 (30.77%)]. Two (15.38%) of the isolates carried both genes. None of the isolates carried the *bla*-SHV gene in our study. All the strains showed resistance to SXT, AMC and CTX. Resistance was most frequently observed against SXT (13), AMC (13), CTX (13), CFM (12), F (8), NA (6), GN (5), CRO (4), OFX (2) and CIP (1). None of the ESBLs-bearing strains showed resistance to IPM. Result showed 34.21% prevalence of ESBLs and suggested the need to be more cautious with the clinical use of third generation Cephalosporins especially for the treatment of acute infections caused by *E. coli* due to the high resistance recorded.**

**Key words:** Extended spectrum beta-lactamase, resistance genes, antimicrobial, plasmids, *Escherichia coli,* HIV-infected, Jos.

## INTRODUCTION

The infections caused by antibiotic resistant microorganisms, may be very difficult to treat due to limited choices of antibiotics. Extended-Spectrum-Beta-Lactamase antibiotics such as third generation cephalosporin (3GC) form the major component of the empiric antibacterial chemotherapy in most clinical setups

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and especially in tertiary care center (Chaudary and Aggarwal, 2004).

Beta-lactamases are enzymes that are major cause of bacterial resistance to the beta-lactam family of antibiotics such as penicillins, cephalosporins, cephamycins, and carbapenems. They catalyze the hydrolysis of the amide bond of four-membered beta-lactam ring and render the antibiotic inactive against its original cellular target, the cell wall transpeptidase. Extended-spectrum beta-lactamases (ESBLs) are derived from the narrow-spectrum beta-lactamases (TEM-1, TEM-2, or SHV-1) by mutations that alter the amino acid configuration around the enzyme active site (Bajpai et al., 2017). They mediate resistance to all penicillins, third generation cephalosporins (e.g. ceftazidime, cefotaxime, and ceftriaxone) and aztreonam, but not to cephamycins (cefoxitin and cefotetan) and carbapenems (Bonnet, 2004).

ESBLs are plasmid-mediated and organisms producing beta-lactamase enzymes exhibit co-resistance to many other classes of antibiotics (Kruse and Sørum, 1994). These enzymes are most commonly produced by the members of the Enterobacteriaceae, especially *Escherichia coli* and *Klebsiella* (Chika et al., 2017). Gram negative Enterobacteriaceae expressing ESBLs are among the most multidrug-resistant pathogens in hospitals and are spreading worldwide. The infections caused by ESBLs-producing organisms have resulted in poor prognosis, prolonged hospital stay and greater hospital expenses (Paterson et al., 2004).

Available literature has demonstrated a risen prevalence of multidrug resistant ESBL-producing *E. coli* globally (Hassuna et al., 2020; Abdulaziz et al., 2018; Falgenhauer et al., 2019). Our study is predicated on the paucity of data from the West African sub-region on ESBLs resistant genes in HIV-infected individuals. Most available reports (Aibinu et al., 2003, 2004; Iroha et al., 2010; Yusuf et al., 2011; Oli et al., 2017) focused on phenotypic detection of ESBLs in HIV-non-infected populations. Such reports have been shown to be inconsistent in revealing the actual prevalence of ESBLs genes in the region, as also earlier advocated by Founou et al. (2018) and Bajpai et al. (2017). Accurate epidemiologic data can enable effective empirical therapy plan and infection control program.

## MATERIALS AND METHODS

### Study area, design and period

The antibiotic resistance and plasmids profile of eighty *E. coli* isolates obtained from HIV-infected individuals attending Bingham University Teaching Hospital and Faith Alive Foundation Hospital Jos, were determined. Identity of the isolates was confirmed using MicrobactTM Gram-Negative Identification System (24E) kits, while ESBLs genes were detected using PCR. This is part of a cross-sectional study conducted between February 2018 and December, 2019. The consenting participants were enrolled and their stool samples screened for *E. coli*. Both prevalence and susceptibility studies of the isolates have been earlier reported. Only isolates showing multidrug resistance were used in this study.

### Ethics approval and consent to participate

This research was ethically cleared and approved by the Jos University Teaching Hospital Institutional Review Board. Written and informed consent was obtained from study participants after explaining the purpose and aim of the study.

### Consent to publish

Informed consent was obtained from all individual participants included in the study.

### Antibiotic susceptibility test

Antibiotic susceptibility was assessed using the disc diffusion method of Bauer et al. (1996) and further described by CLSI (2013). Antibiotics discs used include Imipinem (IPM) 10 µg, Trimethoprim-Sulfamethoxazole (SXT) 25 µg, Gentamycin (GN) 10 µg, AmoxicilinClavulanic acid (AMC) 30 µg, Nitrofurantoin (F) 200 µg, Cefotaxime (CTX) 30 µg, Nalidixic acid (NA) 30 µg, Ofloxacin (OFX) 5 µg, Ceftriazone(CRO) 30 µg, Cefixime (CFM) 5 µg, and Ciprofloxacin (CIP) 5 µg from Oxoid (UK). A cell suspension of organisms equivalent to a 0.5 McFarland standard was used for the susceptibility testing. Tests were standardized using *E. coli* 25922 from the American Type Culture Collection (ATCC) as reference strain. Clear zones of inhibition were measured in mm using a transparent metre rule.

### Plasmids DNA extraction and profiling

Plasmids DNA were extracted using the Plasmid extraction protocol with Zippy plasmid Miniprep kit (Inqaba biotech West Africa Ltd) as follows.

Six hundred microliters of bacterial culture grown in LB medium was added to a 1.5 ml microcentrifuge tube. This was centrifuged for 30 s at 12000 rpm. The supernatant was discarded and the procedure repeated to get a clear pellet. The cell pellet was then resuspended after adding 600 µl of TE to it. This was followed by the addition of 100 µl of 7X lysing buffer. The mixture was inverted for about 4 to 6 times in the tube and then incubated for 1 to 2 min. It was then mixed with 350 µl of cold neutralization buffer thoroughly before centrifuging for 2 to 4 min at 12 rpm. The supernatant was transferred into a Zymo-Spin IIN column placed in a collection tube. This was centrifuged for 15 s for 12000 rpm. The flow-through was discarded and the Zymo-Spin IIN column returned to the collection same tube. 200 µl of the Endo-Wash Buffer was added to the column and centrifuged for 30 s at 12000 rpm. The Zippy Wash Buffer (400 µl) was then added to the column and centrifuged for 1 min at 12000 rpm. The column was then transferred into a sterile RNAse/DNAse-free 1.5 ml microcentrifuge tube. The column was then transferred into a clean 1.5 ml microcentrifuge tube where 30 µl of zippy elution buffer was directly added to the column matrix and incubated for 1 min at room temperature. The whole mixture was finally centrifuged for 30 s at 12 rpm to elute the plasmid DNA.

Plasmids were characterized using agarose gel electrophoresis (Sambrook and Fritsch, 1989) and DNA-Hind 111 Digest as DNA ladder to identify the plasmid copies present in different isolates. For this purpose, an agarose gel of 0.8% was used, while ethidium bromide was used for staining of DNA fragments, which were visualized by UV-Trans illumination. Samples with visible bands

**Table 1.** The primers used for the PCR.

| S/N | Primer name | Sequence ($5^1$-$3^1$) | Base Pair | Annealing temperature (°C) |
|---|---|---|---|---|
| 1 | *bla*$_{SHV}$ F | TGGTTATGCGTTATATTCGCC | 868 | 58 |
| | *bla*$_{SHV}$ R | GGTTAGCGTTGCCAGTGCT | | |
| 2 | *bla*$_{TEM}$ F | TCCGTCATGAGACAATAACC | 972 | 56 |
| | *bla*$_{TEM}$ R | TTGGTCTGACAGTTACCAATGC | | |
| 3 | *bla*$_{CTX}$-M1 | ATGTGCACCAGTAARGT | 593 | 56 |
| | *bla*$_{CTX}$-M2 | TGGGTRAARTARGTSACCAGA | | |

were cut from the gel and purified using the Zymoclean Large Fragment DNA Recovery Kit. Molecular weights of plasmids were calculated using molecular weight calculator by Bikandi et al. (2004) at Insilico.ehu.es.

#### PCR amplification

ESBLs belonging to the CTX, TEM, and SHV families primers (*Bla*-CTX, TEM and SHV) were used for amplification of resistance genes using PCR. The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 20 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates (dNTP) (Solis Biodyne), 20 pMol of each primer (Jena Bioscience, Germany), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5 µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in an Pielter thermal cycler (MJ Research Series) for an initial denaturation of 95°C for 5 min followed by 30 amplification cycles of 30 s at 95°C; 1 min at 56°C and 1 min 30 s at 72°C. This was followed by a final extension step of 10 min at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 h 30 min. After electrophoresis, DNA bands were visualized by ethidium bromide staining using 100 bp DNA ladder (Solis Biodyne) as DNA molecular weight marker. Table 1 shows primers used in the present study.

#### Data processing and analysis

Data were entered into excel sheets and analyzed using SPSS version 2010 software. The correlation analysis was used to determine possible relationship between plasmid copies and number of antibiotics resisted. The 95% confidence limit and probability (P value) of 0.05 were used to determine level of significance of associations.

## RESULTS

Antibiotic resistance of isolates as shown in Figure 1, revealed that SXT had the highest resistance level of 98.75% (79 isolates), while IPM was the least (3.75%). In all, CTX (86.3%) and CFX (83.00%) were the least effective among the cephalosphorins, while there was minimal resistance to the aminoglyosides (OFX=23% and CIP=22.5%). Also, isolates showed 67.5 and 66.3% resistance to NA and F, respectively.

Thirty-eight/eighty (47.50%) of the isolates had plasmids of various sizes ranging from 1.029 to 23.485 kbp. The most frequent plasmid having a molecular weight of 12.371 kbp occurred in 11 of the isolates, three of which also bear the ESBLs genes. Plasmid copies varied from 1 to 7, with those bearing 3 plasmids having the highest frequency (31.58%), while those harboring 7 occurred the least (5.26%) (Figure 2). No significant correlation exist between plasmid copies and number of antibiotics resisted (r= 0.295, P= 0.072).

The PCR results to detect ESBLs belonging to the TEM, SHV and CTX-M families genes are as shown in Figure 3. It revealed that ESBLs genes occurred in 13/38 (34.21%) of the plasmids, with the *bla*-TEM dominating [7/13 (53.85%)] over the *bla*-$_{CTX}$ [4/13 (30.77%)]. Two (15.38%) of the isolates however, carried both *bla*-$_{TEM}$ and *bla*-$_{CTX}$ genes. None of the isolates in our study carried the *bla*-$_{SHV}$ gene. Both *bla*-$_{TEM}$ and *bla*-$_{CTX}$ were detected on the most frequently encountered plasmid in our studies.

Table 2 shows the resistance pattern of ESBLs genes-bearing *E. coli* in this study. They were generally resistant to 5 antibiotics and above. Isolates harboring both *bla*-$_{CTX}$ and *bla*-$_{TEM}$ genes exhibited resistance to more than 8 antibiotics compared to other isolates harboring either of the 2 genes alone. All the strains showed absolute (100%) resistance to SXT, AMC and CTX. Resistance was most frequently observed against SXT 13, AMC 13, CTX 13, CFM 12, F 8, NA 6, GN 5, CRO 4, OFX 2, and CIP 1. Furthermore, resistance to GN and CRO is associated with the *bla*-$_{TEM}$ gene, as none of the *bla*-$_{CTX}$ genes-bearing strains was resistant to the drugs as shown in the table. None of the strains bearing ESBLs genes showed resistance to IPM in this study.

## DISCUSSION

High resistance to SXT and cephalosphorins exhibited by *E. coli* in our study is quite significant since these drugs

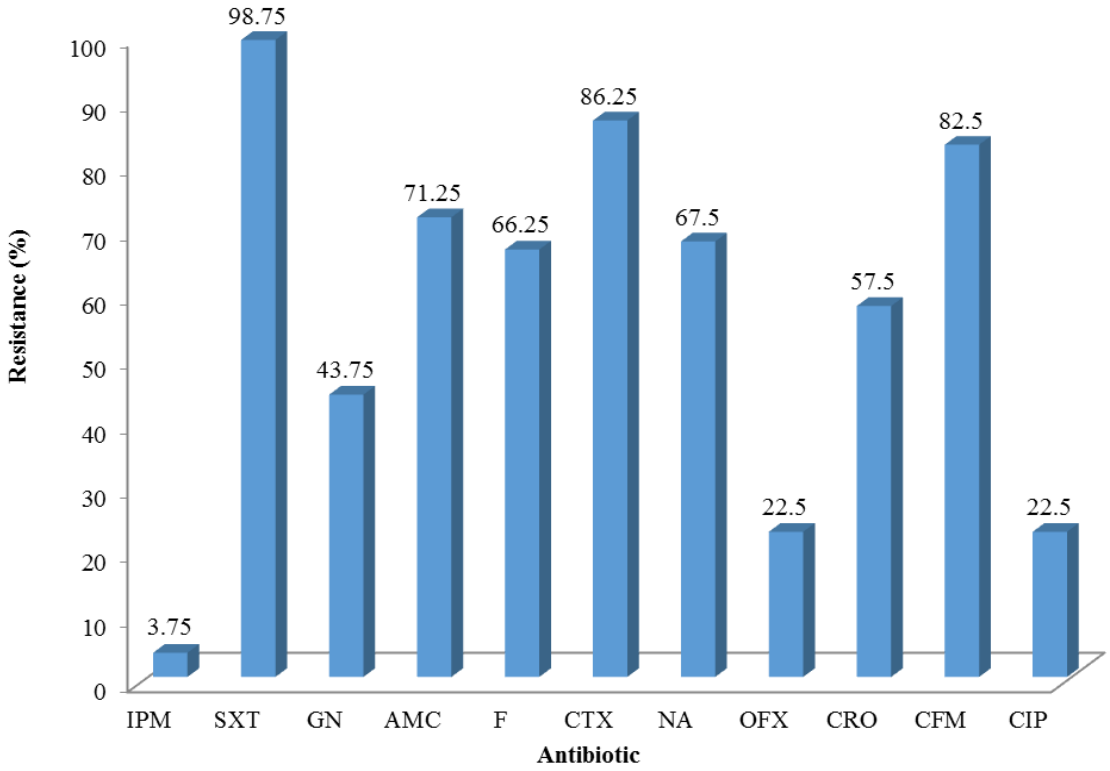


**Figure 1.** Antibiotic resistance profile of *Escherichia coli* from HIV-infected individuals in Jos Nigeria. IPM=Imipinem (10 µg), SXT = Trimethoprim-Sulfamethoxazole (25 µl), GN = Gentamycin (10 µg), AMC = AmoxicilinClavulanic acid (30 µg), F =Nitrofurantoin (200 µg), CTX = Cefotaxime (30 µg), NA = Nalidixic acid (30 µg), OFX = Ofloxacin (5 µg), CRO = Ceftriazone (30 µg), CFM = Cefixime (5 µg), and CIP = Ciprofloxacin (5 µg).

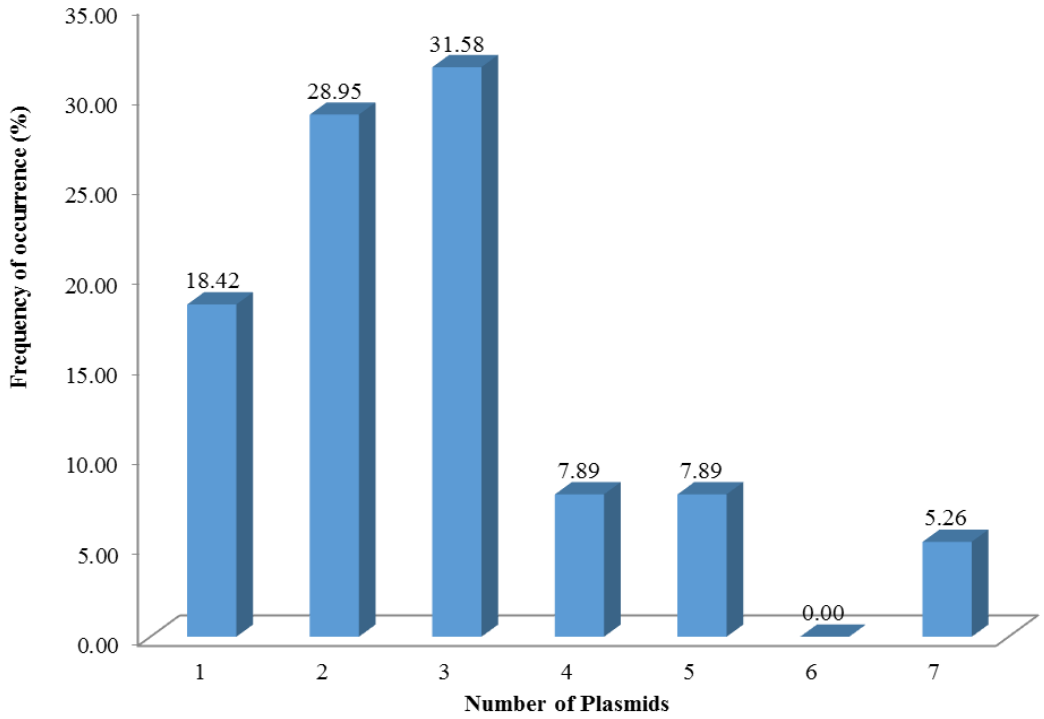


**Figure 2.** Frequency (%) of occurrence of plasmids on isolates (r= 0.295, P= 0.072).

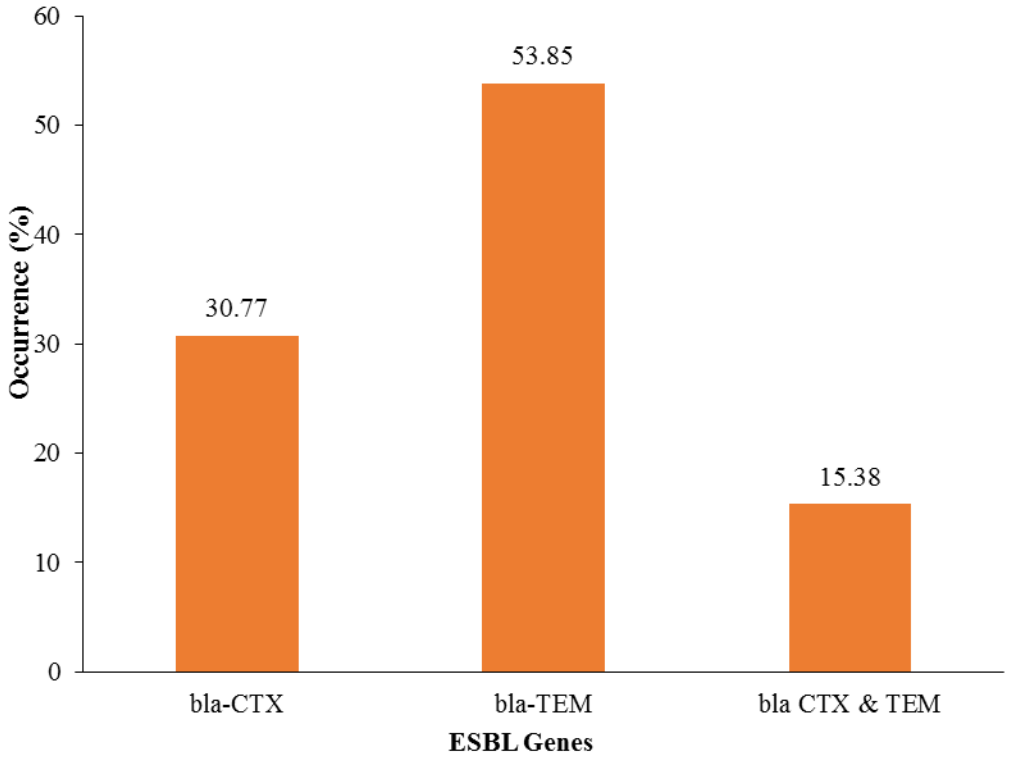


**Figure 3.** Occurrence of ESBL resistance genes in *E. coli* from HIV-infected individuals in Jos.

**Table 2.** Antibiotic resistant pattern of ESBLs genes-bearing *E. coli* from HIV-infected Individuals in Jos.

| ESBL gene | Antibiotic Resistance Pattern | | | | | | | | | Number of antibiotics resisted |
|---|---|---|---|---|---|---|---|---|---|---|
| *bla*-$_{TEM\& CTX}$ | SXT | AMC | F | CTX | NA | OFX | CRO | CFM | CIP | 9 |
| | SXT | GN | AMC | CTX | NA | | OFX | CRO | CFM | 8 |
| *bla*-$_{TEM}$ | SXT | AMC | F | CTX | CFM | | | | | 5 |
| *bla*-$_{TEM}$ | SXT | GN | AMC | F | CTX | | CFM | | | 6 |
| *bla*-$_{TEM}$ | SXT | GN | AMC | CTX | NA | | | | | 5 |
| *bla*-$_{TEM}$ | SXT | AMC | CTX | NA | CRO | | CFM | | | 6 |
| *bla*-$_{TEM}$ | SXT | AMC | F | CTX | CFM | | | | | 5 |
| *bla*-$_{TEM}$ | SXT | GN | AMC | F | CTX | | CFM | | | 6 |
| *bla*-$_{TEM}$ | SXT | GN | AMC | F | CTX | | CRO | CFM | | 7 |
| *bla*-$_{CTX}$ | SXT | AMC | CTX | NA | CFM | | | | | 5 |
| *bla*-$_{CTX}$ | SXT | AMC | CTX | NA | CFM | | | | | 5 |
| *bla*-$_{CTX}$ | SXT | AMC | CTX | NA | CFM | | | | | 5 |
| *bla*-$_{CTX}$ | SXT | AMC | F | CTX | CFM | | | | | 5 |

IPM=Imipinem (10 µg), SXT =Trimethoprim-Sulfamethoxazole (25 µ), GN= Gentamycin (10 µg), AMC = AmoxicilinClavulanic acid (30 µg), F =Nitrofurantoin (200 µg), CTX = Cefotaxime (30 µg), NA = Nalidixic acid (30 µg), OFX = Ofloxacin (5 µg), CRO = Ceftriazone (30 µg), CFM = Cefixime (5 µg), and CIP = Ciprofloxacin (5 µg).

are among the most widely used in the treatment of infectious diseases. Persistent exposure of bacterial strains to a multitude of β-lactams may have induced a selective pressure in favour of the resistant strains having eliminated the sensitive strains in the process. This could expand the activity of the resistant strains, even against newly developed β-lactam antibiotics (Shaikh et al., 2015). Earlier reports (Coudron et al., 1997; Piroth et al.,

1998) have suggested the use of β-lactam antibiotics (including CTX, CFM, CRO, etc.) in combination with AMC to be used in the treatment of resistant bacterial infections. This must be done with caution in the light of our findings. Our result revealed the increasing resistance of *E. coli* to antimicrobials in the region and portends great danger to the treatment of infectious diseases since *E. coli* could transfer resistant genes to enterobacterial pathogens and other normal flora in the body. Our study agrees with that of Igwe et al. (2016), but differ from that of Adenipekun et al. (2016) and Aworh et al. (2019), who reported lower resistance to 3rd and 4th generation cephalosporins in the South-Western and North-Central part of the country, respectively.

The present study showed that most of the plasmids isolated are of smaller to medium sizes (1.029-23.485 kbp), suggesting that majority may be of the mobilizable category (Usually ≤10 kbp). This is further supported by the fact that the most frequent plasmid having a molecular weight of 12.371 kbp occurred in 11 (28.95%) of the isolates, three of which also bear the ESBLs genes.

The present study did not show any significant correlation between plasmid copies and number of antibiotics resisted (r= 0.295, P= 0.072). This is expected as there are isolates resisting fewer (1-3) antibiotics but bearing up to 7 plasmids, just as there were also those resisting 9 antibiotics and bearing up to 7 plasmids, some of which also contain resistant genes. The former could indicate that most of those plasmids are either non-resistant or mobilizable in nature. Such resistance could as well be on the chromosomes as previously suggested (Aibinu et al., 2003).

Strains bearing ESBLs genes in this study were generally resistant to 5 antibiotics and above (Table 1). However, isolates habouring both *bla*-$_{CTX}$ and *bla*-$_{TEM}$ genes exhibited resistance to more (Aibinu et al., 2004; Iroha et al., 2010) antibiotics compared to other isolates habouring either of the 2 genes alone. This could be due to the multiplied effect of the genes on such isolates.

The prevalence of ESBLs genes in our study is high (34.21%). This portends serious risk of resistance to treatment of infections with antibiotics, as earlier reports have shown that organisms producing beta-lactamase enzymes exhibit co-resistance to many other classes of antibiotics (Kruse and Sørum, 1994), leading to highly limited available drugs for the treatment of infectious diseases. The prevalence in our study is however lower than the 56.7, 70.0, 72, 83.0 and 95.5% reported by Irith et al. (2007), Igwe et al. (2016), Horsefall et al. (2017) Husam et al. (2009) and Wani et al. (2009), respectively. It is however higher than the 18.6% earlier reported by Onyedibe et al. (2018) in Jos. This difference may be attributed to the use of molecular technique in our study as against phenotypic detection in theirs, since the latter is known to be less sensitive compared to the former. Our result is very similar to the 33 and 33.5% earlier reported from Saudi Arabia and Egypt by Abdulaziz et al. (2018) and Hassuna et al. (2020), respectively.

Previous workers (Saravanan et al., 2018; Abdulaziz et al., 2018; Hassuna et al., 2020), have also reported higher prevalence of bla-$_{CTX}$ gene in the middle-East and Africa, but our study showed the *bla*-$_{TEM}$ gene dominating over the *bla*-$_{CTX}$ gene with the *bla*-$_{SHV}$ being completely absent. This may be due to the fact that the *bla*-$_{TEM}$ gene was originally known to be associated with *E. coli* where it was originally isolated. Our result however agrees with that of Olugbenga et al. (2015) and Aibinu et al. (2003), who reported higher prevalence of the *bla*-$_{TEM}$ from Osun and Lagos states respectively, in Nigeria. This suggests an increasing regional spread of the resistant genes. This suggests an increasing spread of the *bla*-$_{TEM}$ gene in Nigeria possibly due to human and animal migration.

High resistance of plasmid-borne ESBL positive isolates to 3rd generation Cephalosporins (CTX 100% and CFM 92.31%, AMC 100%) recorded in our study is expected. These are beta lactams, and Beta-lactamases are known to hydrolyze the amide bond of the β-lactam ring resulting in an inactive compound (Bajpai et al., 2017).

