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Research Paper

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Anti-Bacterial and Anti-Fungal Activities of *Citrus sinensis* Leaves Extracts

Nasiru Mohammed[1*], Yusuf Muhammad Sanyinna[2], Ridwan Nuhu Ahmed[3]

[1]Department of Microbiology, Institute of Molecular Biology and Biotechnology, The University of Lahore, Pakistan.
[2,3]Department of Animal and Environmental Biology, Faculty of Life Sciences, Kebbi State University of Science and Technology, Aliero, Nigeria.

[*]*Corresponding Author: elnasirmar@yahoo.com, Tel.: +234-80666-62914.*

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***Abstract*—**The biological activities and medicinal benefits of different plants have been long known. Therefore, humans have ever since researched, tried and utilised them for their betterment. In the present study, an effort was made to perceive the existence of microbial infections alongside with herbal remedy of these infections. The aim of this study was to determine the anti-bacterial and anti-fungal activities of *Citrus sinensis* leaves extracts. *In vitro* anti-bacterial and anti-fungal potentials of *Citrus sinensis* leaves extracts were investigated. Four different solvents i.e. ethanol, ethyl acetate, petroleum ether and water were used for extract preparation from the plant leaves. The anti-microbial activity potency of leaves extracts was evaluated by Standard Kirby-Bauer Disk Diffusion Method against reference strains of *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Salmonella typhi, Aspergillus niger* and *Aspergillus fumigatus.* Four different concentrations (2000 µg, 1000 µg, 500 µg and 200 µg) of each extract were investigated against the test organisms. Significant anti-bacterial activity was displayed by ethanol and ethyl acetate extracts against *Escherichia coli* (ATCC 25922). No activity was displayed by petroleum and aqueous extracts at all concentrations. A significant anti-bacterial activity was displayed against *Salmonella typhi* (ATCC 14028). No activity was displayed by ethyl acetate extract except at 1000 µg. There was no activity displayed by all the extracts against *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228) at all concentrations. Ethanol extract displayed an exceptional anti-fungal activity against *Aspergillus niger* (ATCC 16404) and *Aspergillus fumigatus* (ATCC 16404). No activity was displayed by aqueous extract at all concentrations. This study justifies the claimed uses of *Citrus sinensis* leaves extracts in the Pakistan's traditional system of medicine to treat various diseases. The results of this study show the extracts to possess significant antibacterial and antifungal activities to leaves extracts of *Citrus sinensis*, and are considered appropriate for empirical treatment of various diseases in the study area. It is therefore, recommended that further studies of these extracts should be carried out by agar well dilution and other methods prepared in different solvents and evaluated again through in-depth analysis by fractionation, structure elucidation and docking studies.

***Keywords*—**Anti-Bacterial, Anti-Fungal, Activities, *Citrus sinensis*, Extracts.

I. INTRODUCTION

Humankind and contagion have shared momentous antiquity. As we transpired and developed, so did the contagion. Man has consistently been in struggle to identify and eradicate contagions. Mankind has been recorded to distress from contagions in time periods.

Aspergillus fumigatus is the most frequent cause of invasive fungal infection in immunosuppressed individuals, which include patients receiving immunosuppressive therapy for autoimmune or neoplastic disease, organ transplant recipients and AIDS patients. *A. fumigatus* primarily causes invasive infection in the lung and represents a major cause of morbidity and mortality in these individuals [1].

Aspergillus niger is one of the most common causes of potent mycotoxins otomycosis (fungal ear infections), which can cause pain, temporary hearing loss and in severe cases, damage to ear canal and tympanic membrane.

Escherichia coli is one of the most frequent source of bacteremia and accountable for 15-40% of all notable bacteremia isolates [2]. It is also a major cause of life threatening bloodstream and urinary tract infections [3]. Among the strains of *E. coli,* 0157:H7 strain is responsible for bloody diarrhoea [4]. However, the remaining strains are related with haemolytic uremic syndromes (HUS) [5].

Staphylococcus aureus is a common cause of skin infections including abscesses, respiratory infections such as sinusitis and food poisoning [6], [7]. Pathogenic strains often promote infections by producing virulence factors such as potent protein toxins and the expression of a cell-surface protein that binds and inactivates antibodies. The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem in clinical medicine.

Staphylococcus epidermidis is a Gram-positive bacterium and one of over 40 species belonging to the genus *Staphyloccus.* It is part of the normal human flora, typically the skin flora and less commonly the mucosal flora. It is a facultative anaerobic bacterium.

