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Antibacterial Activity of Saponin Extracted from *Phyllanthus niruri* on Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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Authors' contributions

This work was carried out in collaboration among all authors. Author VAA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OAA and VAA managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

The antimicrobial activity of saponin extracted from Phyllanthus niruri was investigated on methicillin-resistant Staphylococcus aureus (MRSA). The nuclear magnetic resonance (NMR) was used to determine the structure spectra of the extracted purified saponin. The 13carbon NMR predicted on the basis of chemical shift that appeared in the resonances of 20 – 60 ppm gave a structure named Phylagenin-13-O-α-D-glucopyranoside and Phylagenin-25-O-β-D-glucopyranoside. The susceptibility profile of MRSA determined by the agar-diffusion method showed that 97.0% and 90.0% of the test bacterium were resistant to Tetracycline and Cotrimoxazole respectively and 60% of the bacterium was susceptible to saponin extract. The ability of saponin

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extracted from P. niruri to treat clinical manifestation like chest congestion and skin desquamation from which S. aureus resistant to conventional antibiotics have been isolated has been confirmed in this study. The fact that this extract exerted an inhibitory effect on MRSA indicates that they can potentially be further developed into antimicrobial clinically used agents.

Keywords: Antibacterial; methicillin-resistant; Phyllanthus niruri; saponin; Staphylococcus aureus.

1. INTRODUCTION

Historically, plants have provided a source of inspiration for novel drug compounds, as plant-derived medicines have made large contributions to human health and well-being [1]. Their roles are two-fold in the development of new drugs. They may become the base for the development of a medicine, a natural blueprint for the development of new drugs or, a phytomedicine to be used for the treatment of disease [2].

Higher plants especially tropical species, produce secondary metabolites with antibacterial activity [3]. Among higher plants, *Neurolaona lobata* and *Aristolochia* species which are commonly used to treat infections in Belizean folk medicine, have been shown to have activity against *Staphylococcus aureus, Escherichia coli, Proteus vulgaris,* and *Pseudomonas aeruginosa* [3].

Phyllanthus niruri L., (*Syn. P. fraternus* Webster) (Plate 1) is a common kharif (rainy season) weed found in both cultivated fields and wastelands [4]. It carries different nomenclature in different parts of the world. However, in Nigeria it is called *Asasa* or *Arunjeran* in Yoruba, *Majiryar Kurumi* in Hausa, *Asivi* or *Igbehen* in Edo, *Egu eza* in Ibo and *Oyomo-ke-iso-aman-ke-edem* in Efiki [5]. Although considered a problematic weed for farmers, it is a valuable medicine for herbalist [6] and holds a reputable position in both Ayurvedic and Unani systems of medicine. Recently it has attracted the attention of researchers, because of its hepatoprotective properties [6]. Although no effective specific therapy is available for viral hepatitis, *P. niruri* has shown clinical efficacy in the treatment of viral Hepatitis B [7].

Saponins are naturally occurring surface-active glycosides that are produced by plants. They derive their name from their ability to form stable, soap-like foams in aqueous solutions. This easily observable character has attracted human interest from ancient times [8]. Saponins are known to be antimicrobial, to inhibit mold, and to protect plants from insect attack [9,10]. Saponins may be considered a part of plants' defense system, and as such have been included in a large group of protective molecules found in plants named phytoanticipins' or 'phytochemicals' [11].

Plate 1. *Phyllanthus niruri* L. (Syn *P. fraternus* Webster)

A large number of the biological effects of saponins have been ascribed to their action on membranes. In fact, their specific ability to form pores in membranes has contributed to their common use in physiological research [12,13]. Saponins have long been known to have a lytic action on erythrocyte membranes and this property has been used for their detection. The hemolytic action of saponins is believed to be the result of the affinity of the aglycone moiety for membrane sterols, particularly cholesterol [10, 14] with which they form insoluble complexes.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a specific strain of *S. aureus* bacterium which is intrinsically insensitive to methicillin and all beta-lactamase (β-lactamase) antibiotics like dicloxacillin, cloxacillin, nafcillin, penicillin, and oxacillin [15]. Since 1960s, successive waves of epidemic methicillin-resistant *S. aureus* have spread out throughout hospitals and other chronic health care facilities worldwide, to the extent that it is now the most

commonly isolated antimicrobial resistant pathogen in many countries [15,16].