The ESBLs resistant isolates showed the least resistance to the quinolones (CIP 1 or 7.70% and OFX 15.39%) in our study, even as all were sensitive to IPM. Our result differs from that of Igwe et al. (2016) from Zaria, in North-Western Nigeria, where higher levels of resistance were reported against the quinolones (CIP 76.55% and OFX 74.5%). Similar to our findings however, their study and that of Aibinu et al. (2003) also reported no resistance to IPM. This indicates that IPM is still a choice drug for the treatment of MDR bacteria in the region. Nevertheless, other similar studies (Onyedibe et al., 2018, Adesola et al., 2020) reported varying levels of resistance (18.6 and 20.6%, respectively) to the drug. This suggests the need for close monitoring, so as to track and control the spread of resistance.

The results suggest that we could experience high levels of clinical failure when using third generation cephalosporins for the treatment of acute infections caused by *E. coli*. These findings are of concern as *E. coli* are among the most frequent causes of intra-abdominal, soft tissues and community-acquired urinary tract infections word-wide.

## CONCLUSION

Most of the plasmids isolated are of small to medium sizes (1.029-23.485 kbp) and therefore mostly mobilizable. Beta lactamase resistance genes (*bla*-$_{CTX}$ and *bla*-$_{TEM}$) were prevalent (34.21%) in the region. All ESBLs-borne isolates showed absolute resistance to SXT and 100% susceptibility to IPM. The result suggested the need to be more cautious with the clinical use of third generation cephalosporins especially for the

treatment of acute infections caused by *E. coli* as resistance to the drugs by plasmid-borne ESBLs positive isolates was high in the study.

### The limitation and strength of the study

The study was done in hospital setting so that the result may not be representative samples of other patients attending other health sectors in the same area. The number of isolates was small and this may affect the estimation of the prevalence of ESβL producing strains in the study area.

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## CONFLICT OF INTERESTS

The authors have declared any conflict of interests.

### REFERENCES

Abdulaziz A, Ahmad AJ, Abdullah AA (2018). Prevalence of Multidrug Resistance and Extended-Spectrum *β*-Lactamase Carriage of Clinical Uropathogenic *Escherichia coli* Isolates in Riyadh, Saudi Arabia. International Journal of Microbiology pp. 1-9.

Adenipekun EO, Jackson CR, Ramadan H, Iwalokun BA, Oyedeji KS, Frye JG, Barrett JB, Hiott LM, Woodley TA, Oluwadun A (2016). Prevalence and multidrug resistance of Escherichia coli from community-acquired infections in Lagos, Nigeria. Journal of Infection in Developing Countries 10(9):20-931.

Adesola O, Onwugamba F, Iwalokun B, Mellmann A, Becker K, Schaumburg F (2020). High proportion of carbapenemase-producing *Escherichia coli* and *Klebsiellapneumoniae* among extended-spectrum β-lactamase-producers in Nigerian hospitals. Journal of Global Antimicrobial Resistance 21:8-12.

Aibinu I, Aednipekun E, Odugbemi T (2004). Emergence of quinolone resistance amongst *Escherichia coli* strains isolated from clinical infections in some Lagos state hospitals, in Nigeria. Nigerian Journal of Health and Biomedical Sciences 3(2):73-78.

Aibinu IE, Ohaegbulam VC, Ademipekun EO, Ogunsola FT, Odugbemi TO, Mee BJ, (2003). Extended-spectrum beta-lactamase enzymes in clinical isolates of *Enterobacter* species from Lagos, Nigeria. Nigerian Journal of Health and Biomedical Sciences 2:53-60.

Aworh MK, Kwaga J, Okolocha E, Mba N, Thakur S (2019). Prevalence and risk factors for multi-drug resistant Escherichia coli among poultry workers in the Federal Capital Territory, Abuja, Nigeria. PLoS ONE 14(11):e0225379.

Bajpai T, Pandey M, Varma M, Bhatambare GS (2017). Prevalence of TEM, SHV, and CTX-M Beta-Lactamase genes in the urinary isolates of a tertiary care hospital. Avicenna Journal of Medicine 7(1):12-16.

Bauer AW, Kirby WMM, Sherris JKC, Turk MN (1996). Antibiotic susceptibility testing by a standardized single disc method. American Journal of Clinical Pathology 45(4):413-496.

Bikandi J, San Millán R, Rementeria A, Garaizar J (2004). In silico analysis of complete bacterial genomes: PCR, AFLP–PCR and endonuclease restriction.*Bioinformatics* Application Note 20(5):798-799.

Bonnet RG (2004). Group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrobial Agents and Chemotherapy 48(1):1-14.

Chaudary U, Aggarwal R (2004). Extended spectrum beta lactamases; AN emerging threat to clinical therapeutics. Indian Journal of Medical Microbiology 22(2):75-80.

Chika E, Ifeanyichukwu I, Clement OA, Malachy U, Peter E, Iroha CS, Ogene L, Chinedu O (2017). Multiple Antibiotic Resistance, Antibiogram and Phenotypic Detection of Metallo-Beta- Lactamase (MBL) from *Escherichia coli* of Poultry Origin. Journal of Applied Microbiology and Biochemistry 1(4):15-20.

Clinical Laboratory Standard Institute (CLSI) (2013). Performance Standard for Antimicrobial Disk and Dilution Susceptibility Test for Bacteria Isolated from Animals; Approved Standard - Third edition. CLSI document M31-3A, Wayne, PA: C L SI 24 p.

Coudron PE, Moland ES, Sanders CC (1997). Occurrence and Detection of Extended-Spectrum b-Lactamases in Members of the Family Enterobacteriaceae at a Veterans Medical Center. Journal of Clinical Microbiology 35(10):2593-2597.

Falgenhauer L, Imirzalioglu C, Oppong K, Akenten CW, Hogan B, Krumkamp R, Poppert S, Levermann V, Schwengers O, Sarpong N, Owusu-Dabo E, May J, Eibach D (2019). Detection and Characterization of ESBL-Producing Escherichia coli From Humans and Poultry in Ghana . Frontiers in Microbiology 9:3358.

Founou LL, Founou RC, Allam M, Ismail A, Djoko CF, Essack SY (2018). Genome Sequencing of Extended-Spectrum β-Lactamase (ESBL)-Producing *Klebsiellapneumoniae* Isolated from Pigs and Abattoir Workers in Cameroon. Frontiers in Microbiology 9:188-199.

Hassuna NA, Khairalla AS, Farahat EM, Hammad AM, Abdel-Fattah M (2020). Molecular characterization of Extended-spectrum β lactamase- producing *E. coli* recovered from community-acquired urinary tract infections in Upper Egypt. Scientific Report 10:2772.

Horsefall SJ, Abbey SD, Nwokah E, Okonko IO (2017). Prevalence of Extended-Spectrum Beta-lactamases (ESBLs) and Plasmid status of *Escherichia coli* and *Klebsiellapneumoniae* isolates from clinical sources in UPTH, Port-Harcourt, Nigeria. New York Scientific Journal 10(3):29-39.

Husam SK, Khalid MB, Abiola CS, Giuseppe AB (2009). Extended spectrum beta-lactamases (ESBL) in Escherichia coli and Klebsiellapneumoniae: trends in the hospital and community settings. Journal of Infections in Developing Countries 3:295-299.

Igwe JC, Olayinka BO, Ehnimidu JO, Onaolapo JA (2016) Virulent Characteristics of Multidrug Resistant *E. coli* from Zaria, Nigeria. Clinical Microbiology 5(6):268.

Irith W, Heinrich KG, Dietrich M, Enno S, Harald S (2007). Detection of Extended-Spectrum Beta-Lactamases among Enterobacteriaceae by Use of Semi-automated Microbiology Systems and Manual Detection Procedures. Journal of Clinical Microbiology 45:1167-1174.

Iroha IR, Amadi ES, Oji AE, Nwuzo AC, Ejike-Ugwu PC (2010). Detection of Plasmid Borne Extended Spectrum Beta-Lactamse Enzymes from Blood and Urine isolates of Gram-negative Bacteria from a University Teaching Hospital in Nigeria. Current Research in Bacteriology 3:77-83.

Kruse H, Sørum H (1994). Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Applied and Environmental Microbiology 60(11):4015-4021.

Oli AN, Eze DE, Gugu TH, Ezeobi I, Maduagwu, UN, Ihekwereme CP (2017). Multi-antibiotic resistant extended-spectrum beta-lactamase producing bacteria pose a challenge to the effective treatment of wound and skin infections. Pan African Medical Journal 27:66-77.

Olugbenga OA, Adewumi O, Odewale G, Ojurongbe O, Adefioye OJ (2015). Phenotypic and Molecular Characterisation of Extended-Spectrum Beta-Lactamase Producing Escherichia coli Obtained from Animal Fecal Samples in Ado Ekiti, Nigeria. Journal of Environmental and Public Health 7 p.

Onyedibe KI, Shobowale EO, Okolo MO, Iroezindu MO, Afolaranmi TO, Nwaokorie FO, Opajobi SO, Isa SE, Egah DZ (2018). Low

Prevalence of Carbapenem Resistance in Clinical Isolates of Extended Spectrum Beta Lactamase (ESBL) Producing Escherichia coli in North Central, Nigeria. Advances in Infectious Diseases 8:109-120.

Paterson DL, Ko WC, Von Gottberg A, Mohapatra S, Casellas M, Goossens H, Mulazimoglu L, Trenholme G, Klugman KP, Bonomo RA, Rice LB, Wagener MM, McCormack JG (2004). Antibiotic therapy for *Klebsiellapneumoniae* bacteremia: implications of production of extended-spectrum beta-lactamases. Clinical and Infectious Disease 39(1):31-37.

Piroth L, Aube H, Doise JM, Martin MV (1998). Spread of ESBL-producing K. pneumoniae: are β-lactamase inhibitors of therapeutic value?. Clinical and Infectious Disease 27:76-80.

Sambrook J, Fritsch EF (1989). Maniatis T.Isolation of high molecular weight DNA from mammalian cells. Molecular Cloning, a Laboratory Manual 1:9-14.

Saravanan M, Ramachandran B, Barabadi H (2018). The prevalence and drug resistance pattern of extended spectrum β-lactamases (ESBLs) producing Enterobacteriaceae in Africa. Microbial Pathogens 114:180-192.

Shaikh S, Fatima J, Shakil S, Rizvi SM, Kamal MA (2015). Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. Saudi Journal of Biological Sciences 22(1):90-101.

Wani KA, Thakur MA, Siraj FA, Fomdia B, Gulnaz B, Maroof P (2009). Extended Spectrum B-Lactamase Mediated Resistance in Escherichia coli in a Tertiary Care Hospital. International Journal of Health Sciences 3:155-163.

Yusuf I, Arzai AH, Umar A, Magaji N, Salisu N, Tukur A, Hamid KM, Haruna M (2011). Prevalence of Extended Spectrum β-Lactamases (ESBL) producing *Escherichia coli.* Bayero Journal of Pure and Applied Sciences 4(2):182-185.

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# Resistance of *Candida albicans* to antifungal drugs in Abidjan (Cote d'Ivoire)

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**This study aimed to evaluate the resistance levels of strains of *Candida albicans* to the antifungals commonly used in Abidjan, Cote d'Ivoire. This is a prospective study that was carried out from July to October 2017 at the mycology laboratory of the Institut Pasteur of Cote d'Ivoire. 105 *Candida* isolates, obtained from swabs taken from patients receiving out-patient treatment, were seeded on chromogenic medium. Identification of *Candida* species was carried out by MALDI-TOF mass spectrometry (Vitek MS bioMérieux). The susceptibility of *C. albicans* strains to 5-fluorocytosine, amphotericin B, fluconazole, itraconazole and voriconazole was evaluated using the microdilution technique in a semi-solid medium to determine the minimum inhibitory concentration with the ATB1 Fungus 3 kit. Out of 105 *Candida* strains, 68 (64.8%), including *C. albicans*, were identified on the chromogenic medium and confirmed by MALDI-TOF spectrometry. These *C. albicans* strains exhibited varying levels of resistance to the antifungals tested: 1.5% for 5-fluorocytosine, 26.3% for fluconazole, 39.7% for itraconazole, 27.9% for voriconazole. No resistance to amphotericin B was observed. *C. albicans* strains taken from ear pus swabs exhibited greater resistance (*P* = 0.0113). *C. albicans* is developing increasing resistance to common antifungals, hence the need for regular surveillance in resource-poor countries.**

**Key words:** Candidiasis, *Candida albicans*, resistance, mycosis, antifungual drug.

## INTRODUCTION

*Candida albicans* is a yeast that forms part of the commensal flora of healthy individuals. However, when the host-parasite equilibrium is disrupted, the yeast becomes opportunistic and colonises the skin and mucous surfaces in humans and many animal species. In humans, this yeast poses a serious health threat, especially in patients with immune deficiency or undergoing immunosuppressive therapies. It is implicated in more than 80% of yeast infections (Gonsu et al., 2014). Its varied clinical spectrum ranges from superficial infections, in particular of the respiratory, digestive and genital mucosa, to deep (pulmonary mycosis) and

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disseminated (septicaemic mycosis) infections (Badillet et al., 1987).Traditional identification requires yeasts to be grown in biochemical test galleries or on chromogenic media and necessitates an incubation period of between 24 and 72 h (Bernal et al., 1996).

Unlike these so-called classical methods, matrix-assisted laser desorption ionization- time of flight (MALDI-TOF) mass spectrometry directly analyses the various bacterial macromolecules, especially proteins, and therefore yields results more quickly (Lindsay et al., 2010). It meets the need for precise, rapid diagnosis to deal more effectively with candidiasis.

Indeed, it is admitted that antifungal drugs are classified in five groups: (i) Antifungals that affect ergosterol. (ii) Antifungals acting on the fungal cell wall, (iii) Nucleic acid inhibitors, (iv) Mitosis inhibitors and (v) Protein synthase inhibitors. Four types of antifungals are currently used to treat fungal infections (5-fluorocytosine, polyenes, azoles and echinocandins). In limited resource countries, echinocandins, a recent class, is not yet available (Bounouman-Ira et al., 2011). Management of *Candida* infections often runs into a number of issues, including the small number of effective antifungal drugs, the toxicity of available antifungals, *Candida* resistance to common antifungals, recurrence of *Candida* infections, as well as the high cost of antifungal drugs (Khan et al., 2003, Klepser 2001). In addition this fact, in Cote d'Ivoire, antifungals are most often prescribed before susceptibility of the pathogenic fungi to the antifungals has been determined. The aim of this study was to determine the resistance profile of *C. albicans* strains isolated in Abidjan.

## MATERIALS and METHODS

### Patients

This is a prospective study carried out in the mycology laboratory in Institut Pasteur of Cote d'Ivoire (Cocody and Adopodoumé sites) from July to October 2017, on *Candida* isolates obtained from swabs taken from patients receiving out-patient treatment. *Candida* isolates came mainly from vaginal exudates, oropharyngeal and sperm swabs. The other swabs were taken from ear pus, sputum and stools.

### Culture on chromogenic media

The isolates were cultured on chromogenic media (*Candida* Chromogenic agar, Condo S.A.Madrid, Spain), which allowed rapid identification of *Candida* species using the quadrant technique. After seeding, incubation was at 37°C for 24 to 48 h. Colonies were identified on the basis of their colour: *C. albicans* produces pale green colonies, *C. tropicalis* are blue-green, *C. krusei* are pink, and other species are white-pink.

After identification of the *Candida* species, confirmation of the results was sought with MALDI-TOF mass spectrometry (Vitek MS BioMerieux, France) following manufacturer's instructions. A colony of the calibration strain, Escherichia coli ATCC 8739, was spotted onto a MALDI-TOF plate with 1 µl of matrix (α-cyano-4-hydroxycinnamic acid, MS CHCA ref 411071). Using a sterile loop, samples of each colony were then deposited in target wells for testing in duplicate. 0.5 µl of formic acid (Vitek MS-FA, ref 411071) was added to each well. After air drying (approximately 5 min) 1 µl of matrix was added on each spot and these were again dried. Once this was done, the slide was inserted into the Vitek MS, and analysis was instigated after transferring the data from the Prep Station to the Vitek MS. Sample preparation was performed using the Prep Station, a module consisting of a computer and a barcode reader, which are used to enter the various sample data and their sites onto the slide. Measurements were performed with the MALDI BioTyper MYLA® software and the spectra obtained were compared with those from the database for validation. The results were measured by two parameters, namely the degree of confidence or percentage score, and the confidence level of the different colours. Green colour and a score between 99.9 and 60% indicates good identification, orange colour and a score of < 60% indicates a low probability of identification, and when the colour is red with zero percentage, then no identification has been made.

### Resistance of *C. albicans* to antifungal drugs

Anti-fungal susceptibility testing (Zhang et al., 2014) was done for 68 isolates of *C. albicans* by using ATB Fungus 3® of Biomérieux. This method enables to determine the susceptibility of the *C. albicans* isolates to the antifungal agents in a semi-solid medium following the conditions recommended by the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) (National Committee for Clinical Laboratory Standards, 1997). ATB Fungus 3® was performed following manufacturer's instructions. Briefly, ATB Fungus 3®of Biomérieux strip consists of 16 pairs of cupules including two growth control wells and five antifungal drugs at different concentrations: 5-Flucytosine (4, 16 µg/ml), Amphotericin B (0.5 to 16 µg/ml), Fluconazole (1 to 128 µg/ml), Itraconazole (0.125 to 4 µg/ml) and Voriconazole (0.06 to 8 µg/ml). The inoculated strips were used in duplicate (c and C) and were read visually after incubation at 37°C for 24 h. For each antifungal agent, the reading of the strips was started with the lowest concentration. The growth score was recorded for each of the wells and compared with the control wells as follows: No reduction in growth (4), slight reduction in growth (3), distinct reduction in growth (2), very weak growth (1) and no growth (0). For Amphotericin B, the minimum inhibitory concentration (MIC) of the *Candida* species corresponded to its lowest concentration, thus enabling complete growth inhibition. For Fluconazole, Itraconazole and Voriconazole, as the possibility of a trailing growth existed, the MIC corresponded to the lowest concentration of the anti-fungal agent, with which a score of 2, 1 or 0 was obtained. For Flucytosine, a growth was looked for and was quantified in both the wells and tested for two concentrations. The results obtained gave an MIC that helps to classify the strain insensitive, intermediate or resistant. The anti-fungal breakpoints used followed the CLSI guidelines (National Committee for Clinical Laboratory Standards, 1997).

### Statistical analysis

The data were statistically analysed using the Graphpad instat 3 software using the Chi-square test (x2) and the Pearson's correlation test at an α risk of 5%. The $p$ value < 0.05 was considered statistically significant.

## RESULTS

The 105 *Candida* isolates came mainly from vaginal

**Table 1.** *Candida* species identification rates by using MALDI-TOF MS (N=105).

| Species | Total number of samples Identification | | Score |
|---|---|---|---|
| | N=105 | Rates (%) | |
| *C. albicans* | 68 | 64.8 | 99.9 |
| *C. tropicalis* | 15 | 14.3 | 99.9 |
| *C. glabrata* | 9 | 8.6 | 99.9 |
| *C. parapsilosis* | 7 | 6.6 | 99.9 |
| *C. krusei* | 4 | 3.8 | 99.9 |
| *C. guillermondii* | 2 | 1.9 | 99.9 |
| Total | 105 | 100 | 99.9 |

N: Total number of identification; C: *Candida*.

**Table 2.** *C. albicans* in resistance pattern reported to antifungal drugs.

| Pattern | Antifungal drugs [No. (%)] | | | | |
|---|---|---|---|---|---|
| | 5 FC | AMB | FCA | ITR | VRC |
| S | 67 (98.5) | 68 (100) | 41 (60.3) | 34 (50) | 48 (69.2) |
| I | 0 | 0 | 9 (13.2) | 7 (10.3) | 2 (2.9) |
| R | 1 (1.5) | 0 | 18 (26.3) | 27 (39.7) | 18 (27.9) |

**Table 3.** *C. albicans* in resistance pattern reported to antifungal drugs by localization.

| Antifungal drugs (%) | Localisation of samples | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Vaginal | | | Oropharyngeal | | | Ear pus | | |
| | N=47 | | | N=15 | | | N=2 | | |
| | n (%) | | | | | | | | |
| | S | I | R | S | I | R | S | I | R |
| 5FC | 46 (97.9) | 0 | 1(2.1) | 15(100) | 0 | 0 | 2(100) | 0 | 0 |
| AMB | 47(100) | 0 | 0 | 15(100) | 0 | 0 | 2(100) | 0 | 0 |
| FCA | 29(61.7) | 7(14.9) | 11(23.4) | 7(46.7) | 2(13.3) | 6(40) | 1(50) | 0 | 1(50) |
| ITR | 25(53.2) | 4(8.5) | 18(38.3) | 6(40) | 2(13.3) | 7(46.7) | 0 | 0 | 2(100) |
| VRC | 32(68.1) | 2(4.3) | 13(27.7) | 11(73.3) | 0 | 4(26.7) | 1(50) | 0 | 1(50) |

N: Total number of identification; n represents the number front of the percentage.

exudates (70.5%), oropharyngeal (19.1%) and sperm (4.4%) swabs. The other swabs were taken from ear pus (2.9%), sputum (1.5%) and stools (1.5%).

## Culture on chromogenic media and MALDI-TOF MS identification results

A total of 68 strains of *C. albicans* (64.8%), 15 strains of *C. tropicalis* (14.3%) and 4 strains of *C. krusei* (3.8%) were identified by culture on chromogenic media. There were a further 18 strains (17.1%) of other species of *Candida* sp. All results were confirmed and *Candida* spp correctly identified by mass spectrometry with a score of 99.9%. *C. albicans* which was the most prevalent (64.8%) species (Table 1).

## Resistance of *C. albicans* to antifungal drugs results

A total of 68 strains of *C. albicans* were subjected to *in vitro* antifungal susceptibility testing. No resistance to amphotericin B was observed with a minimum inhibitory concentration of 0.5 µg/ml, while 1.5% of strains exhibited resistance to 5-fluorocytosine. Regarding the azoles tested, resistance to itraconazole was particularly high at 39.7%, followed by voriconazole (27.9%) and fluconazole 26.3% (Table 2). Concerning the type of sample, resistance was higher in *C. albicans* strains taken from ear pus ($p = 0.0113$) (Table 3).

## DISCUSSION

Candidosic infections are most frequently caused by *C. albicans*, as evidenced by epidemiological studies carried out in the United States of America (Cleveland et al., 2015), Europe (Klingspor et al., 2015), the Middle East (Sharifzadeh et al., 2013) and Africa (Kechia et al., 2015). *C. albicans* was the most prevalent strain (64.8%) in our series, as in several other studies (Bailly et al., 1995; Djohan et al., 2011; Kechia et al., 2015; Lacroix et al., 2014). The predominance of *C. albicans* could be explained by its considerable ability to adhere to host constituents, as well as by its ability to modify its behaviour according to the environment and the secretion of lytic enzymes (Calderone and Fonzi, 2001), which involves specific ligand/receptor interactions with mannoproteins of the yeast wall (Hoyer et al., 1998). In undergoing dimorphic transition from the blastospore to filamentous state, *C. albicans* increases its adhesion properties, its intercellular penetration capacity and its secretion of proteases. The blastospores appear to initiate the infection, while hyphae are involved in it spreading. Hyphae are less easily phagocytosed because of their morphology, and their large size may cause the death of the macrophages. They are also able to penetrate easily into the epithelial and endothelial layers (Karkowska-kuleta et al., 2009; Roman et al., 2007). Moreover, the secretion of hydrolytic enzymes during infection promotes virulence by degrading the surface of the host's mucous membranes and immune defences. These enzymes are aspartyl proteinases (Saps), phospholipases and lipases (Arslan, 2016; Schaller et al., 2005). Although *C. albicans* is the species most commonly responsible for this infection, there are increasing reports of a rise in candidiasis due to other *Candida* species (Amouri et al., 2010; Bonouman-Ira et al., 2011; Panizo et al., 2009).

*C. albicans* was isolated on *Candida* chromatic chromogenic medium along with *C. tropicalis* (14.3%) and *C. krusei* (3.8%), whereas in the Bernal et al. (1996) study, four *Candida* species: *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata* were identified using CHROMagar *Candida* with a very high percentage of reliability. With respect to *C. albicans* identification, the susceptibility and specificity obtained in another study using the same CHROMagar *Candida* chromogenic medium were 100% similar for each of the above-mentioned parameters (Odds and Bernaerts, 1994).

All strains were identified by MALDI-TOF mass spectrometry with a score of 99.9 (Table 1). Six *Candida* species were identified, in contrast to Lacroix et al. (2014), who took surface and deep swabs from hospital patients in haematology, intensive care and undergoing kidney transplants, and obtained an overall identification rate of 98.2% with four species predominating: *C. albicans* (88%), *C. dubliniensis* (3.2%), *C. krusei* (4.6%) and *C. tropicalis* (4.1%) (Lacroix et al., 2014). Nocon (2013), on the other hand, identified 88.8% of bacteria at the species level: *C. albicans* (93.3%) and *C. glabrata* (66.6%).

Our evaluation of *C. albicans* resistance to antifungals by ATB Fungus 3, a method of microdilution in a semi-solid medium, revealed an increase in the level of *C. albicans* resistance to azole antifungals over the 9 years since Djohan's study on the susceptibility of *C. albicans* strains of vaginal origin from the Institut Pasteur of Côte d'Ivoire. The level of resistance to itraconazole increased from 22.2 to 39.7%, to voriconazole from 11.1 to 27.7% and to fluconazole from 2.2 to 26.3%. However, the data collected during the earlier study provide no indication of whether the patients received treatment before the examination, nor is it possible to clarify whether resistance is primary or secondary.

Furthermore, abusive use of these molecules has led to rising incidences of antifungal resistance (Vandeputte et al., 2012). According to a study carried out in Abidjan in 2008, *C. albicans* accounted for 72.6% of isolated strains of vaginal origin with varying rates of resistance to common antifungals: 2.2% for fluconazole, 11.1% for voriconazole and 22.2% for itraconazole (Djohan et al., 2011). In Cameroon, more than half of *Candida* yeasts were resistant to fluconazole in 2012 (Gonsu Kamga et al., 2014). Elsewhere in the world, high *C. albicans* resistance to azoles has been reported by several authors (Bagg et al., 2005; Chryssanthou, 2001; Nasrollahi et al., 2015; Sandra et al., 2005). These high rates of antifungal resistance provide good reason for regular monitoring of *C. albicans* susceptibility to these drugs to ensure effective treatment of candidosic lesions.