In spite of considerable attempt displayed by the pharmacological industries in designing of various drugs to relieve and avert these infections, microbes have demonstrated resistance to these drugs [8].

The widespread in antimicrobial resistance in *Escherichia coli, Salmonella typhi, Staphylococcus aureus* and *Aspergillus niger* has been adeptly recorded.
The rapid expansion in antimicrobial resistance in *E. coli, Salmonella, Staphylococcus* and *Aspergillus fumigatus* was emphasized by WHO's first global report on antibiotic resistance [9].

These circumstances are frightening due to the interaction of these resistant bacterial isolates with hospital acquired infections as well which can result in increased mortality rates especially in immune compromised persons.
The importance of this matter is emphasized by the circumstances that WHO declared antimicrobial resistance (AMR) to be among the three substantial threats to human health [10].

Distinct footsteps have been taken to eliminate the disputes of antimicrobial resistance such as circumventing extraneous use of antibiotic, visualizing the process of resistance and to explore for different therapeutic managements and cure of diseases.
WHO has also highlighted on the requirement and use of traditional therapies native to a particular region [11].

Citrus sinensis is one of the most important and predominantly grown fruit crop, with total worldwide incense reported to be around 120 million tons.
C. sinensis is eaten up all over the world as an outstanding source of vitamin C, which is a strong inherent antioxidant that strengthen the body's immune system [12].

It has been used conventionally to cure illness such as; constipation, cramps, colic, diarrhoea, bronchitis, tuberculosis, cough, cold, obesity, menstrual disorder, angina, hypertension, anxiety, depression and stress [13].

II. RELATED WORK

A lot of researches were carried out on antimicrobial activities of different extracts of *Citrus sinensis* using various concentrations in the world [14], [15], [16], [17], [18], [19]. For example, Haroen *et al.* reported the "Effect of Different Levels of Orange (*Citrus sinensis*) Waste Juice Extracts on Broiler Chickens Performance" [20], Ebrahimi *et al.* reported the "Effect of Different Levels of Dried Sweet Orange (*Citrus sinensis*) Peel on Broiler Chickens Growth Performance" [21], Kumar *et al.* undertaken research on "Antimicrobial Activity and Phytochemical Analysis of *Citrus* Fruit Peels - Utilization of Fruit Waste" [22] and Kirbaslar *et al.* conducted study on "Antimicrobial Activity of Turkish *Citrus* Peel Oils" [23]. Also, Ekwenye and Edeha carried out research on "The Antibacterial Activity of Crude Leaf Extract of *Citrus sinensis* (Sweet Orange)" [24]. In addition, research was performed on "*In Vitro* Antibiotic Activity of Isolated Volatile Oil of *Citrus sinensis* by Singh *et al.* [25]. Equally, studies on anti-fungal activities of *Citrus sinensis*, grapefruit and Valencia orange were carried out [26], [27]. Also, studies on antioxidant activity [28], [29], [30], [31], [32], [33], anti-carcinogenic property [34], [35], anti-inflammatory activity [36], [37] and anti-typhoid activity [38] were equally conducted.

III. MATERIALS AND METHODS

Study Design
This is a cross-sectional study. The research study period was nine months i.e. from January to September, 2018. The study was carried out in the Microbiology Laboratory of the Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Pakistan.

Bacterial Strains
Staphylococcus aureus (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922), *Salmonella typhi* (ATCC 14028), *Aspergillus niger* (ATCC 16404) and *Aspergillus fumigatus* (ATCC 16404) were adopted in this study.

Storage of Bacterial Strains
Pure culture of bacterial strains used for this study was preserved in glycerol stock solution which was prepared by pouring 10 ml of glycerol in 90 ml PBS solution and was then autoclaved and stored at 4°C till required. Pure culture of each bacterial strain was suspended in it and the suspension was centrifuged at 6000 g for 5 minutes. The suspension was decanted and the deposit was re-suspended in 1.2 ml of 10 % glycerol PBS sterilized solution and stored in 1.5 ml Eppendorf at 4°C for 12 hours and then lifted to 20°C [39].

Solvents used for Extraction
The solvents used included; ethyl acetate, ethanol, petroleum ether and water.

Extracts Preparation
Sukhdev *et al.* [40] method was adopted. *Citrus sinensis* extracts were prepared by dissolving in four different solvents including ethanol, ethyl acetate, petroleum ether and water. *Citrus sinensis* (orange) was obtained from the local market.