The mechanism of methicillin resistance is an altered penicillin-binding protein (PBP2a) in MRSA that markedly reduces affinity for all available beta-lactamase antibiotics, while maintaining effective cell wall binding activity. The PBP2a is encoded by the *mecA* gene that is carried on a mobile DNA element, the staphylococcal cassette chromosome [17].

Healthy individuals may carry MRSA asymptomatically for periods ranging from a few weeks to many years. Patients with compromised immune systems are at a significantly greater risk of symptomatic secondary infection [18]. MRSA may progress substantially within 24 hours to 48 hours of initial topical symptoms, after 72 hours. MRSA can remain in human tissues and eventually become resistant to treatment.

Despite successes achieved in controlling many infectious diseases, efforts to defend against the wide range of microbes that threaten human health continued to be challenged by emerging and re-emerging of infectious pathogens and possible use of a variety of virulent agents as biological weapons [19]. A defensive strategy based solely on developing new vaccines and antimicrobial and antiviral drugs, each specific for only one or a few agents, is unlikely to be successful in dealing with potential microbial threats and these are exceedingly expensive [20]. Therefore, the objective of this study is to extract crude saponin from the whole plant of *P. niruri*, determine its characteristics and the antimicrobial potency and efficacy on strains of MRSA.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

The matured and fresh leaves of the plant, *Phyllanthus niruri* were collected from farmlands in Ado-Ekiti during the raining season between the months of May and October (2004 – 2006). The plant material was air-dried at room temperature (27^{o}C $\pm$ 1^{o}C), was ground into the powdered form using milling machine (Retsch GmbH 5657 HAAH) and stored in air-tight plastic container. Identification and authentication of plant were performed in the Department of Plant Science, University of Ado-Ekiti, Nigeria and a voucher specimen was deposited as FPA/PS/2004:HB-291.

2.2 Extraction of Saponins

The method described by Martson et al. [21] was employed. The dried and powdered plant material (500 g) was defatted in a Soxhlet with petroleum ether at between 40^{o}C and 60^{o}C for 16 h. The residue was added to 100 mL of absolute methanol and left overnight under reflux at 70^{o}C. It was filtered with Whatman No. 2 mm filter paper and the filtrate evaporated to dryness with a rotatory evaporator. The yield was dissolved in 100 mL of distilled water, extracted in a separating funnel with 1-butanol three times and dried by evaporating. Finally, the extract was dissolved in 25 mL of absolute methanol and the saponins compound was precipitated by adding 75 ml of diethyl ether.

2.3 Determination of Saponins

This was determined using the method described by Martson et al. [21]. Thirty grammes of the milled leaves was weighed into 800mL beaker, 55 mL of 95% ethanol was then added, the mixture left for 48hr and filtered with muslin cloth. The filtrate was combined and shaken with a benzene-ether mixture (1:1 v/v) and decanted. The alcohol extract was concentrated under reduced pressure. The crystals that were deposited were removed by filtration with a Whatman No 2MM filter paper. N-butanol was used to dissolve the crystals, filtered, washed with water, dissolved in phosphate buffer solution (pH 7.0) and recrystallized with 95% ethanol to yield white crystals. The crystals were weighed and the saponin content was estimated in mg/100g as shown below; and the purity was determined using the isoelectrophorectic method with a purified commercially obtained saponin for comparison.

$$\frac{Weight\ of\ Crystals}{Weight\ of\ Sample}$$

2.4 ^{13}Carbon Nuclear Magnetic Resonance (NMR) Spectral Analysis of Saponin

The ^{13}C-NMR Model 2007,*47*/(6), was used to determine the structure spectra of the purified saponin extract. A dilution of 10 mg of the saponin was made in 10ml of distilled water. This was injected into the vial of the equipment. The analysis was done at standard conditions of

optical rotations of 20^{o} taken on a Perkin Elmer 241 polarimeter, Pulse of 92.9^{o}, total time of 9hr, 20 min, 51 sec; WALTZ-16 modulated and 19237 repetitions.

