Original Research Article

Identification, Antagonistic Potentials and Plasmid Profiling of Micro-Organisms Associated with Termitarium andMacerated dead termites from Cashew Trees in Ibule-Soro, Akure Nigeria

ABSTRACT

This research was carried out to identify microorganisms associated with termitarium on cashew tree barks and macerated dead termites from Ibule-Soro, Akure, Nigeria. Bacterial isolates such as *Bacillus sp, Micrococcus sp, Corynebacterium sp, Streptococcus sp*were identified,whilefungi isolates such as *Aspergilus niger, Fusarium solani* and *Penicillium nonatum* were identified. The result of antimicrobial sensitivity patterns of the isolates showed that all the bacteria were susceptible to at least three of the antibiotics. However, *Micrococcus sp* and *Bacillus sp* were screened to be multiple antibiotic resistant isolates. Plasmid profiling of these multiple antibiotic resistant bacteria isolates were carried out to determine the size of the bacteria plasmids and genetic basis of their antimicrobial resistance. The isolates were cured of their plasmid and subjected to antibiotic treatments again to determine whether their susceptibility to antibiotic is chromosomal or extra-chromosomal. Antagonistic properties of the isolated bacteria and fungi were determined against known bacterial pathogens such as *Staphylococus aureus, Shigella sp, Salmonella sp,* and *Escherichia coli,* the result showed that only the fungus *Penicillium notatum* showed positive and mild antagonistic potential against the selected pathogens. Findings from this research showed the potentials of termite nest as reservoirs for beneficial microorganisms with great antagonistic properties.

Keywords: Resistance; Antagonistic; Macerated; Plasmid Profiling; Termitarium; Cashew Tree

INTRODUCTION

Termitarium is the nest of termites composed of partly digested food materials and fecal matter of termites, containing minerals and other organic constituents that provides a suitable environment for the existence of a huge diversity of microorganisms (Longair, 2004). The microbial population of dual origins from both termites and neighbouring soil might result in greater microbial diversity in the termitarium than termite gut or termite-associated soil. However (Fall *et al.,* 2007).

Termitarium are associated with cashew trees (*Anacardium occidentale),* with these termites boring holes through the plant and using it as a safe haven.*Anacardium occidentale* is a tropical plant that produces the cashew seed and the cashew apple. The cashew nut, often simply called a cashew, is widely consumed. It is eaten on its own, used in recipes, or processed into cashew cheese or cashew butter. The shell of the cashew seed yields derivatives that can be used in many applications including lubricants, waterproofing, paints, etc. In terms of uses, it is known that every part of the cashew plant is very useful such that they possess medicinal properties (Hamad and Mubofu, 2015).

The bark and the leaf of the tree possess medicinal benefits and have been used as remedy for both diarrhea and colic. Cashews leaf extract is utilized to reduce blood sugar and blood pressure levels. Oils extracted from the seeds prove effective in the preparation of insecticides. The infusion of the bark of the cashew tree has astringent properties and is used as a mouthwash for treating oral ulcers and as a remedy for sore throatand influenza. Leaves of the cashew tree, when boiled with water, serve as an anti-pyretic and are used for the treatment of aches and pains throughout the body (Hamad and Mubofu, 2015).

Materials and Methods

Collection of samples

Termite feeding tubes (Termitarium) containing live termites and cashew tree barks were collected from cashew tree into sterile sample collectors. These samples were collected at a farm settlement in Ibule-soro village, Ondo state, Nigeria. Samples were analyzed within 6hrs of collection.

Preparation of samples for microbial isolation

The method described in Fall *et al*., (2007) was adopted for sample preparation. The diluent used for the samples was sterile distilled water. Using a sterile syringe, a 9ml of sterile distilled water was dispensed into 3 different test tubes under aseptic conditions and a 1g of the termitarium was poured into the first test tube, homogenized and a 1ml was taken out for a serial dilution procedure till the 5^{th} dilution was obtained. A 1ml of the last dilution factor was seeded on already sterilize media for fungal and bacterial isolation (Fawole and Oso, 2007).

Bacteria Isolation from termitarium

A 1ml of dilution of choice from already prepared sample was pour plated into the Petri dish. The plates were swerved gently to allow proper mixture and were allowed to solidify. All the Petri dishes were stacked conveniently for storage in the incubator and were incubated at 37^0C for 24 hours inverted (Fawole and Oso, 2007).