Orange leaves were first washed thoroughly. Then, the oranges were dried and then finely grinded and soaked in the respective solvents for five days with continuous gentle agitation. After five days, the solution was filtered with the help of Whatmann filter paper no. 1. The filtered solution was then air dried [41].

Stock Solutions
The stock solution was prepared by dissolving 100 mg of each extract in 1 ml DMSO. The extract stock solution was stored refrigerated.

Antimicrobial Susceptibility Testing

Standard Kirby-Bauer Disk Diffusion Method on Muller-Hinton Agar (MHA) (Oxoid UK), according to the Clinical Laboratory Standards Institute (CLSI) 2015 guidelines was adopted.

Turbidity Preparation Equivalent to 0.5 McFarland Standard
Standard of $BaSO_4$ is prepared as following:

I. Sulphuric acid solution (1%v/v) was prepared by adding 1 ml of concentrated sulphuric acid to 99 ml of water.
II. Barium chloride solution (1%v/v) was prepared by dissolving 0.5 g of dehydrated Barium chloride in 50 ml of distilled water.
III. Barium chloride solution 0.6 ml was added to 99.4 ml of the sulphuric acid solution.
IV. The turbid solution was stored in capped tube in dark at 20 - 28°C [42].

Preparation of Inoculums
Discrete colonies (n = 3-4) were emulsified in sterilized normal saline to get a homogenous suspension with the aid of a vortex machine. Turbidity of suspensions was visually adjusted to McFarland standard.

Inoculation of Muller-Hinton Agar Plates
Inoculation was done with the help of sterile cotton swab on Muller-Hinton Agar plates which was first soaked in well mixed suspension and pressed along the sides of glass tube to get rid of excessive inoculums and then streaked over agar plates homogeneously. It was ensured that the entire procedure should be done within fifteen minutes after the suspensions were prepared [43].

Disc Preparation
Disc was prepared by punching a Whatman filter paper no. 1 (6 mm in diameter) and then sterilized in hot air oven at 180°C for half an hour. It was then impregnated using micropipette with 2.5 µl, 5 µl, 10 µl and 20 µl from each extract to obtain a concentration of 200 µg, 500 µg, 1000 µg and 2000 µg respectively.

Disc Application
Discs were mounted onto streaked area with the aid of sterile forceps. This was done in a way that all the discs should be 15 mm away from the sides of the plates and sides and the centres of two discs should not be closer than 24 mm. Within 10 - 15 minutes of the application of these plates, they were inverted and incubated at 35°C for 18 - 24 hours [43].

Controls
Amoxicillin – clavulanic acid (AMC 10 µg) was used as positive control for *Escherichia coli* (ATCC 25922) and *Salmonella typhi* (ATCC 14028). Streptomycin (10 µg) was used as a positive control for *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228). Ketoconazole (10 µg) was used as positive control for *Aspergillus niger* (ATCC 16404) and *Aspergillus fumigatus* (ATCC 16404).

Interpretation of Susceptibility Testing
Results were obtained after 18 - 24 hours of incubation. Each plate was thoroughly examined for zone of inhibition. Clear zones of inhibition were observed and measured by Vernier scale.

Characterization and Identification of Bacteria
Following isolation of bacterial strains on nutrient agar, the cultures were characterized morphologically and then subjected to biochemical testing.
The following biochemical tests were performed for identification and confirmation of organisms:

- Gram staining.
- Oxidase test.
- Citrate utilization test.
- Indole test.

Gram Staining
Smear was made on a clean glass slide from cultures with the help of a sterile wire loop. The slide was then air dried and then heat fixed by passing it through the gentle flame [42].

Gram staining technique procedure was described below:

- The smear was air dried and heat fixed.
- It was stained with the crystal violet as the primary dye for 60 seconds.
- The slide was rinsed with water and flooded with Lugol's iodine for 30 seconds.
- The iodine solution was then washed off and decolourized with acetone till the colour stopped coming out of the smear.
- The smear was washed off with water.
- The smear was then counter stained with diluted Carbol Fuchsin for 30 minutes.
- The smear was then washed off with water, blotted with absorbent paper and air dried.

Gram positive bacteria will be stained purple.
Gram negative bacteria will be stained red due to the counter stain used.
Controls: Positive control - *Staphylococcus aureus* (ATCC 25923).
Negative control - *Escherichia coli* (ATCC 25922).

Oxidase Test
A piece of filter paper was placed on a clean petri dish and 3 drops of freshly prepared oxidase reagent were added. A wire loop was used to remove the colony of the test organism and was smeared on the filter paper. Presence of blue-purple colour within 10 seconds indicates positive

oxidase test while the absence of the blue-purple colour indicates negative oxidase test [44].