2.5 Isolation and Identification of Methicillin–Resistant *Staphylococcus aureus* (MRSA)

From the year 2005 through 2007 samples were collected from patients visiting State Specialist hospitals in Ado-Ekiti, Ikere-Ekiti, Ifaki-Ekiti, and Medical Centre, Ido-Ekiti; in Ekiti State, Nigeria. The samples collected included wound/pus, sputum, urine, respiratory swabs, gynecologic specimens, and stool, synovial fluid. Samples for MRSA detection were put on plates. *S. aureus* was confirmed by bound coagulase test. Isolates found to show resistance of ≤ 10 mm in diameter of zone of inhibition to some beta-lactam antibiotics (Amoxicillin, Cotrimoxazole, Nitrofunrantoin, Gentamicin, Nalidixic acid, Oflotarivid, Tobramycin, and Tetracycline) on a Kirby-Bauer disk diffusion assay were further tested by E test on Mueller-Hinton agar with 2% Sodium chloride incubated for 24 h, at 37^{o}C. Those with a MIC $\geq$ 4 µmol/mL were considered to be MRSA [22].

2.6 Determination of Antibacterial Potency of Crude Saponin

The disk diffusion method described by Brady and Katz [23] was employed. Various concentrations of the extract were prepared on test tubes (0.625 – 10 µg/mL). Disks obtained from Whatman No 5 MM filter paper were sterilized in an oven at 60^{o}C for 30 minutes and soaked in the extract for 2 hrs. A loopful of the final dilution (10^{3}) of the test bacterial suspension was spread on dried nutrient agar (Oxoid). The disks of different concentrations of the extracts were placed equidistance on the agar and incubated at 37^{o}C for 24 hrs. Zones of inhibition were measured in millimeter (mm) with a meter rule. Each procedure was repeated three times.

2.7 Determination of Antibacterial Efficacy of Crude Saponin

This was carried out using the agar dilution method as described by Smith et al. [3]. A colony from stock was sub-cultured into 5 mL of nutrient agar (LAB) and incubated at 37^{o}C for 18 h. Overnight broth (0.1 mL) of each organism was pipette into 9.9 mL of the broth to yield 10^{1} dilutions. Serial dilution was carried out to obtain a dilution of 10^{3}. A 2 cm streak of bacteria strains was made on dried nutrient agar plates containing increasing concentrations (0.625 µg/ml – 10 µg/ml) of the extract. The lowest concentration that gave no visible growth after overnight incubation at 37^{o}C was taken as the minimum inhibitory concentration (MIC) of the crude extract.

2.8 Antibacterial Kinetics of Crude Saponin

Kinetics of the antibacterial activity of the crude extract was determined in nutrient broth (LAB) using the methods described by Alade and Irobi [24]. The MRSA were exposed to increasing concentrations of the extract. Bacterial suspension (0.1 mL) was inoculated into 9.9 mL of fresh warm nutrient broth containing different concentrations (0.625 – 10 µg/mL) of the extract. The suspension (1.0 mL) was withdrawn immediately and the absorbance (optical density) was read with a Coleman Spectrophotometer (GallenKamp CH 3053) to give a control zero time absorbance. Samples were then taken at intervals of one hour over 5hours and read with a Coleman Spectrophotometer at wavelength 540 nm.

3. RESULTS AND DISCUSSION

3.1 Results

The ^{13}C-NMR spectra result of the purified saponin extract from *P. niruri* is shown in Fig. 1. The site of attachment of one sugar to another sugar can be predicted on the basis of chemical shifts in resonances of 20 – 60 ppm. A close resemblance of the chemical shifts due to a terminal sugar with respect to a methyl-O-glycoside lead to its immediate characterization whereas the chemical shift of other (inner) sugars differ significantly in comparison to methyl-O-glycosides. In oligoglycosides, the glycosylation causes a downfield shift of 20 – 60 ppm of the α-carbon, the hydroxyl of which has been directly involves in the glycosylation while neighboring β-carbon atoms show an upfield shift of 80 – 100 ppm. These α- and β-shifts are independent of the nature of the monosaccharide and provide a conclusive method for the establishment of interglycosidic linkages.