Bacteria Isolation from cashew tree bark

A 1ml of the prepared sample was pour plated in sterile Petri dish aseptically. The plates were swerved gently to ensure even mixture and then allowed to gel. The plates were incubated after solidifying at 37^0C for 24 hours inverted (Fawole and Oso, 2007).

Bacteria Isolation from macerated dead termite

A 1ml of the suspension was dispensed in to the Petri dish and the prepared media were poured on it. After solidification, the plates were incubated at 37^0C for 24 hours inverted ().

Fungi isolation fromtermitarium

A 1ml of dilution of choice from already prepared sample was pour plated into the Petri dish. The plates were swerved gently to allow proper mixture and were allowed to solidify. All the Petri dishes were stacked conveniently for storage in the incubator and were incubated at 25-27^0C for 72 hours in an un-inverted position (Cheesebrough, 2006).

Fungi Isolation from cashew tree bark

A 1ml of the prepared sample was pour plated in sterile Petri dish aseptically. The plates were swerved gently to ensure even mixture and then allowed to gel. The plates were incubated after solidifying at 25-27^{0}C for 72 hours inverted (Cheesebrough, 2006).

Fungi Isolation from macerated dead termite

A 1ml of the suspension was dispensed in to the Petri dish and the prepared media were poured on it. After solidification, the plates were incubated at 25-27^{0}C for 72 hours inverted (Cheesebrough, 2006).

Identification and characterization of isolated Bacteria and fungi

Standard and conventional methods of cultural, morphological and biochemical characteristics were employed in the identification of the organisms following the method of Sarah.*et al* (2016).

Sub culturing of isolates

Sub culturing of bacterial isolates

From the nutrient agar plate, sterile inoculating loop previously flamed to red-hot and cooled was used to pick different isolates from mixed culture plates and then streaked on an already prepared nutrient agar. This inoculated medium was incubated at 37^{o}C for 24 hours in an inverted position (Cheesbrough, 2006).

Sub culturing of fungal isolates

Sub culture of distinct colonies of fungal growth was done on fresh potato dextrose agar in other to get pure fungi isolates. Sterile inoculating needle flamed to red hot was used to pick a distinct mycelium from the previous plate and the placed at the centre of the fresh culture medium. The medium was incubated at 25-27^{o}C for 48-72 hours inverted (Cheesbrough, 2006).

Preservation of bacterial isolates

A 10ml of already prepared double strength potato dextrose agar was measured into sterile McCartney bottles. After sterilization, it was allowed to cool to about 45^0C and left to solidify in a slant position at an angle of 45^0. On solidification, the inoculum was introduced into the bottle aseptically and incubated at 37^0C for 24 hours. After 24 hours, growth was seen and was stored at 4^0C in the refrigerator until further tests (Cheesbrough, 2006).

Preservation of fungal isolates

A 10ml of already prepared double strength potato dextrose agar was measured into sterile McCartney bottles. After sterilization, it was allowed to cool to about 45^0C and left to solidify in a slant position at an angle of 45^0. After solidification, the inoculum was introduced into the bottle aseptically and incubated at 25-27^0C for 72 hours. After 72 hours, growth was seen and was stored at 4^0C in the refrigerator until further tests (Cheesbrough, 2006).

Antibiotic sensitivity screening of bacterial isolates

This test was carried out to determine the resistance and susceptibility of the isolated bacteria to antibiotics. The various antibiotics impregnated in the gram-positive disc used are as follows: Erythromycin, Amoxicilin, Ofloxacin, Streptomycin, Chloramphenicol, Cefuroxime, Gentamycin, Pefloxacin, Co-trimoxazole, Ciprofloxacin**.** The antibiotic susceptibility testing was carried out using Kirby-Bauer method as described by (Cheesebrough, 2006). A loop full of a bacteria colony was picked and emulsified in a Bijou bottle containing 3.0ml of normal saline. A cotton swab was dipped into the suspension and the swab was pressed against the side of the bottle to remove excess fluid. The inoculated swab was then streaked across the surface of Mueller Hinton agar and allowed to dry for five minutes after which sterile forceps were used to carefully remove the disc from its pack and gently pressed onto the agar surface. The plates were incubated at 37^0C for 24 hours. The zones of inhibition were measured in millimetres using a ruler.

The zones of inhibition were classified into susceptible (16mm and above), intermediate (11mm-15mm), and resistant (0-10mm) based on the specified standard of zone of inhibition as described by

Cheesebrough, 2006. Antibiotic sensitivity screening was also carried out on multiple drug resistant isolates already cured of their plasmids with broad spectrum antibiotics (CM128PR100).