Citrate Utilization Test

As described by Cheesbrough [42].

This test is used for the identification of enterobacteria. This test is based on the ability of an organism to use citrate as its only source of carbon.

Simmons' citrate agar medium was prepared in bijou bottles and was streaked and stabbed with the saline suspension of the test organism. The bijou bottles were then incubated at 35°C for 48 hours. The organisms showing positive citrate test develop a blue colour in the medium. Whereas, no change in colour of medium indicated negative result.

Controls: Positive control - *Klebsiella pneumonia* (ATCC 10031).

Negative control - *Escherichia coli* (ATCC 25922).

Indole Test

This test is based on checking the ability of an organism to form indole from degradation of tryptophan. The organism was inoculated in tryptophan incorporated medium. Indole production was detected by Kovac's reagent which will form red colour after combining with indole.

A red surface layer formed within ten minutes of adding Kovac's reagent indicated a positive reaction.

Controls: Positive control - *Escherichia coli* (ATCC 25922).

Negative control - *Klebsiella pneumonia* (ATCC 10031).

Data Analysis

Data analysis was done by Excel. Values were considered significant at $p \leq 0.05$. Sign α represents significant level.

IV. RESULTS AND DISCUSSION

Anti-Bacterial and Anti-Fungal Activities of *Citrus sinensis* Leaves Extracts

The anti-bacterial and anti-fungal activities of *Citrus sinensis* leaves extracts were evaluated against different strains of bacteria like *Escherichia coli* (ATCC 25922), *Salmonella typhi* (ATCC 14028)**,** *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), and against fungal strains such as *Aspergillus niger* (ATCC 16404) and *Aspergillus fumigatus* (ATCC 16404). The minimum inhibitory concentration (MIC) against four different fractions i.e. ethanol, ethyl acetate, aqueous and petroleum at various concentrations (200 µg, 500 µg, 1000 µg and 2000 µg) was also evaluated. There was no antibacterial activity of *Citrus sinensis* leaves extracts against *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228) at all concentrations.

Table 1: Anti-Bacterial Activity of *Citrus sinensis* Leaves Extracts against *Escherichia coli* (ATCC 25922)

Significant anti-bacterial activity was displayed by ethanol and ethyl acetate extracts against *Escherichia coli* (ATCC 25922). No activity was displayed by petroleum and aqueous extracts at all concentrations.

µg	Csel	Cspl	Cseal	Cswl
2000	13	0	10	0
1000	9	0	7	0
500	7	0	0	0
200	0	0	0	0

Key: µg (micro gram), Csel (*Citrus sinensis* seed ethanol extract), Cspl (*Citrus sinensis* seed petroleum extract), Cseal (*Citrus sinensis* seed ethyl acetate extract), Cswl (*Citrus sinensis* seed aqueous extract).

Table 2: Anti-Bacterial Activity of *Citrus sinensis* Leaves Extracts against *Salmonella typhi* (ATCC 14028)

A significant anti-bacterial activity was displayed against *Salmonella typhi* (ATCC 14028). No activity was displayed by ethyl acetate extract except at 1000 µg.

µg	Csel	Cspl	Cseal	Cswl
2000	10	7	0	7
1000	8	7	7	7
500	0	0	0	0
200	0	0	0	0

Key: µg (micro gram), Csel (*Citrus sinensis* seed ethanol extract), Cspl (*Citrus sinensis* seed petroleum extract), Cseal (*Citrus sinensis* seed ethyl acetate extract), Cswl (*Citrus sinensis* seed aqueous extract).

Table 3: Anti-Bacterial Activity of *Citrus sinensis* Leaves Extracts against *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228)

There was no activity displayed by all the extracts against *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228) at all concentrations.

µg	Csel	Cspl	Cseal	Cswl
2000	0	0	0	0
1000	0	0	0	0
500	0	0	0	0
200	0	0	0	0

Key: µg (micro gram), Csel (*Citrus sinensis* seed ethanol extract), Cspl (*Citrus sinensis* seed petroleum extract), Cseal (*Citrus sinensis* seed ethyl acetate extract), Cswl (*Citrus sinensis* seed aqueous extract).