The susceptibility profile of MRSA is shown in Table 1. Twenty-nine 29(97%) of 30 isolates showed resistance to tetracycline while only 2 (7%) showed resistance to oflotarivid. The

highest susceptibility of 25 (83%) was observed in oflotarivid. The isolates were also susceptible to gentamicin 13(43%) and nitrofunrantoin 12 (40%).

The susceptibility of MRSA to the crude saponin is shown in Fig. 2. Of the 30 isolates, 18 (60%) isolates showed a zone of inhibition of diameter ranging between of ≥ 9.0 mm and 5.0 mm while 12 (40%) of the isolates showed a zone of inhibition of diameter ≤ 4.0 mm.

The antibacterial activity of different concentration of crude saponin extract to MRSA is shown in Fig. 3. At a higher concentration (1.5 mg/mL), the optical density the broth culture decreases from an initial optical density of 0.02 nm to 0.30 nm. The control culture shows a progressive increase in the optical density to 1.20 nm at an interval of 5 hours incubation. There is evidence of increased antibacterial activity as the concentration of the extract increases.

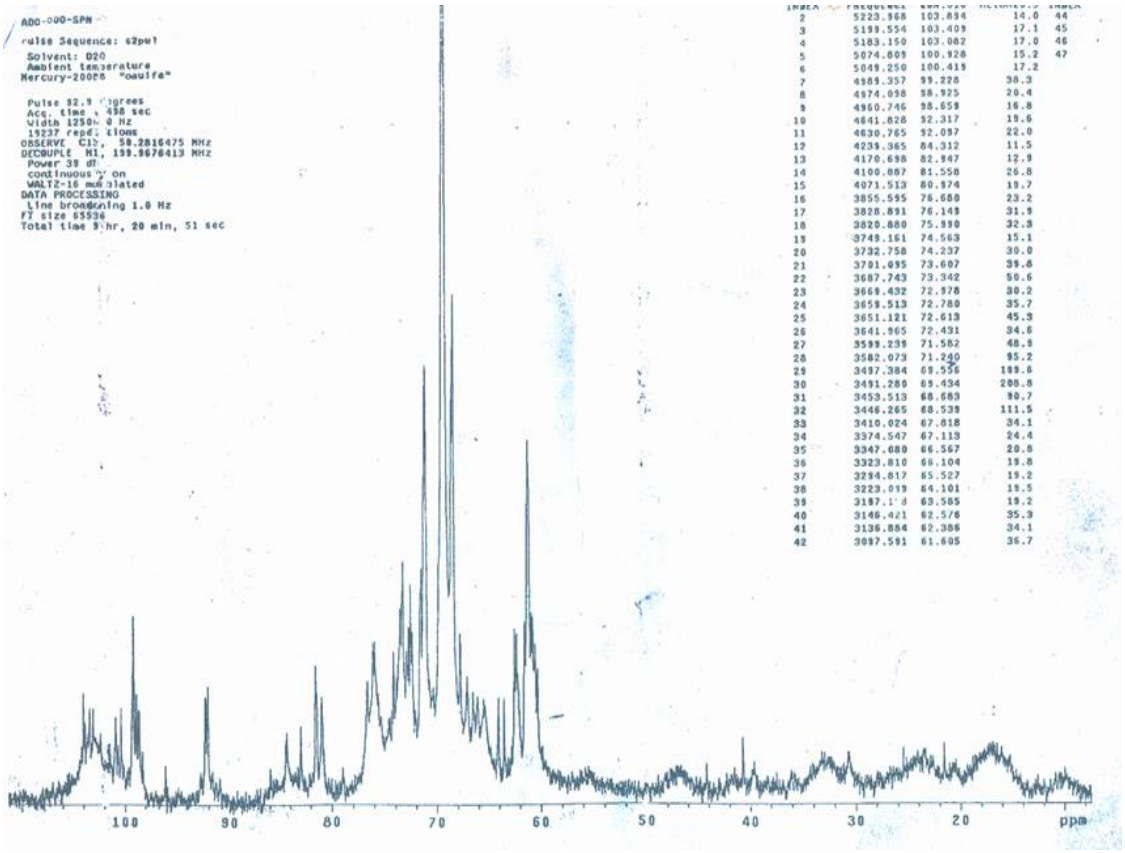

Fig. 1. The ^{13}Carbon NMR Spectra of the crude saponin extract

Table 1. Susceptibility profile of MRSA (n = 30)