Antagonistic properties of isolates against selected pathogens

Bacteria against bacteria

This test was carried out on Mueller Hinton agar on Petri dishes using Fokkema method. Fresh culture (18-hour culture) was used for this test; bacteria isolates previously preserved on nutrient agar slant were sub cultured on freshly prepared nutrient agar medium and incubated for 18 hours before the antagonistic test was carried out. Selected bacteria pathogens such as *Staphylococcus aureus, Streptococcus sp* and *Shigella sp* were sourced as clinical samples from the Ondo State General Hospital, Akure, Nigeria and used against the isolates from the termitarium. It was by streaking the test organism on one side of the agar plate and the known pathogen on the other side of the agar plate. The paired cultures were incubated at 37^{o}C for 24-48hrs and observed for zones of inhibition.

Fungi against bacteria

This was carried out on Mueller Hinton agar too. The fungi isolates from a slant were sub cultured on a fresh Potato Dextrose Agar for 48-72hrs, until the growth is covering the entire plate. Known pathogen used for the bacteria above was also used. A cork borer was used to cut out that diameter from the fungal growth into the center of the fresh Mueller Hinton agar and the known bacterial pathogen was streaked on the side of the fungi about 5mm apart. The paired cultured plates were incubated at 25^{o}C for duration of 7 days and the zones of inhibition observed

Plasmid Profile Analysis

An 18 hours old broth culture was used for this analysis. Theprocedure described by CLSI (2008)was adopted for this analysis.

Plasmid Curing

The plasmid curing was done by exposing the overnight grown culture at 37 °C and 10mg/ml of Etidium bromide. After plasmid curing, isolates were subjected to antibiotic sensitivity test again using broad spectrum antibiotics (CM128PR100) (Brown, 2010).

RESULT

Corynebacterium sp, Bacillus sp, *Streptococcus sp*, and *Micrococcus sp* were isolated from termitarium in this research, gram staining showed the microorganisms to be gram positive, glucose positive with variation in subsequent biochemical tests result obtained

188 Table 1.0 Morphological and Biochemical characteristics of bacterial isolates

I	Gram reaction	Sugar Fermentation				COT	CAT	OX	SP.	MOT	VP/MR	N.I.
		Suc.	Lac.	Glu.	Mann.							
C	+ve (short rods)	+ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve/-ve	3
B	+ve(bacilli rods)	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve/-ve	5
S	+ve (cocci in chains)	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve/+ve	4
M	+ve (cocci)	+ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve/-ve	3

189

190 Keys; I- Isolate , C- *Corynebacterium sp,* B- *Bacillus sp*, S- *Streptococcus sp*, M- *Micrococcus sp*, Glu- glucose, Lac- lactose, Suc- sucrose, Mann- Mannitol,

191 COT- coagulase, CAT- Catalase, OX- oxidase, SP- spore forming, MOT- motility, VP/MR- voges poskauer/methyl red, N.I- number of Isolate.

192

Fungal Isolates Obtained from Cashew tree termitarium

Three different fungi were isolated from the termitarium, their microscopic and macroscopic characteristics vary greatly and were presented in table 2.

Table 2.0: identification of Fungal Isolates

Fungal isolates	Macroscopic characteristics	Microscopic characteristics	Probable Organism
Isolate 1	Colonies are black with a pale yellow reverse side	Hypha is septate. Simple upright canidiophores that terminates in glucoseSwelling, bearing phialides at the apex orradiating form the entire surface. Conidiaare one-celled and globose.	*Aspergillus niger*
Isolate 2	White mycelia with areas of whitish yellow	Aerial mycelium. They appeared as sickle-shaped. Conidiophores arose singly from the mycelium and branched near the apex tip.	*Fusarium solani*
Isolate 3	A yellowish reversed side with black colonies	Hyaline or bright coloured mass that appeared one-celled, ovoid in dry basipetal chains.	*Penicillium notatum*

ANTIMICROBIAL SENSITIVITY RESULT

Test results shows that *Corynebacterium sp, Streptococcus sp*were sensitive to most of the antibiotics used in this study compared with*Bacillus spp*which was resistance to about seven of the antibiotics. Micrococcus spp was totally resistant to the antibiotics hence the need for a plasmid profile analysis using electrophoresis. Table 3 and table 4 shows theantimicrobial characteristics.