Azole antifungals are often the preferred treatments for many *Candida* infections because, on the one hand, they are inexpensive and, on the other, have low toxicity and can be administered orally (Berry et al., 1992; Whaley et al., 2017). Fluconazole is the most frequently prescribed antifungal for most *C. albicans* infections (Pfaller et al., 2002). Its resistance rate varies a lot, so that while a higher resistance rate (94%) was observed in Tehran in 2015 (Nasrollahi et al., 2015). In addition, Gonsu et al. (2014) found that over half of *Candida* yeasts were resistant to fluconazole. But, several authors have found low levels of fluconazole resistance (Jin-sol et al., 2007; Saporiti et al., 2001, Skrodeniene et al., 2006, Sobel et al., 2003), for example, St-Germain et al. (2001) found that only two out of 43 *C. albicans* isolates were fluconazole resistant, and these were isolates from one patient with AIDS and one with leukaemia, both of whom had already been treated with fluconazole. According to these authors, only patients who have already undergone long-term treatment with it are resistance to fluconazole. In contrast, El-Din et al. (2001), Sobel et al. (2004) and Khosravi et al. (2008), all reported no *C. albicans* resistance to fluconazole.

The high levels of resistance to voriconazole and to itraconazole found in our study are not consistent with the results of some other studies. Indeed, several authors have reported no voriconazole resistance (Jin-Sol et al.,

2007; Kronvall and Karlsson, 2001; Panizo et al., 2009; Pfaller et al., 2002; Tortorano et al., 2003). As for Itraconazole, moderate resistance rates of 13, 16.2 and 18% were reported, respectively, by Chryssanthou (2001), Bagg and Sandra (2005), in contrast to Khosravi, who found no *C. albicans* resistance to this drug in 2008.

Resistance to azoles frequently occurs when the target (14-α-demethylase) is modified. This enzyme is involved in synthesis of ergosterol within the membrane and is encoded by the Cyp51 gene (also called ERG11). Spot modifications of Cyp51 reduce the azole's affinity for its target. Only mutations at specific positions lead to resistance to an azole or to all azole drugs. In yeasts such as *C. albicans*, resistance to azoles is also related to increased activity of efflux pumps, which leads to rapid elimination of the antifungals (Guillot and Dannaoui, 1995).

Our study confirmed the excellent *in vitro* activity of amphotericin B on *C. albicans* with an MIC ranging from 0.5 to 1 mg/L (Khosravi et al., 2008; Panizo et al., 2009; Sandra et al., 2005; Skrodeniense et al., 2006). Several authors have also observed low levels of resistance to fluorocytosine (Godoy et al., 2003; Sandra et al., 2005; St-Germain et al., 2001), although Khosravi found increased levels of resistance (83.2%) in Tehran in 2008. Fluorocytosine resistance develops rapidly when the molecule is used alone, which has to do with a combined deficiency of its penetration (alteration of a purine-cytosine permease) or its metabolism (alteration of cytosine deaminase or UMP pyrophosphorylase) in the fungal cells (Dannaoui et al., 2012).

Low levels of fluconazole resistance in *C. albicans* strains of oropharyngeal origin have been observed by Bailly et al. (1995). In their study, nine of the 108 strains (8.3%) exhibited microbiological resistance to fluconazole, a result consistent with previous studies that identified resistant *C. albicans* strains *in vitro* (Regli et al., 1992; Ruhnke et al., 1994). It was not possible to verify whether patients from whom five of the resistant *C. albicans* strains were isolated had taken fluconazole in the 30 days preceding specimen collection. The remaining 4 strains were isolated from patients receiving fluconazole chemoprophylaxis, a situation consistent with secondary resistance.

Previous studies have shown that *C. albicans* is usually sensitive to most azoles (Amouri et al., 2010; Jin-Sol et al., 2007). It would be interesting to realize the resistance of *C. albicans* to antifungals from other tests, based on the principle of MIC, developed according to the protocol of the CLSI or the EUCAST, by incorporating sensitive and resistant reference strains, to compare the data on the resistance of *C. albicans* to antifungals.

## Conclusion

Our study shows that *C. albicans* which was the most prevalent (64.8%) species, is not resistant to Amphotericin B, medicine commonly used to cure candidosic affections in Côte d'Ivoire. However, the relatively high level of resistances observed with itraconazole, voriconazole and fluconazole constitute a real challenge and calls for national strategies to monitor the resistance patterns of the antifungals used and to determine the different underlying mechanisms, particularly in African countries, where the burden of HIV/AIDS is still a problematic issue.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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### REFERENCES

Amouri I, Abbes S, Sellami H, Makni F, Sellami A, Ayadi A (2010). Vulvovaginal candidiasis: A review. Journal of Medical Mycology 20(2):108-115.

Arslan S, Koç AN, Şekerci AE, Tanriverdi F, Sav H, Aydemir G, Diri H (2016). Genotypes and virulence factors of *Candida* species isolated from oral cavities of patients with type 2 diabetes mellitus. Turkish Journal of Medical Sciences 46(1):18-27.

Badillet G, Bievre C, Gueho E (1987). Champignons contaminants des cultures. Champignons opportunistes. Atlas clinique et biologique Varia; 2:132- 216.

Bagg J, Petrina Sweeney M, Andrew N. Davies, Margaret S. Jackson, Susan B (2005). Voriconazole susceptibility of yeasts isolated from the mouths of patients with advanced cancer. Journal of Medical Microbiology 54(10): 959-964.

Bailly C, Vagner O, Aho S, Lopez J, Caillot D, Cuisenier B, Fussy A, Chavanet P, Freysz M, Bonnin A, Camerlynck P (1995). *In vitro* fluconazole susceptibility of 164 *Candida* spp. strains isolated from blood cultures or oropharynx in patients treated or not treated prophylactically with fluconazole. Médecine et Maladies Infectieuses 25(8-9): 919-926.

Bernal S, Martin Mazuelos E, Garcia M, Aller A I, Martinez MA, and Gutiérrez MJ (1996). Evaluation of CHROMagar *Candida* Medium For the Isolation and Presumptive Identification of Species of *Candida* of Clinical Importance. Diagnostic Microbiology and Infectious Disease 24(4):201-204.

Berry AJ, Rinaldi MG, Graybill JR (1992). Use of high-dose fluconazole as salvage therapy for cryptococcal meningitis in patients with AIDS. Antimicrobial Agents and Chemotherapy 36(3):690-692.

Bonouman-Ira V, Angora E , Djohan V, Vanga-Bosson H, Sylla-Thanon K, Beourou S, Touré AO, Faye-Ketté H, Dosso M, Koné M (2011). Resistance of *Candida* no albicans in Abidjan in 2011. Revue Bio-Africa 9:27-31.

Calderone RA, Fonzi WA (2001). Virulence factors of *Candida albicans*. Trends Microbiology 9(7):327-335.

Cleveland AA, Harrison LH , Farley M M ,Hollick R ,Stein,B, Chiller TM, Lockhart SR,, Park BJ (2015). Declining incidence of candidemia and the shifting epidemiology of *Candida* resistance in two US metropolitan areas, 2008-2013: results from population-based surveillance. PLoS ONE 10(3): e0120452.

Chryssanthou E (2001). Trends In antifungal susceptibility among Swedish *Candida* species bloodstream isolates from 1994 to 1998:

Comparison of the E-test and the Sensititre Yeast One Colorimetric Antifungal Panel With the NCCLS M27A reference method. Journal of Clinical Microbiology 39: 4181-4183.
Dannaoui E, Desnos-Ollivier M, Garcia-Hermoso D, Grenouillet F, Cassaing S, Baixench MT, Bretagne S, Dromer F, Lortholary O, French Mycoses Study Group (2012). Candida spp. with acquired echinocandin resistance, France, 2004-2010. Emerging Infectious Diseases 18:86-90.
Djohan V, Angora KE, Vanga-Bosson AH, Konaté A, Kassi FK. Yavo W, Kiki-Barro PC, et col (2011). *In vitro* susceptibility of vaginal Virulence factors of *Candida albicans*. Trends Microbiology to antifungal drugs in Abidjan (Cote d'Ivoire). Journal of Medical Mycology 22(2):129-133.
El-Din S, Reynolds TM, Ashbee HR, Barton RC, Evans EG (2001). An Investigation into the pathogenesis of vulvovaginal candidiasis. Sexually Transmitted Infections 77:179-183.
Gonsu Kamga H, Kechia Agem FA, Tegankam D, Toukam M, Sando Z, Moyou Somo R (2014). Antifungal susceptibility patterns among the clinical isolates of *Candida* spp. in digestive candidiasis in the HIV-positive subjects. Health Sciences and Diseases 15(3).
Godoy P, Tiraboschi IN, Severo LC, Bustamante B, Calvo B, Almeida LP, da Matta DA, Colombo AL (2003). Species distribution and antifungal susceptibility profile of *Candida spp*. Bloodstream isolates from Latin American hospitals. Memórias do Instituto Oswaldo Cruz 98(3):401-405.
Guillot J, Dannaoui E (2015). Resistance to antifungal drugs: importance in human and veterinary medicine. Bulletin de l'Académie Vétérinaire de France Tome 168(4):314-319.
Hoyer LL, Payne TL, Bell M, Myers AM, Scherer S (1998). *Candida albicans* ALS3 and insights into the nature of the ALS gene family. Current Genetics 33:451-459.
Jin-Sol L, Jong HS, Kyungwon L, Mi-Na K, Bo-Moon S, Young U (2007). Species distribution and susceptibility to azole antifungals of *Candida* bloodstream isolates from eight university hospitals in Korea. Yonsei Medical Journal 48:779-86.
Karkowska-Kuleta J, Rapala-Kozik M, Kozik A (2009). Fungi pathogenic to humans: Molecular bases of virulence of *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Acta Biochimica Polonica 56:211-224.
Kechia FA, Dohbit JS, Kouotou EA, Iwewe SY, Dzoyem JP, Mbopuwouo NM, Monamele CG, Moyou SR (2015). Epidemiologic and mycological pattern of vulvo-vaginal candidiasis in pregnancy in Yaounde. Health Sciences and Diseases 16(4):1-6**.**
Khan ZU, Chandy R, Metwali KE (2003). *Candida albicans* strain carriage in patients and nursing staff of an intensive care unit: A study of morphotypes and resistotypes. Mycoses 46:476-486.
Khosravi AR, Shokri H, Mansouri P, Katiraee F, Ziglari T (2008). *Candida* species isolated from nails and their in vitro susceptibility to antifungal drugs in the department of dermatology (University of Tehran, Iran). Journal of Medical Mycology 18:210-215.
Klepser ME (2001). Antifungal resistance among *Candida* species. Pharmacotherapy 21:124-132.
Klingspor L, Tortorano AM, Peman J, Willinger B, Hamal P, Sendid B, Velegraki A, Kibbler C, Meis JF, Sabino R, Ruhnke M, Arikan-Akdagli S, Salonen J, Dóczi I (2015). Invasive *Candida* infections in surgical patients in intensive care units: A prospective, multicentre survey initiated by the European Confederation of Medical Mycology (ECMM) (2006-2008). Clinical Microbiology and Infection 21(1):87.e1-87.e10.
Kronvall G, Karlsson I (2001). Fluconazole and voriconazole multidisk testing of *Candida* species for disk test calibration and MIC estimation. Journal of Clinical Microbiology 39(4):1422-1428.
Lacroix C, Gicquel A, Sendid B, Meyer J, Accoceberry I, Francçois N, Morio F, Desoubeaux G, Chandenier J, Kauffmann-Lacroix C, Hennequin C, Guitard J, Nassif X, Bougnoux M-E (2014). Evaluation of two matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) systems for the identification of *Candida* species. Clinical Microbiology and Infection 20(2):153-158.
Lindsay G, Steven K, Yvonne R, Adrian M, Patrick R (2010). Evaluation of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for Identification of Clinically Important Yeast Species. Journal of Clinical Microbiology 48(10):3482-3486.
Nasrollahi Z, Yadegari MH, Roudbar MS, Roudbary M, Hosseini PM, Nikoomanesh F, Bazl MR (2015). Fluconazole Resistance *Candida albicans* in Females With Recurrent Vaginitis and Pir1 Overexpression. Jundishapur Journal of Microbiology 8(9):e21468.
National Committee for Clinical Laboratory Standards (1997). Reference Method for Broth dilution. Antifungal Susceptibility Testing of Yeasts; Approved Standard. Document M27-A 17, 1/29. 22(15).
Nocon C (2013). Evaluation des performances du spectre de masse MALDI-TOF MICROFLEX LT Bruker comparées à celle du spectre VITEK BIOMERIEUX et demarche d'accréditation. https://pepite-depot.univ-lille2.fr/nuxeo/site/esupversions/f6ce20e2-6556-4e88-a7d7-7e01875b040
Odds FC, Bernaerts R (1994). CHROMagar *Candida*, a new differential isolation medium for presumptive identification of clinically important *Candida* species. Journal of Clinical Microbiology 32:1923-1929.
Panizo MM, Reviakina V, Dolande M, Selgrad S (2009). *Candida spp* in vitro susceptibility profile to four antifungal agents. Resistance surveillance study in Venezuelan strains. Journal of Medical Mycology 47:137-143.
Pfaller MA, Messer SA, Hollis RJ, Jones RN, Diekema DJ (2002). *In vitro* activities of ravuconazole and voriconazole compared with those of four approved systemic antifungal agents against 6,970 clinical isolates of Candida *spp*. Antimicrobial Agents and Chemotherapy 46(6):1723-1727.
Regli P, Blancard A, Goudard M, Moulin-Traffort L, Sarzier JM, Quilici M (1992). Evaluation in vitro susceptibility of yeasts for fluconazole. Pathologie Biologie 40:500-506.
Roman E, Arana DM, Nombela C, Alonso-Monge R, Pla J (2007). MAP kinase pathways as regulators of fungal virulence. Trends in Microbiology 15:181-190.
Ruhnke M, Eigler A, Engelmann E, Geiseler B, Trautmann M (1994). Correlation between antifungal susceptibility testing of *Candida* isolates from patients with HIV infection and clinical results after treatment with fluconazole. Infection 22:72-75.
Sandra SR, Rudolph PG, Shawn AM, Richard JH, Daniel JD, Michael A (2005). Antifungal susceptibilities of *Candida* Species causing vulvovaginitis and epidemiology of recurrent cases. Journal of Clinical Microbiology 43:2155-2162.
Saporiti AM, Gomez D, Levalle S, Galeano M, Davel G, Vivot W, Et al., (2001). Vaginal candidiasis: etiology and sensitivity profile to antifungal agents in clinical use. Revista Argentina de Microbiología 33:217-222.
Schaller M, Borelli C, Korting HC, Hube B (2005). Hydrolytic enzymes as virulence factors of *Candida albicans*. Mycoses 48:365-377.
Sharifzadeh A, Khosravi AR, Shokri H, Asadi Jamnani F, Hajiabdolbaghi, M, Ashrafi Tamami I (2013). Oral microflora and their relation to risk factors in HIV+ patients with oropharyngeal candidiasis. Journal of Medical Mycology 23(2):105-12.
Skrodeniene E Dambrauskiene A, Vitkauskiene A (2006). Susceptibility of yeasts to antifungal agents in Kaunas University of Medicine Hospital. Medicina (Kaunas) 42:294-299.
Sobel JD, Wiesenfeld HC, Martens M, Danna P, Hooton TM, Rompalo A, Malcolm Sperling, Charles Livengood 3rd, Benson Horowitz, James Von Thron, Libby Edwards, Helene Panzer, Teng-Chiao Chu (2004). Maintenance fluconazole therapy for recurrent vulvovaginal candidiasis. New England Journal of Medicine 351:876-883.
Sobel JD, Zervos M, Reed BD, Hooton T, Soper D, Nyirjesy P, Heine MW, Willems J, Panzer H (2003). Fluconazole Susceptibility of vaginal isolates obtained from women with complicated *Candida* vaginitis: Clinical implications. Antimicrobial Agents and Chemotherapy 47:34-38.
St-Germain G, Laverdière M, Pelletier R, Bourgault AM, Libman M, Lemieux C, Noël G (2001). Prevalence and antifungal susceptibility of 442 *Candida* isolates from blood and other normally sterile sites: Results of a 2-Year (1996 to 1998) Multicenter Surveillance Study in Quebec, Canada. Journal of Clinical Microbiology 39(3):949-953.
Tortorano AM, Rigoni AL, Biraghi E, Prigitano A, Viviani MA, FIMUA-ECMM Candidaemia Study Group (2003). The European Confederation of Medical Mycology (ECMM) survey of candidaemia in Italy: Antifungal susceptibility patterns of 261 non-*Candida albicans* isolates from blood. The Journal of Antimicrobial Chemotherapy 52:679-682.

Vandeputte P, Ferrari S, AlixT Coste (2012). Antifungal resistance and new strategies to control fungal. International Journal of Microbiology 2012:26p.

Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS, Rogers PD (2017). Azole Antifungal Resistance in *Candida albicans* and Emerging Non-albicans *Candida* Species. Frontiers Microbiology 7:2173.

Zhang L, Wang H, Xiao M, Kudinha T, Mao LL, Zhao HR, Kong F, Xu YC (2014). The widely used ATB FUNGUS 3 automated readings in China and its misleading high MICs of *Candida* spp. to azoles: Challenges for developing countries' clinical microbiology labs. PLoS One 2014;9:e114004.

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# Arbuscular mycorrhizal fungi (AMF) promote the growth of the pioneer dune plant of coastal areas

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**The establishment of economic enterprises and the demand for coastal areas for leisure purposes exert great pressure on the dunes, stripping them of vegetation and causing the aggravation of coastal erosion processes. The use of arbuscular mycorrhizal fungi (AMF) is a viable alternative to restore dunes, giving their ability to improve soil conditions and plant growth under unfavorable conditions. The aim of this study was to evaluate the effect of AMF on the growth of *Canavalia rosea* (Sw.) DC. For the experiment, two treatment groups were set up (16 pots with arbuscular mycorrhizal fungi inoculum and 16 pots without) over a period of 10 weeks. It was found that *C. rosea* (Sw.) DC. responded positively to the inoculation with the AMF and the percentages of colonization were 6.4 and 10.2% in the eighth and tenth week of plant growth, respectively. Also, the growth of *Canavalia rosea* (Sw.) DC. increased significantly with AMF throughout the experiment. This proved the efficacy of arbuscular mycorrhizal fungi as promoters of dune plants´ growth, and as potential strategy for the rehabilitation of dunes in the coastal areas.**

**Key words:** *Canavalia rosea*, inoculation, mycorrhizal colonization, dune restoration, re-plant.

## INTRODUCTION

The coastal dunes are very important dynamics ecosystems playing different ecological roles such natural barriers and protection of coastal areas from extreme sea activities, natural boundary of the shoreline movement and preservation of beaches (Henrico et al., 2020). There is a pioneer vegetation cover associated with coastal dunes assuming a primordial role in soil (Sigren et al., 2014), protecting it against wind (Dewhurst, 2002; Bar et al., 2016), allowing the protection and maintenance of the coastline (Gomes-Neto et al., 2004) and reducing the occurrence of erosion (Dewhurst, 2002; Sigren et al., 2014; White et al., 2019).

The coastal dunes are affected by various natural and anthropogenic factors (White et al., 2019). The intentional or accidental introduction of alien species in dunes (Sun et al., 2017; Malavasi et al., 2018; Marzialetti et al., 2019) increase in the establishment of economic enterprises, inadequate land parceling (Maueua et al., 2007) and the

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demand for coastal areas for leisure purposes (Langa, 2007; Teixeira et al., 2016) exert great pressure on the dunes, stripping them of vegetation (Langa, 2007; Sperandii et al., 2019) and causing the aggravation of coastal erosion processes (Maueua et al., 2007; Bar et al., 2016).

The escalation of coastal erosion processes affected at least 55% of coastal dune around the world (Bar et al., 2016) will lead to the destruction and loss of habitats, soil erosion and depletion, water pollution, alteration of the coastline configuration, destruction of infrastructure and loss of investment (Hoguane, 2007; Gracia et al., 2018). Can also cause the extinction of plant and animal species occurring in coastal dunes (Prisco et al., 2013), and it is estimated that 85% of the existing coastal dunes are under threat (Henrico et al., 2020).

Around the world diverse techniques have been applied to restore coastal dunes, but the majority are expensive to implement (Teixeira et al., 2016). One of the interventions used to reduce the observed erosion process was the re-colonization of the dunes (Langa, 2007; Assis et al., 2016). Studies showed that using plants in coastal dune restoration has an enormous potential to reduce erosion under wind and wave action (Sigren et al., 2014). However, this was difficult and slow (Bécard et al., 2004; Teixeira et al., 2016), because of the high mortality rates of seedlings as a consequence of the stress to which they were subjected on the dunes (Gomes-Neto et al., 2004; Teixeira et al., 2016).

The use of mycorrhizae is a viable alternative to restore dunes (Bécard et al., 2004; Asmelash et al., 2016; Assis et al., 2016), giving their ability to improve soil conditions and plant growth under unfavorable conditions (Bever, 2003; Al-Karaki, 2013; Amir et al., 2013; Winagraski et al., 2019), and it is known that AMF are highly prevalent in coastal dune plants (Sigren et al., 2014). Thus, since AMF offered a possible solution to the identified problem, it was relevant to study their possible effects on the growth of *Canavalia rosea* (Sw.) DC., a typical dune pioneer, which was of extreme importance in the stabilization process of dunes (Kitajima et al., 2008; Mendoza-González et al., 2014) and widely used to control soil erosion in several countries due to dense cover, root binding the substrate and the quick growth (Mendoza-González et al., 2014). Therefore, the present study aimed to provide information about the mycorrhizal association with *C. rosea*, as no study has been published to date on mycorrhizal association with this plant. Also, to evaluate the effect of the use of AMF inoculum on *C. rosea* growth and to determine the percentage of colonization in its roots.

## MATERIALS AND METHODS

### Study area

The experiment was conducted in the greenhouse and in the Plant Physiology Laboratory of the Department of Biological Sciences of the Eduardo Mondlane University, Maputo, Mozambique, over a period of three months. The experimental design was completely randomized, with two treatments (with inoculum-propagules of *Glomus intraradices* N.C. Schenck and G.S. Sm. and control) with eight replicates for each treatment.

### Sampling and laboratory procedure

The seeds were collected from dry pods of *C. rosea* (Sw.) DC on the dunes of Muntanhana beach in Maputo. The soil was collected to a depth of about 20 cm and then autoclaved at 120°C for 90 min to eliminate microorganisms in the soil that could influence the results of the experiment (Miyasaka et al., 2003). The samples were immersed in 40% sodium hypochlorite for 15 min, to disinfect them according to the protocol described by Zorato et al. (2001). Subsequently, the samples were immersed in 100% sulfuric acid for 110 min to break dormancy (Hartmann et al., 2001). The seeds were pre-germinated in Petri dishes lined with filter paper moistened with distilled water, and those with a radicle equal to or greater than 2 mm in length were used for sowing.

In the first phase, the sowing was carried out in 1kg pots containing autoclaved soil, and these were divided into two treatments: 1) control (16 pots without inoculum) and 2) inoculum (16 pots with *Glomus intraradices*). In the "control" treatment, the seeds were placed in opened clumps in the pots, and in the "inoculum" treatment, a 9 g of inoculum was added per pot, followed by the seed. Irrigation was done with 20 ml of distilled water, in other days for five weeks. After five weeks, all the seedlings (control and inoculum) were transplanted to 5 kg pots, where watering per pot was done with 100 ml of running water every two days. In the third and fifth week after transplantation, the eight and tenth weeks of growth after sowing, the plants were harvested in both treatments. The plants were separated into roots, stems and leaves for determination of growth parameters (dry weight, root length, plant height, number of leaves and leaf area) and percentage of roots colonized by AMF. Dry weight was obtained after drying for 72 h in a 65°C oven, and weighed on a root, stem and leaf electronic scale. The maximum root length and plant height were measured with a ruler. The number of leaves was determined by manual counting, and the leaf area was measured by using a leaf area meter (Model 3100 LI-Cor Inc., Licoln, NE, USA). The percentage of colonized root was determined by the Locatelli and Lovato (2002) root staining method.

### Data analysis

The data were analyzed using the statistical package SPSS Statistics. The non-parametric Mann-Whitney test was used to compare growth averages between inoculated and control plants, and it was considered significant at $p \leq 0.05$.

## RESULTS AND DISCUSSION

The *C. rosea* (Sw.) DC associated with mycorrhizal fungi responded positively to the mycorrhizal inoculation, and presented 60% higher dry weight production ($p \leq 0.05$) compared to the non-inoculated bean (Figures 1B and 3A). This corroborates with the results of De Oliveira et al. (2009), in a greenhouse experiment using Brasilian dunar native soil with AMF who found an increase of 75.93% of aerial biomass and 76.79% of radicular biomass in *Tabebuia roseo-alba* (Ridl.) Sandw. and 88.34% increment of aerial biomass and 87.17% of

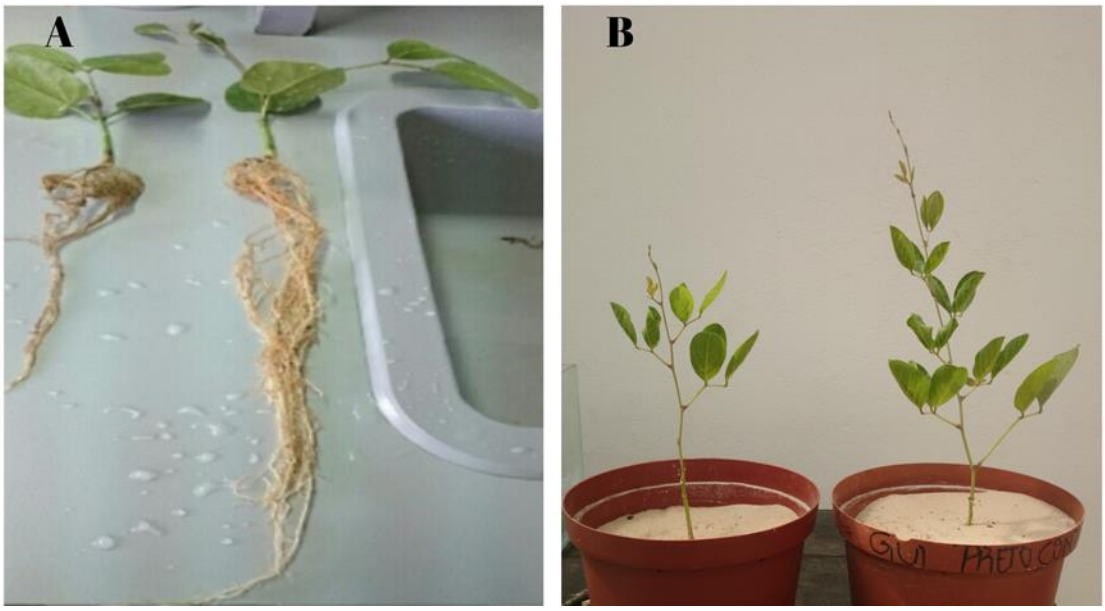


**Figure 1.** Effect of inoculation with arbuscular mycorrhizal fungi on plant root length (A), and plant height (B) in *C. rosea* (Sw.) DC. On the left, non-inoculated plant, and on the right, inoculated plant.

radicular biomass in *Tocoyena selloana* Schum. The biomass increase may be a consequence of the improvement of the nutritional status due to the AMF inoculation as elucidated in a review by Chen et al. (2018). This fact was confirmed by Wang *et al*. (2019) who found an increase in phosphorus, potassium and magnesium uptake in a study with *Zelkova serrata* seedilings inoculated with *Funneliformis mosseae*.