Antimicrobial agent (µg)	Resistance pattern		
	S (%)	I (%)	R (%)
Amoxicillin	2 (6.8)	9 (30)	19 (63.3)
Cotrimoxazole	1 (3.3)	2 (6.8)	27 (90)
Nitrofunrantoin	12 (40)	4 (13)	14 (47)
Gentamicin	13 (43)	12 (40)	5 (17)
Nalidixic acid	11 (37)	11 (37)	8 (27)
Oflotarivid	25 (83)	3 (10)	2 (7)
Tobramycin	6 (20)	8 (27)	16 (53)
Tetracycline	0 (0)	1 (3.3)	29 (97)

S – Sensitive, I – Intermediate, R – Resistant

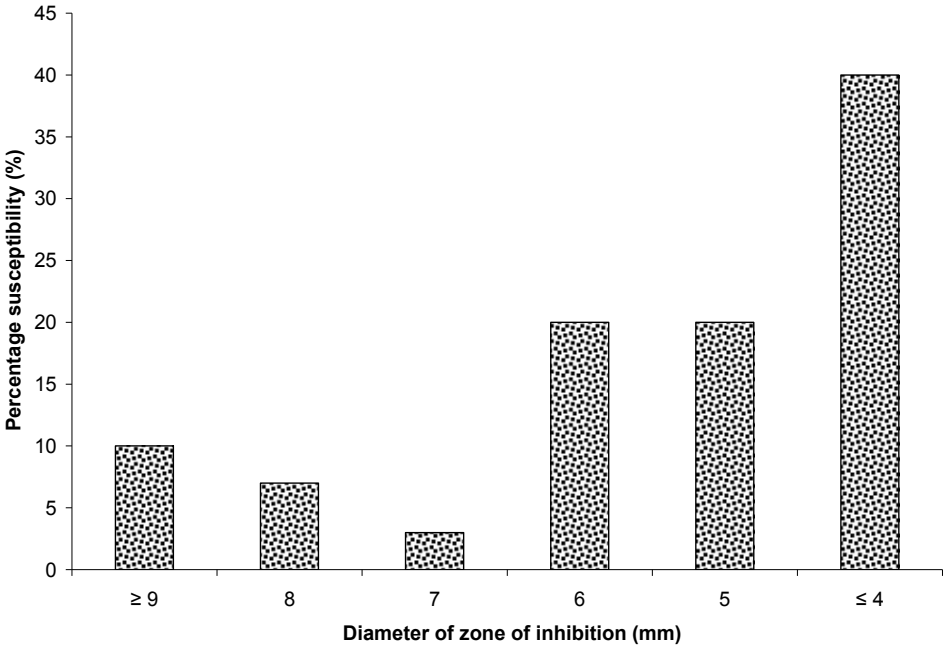

Fig. 2. Susceptibility of strains of MRSA to saponin extract of *Phyllanthus niruri* at a concentration of 1.5 mg/mL

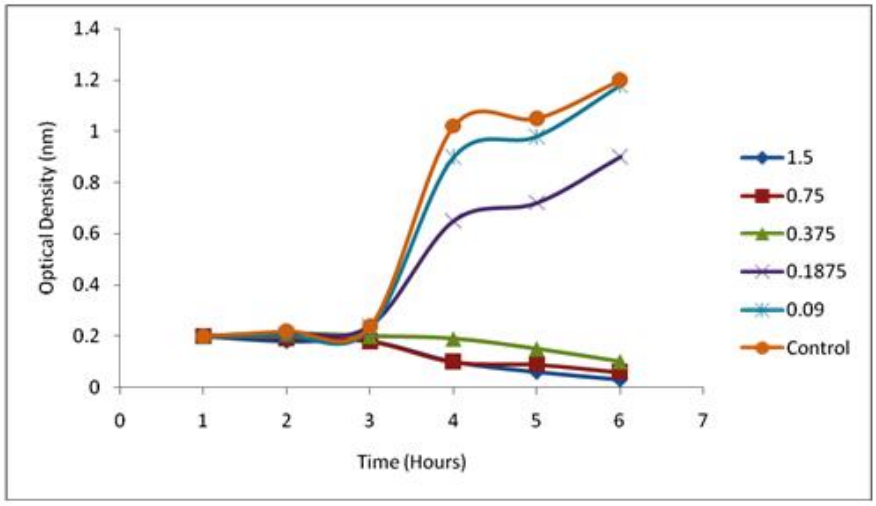

Fig. 3. The optical activity of different concentration of crude saponin extracts on MRSA