Table 3.0 Zones of inhibition (in mm) of isolates bacteria against antibiotics

I.C	Antibiotic used with zones of inhibition (mm)										N.I
	ERY	CPX	COT	AMX	OFL	STR	CHL	CEF	GEN	PEF	
C	17.33± 0.5	12.22±0.7	16.86±0.3	10.02±0.1	16.23±0.5	17.25±0.9	12.33±1.2	18.13±0.8	00.00	13.37±1.4	3
B	13.10±1.6	00.00	8.15±1.2	00.00	00.00	00.00	00.00	16.23±0.9	00.00	11.13±0.6	4
S	14.05±0.7	00.00	18.05±1.4	17.33±0.8	16.75±0.5	17.05±0.7	16.23±0.3	16.03±0.2	00.00	15.45±0.3	5
M	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	3

Keys; I.C- isolate codes, ERY= Erythromycin, AMX=Amoxicilin, OFL=Ofloxacin, STR=Streptomycin, CHL=Chloramphenicol, CRO= Cefuroxime, GEN=Gentamycin, PFX =Pefloxacin, COT = Co-trimoxazole, CPX=Ciprofloxacin, C- *Corynebacterium sp,* B- *Bacillus cereus*, S- *Streptococcus sp*, M- *Micrococcus sp*, N.I- number of isolates, 0-10mm- Resistant, 11-16- Intermediate, 16-above- Susceptible. (Cheesebrough, 2006).

Table 4.0 Antibiotic sensitivity patterns of bacteria isolates

I.C	Antibiotics used										N.I
	ERY	CPX	COT	AMX	OFL	STR	CHL	CEF	GEN	PEF	
C	S	I	S	R	S	S	I	S	R	I	3
B	I	R	R	R	R	R	R	S	R	I	4
S	I	R	S	S	S	S	S	S	R	I	5
M	R	R	R	R	R	R	R	R	R	R	3

Keys; I.C- isolate codes, ERY= Erythromycin, AMX=Amoxicilin, OFL=Ofloxacin, STR=Streptomycin, CHL=Chloramphenicol, CRO= Cefuroxime, GEN=Gentamycin, PFX =Pefloxacin, COT = Co-trimoxazole, CPX=Ciprofloxacin, C- *Corynebacterium sp,* B- *Bacillus cereus*, S- *Streptococcus sp*, M- *Micrococcus sp*, N.I- number of isolates, 0-10mm- Resistant, 11-15- Intermediate, 16-above- Susceptible (Cheesebrough, 2006).

Antagonistic result of Fungi

Results indicate that only *Penicillium notatum* had positive antagonistic effect on *Staphylococcus aureus* and mild antagonistic effect on *Shigella sp* and *Salmonella sp*. Table 5 shows the antagonistic pattern.

Table 5.0: Antagonistic patterns of identified fungi against selected pathogens

I.C	Selected pathogens				N.I
	S.A	SH	S	B.S	
A.N	-ve	-ve	-ve	-ve	4
P.N	+ve	I	I	-ve	3
F.S	-ve	-ve	-ve	-ve	3

Keys: I.C- isolate codes, A.N- *Aspergillus niger,* P.N-*Penicillium nonatum*, F.S-*Fusarium solani* S.A- *Staphylococcus aureus,* SH-*Shigella sp,* S-*Salmonella sp,* B.S-*Bacillus subtilis,* N.I- number of isolates, 0-10mm- -ve (no antagonism), 11-16mm- I (mild antagonism), 16-above- +ve (strong antagonism), (Cheesebrough,2006).

Antagonisitic result for bacteria isolatesfrom Cashew Trees

Test results shows that none of the bacterial isolate had antagonistic effect on selected pathogenic test organisms. Table 6 shows the antagonistic pattern of identified bacterial against selected pathogen.

Table 6.0: Antagonistic patterns of identified bacteria against selected pathogens

I.C	Selected pathogens				N.I
	S.A	SH	S	E.C	
C	-ve	-ve	-ve	-ve	3
B	-ve	-ve	-ve	-ve	4
S	-ve	-ve	-ve	-ve	5
M	-ve	-ve	-ve	-ve	3

Keys: I.C- isolate codes, C- *Corynebacterium sp,* BC- *Bacillus cereus*, S- *Streptococcus sp*, M-*Micrococcus sp*, S.A- *Staphylococcus aureus,* SH-*Shigella sp,* S-*Salmonella sp,* E.C- *Escherichia coli,* N.I- number of isolates, 0-10mm- Negative (no antagonism), 11-16mm- Intermediate (mild antagonism), 16-above- Positive (strong antagonism), +ve- positive, -ve- negative (Cheesebrough, 2006).