The mycorrhizal inoculation resulted in a higher root growth, reflecting the increase by 28% of the maximum length of the *C. rosea* root compared to the non-inoculated ($p \leq 0.05$) (Figures 1A and 3B). A higher growth of inoculated plant roots was expected, since several studies reported the benefits of mycorrhizal association for root growth (Carneiro et al., 2004; Júnior and Da Silva, 2006; Balota et al., 2011; Hidalgo, 2015; Sharma et al., 2017).

According to Smith and Read (2008), the response is related to the scarcity of water and nutrients in the soil, causing AMF stimulus of root branching and better development and consequently giving the host plant a greater capacity to absorb water and nutrients. Similar results to those verified in the present study were also observed by Little and Maun (1996) and Feagin et al. (2008) when studying the efficiency of the mycorrhizal association in pioneer dune species, *Ammophila breviligulata* and *Uniola paniculata* for the restoration of degraded dunes in the United States. Pérez-de-Luque et al. (2017) also showed the AMF stimulation on root growth.

An increase of 20% in height was also observed in inoculated bean compared to non-inoculated, although it was a statistically non-significant difference ($p \geq 0.05$) (Figure 1B and Figure 3C). This is according to Peña-Becerril et al. (2016) who observed an increase 3 times greater in *Mimosa biuncifera* Benth. height when inoculated with native AMF compared to non-inoculated. The increase in height is a positive point because combining the positive responses obtained during the experiment in the other parameters showed the effect of the AMF on the plant and how this higher uptake of nutrients resulted in increased plant growth (Marschner and Dell, 1994; Lanfranco et al., 2018; Begum et al., 2019). The fact that it has not differentiated from non inoculated plants in the study by Brandon and Shelton (1993), who reported that there was a latency phase between mycorrhizal inoculation and the time when its effects were manifested in the plant and this fact was also corroborated by Lanfranco et al. (2018). Even having a height above the control may not be significant until 45 days, the difference being significant from 60 days until the end of the experiment.

The results of the experiment by Oliveira et al. (2009) are in agreement with those of previous authors, since the native Brazilian dune plant, *Tabebuia roseo-alba,* used in the study grew to a higher height than non-inoculated plants; however, the superiority was shown to be significant only at 75th day. This suggested that the same effect probably would happen in this experiment by extending the plant observation time and the differences would become significant. Besides the experiment of Oliveira et al. (2009), similar results were verified by Dos Santos et al. (2016), studying AMF effects on a leguminous species called *Albizia polycephala*, where

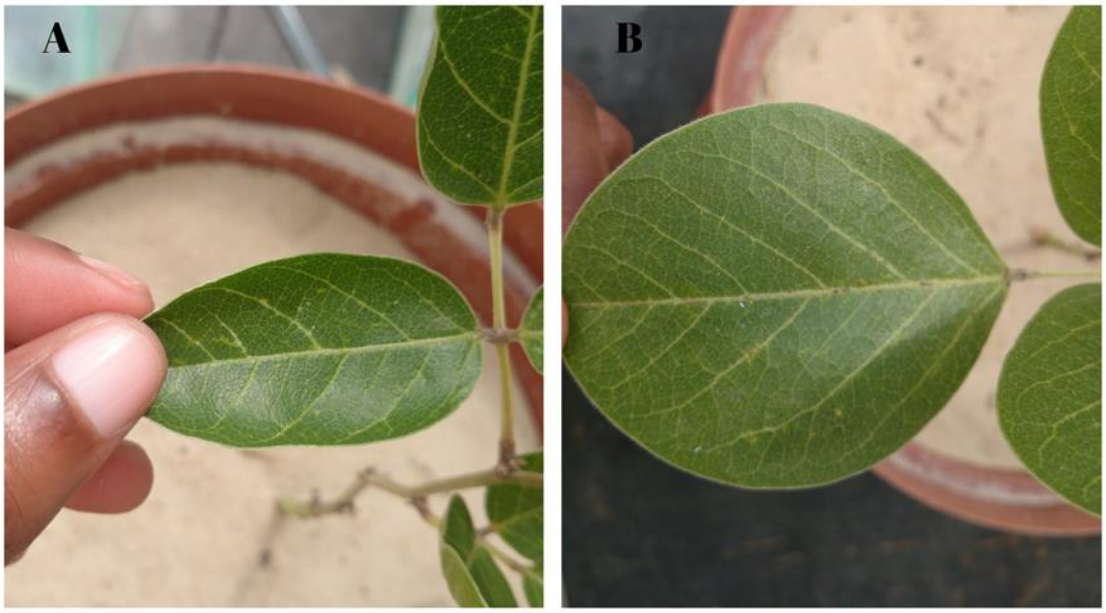


**Figure 2.** Effect of inoculation with arbuscular mycorrhizal fungi on leaf area in *C. rosea* (Sw.) DC. **A**: Non-inoculated plant; **B**: Inoculated plant.

they found that *Acaulospora colombiana* (AMF species) had increased positively and significantly the neck diameter, leaf size and dry matter. On the other hand, it hadn't increased significally the height at 135 days of experiment, although it had shown an increase in height of 20% more than non-inoculated plants. This suggested that these criteria probably would not change even if the experiment had continued for an extended period.

In contrast, Lu et al. (2015) found different results studying AMF effects of *Glomus intraradices* and *Glomus mosseae* on the growth of *Morus alba* L. There were significant increases in dry matter, number of roots, as well as the height of the plant compared to non-inoculated plants. Since the same species (*G. intraradices*) was used in the present study, but in plant belonging to other family, this result suggested that it is not correct to assume that the same fungus species can contribute in the same way to all hosts, the same species can contribute in a different way on the growth of different hosts.

At leaf level, mycorrhizal inoculation resulted in the production of a larger number of leaves and a larger leaf area compared to the non inoculated bean, although this was statistically significant only at the tenth week of growth ($p \leq 0.05$) with increases of 56 and 36%, respectively (Figures 2 and 3D and E). The same result was verified by Lu et al. (2015) studying AMF effects of *G. intraradices* on the growth of *M. alba* L. Shi et al. (2016) observed in all tested plant growth parameters, including aboveground criteria, an increase of 31 to 121% in AMF inoculated mulberry plants.

For Oliveira et al. (2005), the processes of faster leaf growth and the production of greater numbers of leaves and leaf areas may be related to greater energy reserving (phosphorus) in inoculated plants. As a result of this, AMF stimulate these plants to increase their exploitation of solar irradiation and their potential for photosynthesis (because their leaves have greater chlorophyll a+b), consequently an increase in the biomass accumulation occurs (Oliveira et al., 2005; Tristão et al., 2016). This is corroborated by Gao et al. (2020) in a study with *Gossypium hirsitum* L. demonstrating the positive effect of AMF in phosphorus absorption and consequent enhance of plant growth. The observed productivity is a consequence of functional efficiency as a response to the higher uptake of water and nutrients verified in AMF inoculated plants (Romero, 2012), which allows greater development of leaves than non-inoculated plants (Atwell et al., 1999; Peña-Becerril et al., 2016; Wang et al., 2019).

The colonization of the bean roots by AMF was verified from the eighth week of growth, evidenced by the presence of hyphae and vesicles in the cortex of the root segments. Root colonization aroused probably as a result of the germination of AMF spores present in the soil associated with low nutrient availability in the soil (Avila, 2004; Silva-Flores et al., 2019) and other soil factors such as water availability, temperature and relative humidity (Dell-Santo, 2011; Kilpeläinen et al., 2020).

The spore germination starts when the plant (host) under low phosphate or nitrogen conditions exudes or excretes strigolactones from its roots into the rhizosphere, and these are recognized by AMF, inducing the spore germination and then enhancing fungal

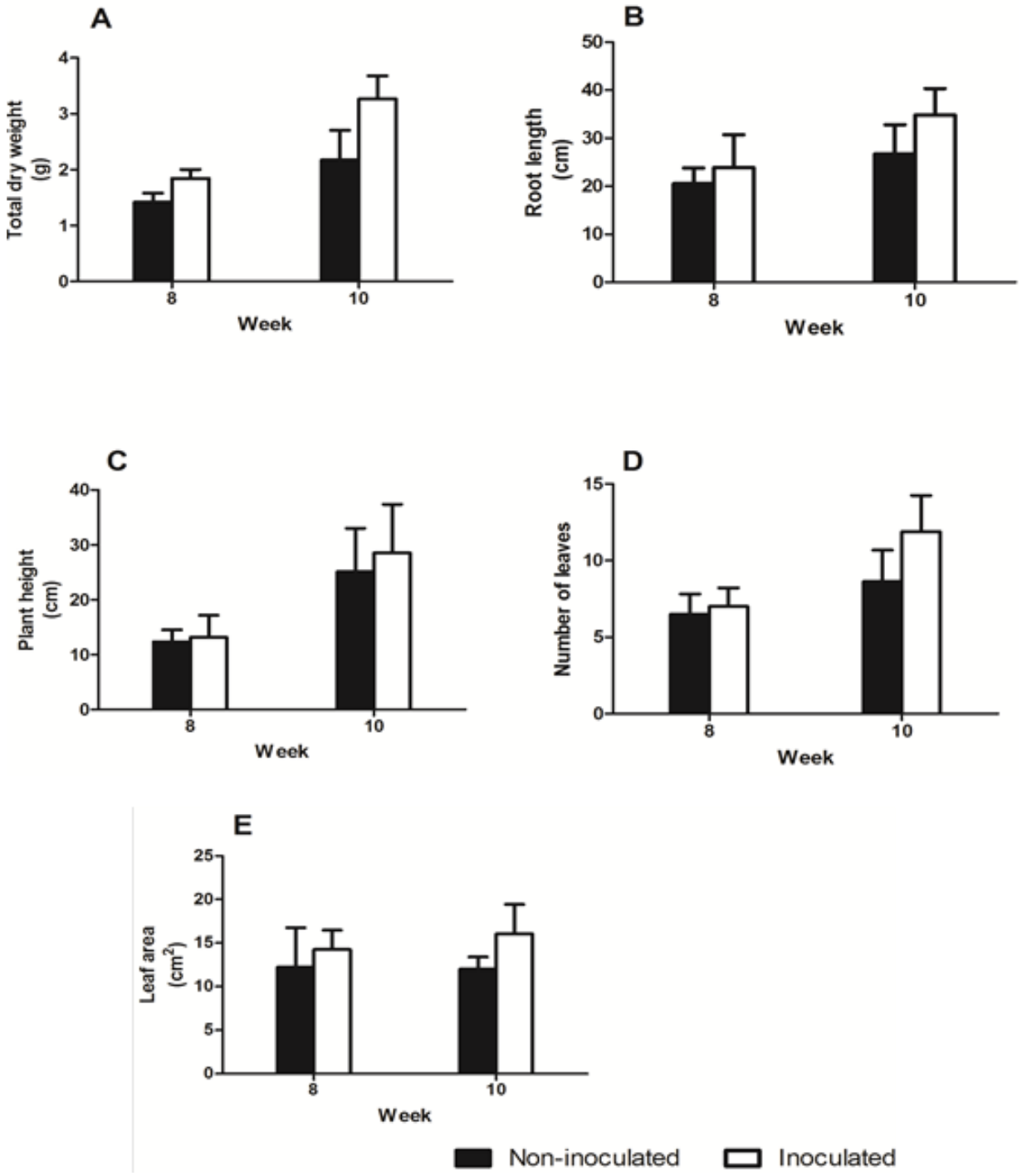


**Figure 3.** Effect of inoculation with arbuscular mycorrhizal fungi on plant dry weight (A), root length (B), plant height (C), number of leaves (D) and leaf area (E), in the eighth and tenth week of growth in *C. rosea* (Sw.) DC. Each bar represents an average of eight plants. The vertical lines indicate the standard deviation. In each week, the Mann-Whitney Test was performed for comparison at 5% probability.

metabolic activity, hyphal growth and branching, and exudation of a short chain of chitin oligosaccharides (Pimprikar and Gutjahr, 2018).

The justification presented above may be applicable to the present work, since dune soil, characteristically known for low organic matter decomposition and low soil fertility (Camprubíet et al., 2010; Oliveira and Landim, 2020), the same characteristics were presented by the

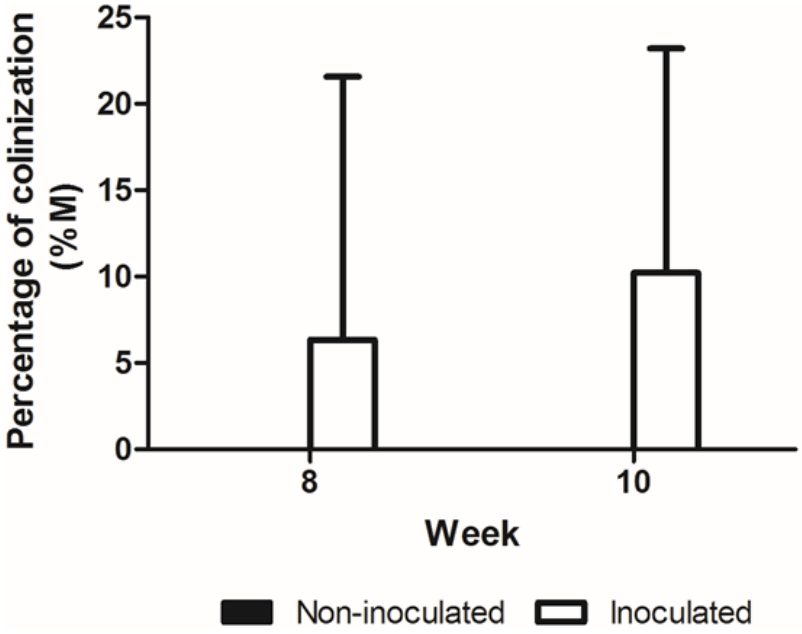


**Figure 4.** Colonization percentage (% M) in *C. rosea* (Sw.) DC. roots in the eighth and tenth week of growth. Each bar represents an average of eight plants. The vertical lines indicate the standard deviation.

substrate used, and as suggested by Smith and Read (2008) and Nayak et al. (2019), the AMF were probably stimulated in soils with low fertility.

The percentage of root colonization (% M) by AMF increased from 6.4 to 10.2% throughout the experiment (Figure 4). Similar percentages were also found by Santos et al. (2004) in *Ammophila arenaria* (L.) plants associated to AMF, having observed values of 5.9 and 1.9% in dune systems of two different regions, and by Mocuba (2005) in *Carpobrotus dimidiate* (L.) L. Bolus, a dune plant, having observed 3.5 and 17.5% of colonization throughout the experiment. However, the percentages over 50% of root colonization were observed by Shi et al. (2016) in mulberry seedlings with *Acaulospora scrobiculata, Funneliformis mosseae*, and *Glomus intraradices* and by Peña-Becerril et al. (2016) in a study with *Mimosa biuncifera* (Benth.) inoculated with native AMF.

Simpson and Daft (1990) reported that the development of AMF colonization in the roots initially showed a period of colonization delay and low intensity due to the need for perfect symbiotic integration between the mycorrhizae and plant (fungi and roots), followed by a growing phase involving colonization and stabilization thereofore over the growing time. This fact was also referred by Lanfranco et al. (2018) explaining the different stages of the communication between fungi and plant roots in AMF symbiosis.

The results of the present study corroborated these observations, since the percentage of colonization was low, with an increase over the experiment, suggesting that the percentage of colonization probably followed the trend reported by Simpson and Daft (1990) and could increase over time to a stabilization phase. A different pattern of colonization was found by Juntahum et al. (2019), in a study with sugarcane inoculated with AMF decreasing the colonization percentage over the time. These results showed once again the dynamic complexity of AMF symbiosis in field conditions.

## Conclusion

From this study, it was found that *C. rosea* (Sw.) DC. have association with *G. intraradices* and the mycorrhizal fungi have a positive effect on *C. rosea* (Sw.) DC. growth and the levels of root colonization were 6.4 and 10.2% with a tendency to increase along its growth.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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**REFERENCES**


Al-Karaki GN (2013). The role of mycorrhiza in the reclamation of degraded lands in arid environments. In: Developments in soil classification, land use planning and policy implications: Innovative thinking of soil inventory for land use planning and management of land resources Shahid SA, Taha FK, Abdelfattah MA (eds.), Dordrecht: Springer Science+Business Media, pp. 823-836.

Assis DMA, Oehl F, Gonçalves CM, Silva DKA, Silva GA (2016). Community structure of arbuscular mycorhizal fungi in fluvial and maritime dunes of Brazilian Northeast. Applied Soil Ecology 108:136-146.

Asmelash F, Bekele T, Birhane E (2016). The potential role of arbuscular mycorrhizal fungi in the restoration of degraded lands. Frontiers in Microbiology 7(1095):1-15.

Amir H, Lagrange A, Hassaïne N, Cavaloc Y (2013). Arbuscular mycorrhizal fungi from New Caledonian ultramafic soils improve tolerance to nickel of endemic plant species. Mycorrhiza 23:585-595.

Atwell BJ, Kriedemann PE, Turnbull CGN (1999). Plants in action: adaptation in nature, performance in cultivation. Melbourne: Macmillan Education Australia, P. 664.

Avila AL (2004). Ocorrência de fungos micorrízicos arbusculares em cultivo de videiras (*Viti*ssp.) Sob manejo orgânico e comercial. Dissertação (Mestrado). Universidade Federal do Rio Grande do Sul, Brasil.

Balota EL, Machineski O, Stenzel NMC (2011). Resposta da acerola à inoculação de fungos micorrízicos arbusculares em solo com diferentes níveis de fósforo. Revista Bragantina 70(1):166-175.

Bar P, Becker N, Segev M (2016). Sand dunes management: A comparative analysis of ecological versus economic valuations applied to the Coastal region in Israel. Regional Environmental Change 16(4):941-950.

Bécard G, Kosuta S, Tamasloukht M, Séjalon-Delmas N, Roux C (2004). Partner communication in the arbuscular mycorrhizal interaction. Canadian Journal of Botany 82:1186-1197.

Begum N, Qin C, Ahanger MA, Raza S, Khan MI, Ashraf M, Ahmed N, Zhang L (2019). Role of Arbuscular Mycorrhizal Fungi in Plant Growth Regulation: Implications in Abiotic Stress. Tolerance Frontiers in Plant Science 10(1068):1-15.

Bever JD (2003). Soil community feedback and the coexistence of competitors: conceptual frameworks and empirical tests. New Phytologist 157:465-473.

Brandon NJ, Shelton HM (1993). Role of vesicular-arbuscular mycorrhizae in *Leucaena* establishment. Proceedings of the XVII International Grassl and Congress. pp. 2064-2065.

Camprubí A, Calvet C, Cabot P, Pitet M, Estaún V (2010). Arbuscular mycorrhizal fungi associated with psammophilic vegetation in Mediterranean coastal sand dunes. Spanish Journal of Agricultural Research 8:96-102.

Carneiro MAC, Siqueira JO, Davide AC (2004). Fósforo e inoculação com fungos micorrizicos arbusculares no estabelecimento de mudas de embaúba (*Cecropia pachstachya* Trec*).* Pesquisa Agropecuária Tropical 34(3):119-125.

Chen M, Arato M, Borghi L, Nouri E, Reinharft D (2018). Beneficial Services of Arbuscular Mycorrhizal Fungi-From Ecology to Application 9(1270):1-14.

Dell-Santo VBR (2011). Respostas de plantas de tomate (S*olanum lycopersicum* L.) com superexpressão da $H^+$ pirofosfatase à inoculação com fungos micorrízicos arbusculares. Trabalho de culminação de curso (Licenciatura) - Centro Universitário Vila Velha, Brasil.

Dewhurst D (2002). Coastal dunes: Dune protection and improvement manual for the Texas Gulf coast. Texas Editor. P. 28.

Dos Santos RS, Scoriza RN, Da Silva EMR, Júnior OJS (2016). Selection of mycorrhizal fungi for the initial growth of *Albizia polycephala*. Revista Brasileira de Ciências Agrárias 11:98-103.

Feagin RA, Koske RE, Gemma JN, Williams AM (2008). Restoration of sea oats (*Uniola paniculata*) with mycorrhizae on Galveston Island. Coastal Management Program. P. 16.

Gao X, Guo H, Zhang Q, Guo H, Zhang L, Zhang C, Gou Z, Liu Y, Wei J, Chen A, Chu Z, Zeng F (2020). Arbuscular mycorrhizal fungi (AMF) enhanced the growth, yield, fiber quality and phosphorus regulation in upland cotton (*Gossypium hirsutum L*.) Scientific Reports 10(1):1-12.

Gracia A, Rangel-Buitrago N, Oakley JA, Williams AT (2018). Use of ecosystems in coastal erosion management. Ocean and Coastal Management 156:277-289.

Gomes-Neto A, Emílio TC, Bosa P, Cunha SR (2004). Plantio de *Ipomoea pes-caprae* nas dunas da praia brava (itajaí, sc): Comparação de duas técnicas. Notas Técnicas da Facimar 8:33-38.

Hartmann HT, Kester DE, Davies FT, Greeve RL (2001). Principles and practices of plant propagation. London: Prentice Hall Publishers, 6th Edition. 770p.

Henrico I, Ledwaba T, van Zyl G (2020). Measuring the effect of wind-driven processes on coastal dunes: A study of the Atlantis and Geelbek dune fields along the West Coast of South Africa. Spatial Information Research 2020:1-9.

Hidalgo ABL (2015). Efecto de la coinoculación de hongos micorrízicos arbusculares y *Bradyrhizobium japonicum* sobre la producción de soya (*Glycine max* (L.) Merr) en la provincia Los Ríos, República del Ecuador. Tesis (Doctor), Universidad Agraria de La Habana, Cuba.

Hoguane AM (2007). Perfil diagnóstico da zona costeira de Moçambique. Revista de Gestão Costeira Integrada 7(1):69-82.

Júnior OJS, Da Silva EMR (2006). Micorriza Arbuscular–Papel, Funcionamento e Aplicação da Simbiose. Miolo_Biota 12(32):101-150.

Juntahum S, Jongrungklang N, Kaewpradit W, Lumyong S, Boonlue S (2019). Impact of arbuscular mycorrhizal fungi on growth and productivity of sugarcane under field conditions. Sugar Tech 22(3):451-459.

Kitajima EW, Alcântara BK De, Madureira PM, Alfenas-Zerbini P, Rezende JAM, Zerbini FM (2008). A mosaic of beach bean (*Canavalia rosea (Sw.) DC.*) caused by anisolate of cowpea aphid-borne mosaic virus (cabmv) in Brazil. Archives of Virology Journal 153:743-747.

Kilpeläinen J, Aphalo PJ, Lehto T (2020). Temperature affected the formation of arbuscular mycorrhizas and ectomycorrhizas in *Populus angustifolia* seedlings more than a mild drought. Soil Biology and Biochemistry 146(10779):1-12.

Lanfranco L, Fiorilli V, Gutjahr C (2018). Partner communication and role of nutrients in the arbuscular mycorrhizal symbiosis. New Phytologist 220:1031-1046.

Langa JVQ (2007). Problemas na zona costeira de Moçambique com ênfase para a costa de Maputo. Revista de Gestão Costeira Integrada 7(1):33-44.

Little LR, Maun MA (1996). The *Ammophila* problem' revisited: A role for mycorrhizal. Journal of Ecology 84(1):1-7.

Locatelli LM, Lovato PE (2002). Inoculação micorrízica e aclimatização de dois porta-enxertos de macieira micropropagados. Pesquisa Agropecuária Brasileira 37(2):177-184.

Lu N, Zhou X, Cui M, Yu M, Zhou J, Qin Y, Li Y (2015). Colonization with arbuscular mycorrhizal fungi promotes the growth of *Morus alba* L. seedlings under greenhouse conditions. Forests 6(3):734-747.

Malavasi M, Acosta ATR, Carranza ML, Bartolozzi L, Basset A, Bassignana M, Campanaro A, Canullo R, Carruggio F, Cavallaro V (2018). Plant invasions in Italy. An integrative approach using LifeWatch infrastructure database. Ecological Indicators 91:182-188.

Marschner H, Dell B (1994). Nutrient uptake in mycorrhizal symbiosis. Plant and Soil 159:89-102.

Marzialetti F, Bazzichetto M, Giulio S, Acosta ATR, Stanisci A, Malavasi M, Carranza ML (2019). Modelling *Acacia saligna* invasion on the Adriatic coastal landscape: An integrative approach using LTER data. Nature Conservation 34:127-144.

Maueua C, Cossa O, Mulhovo G, Pereira M (2007). Vulnerabilidade climática nas zonas costeira: Delta do Zambeze. dot Project. P. 15.

Mendoza-González G, Martínez ML, Lithgow D (2014). Biological Flora of Coastal Dunes and Wetlands: *Canavalia rosea* (Sw.) DC Journal of Coastal Research 30(4):697-713.