Table 4: Anti-Fungal Activity of *Citrus sinensis* Leaves Extracts against *Aspergillus niger* (ATCC 16404) and *Aspergillus fumigatus* (ATCC 16404)

Ethanol extract displayed an exceptional anti-fungal activity against *Aspergillus niger* (ATCC 16404) and

Aspergillus fumigatus (ATCC 16404). No activity was displayed by aqueous extract at all concentrations.

µg	Csel	Cspl	Cseal	Cswl
2000	14	7	8	0
1000	12	7	7	0
500	7	0	0	0
200	7	0	0	0

Key: µg (micro gram), Csel (*Citrus sinensis* seed ethanol extract), Cspl (*Citrus sinensis* seed petroleum extract), Cseal (*Citrus sinensis* seed ethyl acetate extract), Cswl (*Citrus sinensis* seed aqueous extract).

Discussion

In our present study, *Citrus sinensis* leaves extracts in four different solvents displayed exceptional activity at various concentrations. The results are in line with the findings of previous studies by Chah *et al.* [45]. This is also consistent with the study conducted by Strange *et al.* who observed that drinking extracts of dried leaves or shell of orange seeds have been used as anti-bacterial, anti-fungal, anti-spasmodic and anti-emetics in Italy, France and China [26]. According to Zugic *et al.* [33], most of the inhibitory capacity of *Citrus sinensis* and vegetables is presented by its content of vitamin E, C and carotenoids, as well as different polyphenols which are vital for life. Scalbert *et al.* [29] as cited by Huang *et al.* [31], observed that polyphenols as antioxidants can safeguard cells against decomposition and therefore, reduce the risk of various degenerative diseases associated with oxidative stress caused by free radicals. Ko *et al.* as cited by Zugic *et al.* [33], observed that most of the antioxidant capacity of fruits and vegetables were provided by their content of vitamin E, C and carotenoids, as well as different polyphenols which are essential for life. Tanaka *et al.* [34], summarised the anti-carcinogenic property of *Citrus sinensis* as follows: "Limonene, diosmin, hesperidin, as well as polymethoxylated flavone analogue shows strong anti-proliferative action against cancer and activated T-lymphocytes in various *in vivo* studies". Relieving, anti-arthritic and anti-inflammatory activity of *Citrus sinensis* is due to the presence of polymethoxyflavones. The polymethoxyflavones content, especially nobiletin, appears to be responsible for the anti-inflammatory activities of certain *Citrus* peel extracts, which have anti-inflammatory, antioxidant and hypolipidemic effects [36]. Oben *et al.* [37], observed that individual's high uptake of zeaxanthin and cryptoxanthin presents 52% less chances to experience rheumatoid arthritis. Vivek *et al.* [38], reported that constituents of orange fruit are responsible for anti-typhoid activity which include flavonoids like citacridone, citbrasine and saponins.

The overall antibacterial activity rates obtained in this study was 18 mm per disc, which is relatively lower as compared to the susceptibility patterns reported from previous studies. This may be due to decreased test concentrations per disc.

Furthermore, another reason for inactivity result may be attributed to the nature of solvent used for the extraction of plant extract for this research. In accordance to Dey *et al.* [46], plant extracts in methanol display greater antimicrobial activity as compared to those extracted in water.

Significant antibacterial activity was displayed by ethanol and ethyl acetate extracts against *Escherichia coli* (ATCC 25922). No activity was displayed by petroleum and aqueous extracts at all concentrations (Table 1). Similarly, a significant antibacterial activity was displayed against *Salmonella typhi* (ATCC 14028). No activity was displayed by ethyl acetate extract except at 1000 µg (Table 2). Therefore, the leaves extracts of *C. sinensis* displayed exceptional susceptibility against *E. coli* and *Salmonella typhi*. These results agree with the reports by Ibezim that demonstrated the Gram negative bacteria usually resistant to many antibiotics that are effective against Gram positive bacteria because they are associated with permeable membrane (PM) that limit the transport system and the target sites of the antibiotics [47].

There was no activity displayed by all the extracts against *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228) at all concentrations (Table 3). This is also in agreement with the results reported by Ibezim [47]. However, the results of this study are in disagreement with the findings of other studies conducted by previous researchers [48].

In this study, the significant antifungal activity was displayed by different leaves extracts of *C. sinensis* against *Aspergillus niger* and *Aspergillus fumigatus.* Ethanol extract displayed an exceptional antifungal activity against *Aspergillus niger* (ATCC 16404) and *Aspergillus fumigatus* (ATCC 16404). However, no activity was displayed by aqueous extract at all concentrations (Table 4). Sharma and Tripathi provided a number of reasons for the anti-fungal activity of *Citrus sinensis* by observing: "the vital oil and its components in *Citrus sinensis* as a potent preventer of deteriorating and storage-adulterating fungus *A. niger*, and the major anti-fungal components of orange are limonene (84.2%), linalool (4.4%) and myrcene (4.1%)" [27].