Plasmid profile of bacteria isolates from Cashew Trees

The results obtained revealed the presence of plasmid bands ofdifferent molecular weights. The molecular weights of the plasmidswere determined using DNA- Hind III molecular weight marker(fig- 1). It was observed that *Bacillus sp* and *Micrococcus* spcontains plasmid with an estimated molecular weight of 1000 bpand 980bp respectively.

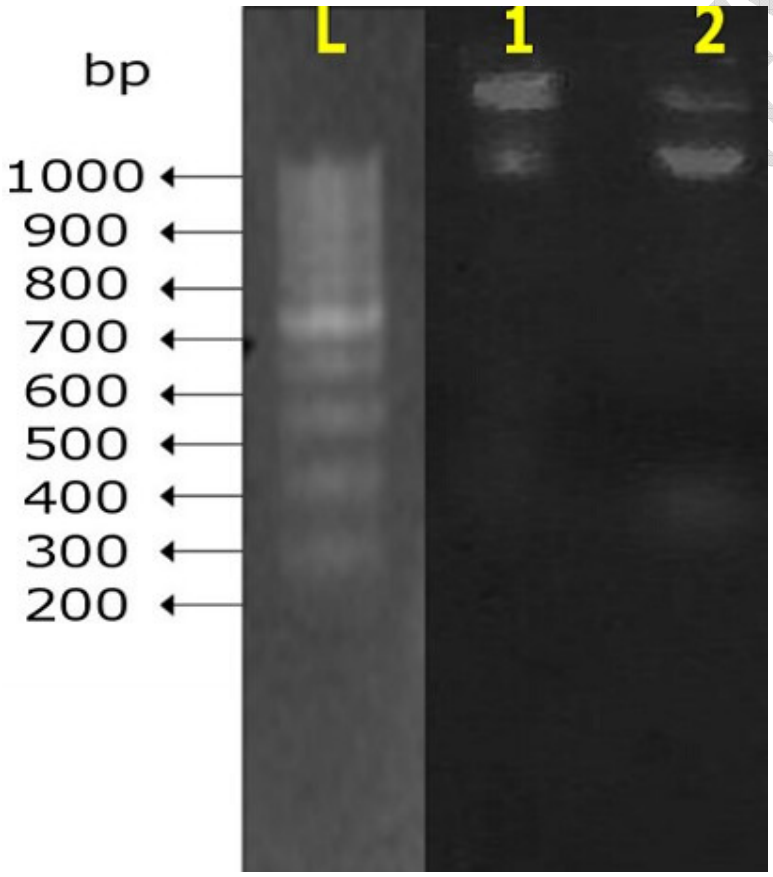

Fig 1.0: Electrophorogram of isolated bacteria plasmid DNA

KEY; L – Gene ladder, 1 - *Micrococcus sp,* 2- *Bacillus sp*

279

280 **Sensitivity result of bacteria isolates after plasmid curing**

281 Result shows that *Bacillus sp and Micrococcus sp* were both sensitive to the generally antibiotics.
282 This makes the initial resistance of this isolates to be plasmid mediated. Thus, resistivity is extra
283 chromosomal in nature.

284

285 Table 7:0 Antibiotic sensitivity patterns of bacterial isolates after plasmid curing

I.C	Antibiotic sensitivity patterns after plasmid curing								N.I
	ERY	CXC	OFL	AUG	CAZ	CRX	GEN	CTR	
M	S	I	S	S	S	I	S	I	3
B	S	S	I	S	S	S	S	S	4

286 Keys: I.C- isolate code, ERY: Erythromycin, CXC: Cloxacillin, OFL: Ofloxacin, AUG: Augumentin, CAZ: Ceftrazidine, CRX: Cefuroxime, GEN:
287 Gentamicin, CTR: Ceftriaxone, B- *Bacillus sp,*M- *Micrococcus sp*, N.I- number of isolates, 0-10mm- Resistant, 11-16- Intermediate, 16-above-
288 Susceptible (Cheesebrough, 2006). S- Susceptible, I- intermediate, R- resistant.

DISCUSSION

The microbial load obtained in this study shows the importance of termitarium sampled from cashew trees as suitable habitats for microorganisms. Relevant studies have opined the rich mineral and nutrient contents of cashew tree gum which is composed of polysaccharides such as glucose, mannose, galactose and cellulose; this affords termite nests, bark sheaths and termites inhabiting the tree environments enough growth factors for wide arrays of microorganisms (Nicoletti *et al.,* 2009 and Adeigbe *et al.,* 2015). However, some fungi isolates obtained especially *Fusarium sp* have also been implicated in causing damping off disease in cashew plant hence, this justifies the presence of this fungi in the samples analyzed; this also bears similarities to the findings of Adeigbe *et al.,* (2015).