Miyasaka SC, Habte M, Friday JB, Johnson EV (2003). Manual on arbuscular mycorrhizal fungus production and inoculation techniques. Available at: https://scholarspace.manoa.hawaii.edu/bitstream/10125/12454/SCM-5.pdf

Mocuba JJ (2005). Efeito de endomicorriza no crescimento de plantas das dunas. Course culmination work (Licenciatura) – Universidade

Eduardo Mondlane, Mozambique.
Nayak S, Behera S, Dash PK (2019). Potential of Microbial Diversity of Coastal Sand Dunes: Need for Exploration in Odisha Coast of India The Scientifc World Journal 2019:1-9.
Oliveira JRG, De Souza RG, Da Silva FSB, Mendes ASM, Yano-Melo AM (2009). O papel da comunidade de fungos micorrízicos arbusculares (FMA's) autóctones no desenvolvimento de espécies vegetais nativas em área de dunas de restinga revegetadas no litoral do estado da paraíba. Revista Brasileira de Botânica 32(4):663-670.
Oliveira RS, Castro PML, Dodd JC, Vosátka M (2005). Synergistic effect of *Glomus intraradices* and *Frankia* spp. on the growth and stress recovery of *Alnus glutinosa* in alkaline anthropogenic sediment. Chemosphere 60(10):1462-1470.
Oliveira EVS, Landim MF (2020). Dunes in the North coast of Sergipe, Brazil: plant species and their ecological traits. Rodriguésia 71:1-18.
Peña-Becerril JC, Monroy-Ata A, Orozco-Almanza MS, García-Amador EM (2016). Establishment of *Mimosa biuncifera* (Fabaceae) inoculated with arbuscular mycorrhizal fungi in greenhouse and field drought conditions. International Journal of Tropical Biology and Conservation 64(2):791-803.
Pérez-de-Luque A, Tille S, Johnson I, Pascual-Pardo D, Ton J, DD Cameron (2017). The interactive efects of arbuscular mycorrhiza and plant growth-promoting rhizobacteria synergistically enhance host plant defences against pathogens. Scientific Report 7:1-10.
Pimprikar P, Gutjahr C (2018). Transcriptional regulation of arbuscular mycorrhiza development. Plant and Cell Physiology 59(4):678-695.
Prisco I, Carboni M, Acosta ATR (2013). The fate of threatened coastal dune habitats in Italy under climate change scenarios. PLoS One 8(7):1-14.
Romero CGK (2012). Influencia de las micorrizas arbusculares sobre el crecimiento y respuesta a la sequía de *Piscidia carthagenensi*s Jacq.: Implicaciones en la recuperación de un bosque seco de la península de Macanao, Isla de Margarita. Tesis (Doctor) - Universidade Central de Venezuela, Venezuela.
Santos J, Schreck RC, Gonçalves MT, Freitas H, Santos J, Reis CS, Gonçalves MT, Freitas H (2004). Micorrizas em *Ammophila arenaria* – comparação entre dois sistemas dunares. Revista de Biologia 22:101-108.
Sharma N, Aggarwal A, Yadav K (2017). Arbuscular mycorrhizal fungi enhance growth, physiological parameters and yield of salt stressed *Phaseolus mungo* (L.) Hepper. European Journal of Environmental Sciences 7(1):5-13.
Sigren JM, Figlus J, Armitage AR (2014). Coastal and dunes and dune vegetation: Restoration, erosion, and storm protection. Shore and Beach 82(4):5-12.
Silva-Flores P, Bueno CG, Neira J, Palfner G (2019). Factors Affecting Arbuscular Mycorrhizal Fungi Spore Density in the Chilean Mediterranean-Type Ecosystem. Journal of Soil Science and Plant Nutrition 19:42-50.
Shi S, Chen K, Gao Y, Liu B, Yang X, Huang X, Liu G, Zhu L, Hua-He X (2016). Arbuscular mycorrhizal fungus species dependency governs better plant physiological characteristics and leaf quality of mulberry (*Morus alba* L.) seedlings. Frontiers in Microbiology 7:1030.
Simpson D, Daft MJ (1990). Interactions between water stress and different mycorrhizal inocula on plant growth and mycorrhizal development in maize and sorghum. Plant Soil Journal 21:179-186.
Smith SE, Read DJ (2008). Mycorrhizal symbiosis. Academic Press. San Diego, USA. P 800.
Sperandii MG, Bazzichetto M, Gatti F, Acosta ATR (2019). Back into the past: Resurveying random plots to track community changes in Italian coastal dunes. Ecological Indicators 96:572-578.
Sun C, Liu Y, Zhao S, Li H, Sun J (2017). Saltmarshes response to human activities on prograding coast revealed by a dual-scale time-series strategy. Estuaries and Coasts 40:522-539.
Teixeira LH, Weissen W, Ganade G (2016). Facilitation and sand burial affect plant survival during restoration of a tropical coastal sand dune degraded by tourist cars. Society for Ecological Restoration 24:390-397.
Tristão LE, De Figueiredo PAM, Zied DC, Lisboa LAM, Alves VG (2016). Parâmetros agronômicos iniciais de cana-de-açúcar inoculadas com fungos micorrizicos arbusculares. 1º Encontro Internacional de Ciências Agrárias e Tecnológicas, pp. 529-537.
Wang J, Fu Z, Ren Q, Zhu L, Lin J, Zhang J, Cheng X, Ma Ji, Yue J (2019). Effects of Arbuscular Mycorrhizal Fungi on Growth, Photosynthesis, and Nutrient Uptake of *Zelkova serrata* (Thunb.) Makino Seedlings under Salt Stress Forests 10(186):1-16.
White RA, Piraino K, Shortridge A, Arbogast AF (2019). Measurement of Vegetation Change in Critical Dune Sites along the Eastern Shores of Lake Michigan from 1938 to 2014 with Object-Based Image Analysis. Journal of Coastal Research 35(4):1-11.
Winagraski E, Kaschuk G, Monteiro PHR, Auer G, Higa AR (2019). Diversity of arbuscular mycorrhizal fungi in forest ecosystems of Brazil: A review. Cerne 25:25-35.
Zorato MF, Homechin M, Henning AA (2001). Efeitos da assepsia superficial com diferentes agentes químicos na incidência de microrganismos em sementes de soja. Revista Brasileira de Sementes 23(1):159-166.

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# Prevalence and Antibiotic resistance profile of Avian Pathogenic *Escherichia coli* (APEC) strains isolated from poultry feeds in Abidjan District, Côte d'Ivoire

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**The aim of this study was to isolate avian pathogenic *Escherichia coli* (APEC) strains from poultry feeds for assessing their susceptibility patterns to antibiotic agents. From November 2018 to March 2019, one hundred eighteen poultry feeds samples were collected in farms of Abidjan District and *E. coli* strains were isolated on TBX (Tryptone Bile Glucuronic) agar medium, followed by biochemical identification and APEC virulence genes detection via polymerase chain reaction (PCR) analysis. Among these samples, 44 (37.29%) were positive to *E. coli*. Municipalities of Anyama, Bingerville and Port-Bouët provided most contaminated poultry feeds with respectively 100, 54.04 and 30.76% of prevalence rate. Moreover, increased serum survival (*iss*) and iron-acquisition system (*iucD*) genes were respectively detected in 10 (15.87%) and 15 (23.81%) tested strains while seven *E. coli* isolates were positive for both genes. Antibiotic susceptibility tests by the disk diffusion method in Mueller-Hinton agar medium showed high resistance level to tetracycline (100%) and nalidixic acid (61.90%) while moderate resistance rates was observed with amoxicillin +clavulanic acid (28.57%) and ciprofloxacin (16%). Moreover, all the tested strains were susceptible to gentamicin. This study indicate the necessity to control the quality of poultry feeds in Côte d'Ivoire and especially to research alternative methods to reduce extensive antibiotics use in this sector in Côte d'Ivoire.**

**Key words:** Avian pathogenic *Escherichia coli* (APEC), poultry feeds, antibiotic, poultry farm, Côte d'Ivoire.

## INTRODUCTION

In Côte d'Ivoire, poultry production systems have significant effect on national economy (Koné and Danho, 2008). Indeed, a recent report by Interprofessional Ivorian Poultry sector organization in 2017 showed that total income in 2015 was estimated at about 412.8 millions US Dollar (IPRAVI, 2017). This sector accounts for 4.5% of agricultural GDP and 2% of total GDP. In addition, Côte d'Ivoire's government intends to increase this performance by reaching 60000 tons of poultry meat and more than 1.678 billion of eggs / year in 2020 to fully

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cover population needs of animal proteins (IPRAVI, 2017). However, poultry production sector is affected by many factors including several illnesses which lead to a significant decrease of chicken meat and eggs production. For example, in 2012, the loss of production was estimated to 39.45% corresponding to more than 240 000 USD. Generally, these diseases are due to various microorganisms including virus, fungi, parasites and bacteria agents (Zhao et al., 2005). Among poultry diseases due to bacteria, colibacillosis is the primary cause of morbidity, mortality, and condemnation of carcasses in the poultry industry worldwide. It is, thus, an economically devastating disease for poultry industry in many parts of the world (Zhao et al., 2005). Strains causing these systemic diseases in poultry are termed avian pathogenic *Escherichia coli* (APEC) (Zhuang et al., 2014; Schouler et al., 2018). *E. coli* is, normally, one of the common microbial flora of gastrointestinal tract of poultry but may become pathogenic because of specific virulence attributes that have been associated with a systemic disease, colibacillosis (Jawetz et al., 1984; Levine, 1987; Yang et al., 2004). Colibacillosis of poultry is characterized in its acute form by septicemia resulting in death (Calnek et al., 1997). At days, approaches to prevent and control this pathology in the poultry industry include improved hygienic methods, good practices of micro-environment management, vaccination and use of antimicrobial agents. However, many reports have described increased multidrug resistance of *E. coli* to commonly used antimicrobial agents for treatment (Yang et al., 2004, Zhao et al., 2005). One of the potential source of *E. coli* contamination is chicken feeds. The latter has a significant impact on poultry health and its zootechnical performances. Unfortunately, data on microbiologic qualities of these feeds in Côte d'Ivoire remain unavailable. Moreover, antibiotics are extensively used as growth promoters in poultry production or to control infectious diseases. This misuse of antibiotics is considered the most vital selection force to antimicrobial resistance of bacteria (Okeke et al., 1999; Moreno et al., 2000; Ouattara et al., 2013). Moreover, the resistant *E. coli* could be passed from poultry to people via handling of feeds or direct contact with infected chicken. In addition, acquired resistance to antimicrobial agents creates an extensive trouble in case of management of intra and extra intestinal infections caused by *E. coli*, which are a major source of illness, death, and increased healthcare costs both in poultry and in human (Gupta et al., 2001). Therefore, the present study was designed to isolate avian pathogenic *E. coli* strains from poultry feeds for assessing their susceptibility and resistance patterns to some selected antimicrobials in Côte d'Ivoire.

## MATERIALS AND METHODS

### Study area and sampling

This study was conducted in five municipalities including Yopougon, Songon, Bingerville, Port-Bouët and Anyama because of their high poultry production capacity in the District of Abidjan, Côte d'Ivoire. The geographical location of each municipality is shown in Figure 1. A preliminary survey was conducted in the Abidjan District studying the major pathologies such as affecting poultry production systems (Doumbia, 2018). According to all information recorded, we hypothesized that foods used for animal could be contamination source of poultry by pathogenic microbial flora such as Avian pathogenic *Escherichia coli* (APEC), and reported in breeder chickens in the District of Abidjan. Therefore, a total of 118 dehydrated samples of industrial and farmers formulated feeds were collected from August 2018 to March 2019. Modern breeding farms including at least 1000 chicken heads, based on the previous survey (Doumbia, 2018; Goualié et al., 2020), were selected for this study.

In each farm, three to four samples were collected according to the number of building and feeders by farmers in the farm. Approximately 200 to 300 g of samples were directly taken from feeders and put in sterile boxes. After collection, all the samples were labeled and rapidly transported to the laboratory in a cooler containing ice.

### Bacteriological analysis

Isolation of *E. coli* was performed by culture on TBX agar (Conda, Madrid, Spain) preceded by enrichment in buffered peptone water (BioRad, La Marnes-la-Coquette, France). Briefly, 10 g of foods sample were transferred to 90 ml of buffered peptone water. After manual homogenization, and overnight incubation was carried out for 24 h at 37°C and 100 µl of the enriched broth were spread on a solid surface of TBX agar. Then, all plates were incubated at 37°C for 24 h. After incubation, one typical *E. coli* colony (a blue colony on TBX agar) was selected from each plate and identified according to Buchanan and Gibbons (1974) following a series of biochemical tests included gram staining, tests for oxidase, methyl red, Voges-Proskauer reactions, indole, citrate, catalase, urea hydrolysis, gelatin hydrolysis, lactose fermentation, nitrate reduction, casein hydrolysis and sugar fermentation. All the process was conducted under sterile conditions by using Bunsen burner.

### APEC strains molecular identification

Molecular identification of APEC in *E. coli* isolates was performed by detection of two virulence genes (Ewers et al., 2005). The investigations were based on the detection of virulence-associated genes including *iucD* and *iss* respectively coding for iron-acquisition system (aerobactin) and a protein for increased serum survival. Indeed, *iss* and *iucD* genes have generally been recognized to be associated with virulence factors of *E. coli* isolated in colibacillosis cases in poultry farms (Nakazato et al., 2009; Ashraf et al., 2020).

The simplex polymerase chain reaction (PCR) was performed in final volume of 50 µl mix containing 0.6 µl of each dNTP (10 mM), 3 µl of $MgCl_2$ (25 mM), 10 µl of Buffer 5X DNA Taq polymerase, 0.2 µl of Taq polymerase (Promega, WI USA), 1.4 µl of each primer (100 µM). Amplification reactions were carried out using thermal cycler (Gene Amp PCR system type 9700, Applied Biosystems, Villebon-sur-yvette, France) with the following program: an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and polymerization at 72°C for 90 s. A final extension was performed at 72°C for 5 min. The amplification generated 309 bp and 714 bp DNA fragments corresponding, respectively, to *iss* and *iucD* genes. Table 1 shows primer sequences used in this study. For visualization of PCR products, 15 µl samples of the reaction mixtures were analyzed by gel electrophoresis in a 1% agarose, dissolved in 1 X TBE (8.9 M Tris, 8.9 M boric acid, 0.2 M EDTA), for 90 min at 90 V. The

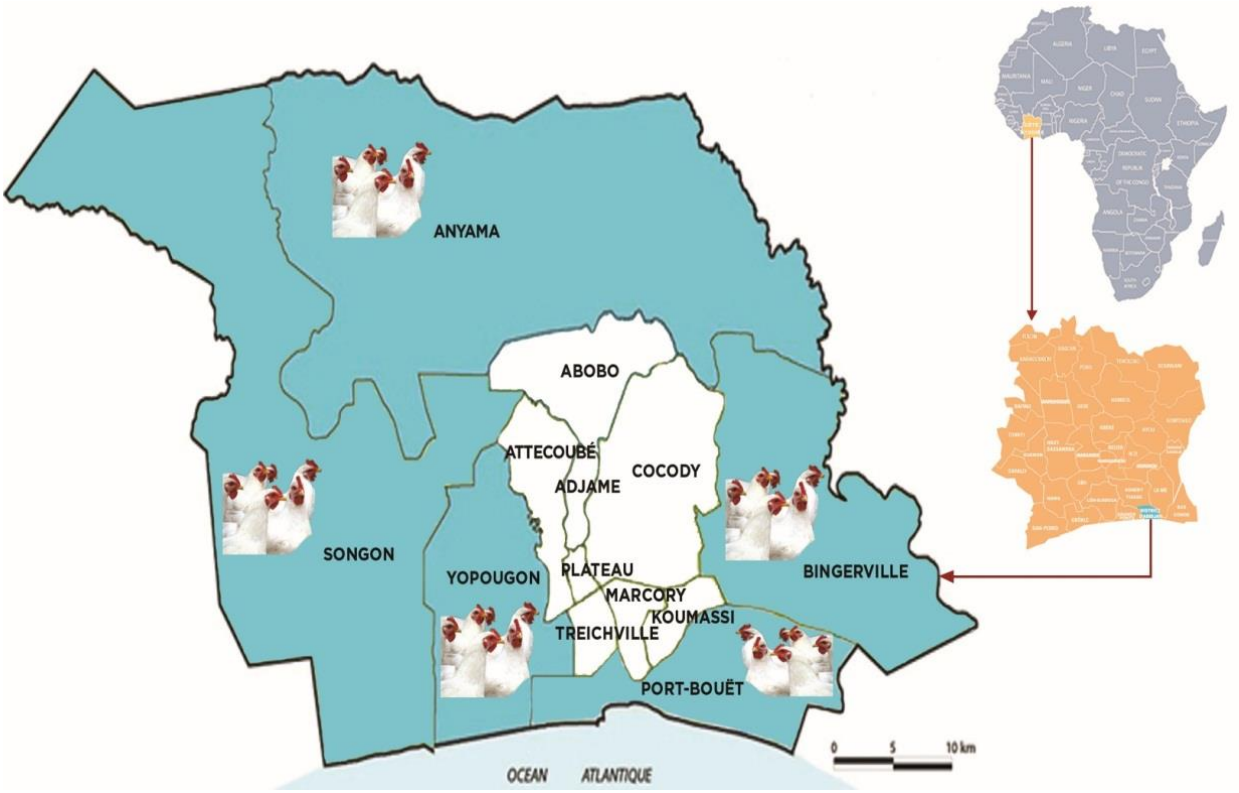


**Figure 1.** Abidjan District map indicating study areas.

**Table 1.** *iss* and *iucD* primer sequences used in PCR.

| Gene | Primer sequence | Amplicon size (bp) |
|---|---|---|
| *iss* | F 5' ATCACATAGGATTCTGCCG 3'<br>R 5' CAGCGGAGTATAGATGCCA 3' | 309 |
| *iucD* | F 5' ACAAAAAGTTCTATCGCTTCC 3'<br>R 5' CCTGATCCAGATGATGCTC 3' | 714 |

Primer source: Ewers et al. (2005).

were stained with safe SYBR green and photographed under UV exposure.

### Antibiotics sensitivity test

Antibiotic sensitivity was determined by the disk diffusion method on Mueller-Hinton agar medium (BioRad, France) according to the guidelines of the "Comité de l'Antibiogramme de la Société Française de Microbiologie" (CASFM, 2018). Standard paper disks containing antibiotics widely used in the poultry industry in Côte d'Ivoire including tetracyclin (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), combination of amoxicilin and clavulanic acid (20/10 µg), gentamicin (10 µg) were laid on the medium. All commercial antibiotic disks were purchased from BioRad (France). The plates were aerobically incubated for 24 h at 37°C. Inhibition zones were measured and analyzed according to the CASFM (2018). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as reference strains.

## RESULTS

### Prevalence of APEC

A total of 118 chicken feeds samples were collected and analyzed for *E. coli* isolation. Among them, 44 (37.29%) were positive for APEC consisting of a total 63 strains. Moreover, most contaminated samples were collected from Anyama, Bingerville and Port-Bouët areas with prevalence of 100%, 54.04 and 30.76% respectively. Among the 63 isolated strains, 10 (15.87%) and

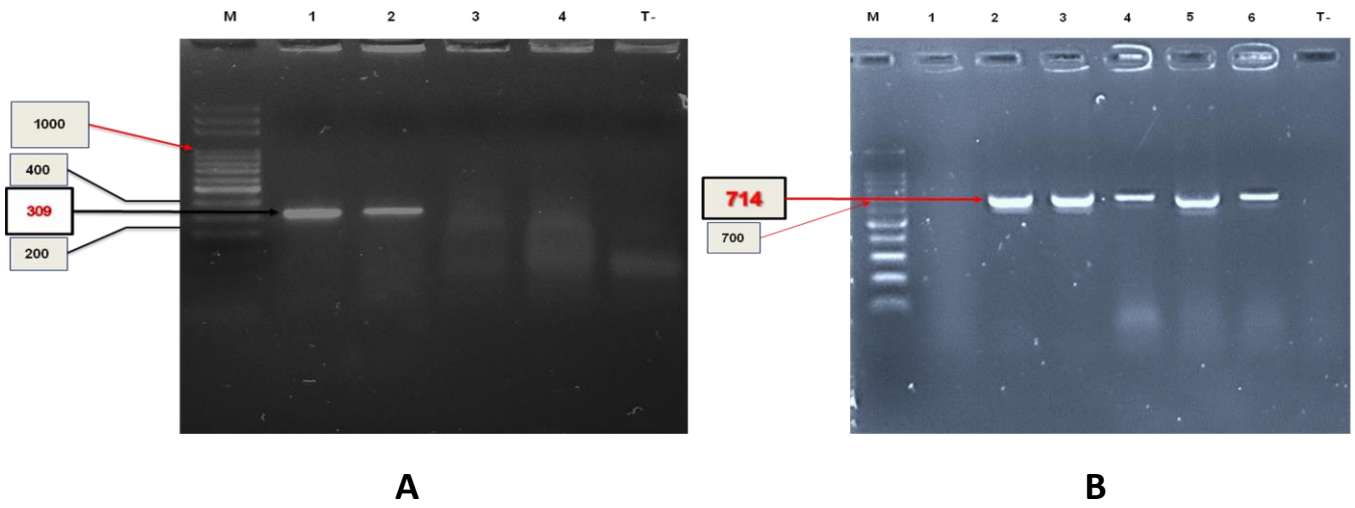


**Figure 2.** Electrophoretic profile obtained after amplification by PCR. (A) using *iss* virulence gene primers, (B) using *iuc*D virulence gene primers. M: DNA Ladder, 1 (A) and 2 (B): positive control, T- Nagative control.

**Table 2.** Antibiotic resistance of *APEC* isolates.

| Antibiotics used | Resistance percentage | Number of drugs families |
|---|---|---|
| CIP | 16 | 1 |
| NAL | 61.9 | 1 |
| AMC | 28.57 | 1 |
| GMN | 0 | 1 |
| TET | 100 | 1 |
| CIP/NAL | 16 | 1 |
| NAL/TET | 61.9 | 2 |
| CIP/NAL/TET | 4.76 | 2 |
| NAL/AMC/TET | 28 | 3 |
| NAL/CIP/AMC/TET | 9.52 | 3 |

CIP (Ciprofloxacin), NAL (Nalidixic acid), AMC (combination of Amoxicillin and clavulanic acid), GMN (Gentamicin), TET (Tetracyclin).

15 (23.81%) *E. coli* strains were *iss* and *iucD* genes respectively corresponding to the DNA bands sizes (Figure 2). Among these 25 isolates, seven (11.11%) strains were positive for both *iss* and *iucD*.

### Antimicrobial susceptibility profiles of tested strains

Antimicrobial susceptibility was tested for all the 25 *E. coli* isolates which include one or two virulence genes and antimicrobial resistance profiles were shown in Table 2. All of these strains were resistant to one or more antimicrobial agents.The highest rate of antimicrobial resistance was detected with tetracyclin (100%) and nalidixic acid (61.90%). Comparatively, resistance levels to ciprofloxacin and combination of amoxicilin and clavulanic acid were low with 16 and 28.57% respectively. Moreover, cross resistance were observed for both nalidixic acid and ciprofloxacin belonging both to fluoroquinolones family with 16% (3/25) rate. Multidrugs resistance (MDR) concerning three drugs families was detected in 28% (7/25) of the tested APEC strains. However, all tested strains (100%) were sensitive to gentamicin.

## DISCUSSION

The aim of this study was to isolate and identify APEC strains from poultry feeds for assessing their resistance patterns to selected and locally used antimicrobials. The results indicate high prevalence of *E. coli* in analyzed

samples. Our findings are in accordance to previously reported works. Indeed, other studies conducted in Algeria and in Chad indicated high prevalence of *E. coli* in poultry and swine feeds (Aimeur et al., 2014; Bodering et al., 2018). However, it is reported a low feeds contamination by ANSES (2018) in France with prevalence ranged from 0.1 to 2%. In general, a high prevalence of bacteria in feeds could be associated to poor quality of raw materials, handling of feed by producers and farmers, on-farm storage and poor practices in buildings and breeding equipment (ANSES, 2018). *E. coli* is one of the commensals microbial floras of poultry gastrointestinal tract but some serotypes are pathogenic (Jawetz et al., 1984; Levine, 1987) such as APEC associated with avian colibacillosis which is implicated in recognized economical loses in poultry production systems worldwide.

The identification of these pathogens is based on the detection of specific markers involved in their pathogenicity. Indeed, investigations have indicated that the distribution of various virulence factors are useful markers for the detection and characterization of APEC, and could, therefore, be used in the diagnosis of colibacillosis in poultry (Jansen et al., 2001).

In fact, the majority of APEC strains have been characterized by possession of *fimC, iucD*, *irp2, iss* and *tsh* virulence genes. Moreover, previous studies reported that episomal *iss*, the increased serum survival gene, was identified as significantly more associated with the APEC strains than with fecal isolates from healthy birds (Pfaff-McDonough et al., 2000; Rodriguez-Siek et al., 2005, Johnson et al., 2008a; b). Moreover, iron acquisition systems such as iucD have been recognized to be associated with bacterial virulence especially in those bacterial pathogens causing septicemia (Nakazato et al., 2009). In our study, seven strains were identified to harbor both of the studied virulence-associated genes. Thus, these feed could be a potential source of colibacillosis in the farms because of the key role of both *iucD* and *iss* in avian *E. coli* pathogenesis (Pfaff-McDonough et al., 2000).

Indeed, it has been reported that the presence of several virulence genes in an isolate would increase the pathogenicity of the strains as there is a real interaction between APEC virulence factors (Ashraf et al., 2020).

In general, *E. coli* are considered as indicator of faecal contamination in food and about 10 to 15% of intestinal coliforms are opportunistic and could be induced various diseases in poultry as well as in human (Barnes and Gross, 1997). Thus, presence of *E. coli* non-APEC observed in this study indicates poor conditions of poultry feeds production and existence of over health risk for the visited farms.

On the other hand, the absence of *E. coli* found in samples from Yopougon and Songon municipalities may be due to good hygiene practices in the sampled poultry farms. In this study, high percentage of antimicrobial resistance was observed for tetracyclin and nalidixic acid in our studied isolates. Similar results were previously reported by Johnson et al. (2007) and by Akond et al. (2009). Moreover, results concerning increase of avian *E. coli* resistance to antibiotics were shown by many researchers such as Salehi and Farashi (2006) in Iran; Saidi et al. (2013) in Zimbabwe, Messaï et al. (2014) in Algeria; Garcia-graells et al. (2014) in Belgium; and Bodering et al. (2017) in Chad. These authors reported high resistance to tetracyclines ranged from 67 to 100% and to nalidixic acid with 23 to 100% resistance level.

The resistance observed to tetracycline could be due to mutations in porin structures or the decrease of their synthesis. Moreover, one or more modifications in porins could induce to drug resistance like beta-lactams, quinolones, chloramphenicol, sulfonamides, trimethoprim and tetracyclines (Fauchère and Avril, 2002).