Hunte and Sultana [49], summarized the significance and use of medicinal plants in Pakistan as follows: "In Pakistan, quite number of the population lives in rural areas of the country [50]; and most of them choose plants, herbs and spices for remedy purpose due to their poverty level, inadequate understanding of health and disease, cultural household cures, perceptions about health services and providers, low cost, challenges in obtaining proper health care services and the strong belief and trust of the community on these methods. Pakistan is considered number eight ranking leading exporters of medicinal plants [51]. Bogers *et al.* [52], reported that Pakistan was amongst the top ranked number on the basis of average import trade volume of medicinal plants during the time period of 1991-2003.

V. CONCLUSION AND FUTURE SCOPE

This study justifies the claimed uses of *Citrus sinensis* leaves extracts in the Pakistan's traditional system of medicine to treat various diseases.

The results of this study show the extracts to possess significant antibacterial and antifungal activities to leaves extracts of *Citrus sinensis*, and are considered appropriate for empirical treatment of various diseases in the study area.

It is therefore, recommended that further studies of these extracts should be carried out by agar well dilution and other methods prepared in different solvents and evaluated again through in-depth analysis by fractionation, structure elucidation and docking studies. This is important because the tested plants are known to have medicinal properties which shall come to be known when evaluated on other body systems in functioning of body's metabolic pathways. Meaning, the plant extracts may have immunomodulation, anti-inflammatory, wound healing effects rather than the effects for which they were tested.

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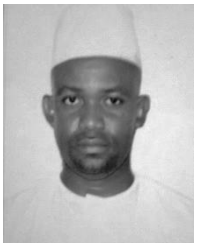
AUTHORS' PROFILE

Mr. Nasiru Mohammed had a B. Sc. (Hons.) Microbiology degree from Usmanu Danfodiyo University, Sokoto, Nigeria, M. Sc. Environmental Science of Islamic University in Uganda and M. Sc. Microbiology and Biotechnology, Department of Microbiology, Institute of Molecular Biology and Biotechnology, The University of Lahore, Pakistan. He had worked with Infectious Diseases Hospital and Public Health Laboratory, Amanawa, Sokoto and currently a Lecturer in Umaru Ali Shinkafi Polytechnic, Sokoto, Nigeria. He has a lot of experiences in different fields.

Mr. Yusuf Muhammad Sanyinna pursued B. Sc. Ed. (Hons.) Biology degree from Usmanu Danfodiyo University, Sokoto, Nigeria and currently undergoing M. Sc. Zoology with specialization in Parasitology from Department of Animal and Environmental Biology, Faculty of Life Sciences, Kebbi State University of Science and Technology, Aliero, Nigeria. He is currently working as

the Principal, Secondary Conventional Section as well as Biology and Animal Husbandry Teacher with Imam Malik Academy Nigeria (IMAN), Sokoto State, Nigeria. He is a member of Teachers' Registration Council of Nigeria (TRCN), Parasitology and Public Health Society of Nigeria (PPSN) and Zoological Society of Nigeria (ZSN). His main research works focus on Medical and Veterinary Parasitology, Bacteriology, Protozoology, Helminthology, Molecular Epidemiology, Entomology, Animal Physiology and Microbiology. He had taught in Government Secondary School, Sanyinna; Government Girls Day Secondary School, Sanyinna; Shehu Shagari College of Education Staff Secondary School, Sokoto; Sani Dingyadi Unity Secondary School, Sokoto; Bichi Academy, Kano; Nady Academy Schools, Sokoto and Brilliant Footsteps Int'l Academy, Sokoto. He has 5 years of teaching experience and 2 years of research experience. He has a lot of experiences in diverse fields of endeavour and leadership qualities.



Mr. Ridwan Nuhu Ahmed held a B. Sc. Ed. (Hons.) Biology degree from Usmanu Danfodiyo University, Sokoto, Nigeria and Postgraduate Diploma in Biotechnology of Department of Biological Sciences, Faculty of Science, Nigerian Defence Academy, Kaduna, Nigeria. He is also currently pursuing M. Sc. Zoology (Parasitology) degree from Kebbi State University of Science and Technology, Aliero, Nigeria. He has a lot of experiences and leadership capabilities.