The antagonistic test carried out on the fungi isolates against selected pathogen showed mild antagonism in the fungus *Penicillum notatum* especially against *Salmonella sp*. Since species of Penicillium are ubiquitous as soil and air fungi, their presence in the termitarium indicates a positive mutualism of these fungi isolates with the termite guts or the termitarium microenvironment themselves considering the known potentials of *Penicillum notatum* in production of antimicrobials against pathogenic bacteria (Nicoletti *et al.,* 2009).

The bacteria isolates showed varying degrees of resistance to the antibiotics used against them. This could be as a result of the microorganisms being exposed to several chemicals used by the farmers on their crops. The termites on cashew trees may have also been exposed to some insecticides and their active ingredients which are similar analogues to many of the antibiotics used to evaluate their sensitivity patterns; resulting in possession of resistant (R-factor) plasmids as survival mechanisms against these antimicrobials (Adeigbe *et al.,* 2015).

Bacteria isolates such as *Bacillus sp,* and *Micrococcus sp,* were screened out to be multiple drug resistant isolates displaying stellar antibiotic resistance against antibiotics used. These isolates were analyzed via plasmid profiling to determine if they possess resistant gene encoding plasmids in their cell structures and if their genetic basis of antimicrobial resistance was extra-chromosomal or not. They were discovered to possess heavy chained resistant factor chromosomes that encode for

antibiotic resistance, after which they were cured of their plasmids and then subsequent exposure to broad spectrum antibiotic treatments again showed they were susceptible to antibiotic treatments, this also agrees with the findings described in Nicoletti *et al.,* (2009).

CONCLUSION

This study has shown that the termitarium is a microbial habitat that is rich in many nutrients that enables optimum growth of many microbes, revealed the mild antagonistic potentials of isolated microorganisms obtained from test samples against known selected pathogens and shown that the possession of resistant factor plasmids is responsible for the antibiotic resistance patterns of isolated bacteria to antibiotic used.

Bibliography

1. Adeigbe, O., Olasupo, F., Adewale, B., and Muyiwa, A. (2015) Review on cashew research and prodection in Nigeria in the last four decades. *Scientific Research and Essays* 10(5): 196-209.
2. Brown, T. (2010) Vectors for Gene Cloning: Plasmids and Bacteriophages. Gene Cloning and DNA Analysis: An Introduction. Academic Press, U.S.A; 64-76.
3. Chessbrough, M. (2006) District laboratory practice in tropical countries. Cambridge University press, United Kingdom, 124-176.
4. Clinical and Laboratory Standards Institute (CLSI). “Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals Approved standard

Third edition, CLSI document M31-A3, Clinical and Laboratory Standards Institute, 940 West Valley Road, WaynePennsylvania, USA, 28 (2008): 1-99.

5. Fall, S., Hamelin, J., Ndiaye, F., Assigbetse, K., Aragno, M., Chotte, J. and Brauman, A., (2007) Differences between bacterial communities in the gut of a soil-feeding termite (*Cubitermes niokoloensis*) and its mounds. *Appl. Environ. Microbiol.*, 73(4):5199–5208.
6. Fawole, M. and Oso, B., (2007) Laboratory manual on microbiology. Spectrum books limited, Ibadan, Nigeria, 127.
7. Hamad, F., and Mubofu, E., (2015) "Potential biological applications of bio-based anacardic acids and their derivatives". Int J Mol Sci. 16 (4): 85–90.
8. Longair, R. (2004) Tusked males, male dimorphism and nesting behaviour in a sub-social Afro tropical wasp, Synagris cornuta and weapons and dimorphism in the genus (Hymenophera, vespidea, enumenie), Journal of the Kansas Entromological Society, 77(4), 528 – 557.
9. Nicoletti, R., Buommino, E., De Filippis, A., Lopez-Gresa, M., Manzo, E., Carella, A., Petrazzuolo, M., and Tufano, M., (2009) "Bioprospecting for antagonistic Penicillium strains as a resource of new antitumor compounds". World Journal of Microbiology. 24 (2): 185–95.
10. Sarah K., *et al*. “Descriptions of Medical Fungi”. Department ofMolecular and Cellular Biology, School of Biological Sciences, University of Adelaide, Australia. 3rd edition (2016).