3.2 Discussion

The resistance pattern of *S. aureus* showed that a high percentage of this isolate is resistant to most of the commonly used antibiotics in the classes of β-lactams (amoxicillin), tetracycline, folate inhibitors (co-trimoxazole), aminoglycosides (tobramycin and nitrofunrantoin). It has been reported that similar to pathogenic bacteria, commensals are exposed to the selective pressure of antimicrobial agents [25]. The findings of this study were in substantial agreement with previous reports of high resistance rates of bacteria from low resource settings [22,26].

In recent years, outbreaks of MRSA infection have been reported in different settings,

outpatients and settings visiting hospitals, including hospitalized or surgery patients [27], as well as nursing homes and convectional facilities [22]. The problems of MRSA are increasing worldwide. MRSA is no longer restricted to hospital settings but is found in homes, places of work and kindergartens [28]. A number of risk factors for MRSA infection have been identified in those studies to include antimicrobial drug use, close contact with persons colonized with MRSA and barriers to medical care. Antibiotic drug self-medication is a cause for concern because it has contributed to the spread of antimicrobial drug resistance. Self-treatment with a drug that is ineffective against a disease causative organism or with an inappropriate dosage may increase the risk of selection of resistant organism that may be difficult to eradicate. These resistant organisms may then be transferred into the community. Unrecognized community associated-methicillin resistant *Staph aureus* (CA-MRSA) colonization during hospitalization could become an additional method of its dissemination in the community. Increased prevalence of CA-MRSA has been reported in Chicago, Los Angeles, Texas, and Minnesota [29].

Over the last 60 years or more, bacteria and, in particular, those pathogenic for humans have evolved toward antimicrobial drug resistance. Humans cannot control emergence because it occurs by chance and represents a particular aspect of bacterial evolution. Emergence can result from mutations in house-keeping, structural or regulatory genes or from acquiring foreign genetic information [20]. However, much can be done to delay the subsequent spread of resistance. Dissemination can occur at the level of the bacteria (clonal spread), replecons (plasmid epidermis), or of genes (transposons). These three levels of dissemination, which occur in nature, are not only infectious but also exponential, since all are associated with DNA duplication.

The activity of crude saponin extract showed high efficacy on multidrug-resistant *S. aureus* 60% of the isolates showing susceptibility. The highest percentage of resistance (40%) was observed. These findings showed that saponin extract from *P. niruri* is potent against multidrug-resistant *S. aureus* and it further substantiates the findings of Sen et al. [30] where it was observed that the growth of *S. aureus* was lowered at levels of 0.25% (w/v) by *Quillaja saponaria* saponin. Saponin was reported to have the potential to modulate microbial growth [31]. In this work it was observed that the crude saponin exhibited modulatory activities on MRSA, hence, it complements the earlier report. Membrane fluidity controls the enzyme activity of biological membranes and plays an important role in ion transport [32] and also controls the ability of saponins to affect the cellular function of bacteria. It has been postulated that higher concentration of saponin from whatever extract can be toxic. The involvement of body fluids e.g. enzymes possess the ability to alter the chemical structure of saponin thereby reducing its toxicity.

4. CONCLUSION

The ability of saponin extracted from *P. niruri* to treat skin infection and pneumonia has been observed in this study. These favorable effects point to the potential of the saponin as a remedy against these two major health hazards in many countries including Nigeria. The fact that this extract exerted an inhibitory effect on MRSA indicates that they can potentially be further developed into antimicrobial agents that can be used as antidotes to treat ailments from where isolation of *S. aureus* has been significant.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical permission has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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