Generally, the overuse or misuse of antibiotics is considered to be the key factor promoting the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine (Neu, 1992; Witte, 1998; Ungemach et al., 2006). In Côte d'Ivoire, antibiotics such as tetracycline are intensively used in animals for therapy and control of bacterial infections (Ouattara et al., 2013) and as growth promoters. Unlike other antibiotics tested, no resistance was observed with gentamicin. This result is in agreement with those indicated by Doumbia (2018) during his study of poultry contamination risk factors by enteropathogenic microorganisms in the municipality of Bingerville. Its probably indicates that this drug is rarely used in poultry production in Côte d'Ivoire. However, the most relevant aspect of antimicrobial resistance remains multiresistance, which leads to therapeutic failure in cases of bacterial infection. In this study, multiresistance concerning fluoroquinolones, penicillins and tetracyclines families was found in 28% of APEC isolates. Occurrence of this multidrug resistance is directly related to the extensive use of several of these antimicrobial agents in poultry farming in the District of Abidjan. In addition, these results indicate a risk of therapeutic failure in the treatment of avian colibacillosis and other bacterial infections, since tetracyclines are to date one of the most used antibiotic families in the treatment of poultry illnesses. Hence, it becomes urgent to avoid the overuse and misuse of antibiotics and promote alternative methods to control and reduce bacterial related pathologies in poultry farming.

## CONCLUSION

The results of this study showed high contamination level of poultry feeds on farms in the District of Abidjan by APEC. However, *fimC, irp2* and *tsh* virulence genes also specific to APEC must be detected in these strains to better evaluate the sanitary risk due to these feeds. They

also showed high resistance to antimicrobials of the fluoroquinolone and tetracycline families. In short, this study highlights the need to control the microbiological quality of poultry feed, improve hygiene conditions during poultry feeds production and the urgent requirement to seek alternatives to avoid the overuse and misuse of antibiotics in the poultry sector of Côte d'Ivoire.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

### REFERENCES


Aimeur R, Bouaziz A, Boudjemaa S, Dria O, Boussena S, Hireche S (2014). Study of the bacteriology and physico-chemical quality of drinking in poultry farms (Article in French). III$^{th}$ Poultry Research Symposium (Batna-Algeria). ed. Titouche Y, *ResearchGate* 62 p.

Akond MA, Alam S, Hassan SMR, Shirin M (2009). Antibiotic Resistance of *Escherichia Coli* Isolated From Poultry and Poultry Environment of Bangladesh. Internet Journal of Food Safety 11:19-23.

Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) (2018). *Salmonella spp.* in animal feed. Opinion of the Anses (Article in French). Collective expertise report. Scientific edition 180 p.

Ashraf MA, Nahed AE, Doha S K, Mohamed EAE, Ayman AS, Ahmed HM, Hossam E, Ahmed K, Reda HS, Mahmoud ES (2020). Incidence, Pathotyping, and Antibiotic Susceptibility of Avian Pathogenic *Escherichia coli* among Diseased Broiler Chicks. Pathogens 9(2):114-129.

Barnes HJ, Gross WB (1997). Colibacillosis. In B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald & Y.M. Saif (Eds), Diseases of Poultry 10th edn (pp. 131-141). Ames, IA: Iowa State University Press.

Bodering A, Ndoutamia G, Ngandolo BN, Ngakou A (2017). Antibiotic use and resistance profile of *Salmonella spp*. and *Escherichia coli* strains isolated from poultry farms in N'Djamena and Doba cities in Chad (Article in French). International Journal of Biology and Chemical Sciences 11(4):1669-1684.

Bodering A, Ndoutamia G, Ngandolo BN, Mopate LY, Ngakou A (2018). Characteristics of poultry farms and assessment of their level of contamination by *Salmonella spp*. and *Escherichia coli* in the towns of N'Djamena and Doba in Chad (Article in French). International Office of Epizootics 37(3):1029-1038.

Buchanan RE, Gibbons NE (1974). Bergey's manual of determinative Bacteriology. 8th ed. Baltimore. The Williams & Wilkins Co., Baltimore, Md. 21202:1246.

Calnek BW, Barnes HJ, Beard CW, McDougald LR, Saif YM (1997). Diseases of Poultry. 10th ed. Iowa State University Press; Ames, IA, USA.

Comité de l'Antibiogramme de la Société française de Microbiologie (CASFM) (CASFM, France) (2018). Available on : http://www.sfm.asso.fr/publi/general.php?pa=1.

Doumbia M (2018). Risk factors for contamination of poultry farms by enteropathogenic microorganisms in the Bingerville department (Article in French). Bachelor's degree in Quality Control from ETS LOKO, Abidjan, Côte d'Ivoire. 38 p.

Ewers C, Janßen T, Kießling S, Philipp HC, Wieler LH (2005). Rapid Detection of Virulence-Associated Genes in Avian Pathogenic *Escherichia coli* by Multiplex Polymerase Chain Reaction. Avian Diseases 49(2):269-273.

Fauchère JL, Avril JL (2002). General and medical bacteriology (Article in French). Ellipses Edition 15:252-253.

Garcia-Graells C, Botteldoorn N, Polet M, Dierick K (2014). Antimicrobial resistance in commensal *E. coli*, *Campylobacter* spp. and Salmonella spp. isolated from carcasses and meat of poultry, beef and pork. Report 2013. 35 pages. (Article in French). Available on: http://www.favv.be/productionanimale/antibioresistance/resultats/_documents/2013AMRFood_FR.pdf

Goualié GB, Bakayoko S, Coulibaly KJ (2020). Practices of biosecurity measures and their consequences on poultry farms in Abidjan district. Food and Environment Safety 29(1):84-91.

Gupta K, Hooton TM, Stamm WE (2001). Increasing antimicrobial resistance and the management of uncomplicated community-acquired urinary tract infections. Annals of Internal Medicine 135:41-50.

Interprofessionel Avicole Ivoirien (IPRAVI) (2017). The poultry letter, newsletter of the poultry of Côte d'Ivoire (Article in French). Special issue 4:20.

Jansen T, Schwarz C, Preikschat P, Voss M, Philipp HC, Wieler LH (2001). Virulence-associated genes in avian pathogenic (APEC) isolated from internal organs of poultry having died from colibacillosis. International Journal of Medical Microbiology 291(5):371-378.

Jawetz E, Melnick J, Adelberg EA (1984). Review of Medical Microbiology. 16th ed. Los Altos, California: Long Medical Publication pp. 122-144.

Johnson TJ, Wannemuehler Y, Doetkott C, Johnson SJ, Rosenberger SC, Nolan LK (2008a). Identification of Minimal Predictors of Avian Pathogenic *Escherichia coli* Virulence for Use as a Rapid Diagnostic Tool. Journal of Clinical Microbiology 46(12):3987-3996.

Johnson JR, Sannes MR, Croy C, Johnston B, Clabots C, Kuskowski MA, Bender J, Smith KE, Winokur PL, Belongia EA (2007). Antimicrobial Drug–Resistant *Escherichia coli* from Humans and Poultry Products, Minnesota and Wisconsin, 2002–2004. Emerging Infectious Diseases 13(6):838-846.

Johnson TJ, Wannemuehler YM, Nolan LK (2008b). Evolution of the iss Gene in *Escherichia coli*. Applied and Environmental Microbiology 74(8):2360-2369.

Koné S, Danho T (2008). Structure of the poultry sector. In: Poultry sector review – Côte d'Ivoire (Article in French). FAO, Rome (Italy) pp. 3-15.

Levine M (1987). *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroadherent. Journal of Infectious Diseases 155:377-390.

Messaï CR, Faci FZ, Benbegri S, Ait Oudhia K, Hamdi MT, Goucem R, Khelef D (2014). Detection of antibiotic residues in broiler meat by the microbiological method (Article in French). III$^{th}$ Poultry Research Symposium (Batna-Algeria). ed. Titouche Y, *ResearchGate* 62 p.

Moreno MA, Dominguez L, Teshoger T, Herrero IA, Perrere ME (2000). Antibiotic resistances monitoring: The Spanish Programme. International Journal of Antimicrobial Agents 14:285-290.

Nakazato G, Brocchi M, de Campos TA, Stehling EG, Dias da Silveira W (2009). Virulence factors of avian pathogenic *Escherichia coli* (APEC). Pesquisa Veterinária Brasileira 29(7):479-486.

Neu HC (1992). The crisis in antibiotic resistance. Science 257:1064-1073.

Okeke IN, Lamikanra A, Edelman R (1999). Socioeconomic and behavioral factors leading to acquired bacterial resistance to antibiotics in developing countries. Emerging Infectious Diseases 5:13-27.

Ouattara ND, Guessennd N, Gbonon V, Toe E, Dadié T, Tiécoura B (2013). Using of Antibiotics in avian sector in Abidjan: Case of Semi-Industrial Farms (Article in French). European Journal of Scientific Research 94:80-85.

Pfaff-McDonough SJ, Horne SM, Giddings CW, Ebert JO, Doetkott C, Smith MH, Nolan LK (2000). Complement resistance related traits among *Escherichia coli* isolates from apparently healthy birds and birds with colibacillosis. Avian Diseases 44:23-33.

Rodriguez-Siek KE, Giddings CW, Doetkott C, Johnson TJ, Fakhr MK, Nolan LK (2005). Comparison of Escherichia coli isolates implicated in human urinary tract infection and avian colibacillosis. Microbiology 151:2097-2110.

Saidi B, Mafirakureva P, Mbanga J (2013). Antimicrobial Resistance of *Escherichia coli* Isolated from Chickens with Colibacillosis in and around Harare, Zimbabwe. Avian Diseases 57(1):152-154.

Salehi ZT, Farashi BS (2006). Antibiotic susceptibility pattern of

*Escherichia coli* strains isolated from chickens with colisepticemia in Tabriz Province, Iran. International Journal of Poultry Science 5:677-684.

Schouler C, Thiriet A, Viardot A, Trotereau A (2018). Exploring the therapeutic potential of coliphages: a curative approach to *Escherichia coli* infections in birds (Article in French). Innovations Agronomiques 66:9-17.

Ungemach FR, Müller-Bahrdt D, Abraham G (2006). Guidelines for prudentuse of antimicrobials and their implications on antibiotic usage in veterinary medicine. International Journal of Medical Microbiology 296(S2):33-38.

Witte W (1998). Medical consequences of antibiotic use in agriculture. Science 279(5353):996-997.

Yang H, Chen S, White DG, Zhao S, McDermott P, Walker R, Meng J (2004). Characterization of multiple-antimicrobial resistant *Escherichia coli* isolates from diseased chickens and swine in China. Journal of Clinical Microbiology 42:3483-3489.

Zhuang QY, Wang SC, Li JP, Liu D, Liu S, Jiang WM, Chen JM (2014). A clinical survey of common avian infectious diseases in China. Avian Diseases 58:297-302.

Zhao S, Maurer JJ, Hubert S, De Villena JF, McDermott PF, Meng J, Ayers S, English L, White DG (2005). Antimicrobial susceptibility and molecular characterization of avian pathogenic *Escherichia coli* isolates. Veterinary Microbiology 107(3-4):215-224.

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# Isolation and morphological characterization of endophytic fungi isolated from mangrove plants along the Kenyan coastline

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**Microorganisms in marine ecosystems are exposed to harsh conditions, thus such systems are of interest in bioprospecting for useful secondary metabolites. The aim of the study was to isolate and identify the fungal endophytes that colonize mangroves. The fungal endophytes were isolated from the leaves, roots, and branches of different mangrove plants (*Bruguiera gymnorrhiza*, *Heritiera littoralis*, *Xylocarpus granatum, Rhizophora mucronata,* and *Avicennia marina*) from Mida creek, Tudor creek and Gazi bay using Point-Centered Quarter Sampling method and then, morphologically characterized. A total of 76 fungal isolates were obtained and identified using macro- and micro-morphological features. The isolates were affiliated with eight different genera (*Aspergillus, Cladosporium, Nigrospora, Fusarium, Alternaria, Lasiodiplodia, Chaetomium and Penicillum*). *Aspergillus* spp. were the most prominent with a colonization frequency of 38.9 and 55.6% in root and branch tissues, respectively while *Chaetomium* species were the least frequent appearing only in one branch tissue. Mida creek had the highest total number of endophytic isolates (52.6%) followed by Gazi bay (27.6%). Majority (30.3%) of the endophytic fungal isolates were obtained from *Avicennia marina.* The results indicate that mangrove species are a source of diverse endophytic fungi that may have useful biotechnological applications.**

**Key words:** Mangrove species, endophytic fungi, fungi diversity, colonization frequency.

## INTRODUCTION

In Kenya, Mangrove forests cover approximately 61,271 ha and are estimated to make up 3% of the total area occupied by natural forest (Kairo et al., 2017). The forests are spatially fragmented, spanning from Vanga to Lamu along the Kenyan Coastal strip (Kirui et al., 2011). Mangroves are known to play a significant role in

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providing nursery habitat and shelter for juvenile fish, sequester carbon (Chmura et al., 2003), and protect the shoreline from soil erosion and tsunamis (Kathiresan and Rajendran, 2005). Nine species of mangroves (*Rhizophora mucronata, Bruguiera gymnorhiza, Ceriops tagal, Sonneratia alba, Avicennia marina, Lumnitzera racemose, Xylocarpus granatum, Xylocarpus moluccensis,* and *Heritiera littoralis)* have been recorded in Kenya (Abuodha and Kairo, 2001).

Harsh conditions (saline and low oxygen) make mangrove ecosystems ideal in the hunt for novel and unique endophytic fungi (Debbab et al*.,* 2010). Endophytic microorganisms are fungi and bacteria that colonize inter- or intra-cellular spaces of plant tissues during at least one phase of their life cycle as described by Compant and Vacher (2019). Endophytic microorganisms can protect the hosts against several biotic and abiotic factors, such as the attack of insects, pathogens, and herbivores (Bamisile et al., 2018). The interaction between plants and endophytic micro-organisms produce several substances of biotechnological interest. For instance, it has been reported that endophytic fungi are known to produce secondary metabolites with bioabsorption systems application in removing heavy metal ions from water. *Aspergillus niger* has been shown to remove lead, cadmium, copper, and nickel ions from wastewater (Ling et al., 2016). Also, it has pharmaceutical application in the production of antimicrobials that inhibit the development of pathogens (Rao et al., 2020). Fungal endophytes colonize the interior parts of healthy plant tissues without causing symptoms of a disease (Kaul et al., 2014).

Plants and microorganisms provide a leading source of natural products with desirable bioactive properties. Fungi are among the most significant eukaryotic organisms that are being explored for their bioactive secondary metabolites in pharmaceutical applications (Keller, 2019). Previous studies suggest that geographical, rather than plant-linked factors contribute to the composition of endophytes in plants (Cannon and Simmons, 2002). Endophytic fungi have been reported to be a source of anti-inflammatory, antibacterial, antiviral, antitumor, antifungal, and other substances found in terpenoid, alkaloid, flavonoid, and steroid extracts (Selvakumar and Panneerselvam, 2018). Mangrove endophytic fungi constitute the second largest group of marine endophytes (Sridhar et al., 2012) with their leaves harboring more diverse fungal endophytes community compared to other parts of the plant (Hamzah et al., 2018). Endophytic fungi have been reported to be abundant in all tissues such as flowers, fruits, stems, roots, and leaves that are potential sources of natural products (Rana et al*.,* 2019). For instance, nigerasterol A and B compounds obtained from *A. niger,* an endophytic fungus residing inside the inner tissues of *Avicennia marina* collected in Hainan Island, China showed cytotoxic activities against the human A549 cell line with $IC_{50}$ values of 1.82 µM (Deshmukh and Prakash, 2018). Endophytic fungi isolated from sponges, seaweeds, and plants from the marine environment have also been reported to produce secondary metabolites with therapeutic potential (Kusam et al., 2019). Mangrove endophytes have been identified as potential producers of novel molecules with diverse biological activities (Deshmukh and Prakash, 2018). Therefore this study aimed to characterize the endophytic fungi that colonize some selected mangrove species which will consequently enhance the conservation and value of the mangroves.

## MATERIALS AND METHODS

### Study sites

This study was conducted on three creeks along the Kenyan Coastline Tudor, Mombasa County (040 00 ' 18.2" S, 0390 38' 17.1" E) located on Mombasa Island and its surround. The Creek extends to about 10 km inland and bounds Mombasa Island on the North West. It is fringed by well-developed mangrove forests composed mainly of *R. mucronata and A. marina* species*.* The area has human settlements with rural villages that are sparsely populated and lacks formal infrastructures such as sewage and solid waste handling facilities (Mohamed et al*.,* 2008).

Mida creek is situated about 100 km North of Mombasa with its mangrove area estimated to cover 1657.8 ha. The creek has no overland freshwater input and hence, benefit from a high groundwater flow. The area (030 21' 07.5" S, 039 0 56' 30.1" E) is composed of *Ceriops tagal*, *R. mucronata* and *A. marina* as the dominant species which grow on mangrove swamps in soils that are excessively saline, deep, and poorly drained (Alemayehu *et al.,* 2014). Gazi bay is located about 55 km south of Mombasa area (040 25' 09.1" S, 039 0 30' 41.0" E) and is sheltered from storms by a coral reef to the South and Chale Peninsula to the East (Maina et al*.,* 2008). These two natural barriers support the mangroves' growth in the protected bay. The area is surrounded by 6.2 $km^2$ of mangroves (Hoberg, 2011) (Figure 1).

### Sample collection

Sampling was done according to Mitchell (2010) with some modifications during the low tides of the day in the dry season (July) on sunny afternoons. The Kenya Marine and Fisheries Research Institute (KMFRI) High-Low and Hourly Tide Predictions chart was used for planning the sampling time (2019 High-Low and Hourly Tide Predictions for Mombasa and Lamu, 2019). A transect of 150 m was established in each mangrove forest cluster and a prop placed every 50 m to represent the center of four compass directions thus dividing the sampling sites into four quarters. In each quarter, the distance from the prop to the nearest large mangrove tree was measured and recorded. A total of 16 trees were recorded in each transect. With the help of a botanist, the selected mangrove trees were identified and recorded. The mangrove species samples were; *B. gymnorrhiza* (11)*, H. littoralis* (8)*, X. granatum* (8), *R. mucronata* (11), and *Avicennia marina* (10). Five healthy leaves, two aerial branches, and two submerged roots were randomly selected and cut using sterile shears. The samples were further chopped into 5 cm pieces before being packed into labeled sterile Ziploc® bags. All samples were transported to Kenya Marine and Fisheries Research Institute (KMFRI, 2019), Mombasa in cooling boxes and then stored in a refrigerator at 4°C until analyses.

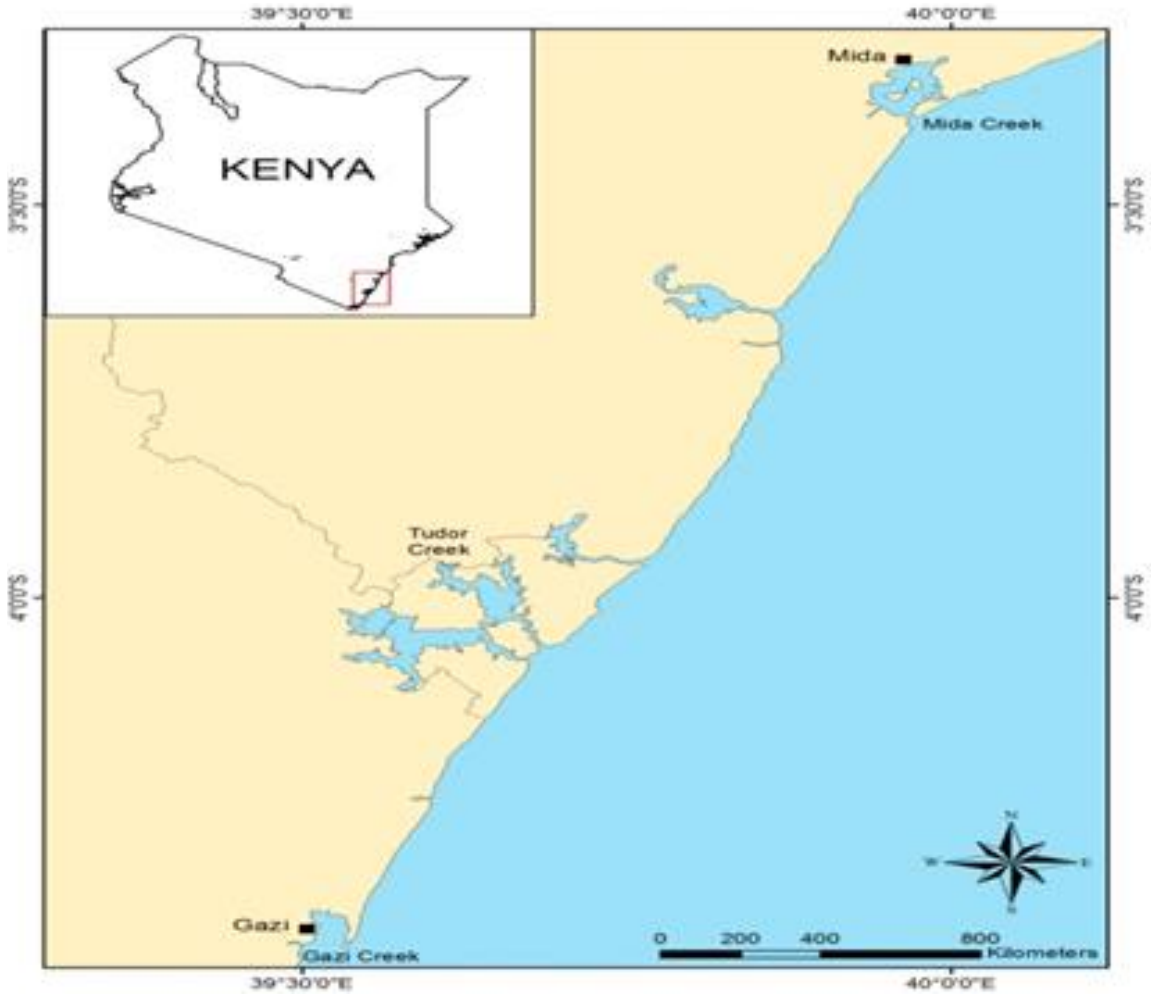



**Figure 1.** Map of Kenyan coastline showing the locations of selected study sites where mangroves grow.

**Sample preparation**

The samples were washed with running water for 2 h to remove mud and debris before rinsing thoroughly with sterile distilled water. All the sample surfaces were sterilized by immersing in 75% ethanol for 1 min followed by 5% sodium hypochlorite for 3 min (leaves) and 5 min (branch and root), 75% ethanol for 30 s and finally rinsed three times with sterile distilled water. The leaf samples were aseptically cut into small pieces of approximately 5 × 5 mm while the root and branch samples were cut into 1 cm cross-section and further split longitudinally to expose the interior section under a biological safety cabinet to prevent contamination (Liu et al., 2007).

**Isolation of fungi**

Sterile Potato Dextrose Agar (PDA) (HiMedia, Mumbai, India) was the medium of choice for this project since it is a general-purpose medium for fungi isolation and was prepared according to the manufacturer's instructions (39 g in 1 L distilled water). The pH of the medium was adjusted to about 4.8, which is low enough to inhibit most bacteria growth by the addition of lactic acid (PDA; with 1:100 lactic acid). Pieces of each sample were placed on labeled PDA Petri plates in triplicates with 3 negative controls and incubated at room temperature for 5 days. Each distinct fungal colony was sub-cultured onto a fresh labeled PDA Petri plate twice to obtain pure isolates which were inoculated into 50 ml sterile Potato Dextrose Broth (TM Media, Rajasthan, India) (24 g in 1 L sterile seawater) and incubated for two weeks at room temperature. For the highest viability when reviving fungal isolates, 80% sterile glycerol was prepared, and an aliquot of 1 ml fungal culture broth and glycerol each were mixed, labeled, and stored at -86°C (Mwamburi et al., 2019).

**Morphological characterization of isolates**

The pure fungal isolates were grouped based on their morphological features (color, texture) and growth rate. Microscopic analysis was carried out using Lacto phenol cotton blue stain. Observations were made using an Image analyzer microscope (Primo Star ZEISS, JenaGermany) supported by Axiocam camera (ERc5s). The following features were observed, recorded and captured; -type of hyphae, mycelium color, type of spores, characteristics of hyphae and sporangia, features of conidia and arrangement of sporangiophore and conidiophores. Identification of the fungal isolates was aided by an identification guide (Dugan, 2017).

**Data analysis**

The colonization frequency (CF%) of endophytic fungi was calculated using the formula according to Deepthi et al. (2018). One-way ANOVA was performed to test whether there were significant differences in the colonization rates between the different genera of endophytic fungi obtained from the mangrove species.

## RESULTS AND DISCUSSION

Endophytic fungal isolates (76) were obtained from five mangrove species namely; *Bruguiera gymnorrhiza, Rhizophora mucronata*, *Avicennia marina, Xylocarpus granatum*, and *Heritiera littoralis* species. All the negative controls did not show the growth of fungi indicating the absence of contamination in the sample preparation and subsequent inoculation steps. Of the 76 fungal isolates recovered, 17.1% were from leaf samples, 59.2% from branch samples, and 23.7% from root samples (Figure 2). Initially, fungal colonies were grouped according to color, shape, and topography followed by microscopic examination of the isolates for characterization of hyphae, mycelium color, type of spores, sporangia,

$$CF\% = \frac{\text{Number of plant tissue colonized by each endophytic fungi } (N_{col})}{\text{Total number of plant tissue studied } (N_{t})} \times 100$$

conidia, sporangiophore and conidiophores arrangement (Figure 3). This examination led to the classification of the 76 isolates into 8 genera (*Aspergillus, Cladosporium, Nigrospora, Fusarium, Alternaria, Lasiodiplodia, Chaetomium,* and *Penicillum*).

A total of eight isolates were placed in the genus *Alternaria* based on their macro- and micro-morphological features. The colony ranged from white to brown in colour on PDA media at 27°C and was relatively rapid-growing. Some of the white colonies were covered in black or yellow pigmentation in the middle while the brown colonies appeared white at the beginning and later darkened to brown by day 14. These isolates belonging were distinguished by brown hyphae with brown septate conidiophores (Figure 7a). The conidia were branched and large (Meena et al., 2017). These isolates were obtained from leaf and branch tissues of *B. gymnorrhiza, R. mucronata, A. marina,* and *X. granatum* species and were found in the three study sites. Isolates have also been obtained from *R. mucronata* species in the Malaysian mangrove forest (Hamzah et al., 2018) .The genus *Alternaria,* have commercially important species, and is one of the most common fungal genera found ubiquitously (Tibpromma et al., 2018). This genus displayed a high divergence in culture and morphology. In addition, the genus has been reported to produce important bioactive compounds useful in the pharmaceutical industry (Yadav et al., 2019).

In the genus *Penicillium*, five isolates were identified based on the observed macro- and micro-morphological features. The colony ranged from grey to brown in color on PDA media at 27°C. Grey colonies were rapidly growing and were observed by day 3 and appeared to be cottony in texture. The growth rate of the brown colonies was slow and was observed by day 7. The genus *Penicillium* was distinguished by septate hyphae, conidiophores, metulae, phialides, and conidia (Figure 7b). Metulae branches that formed on conidiophores with attached flask-shaped phialides were also observed. These results were consistent with a report by Liu et al. (2007). *Penicillium* was isolated from leaf and root tissues of *B. gymnorrhiza* and *A. marina* species from Mida and Tudor Creeks along the Kenyan coast. The genus has also been isolated from *A. marina* and *R. mucronata* species in mangrove plants of Northeast Brazil (Costa et al., 2012). *Penicillium* is a diverse genus belonging to the Ascomycota phylum which occurs worldwide. Its species play important roles as decomposers of organic materials, destructive rots in the food industry, and source of important drugs (Visagie et al., 2014). *Penicillium,* from earlier studies has been found to produce important bioactive compounds with anticancer, antibacterial, antifungal, and cytotoxic activities (Yadav et al., 2019).

Eight isolates were placed in the genus *Fusarium* based on the observed macro- and micro-morphological features. The colony of the isolates ranged from white, brown to purple, and white in PDA media at 27°C. The growth rate of the colonies was slow and appeared on day 6. They were observed to be cottony in texture. The purple and white colonies showed a white ring formed around the purple colony. Members from this genus *Fusarium* were distinguished by branched conidiophores and sickle-shaped macro conidia (Figure 8a) as observed by Munkvold (2017) and (Deepthi et al., 2018). *Fusarium* was isolated from leaf, root, and branch tissues of *B. gymnorrhiza, R. mucronata, A. marina,* and *H. littoralis* species. It was found in Mida Creek and Gazi Bay along the Kenyan coast. The genus *Fusarium* has also been isolated from *R. mucronata* species in the Malaysian mangrove forest (Hamzah et al., 2018) and in *A. marina* from two different locations of Red Sea mangrove forest (Sea and Shebany, 2012).The genus *Fusarium* includes numerous toxigenic species that are pathogenic to plants or humans and can colonize a wide range of niche. The genus comprises around 70 described species and is also one of the most economically important fungal genera because of yield loss due to plant pathogenic activity (Liu et al., 2007). *Fusarium* has been found to produce bioactive compounds of importance in the medical industry. These include anticancer antimicrobial, immunosuppressive and insecticidal compounds

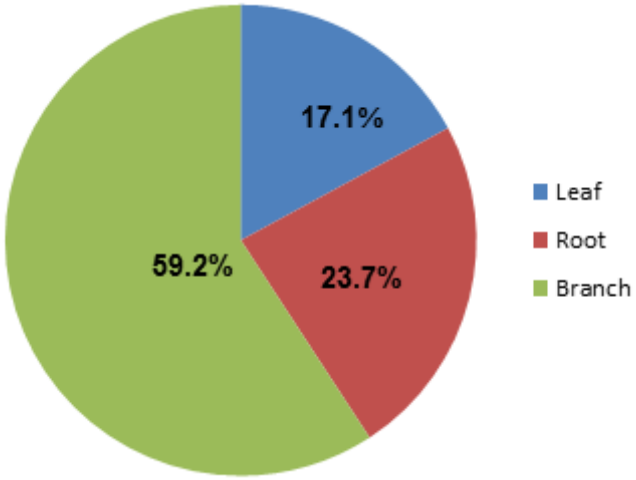



**Figure 2.** Endophytic fungi (%) isolated from different parts of a mangrove tree.

(Rana et al., 2019).

Thirty-five isolates of the endophytic fungi were placed into the genus *Aspergillus* based on the observed macro- and micro-morphological features. The colony of the isolates ranged from grey, green, brown, white, black to brown, and grey in PDA media at 27°C. The colonies grew rapidly and appeared by day 3 and were powdery in texture. The brown and grey colonies formed a grey ring around the brown colony. The genus *Aspergillus* was distinguished by the unifying feature of the asexual reproductive structure, vesicle formation, and septate hyphae (Figure 8b). The phialides appeared to be flask-shaped and covered the surface of the vesicle. Above the phialides, the conidia formed radial chains (Liu et al., 2007; Makhuvele et al., 2017).

*Aspergillus* species were isolated from leaf, root, and branch tissues of *B. gymnorrhiza, R. mucronata, A. marina, H. littoralis, and X. granatum* species from all the study sites. The genus *Aspergillus* has also been isolated from two different locations of the Red Sea mangrove forest (Sea and Shebany, 2012) and in *A. marina* from the mangrove rich area of Thazhekavu in Madakkara (Gilna and Khaleel, 2011). *Aspergilli* show a large taxonomic divergence in terms of their morphology (Varga and Samson, 2008). They have been reported as the most dominant endophytic fungal inhibiting internal plant tissues and are an enormous source of chemical compounds with promising biological activities (El-Hawary et al., 2020). Some members of this genus are used in the fermentation industry but are also responsible for various plant diseases (Perrone et al., 2007). *Aspergilli* grow in a wide range of habitats, mostly in soil, dead matter and some are capable of colonizing living animal or plant hosts. In total, approximately 350 species have been identified in this genus (Samson et al., 2014). *Aspergillus* has been reported to be useful in the pharmaceutical industry as a source of anticancer, antitumor, cytotoxic, and antimicrobial compounds (Rana et al., 2019).

In the *Cladosporium* genus, ten isolates were identified based on the observed macro- and micro-morphological features. The colony of the isolates ranged from greyish white to brown in PDA media at 27°C. The growth rate of colonies was slow and appeared by day 7. The texture was observed to be velvety. Septate brown hyphae, erect and pigmented conidiophores and conidia were observed (Figure 9a). The conidia were brown and appeared in branching chains (Wijayawardene et al., 2017). *Cladosporium* was isolated from leaf, root, and branch tissues of *B. gymnorrhiza, R. mucronata,* and *A. marina species.* It was found in Mida and Tudor Creeks along the Kenyan coast. The genus *Cladosporium* has also been isolated from *R. mucronata* species in the Malaysian mangrove forest (Hamzah et al., 2018). It is a large genus comprising species that are saprobes, endophytes, and pathogens (Tibpromma et al., 2018). *Cladosporium* has also been reported to produce antifungal compounds (Selvakumar and Panneerselvam, 2018).

One isolate was identified in the genus *Chaetomium* based on the observed macro- and micro-morphological features. Colony colour ranged from white to chocolate-brown in PDA media at 27°C while the growth rate was slow and appeared by day 7. The colony texture was powdery with non-sporulating hyphae (Figure 9b). Terminal hairs were brown with paler tips, wavy or loosely coiled, and intertwined (Zhai et al., 2018). *Chaetomium* was found in only one of the study sites; Gazi Bay along the Kenyan coast. It was isolated from a branch tissue of *A. marina.* The genus *Chaetomium* has also been isolated from two different locations in the Red Sea mangrove forest (Sea and Shebany, 2012). It has been reported to produce secondary metabolites with

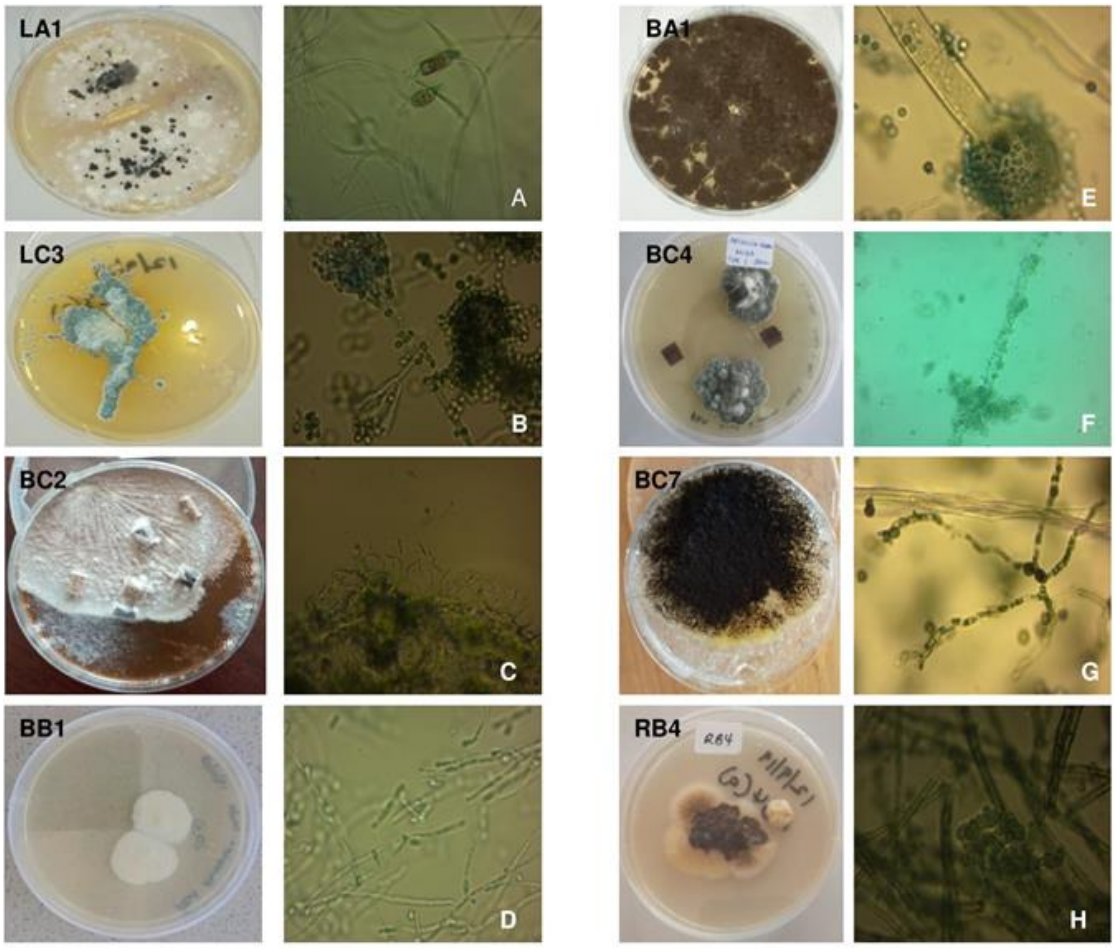



**Figure 3.** Endophytic fungi isolated from the leaves, roots and branches of mangrove species. Shown here are some representatives of the fungal isolates from the 8 genus identified; **a:** *Alternaria;* b: *Penicillum;* c: *Chaetomium;* d: *Fusarium;* e: *Aspergillus* f: *Cladosporium*; g: *Nigrospora;* h: *Lasiodiplodia.* All fungal isolates were from the inside tissues (leaf, root and branch) of the mangrove species and cultivated on Potato Dextrose Agar media for 7 to 15 days at 27°C.

potential bioactivity (Selvakumar and Panneerselvam, 2018).

Some six isolates were placed in the genus *Nigrospora* based on the observed macro- and micro-morphological features. Colony colour ranged from grey, white to black in PDA media at 27°C. The grey colonies were observed to be velvety, white colonies cottony, and black colonies powdery in texture. Septate mycelia and conidia which were on the swollen conidiophores were observed (Figure 10a) (Gond et al., 2007; Rathod et al., 2014 and Deepthi et al., 2018). *Nigrospora* was isolated from the root and branch tissues of *B. gymnorrhiza* and *A. marina species.* It was found in Mida Creek and Tudor Creek along the Kenyan coast. The genus *Nigrospora* has also been isolated from *A. marina* plant collected from different coastal areas of Pakistan (Tariq et al., 2006). *Nigrospora* is a monophyletic genus belonging to Apiosporaceae. The species in this genus are phytopathogenic, endophytic, and saprobic on different hosts (Hao et al., 2020). Antifungal bioactive compounds have been found in *Nigrospora* (Rana *et* al*.,* 2019; Deshmukh and Prakash, 2018).

Based on the observed macro- and micro-morphological features, 3 isolates were placed in the genus *Lasiodiplodia*. The colour of the colony ranged from white to brown in PDA media at 27°C and grew rapidly appearing on day 3. Observed to be woolly in texture, hyaline and brown conidia bearing longitudinal striations and conspicuous conidiomatal paraphyses were also observed (Figure 10b) (Abdollahzadeh et al. (2010)). The genus *Lasiodiplodia* was found in Mida and Tudor Creeks and was isolated from the root and branch tissues of *B. gymnorrhiza* and *A. marina* species*.* This genus has also been isolated from *B. gymnorrhiza* and *A. marina* species in South Africa (Osorio et al., 2017)*.* Deshmukh et al. (2018) reported the presence of bioactive

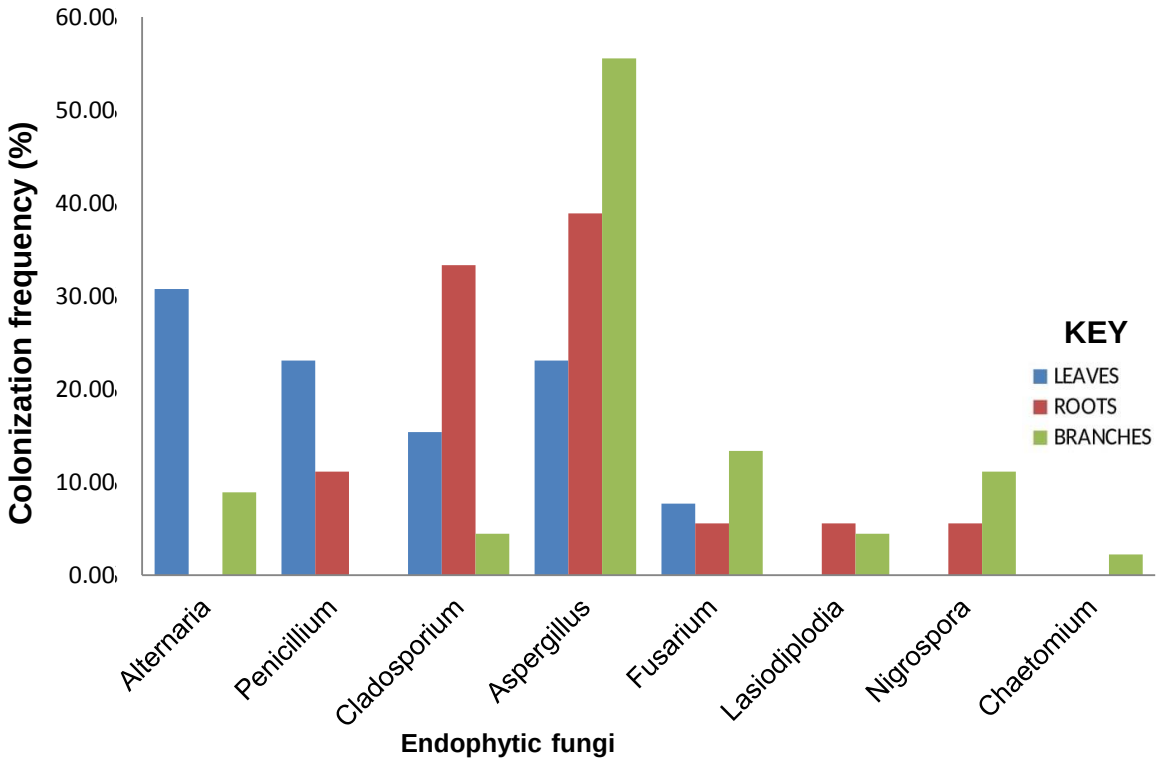



**Figure 4.** Colonization frequency of endophytic fungi on leaf, root and Branch tissues of mangrove species.

**Table 1.** Colonizing frequency of endophytic fungi isolated from mangrove trees.

| No. of Isolates | Name of Endophytic Fungi (sp.) | Leaves | | Roots | | Branches | |
|---|---|---|---|---|---|---|---|
| | | Colonization frequency (%)($N_{t=13}$) | | Colonization frequency (%)($N_{t=18}$) | | Colonization frequency (%)($N_{t=45}$) | |
| | | ($N_{col}$) | % CF | ($N_{col}$) | % CF | ($N_{col}$) | % CF |
| 8 | *Alternaria* | 4 | 30.77 | - | - | 4 | 8.89 |
| 5 | *Penicillium* | 3 | 23.08 | 2 | 11.11 | - | - |
| 10 | *Cladosporium* | 2 | 15.38 | 6 | 33.33 | 2 | 4.44 |
| 35 | *Aspergillus* | 3 | 23.08 | 7 | 38.89 | 25 | 55.56 |
| 8 | *Fusarium* | 1 | 7.69 | 1 | 5.56 | 6 | 13.33 |
| 3 | *Lasiodiplodia* | - | - | 1 | 5.56 | 2 | 4.44 |
| 6 | *Nigrospora* | - | - | 1 | 5.56 | 5 | 11.11 |
| 1 | *Chaetomium* | - | - | - | - | 1 | 2.22 |

$N_t$ =Total number of plant tissue studied; $N_{col}$ = Number of plant tissue colonized by each endophytic fungi%; CF =Colonization frequency**_=** Absence of isolates**.** The isolates were coded (A-E)according to the mangrove species *B. gymnorrhiza, R. mucronata, A. marina, X. granatum,* and *H. littoralis,* respectively and part of the tree that was sampled (L, B & R), representing leaf, branch, and root, respectively.

compounds in *Lasiodiplodia* with medical importance. From this study, the overall mean colonization frequency in the genus *Aspergillus* was found to be significantly higher compared to the other genera identified (P<0.05) (Figure 4). *Alternaria* recorded the highest colonization frequency in leaves (30.8%) while *Aspergillus* had the highest colonization frequency in both branches and roots (55.6 and 38.9%, respectively). The genus *Chaetomium* had the least colonization frequency of 2.2% appearing only in a branch tissue (Table 1 and Figure 5).

Of the five mangrove species investigated, *A. marina* and *B. gymnorrhiza* recovered the highest number of endophytic fungal isolates at 30.3 and 26.3%, respectively,

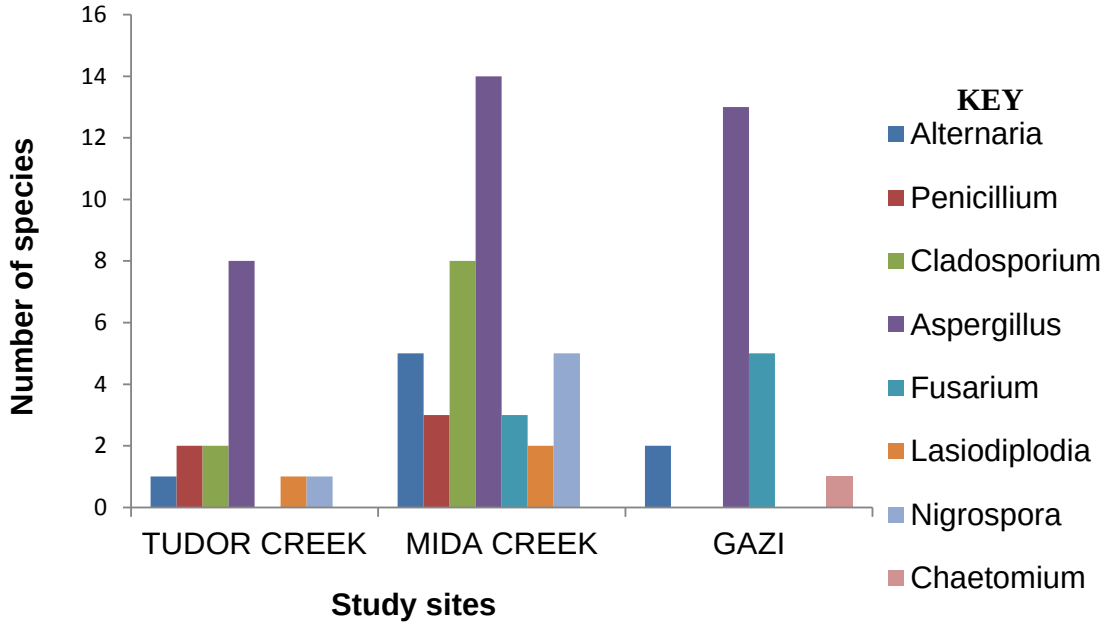



**Figure 5.** Mangrove endophytic fungi isolated from different sites of the Kenyan coast.

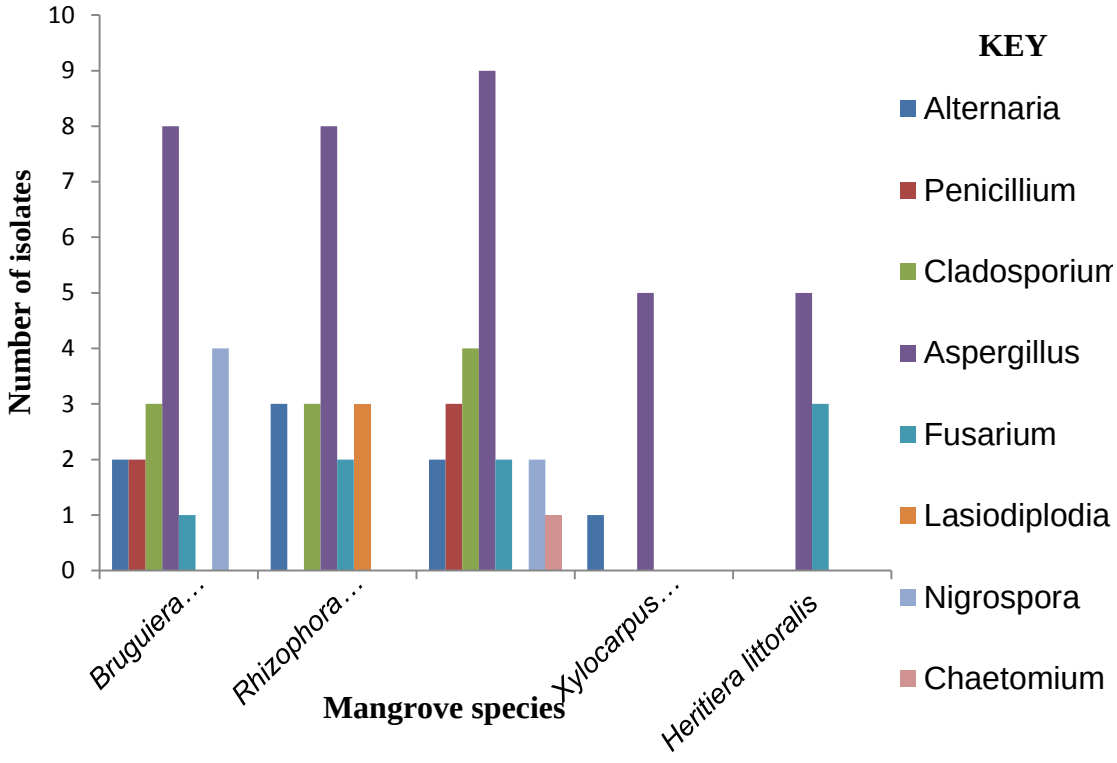
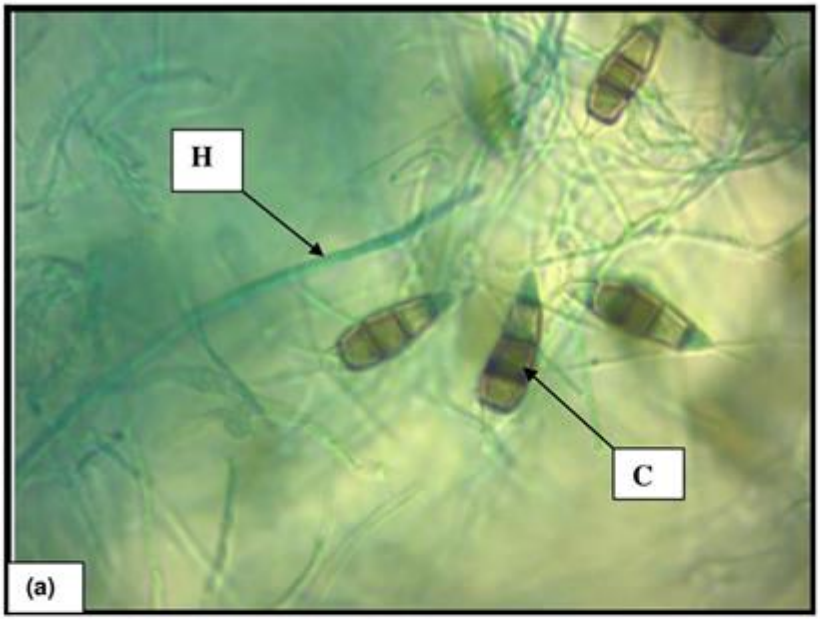
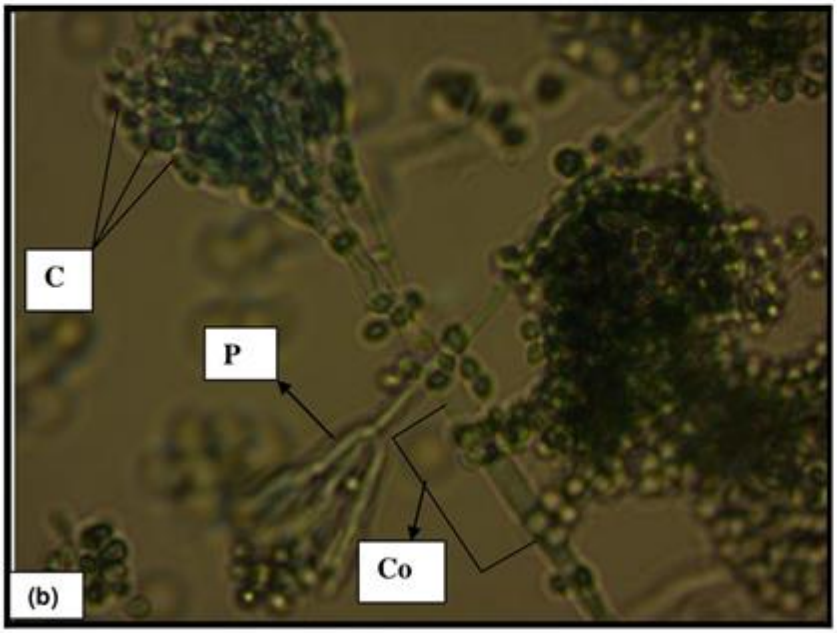



**Figure 6.** Mangrove endophytic fungi isolated from different mangrove species in the Kenyan coast.

while *X. granatum* had the least recovery rate of 7.9% (Figure 6). *Aspergillus* was dominant in the five mangrove species: *A. marina* (25.7%), *B. gymnorrhiza* (22.9%), *R. mucronata* (22.9%) and *H. littoralis* (14.3%). This was followed by *Nigrospora* from *B. gymnorrhiza* species (66.7%). *Penicillum* was isolated from *A. marina* (60%)




**LB1**: Microscopic features of ***Alternaria*** sp. (10x 40X) H= Hyphae, C=conidia.

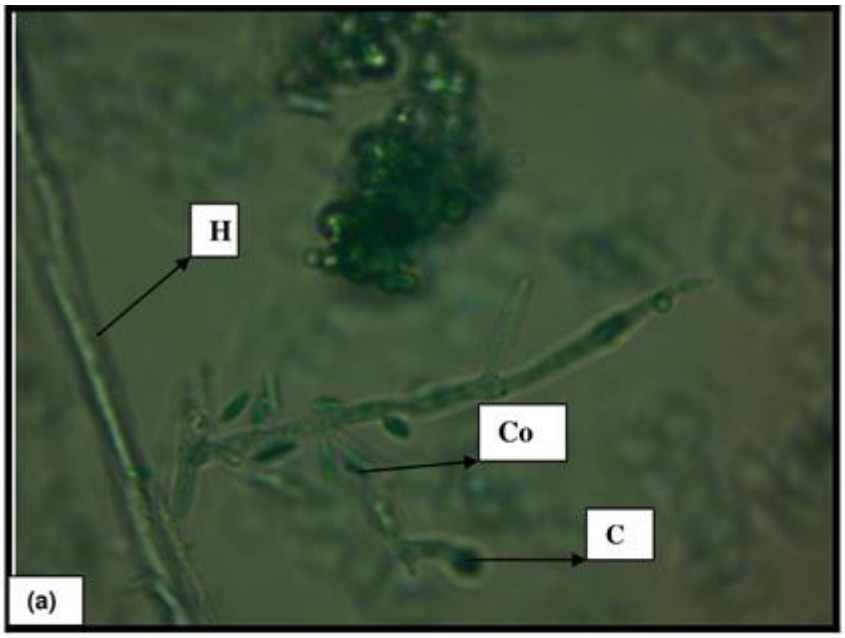



**LC3:** Microscopic features of ***Penincillum*** sp. (10x 40X) conidiophores bearing metulae and chains of conidia, C=Conidia, Co=Conidiophore, P=Philades

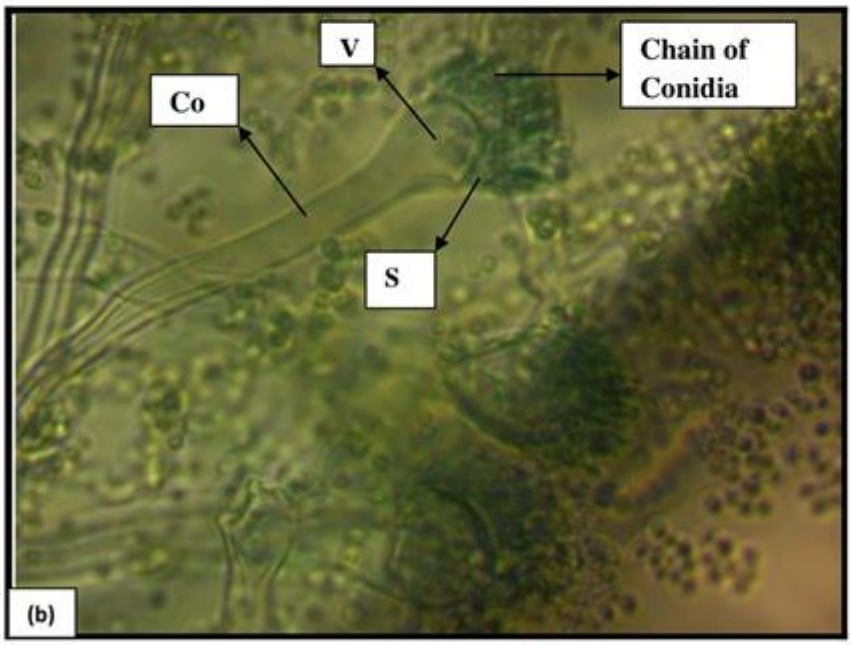
**Figure 7.** (a) and (b) Microscopic features of Genus Alternaria and Penicillium respectively.

*and B. gymnorrhiza* (40%).

The genus *Alternaria* was recovered *from R. mucronata (*37.5%) and *X. granatum (*12.5%) while genus *Fusarium (*37.5%) was recovered from *H. littoralis.* The genus *Cladosporium* (30%) was isolated from *R. mucronata.* The genus *Lasiodiplodia* and *Chaetomium* were recovered from *R. mucronata* and *A. marina*, respectively. Mida Creek had the highest number of fungal isolates (52.6%) as compared to Gazi bay (27.6%) and Tudor Creek (19.7%) (Figure 5). The factors influencing the variations




**RB3**: Microscopic features of ***Fusarium*** sp. (10x40X) H=Hyphae, C=Conidia, Co= Conidiophore.

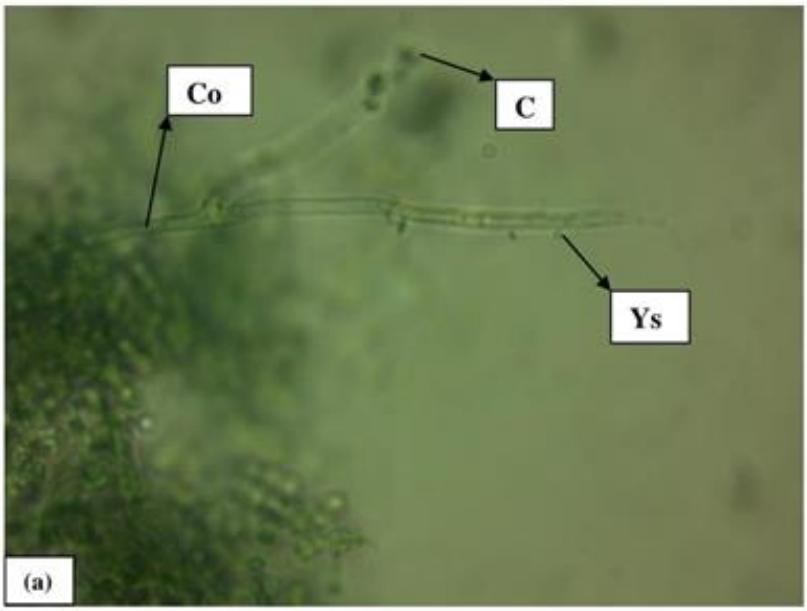



**BE3**: Microscopic features of ***Aspergillus*** sp. (10x 40X) conidiophores bearing sterigmata and chains of conidia C=Conidia, Co=Conidiophore, S=Sterigmata, V=Vescile.

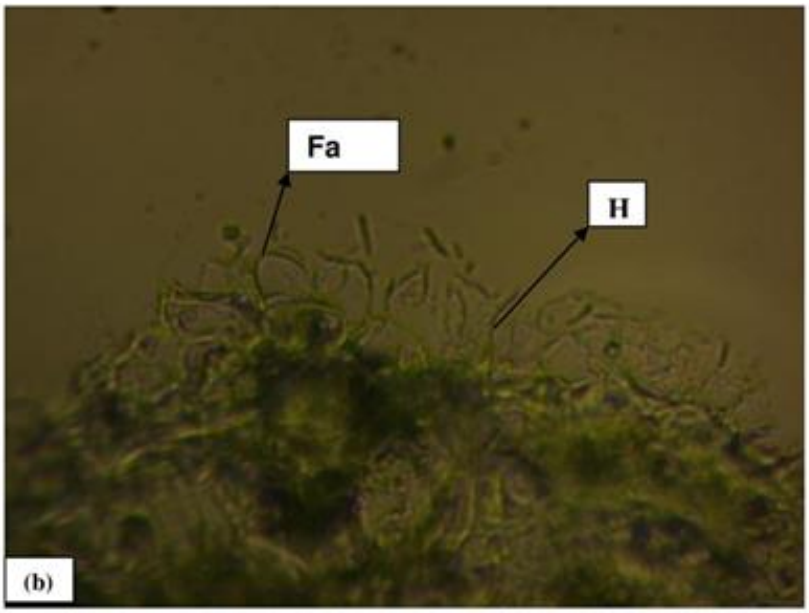
**Figure 8. (**a) and (b) Microscopic features of genus *Fusarium* and *Aspergillus* respectively.

of these fungal species on different plant parts, mangrove trees and locations are still obscure.

Biodiversity analysis of endophytic fungi showed that all the recovered endophytes belong to the division Ascomycota and three classes (Figure 11). *Alternaria, Cladosporium* and *Lasiodiplodia* species belong to class




**RC8:** Microscopic features of ***Cladosporium*** sp. (10x 100X) Ys=Young spore, C=Conidia, Co=Conidiophore.



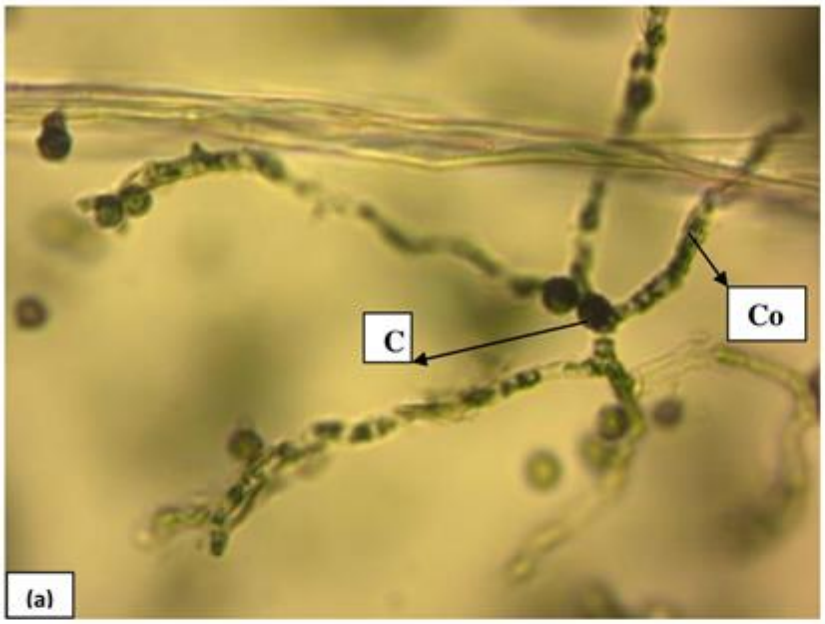

**BC2:** Microscopic features of ***Chaetomium*** sp. (10x 100X) Fa= Floccose aerial, H=Hyphae,

**Figure 9.** (a) and (b) Microscopic features of Genus Cladosporium and Chaetomium respectively.

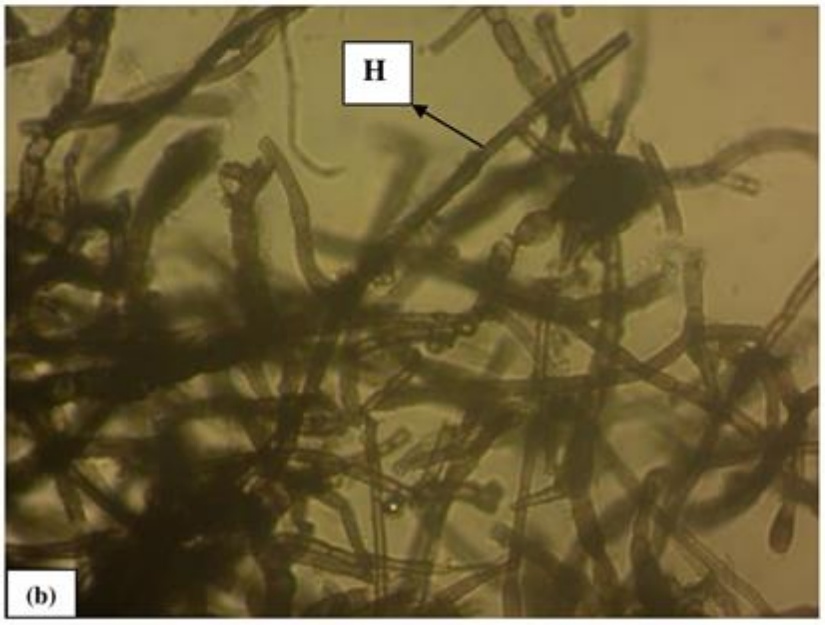
Dothiodeomycetes, *Fusarium, Nigrospora* and *Chaetomium* species belong to class Sordariomycetes while *Penicillium* and *Aspergillus* species belong to class Eurotiomycetes. Of the seventy-six recovered isolates, 40 belong to Eurotiomycetes, 21 to Dothideomycetes and 15 to Sordariomycetes.

## Conclusion

In this study, the 76 endophytic fungi recovered were associated with eight genera (*Aspergillus, Cladosporium, Nigrospora, Fusarium, Alternaria, Lasiodiplodia, Chaetomium,* and *Penicillum), and were obtained* from




**BC7:** Microscopic features of ***Nigrospora*** sp. (10x 100X) C=Conidia, Co=Conidiophore




**BB7:** Microscopic features of ***Lasiodiplodia*** sp. (10x 100X) H=Hyphae.

**Figure 10.** (a) and (b) Microscopic features of Genus Nigrospora and Lasiodiplodia respectively.

leaves, roots, and branches of *B. gymnorrhiza, R. mucronata*, *A. marina, X. granatum*, and *H.* colonization frequency in both branches and roots (55.6% and 38.9%, respectively), while the genus *Alternaria* recorded the highest colonization frequency in leaves (30.8%). The results show the distribution of mangrove fungal endophytes within the studied sites and contribute *littoralis* species. Eurotiomycetes was the most dominant fungal class. The genus *Aspergillus* had the highest to the growing inventory of mangrove endophytic fungi. Further characterization of mangrove fungal endophytes might offer valuable information about their biotechnological potential, a baseline for subsequent functional and bioprospecting studies.

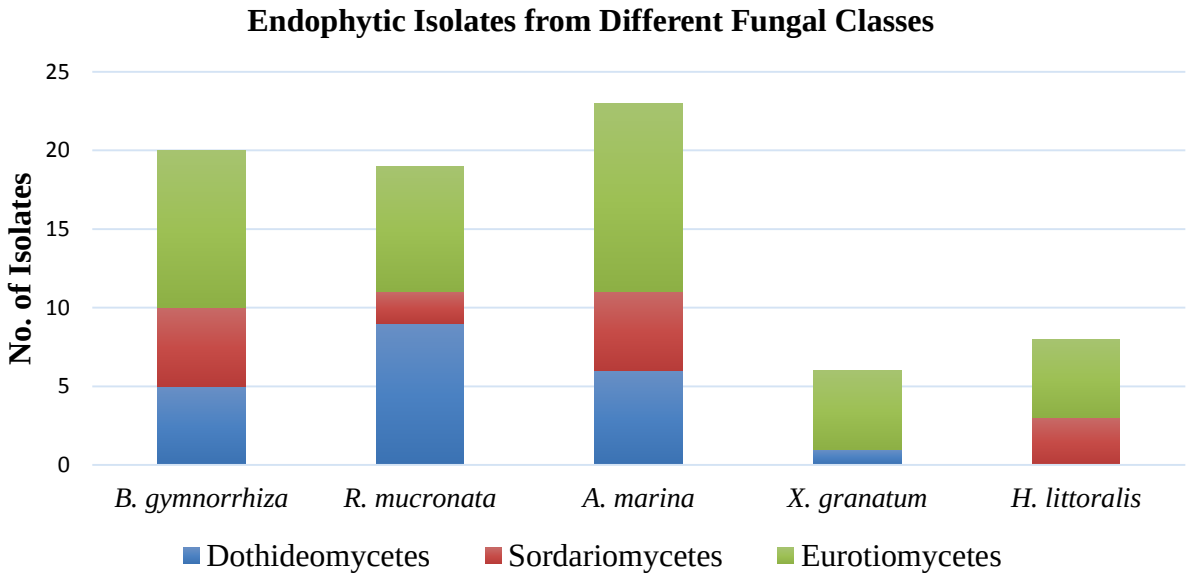



**Figure 11.** Mangrove endophytic fungi biodiversity.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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## REFERENCES

Abdollahzadeh J, Javadi A, Goltapeh EM, Zare R, and Phillips AJL (2010). Phylogeny and morphology of four new species of *Lasiodiplodia* from Iran. Journal of Persoonia: Molecular Phylogeny and Evolution of Fungi 25:1.

Abuodha PAW, Kairo JG (2001). Human-induced stresses on mangrove swamps along the Kenyan coast. Hydrobiologia 458:255-265.

Alemayehu F, Richard O, James KM, and Wasonga O (2014). Assessment of Mangrove Covers Change and Biomass in Mida creek, Kenya. Open Journal of Forestry 4(4):398-413.

Bamisile BS, Dash CK, Akutse KS, Keppanan R, Wang L (2018). Fungal endophytes: beyond herbivore management. Frontiers in Microbiology 9(544):1-11.

Cannon P, Simmons C (2002). Diversity and Host Preference of Leaf Endophytic Fungi in the Iwokrama Forest Reserve, Guyana. Mycologia 94:210-220.

Chmura GL, Anisfeld SC, Cahoon DR, Lynch JC (2003). Global carbon sequestration in tidal, saline wetland soils. Global Biogeochemical Cycles 17(4).

Compant S, Vacher C (2019). Sources, niches and routes of colonization by beneficial bacterial endophytes. Endophyte Biotechnology: Potential for Agriculture and Pharmacology 8:32.

Costa I PMW, Maia LC, Cavalcanti MA (2012). Diversity of leaf endophytic fungi in mangrove plants of Northeast Brazil. Brazilian Journal of Microbiology 43(3):1165-1173.

Debbab A, Aly AH, Lin WH, Proksch P (2010). Bioactive compounds from marine bacteria and fungi. Microbial Biotechnology 3(5):544-563.

Deepthi VC, Sumathi S, Faisal M and Elyas KK (2018). Isolation and identification of endophytic fungi with antimicrobial activities from the leaves of Elaeocarpus sphaericus (gaertn.) K. Schum and Myristicafragranshoutt. International. Journal of Pharmaceutical Sciences and Research 9(7):2783–2791.

Deshmukh S, Prakash V (2018). Mangrove-Associated Fungi: A Novel Source of Potential Anticancer Compounds. Journal of Fungi — Open Access Mycology Journal 4:101.

Dugan F (2017). The identification of fungi : an illustrated introduction with keys, glossary, and guide to literature. Available at: https://apsjournals.apsnet.org/doi/pdf/10.1094/9780890545041.fm

El-Hawary SS, Moawad AS, Bahr HS, Abdelmohsen UR, Mohammed R (2020). Natural product diversity from the endophytic fungi of the genusAspergillus. RSC Advances 10(37):22058-22079.

Gilna VV, Khaleel AKM (2011). Diversity of fungi in mangrove ecosystem. Journal of Experimental Sciences 2(2):47-48.

Gond SK, Verma VC, Kumar A, Kumar V, Kharwar RN (2007). Study of endophytic fungal community from different parts of Aegle marmelos Correae (Rutaceae) from Varanasi (India). World Journal of Microbiology and Biotechnology 23(10):1371-1375.

Hamzah TNT, Lee SY, Hidayat A, Terhem R, Faridah-Hanum I, Mohamed R (2018). Diversity and characterization of endophytic fungi isolated from the tropical mangrove species, Rhizophora mucronata, and identification of potential antagonists against the soil-borne fungus, Fusarium solani. Frontiers in Microbiology https://doi.org/10.3389/fmicb.2018.01707

Hao Y, Aluthmuhandiram JVS, Chethana KWT, Manawasinghe IS, Li X, Liu M, Hyde KD, Phillips AJL, and Zhang W (2020). Nigrospora Species Associated with Various Hosts from Shandong Peninsula, China. Mycobiology 48(3):169-183.

Hoberg J (2011). Economic Analysis of Mangrove Forests: A Case Study in Gazi Bay, Kenya. United Nations Environment Programme.

Kairo JG, Nyabuti A, Mkazalla A, Omar M, Musingo M, and Pakia M (2017). Republic of Kenya on Forest Policy Summary for Policy Makers 1:1-8.

Kathiresan K, Rajendran N (2005). Coastal mangrove forests mitigated tsunami. Estuarine, Coastal and shelf Science 65(3):601-606.

Kaul S, Ahmed M, Sharma T, Dhar MK (2014). Unlocking the Myriad Benefits of Endophytes: An Overview. Microbial Diversity and Biotechnology in Food Security pp. 41-57.
Keller NP (2019). Fungal secondary metabolism: regulation, function and drug discovery. Nature Reviews. Microbiology 17(3):167-180.
Kenya Marine and Fisheries Research Institute (KMFRI) (2019). 2019 high-low and hourly tide predictions for Mombasa and Lamu. Available at: https://www.kmfri.co.ke/images/pdf/2019_Kenya_Tide_Predictions.pdf
Kirui B, Kairo JG, Bosire J, Viergever K, Rudra S, Huxham M, Briers R (2011). Mapping of mangrove forest land cover change along the Kenya coastline using Landsat imagery. Ocean and Coastal Management 83:19-24.
Kusam LR, Divjot K, Imran S, Neelam Y, Ajar NY, Vinod K, Bhim PS, Harcharan SD, Anil KS (2019). Biodiversity of Endophytic Fungi from Diverse Niches and Their Biotechnological Applications. Advances in Endophytic Fungal Research pp. 105-144.
Ling OM, Lim PT, Mujahid A, Proksch P, Müller M (2016). Initial screening of mangrove endophytic fungi for antimicrobial compounds and heavy metal biosorption potential.Sains Malaysiana 45(7):1063-1071.
Liu A, Wu X, Xu T (2007). Research advances in endophytic fungi of mangrove. The Journal of Applied Ecology 18(4):912-918.
Maina GW, Obura D, Alidina H, Munywoki B, Program F, Author C (2008). Increasing catch in an Over-exploited Reef Fishery: Diani-Chale, Kenya, from 1998 to 2006. East 1234:12.
Makhuvele R, Ncube I, Jansen van Rensburg EL, La Grange DC (2017). Isolation of fungi from dung of wild herbivores for application in bioethanol production. Brazilian Journal of Microbiology 48(4):648-655.
Meena M, Swapnil P, Upadhyay RS (2017). Isolation, characterization and toxicological potential of Alternaria-mycotoxins in different Alternaria species from various regions of India. Scientific Reports 7(1):8777.
Mitchell K (2010). Quantitative analysis by the point-centered quarter method. arXiv preprint arXiv:1010.3303.
Mohamed MO, Neukermans G, Kairo JG, Dahdouh-Guebas F, Koedam N (2008). Mangrove forests in a Peri-urban setting: The case of Mombasa (Kenya). Wetlands Ecology and Management 17(3):243-255.
Munkvold G (2017). Fusarium Species and Their Associated Mycotoxins. In Methods in molecular biology 1542:51-106.
Mwamburi SM, Mbatia BN, Remmy K, Kirwa EM, Naumih N (2019). Production of polyhydroxyalkanoates by hydrocarbonaclastic bacteria. African Journal of Biotechnology 18:352-364.
Osorio JA, Crous CJ, Beer ZW, Wingfield MJ, and Roux J (2017). Endophytic Botryosphaeriaceae, including five new species, associated with mangrove trees in South Africa. Fungal Biology 121(4):361-393.
Perrone G, Susca A, Cozzi G, Ehrlich K, Varga J, Frisvad JC, Meijer M, Noonim P, Mahakarnchanakul W, Samson RA (2007). Biodiversity of Aspergillus species in some important agricultural products. Studies in Mycology 59:53-66.
Rana KL, Kour D, Sheikh I, Yadav N, Yadav AN, Kumar V, Singh B, Dhaliwal H, Saxena A (2019). Biodiversity of Endophytic Fungi from Diverse Niches and Their Biotechnological Applications. Advances in Endophytic Fungal Research pp. 105-144.
Rao HY, Kamalraj S, Jayabaskaran C (2020). Fascinating fungal endophytes associated with medicinal plants: Recent advances and beneficial applications. Microbial Endophytes 263-289.
Rathod D, Dar MA, Gade AK, Rai M, Baba G (2014). Griseofulvin Producing Endophytic Nigrosporaoryzae from Indian Emblica officinalis Gaertn: a New Report.
Samson RA, Visagie CM., Houbraken J, Hong SB, Hubka V, Klaassen CHW, Perrone G, Seifert KA, Susca A, Tanney JB, Varga J, Kocsubé S, Szigeti G, Yaguchi T, Frisvad JC (2014). Phylogeny, identification and nomenclature of the genus Aspergillus. Studies in Mycology 78(1):141-173.
Sea R, Shebany YM (2012). Antimicrobial Activity of Endophytic Fungi Isolated from Avicennia marina Plant, Red Sea, Egypt. Egyptian Journal of Microbiology 47(1):141-152.
Selvakumar V, Panneerselvam A (2018). Bioactive compounds from endophytic fungi. Fungi and their Role in Sustainable Development: Current Perspectives pp. 699-717.
Sridhar KR, Alias SA, Pang KL (2012). 14 Mangrove fungi. Marine Fungi and Fungal-like Organisms P 253.
Tariq M, Dawar S, Mehdi FS (2006). Isolation of fungi from Avicennia marina. Pakistan Journal of Botany 38(3):805-810.
Tibpromma S, Hyde K, Mckenzie E, Bhat D, Phillips A, Wanasinghe D, Samarakoon MC, Jayawardena R, Dissanayake A, Tennakoon D, Doilom M, Phookamsak R, Tang A, Xu J, Mortimer P, Promputtha I, Maharachchikumbura S, Khan S, Karunarathna S (2018). Fungal diversity notes 840-928: micro-fungi associated with Pandanaceae. Fungal Diversity 93(1):1-160.
Varga J, Samson RA (2008). Aspergillus in the genomic era. Wageningen Academic Publishers.
Visagie CM, Houbraken J, Frisvad JC, Hong SB, Klaassen CHW, Perrone G, Seifert KA, Varga J, Yaguchi T, Samson RA (2014). Identification and nomenclature of the genus Penicillium. Studies in Mycology 78:343-371.
Wijayawardene NN, Hyde KD, Rajeshkumar KC, Hawksworth DL, Madrid H, Kirk PM, Braun U, Singh RV, Crous PW, Kukwa M, Luecking R (2017). Notes for genera: Ascomycota. Fungal Diversity 86(1):1-594.
Yadav AN, Singh S, Mishra S, Gupta A (2019). Recent Advancement in White Biotechnology Through Fungi. Volume 3: Perspective for Sustainable Environments. https://www.springer.com/gp/book/9783030255053
Zhai X, Luo D, Li X, Han T, Jia M, Kong Z, Ji J, Rahman K, Qin L, Zheng C (2018). Endophyte Chaetomium globosum D38 promotes bioactive constituents accumulation and root production in Salvia miltiorrhiza. Frontiers in Microbiology 8:2694.

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