Evaluation of the Anti-virulent Potentials of Ginger (*Zingiber officinale* L) and Garlic (*Allium sativum*) on *S. aureus* and *P. aeruginosa*

Abstract

The degree of a pathogen to cause infection is termed virulence. Anti-virulence substances are substances that could inhibit the expression of virulence by these pathogens thereby limiting the degree of diseases caused. The study aimed to evaluate the anti-virulence of Zingiber officinale and Allium sativum on S. aureus and P. aeruginosa. The Methicillin-resistant Staphylococcus aureus and Carbapenem-resistant Pseudomonas aeruginosa associated with clinical specimens were collected from the Microbiology Research Laboratory, Department of Microbiology, Rivers State University. The extracts of Zingiber officinale and Allium sativum were prepared using standard method. The virulence considered included lecithinase, biofilm formation, coagulase, swarming, haemolysis and pyocyanin. Thus, the inhibition of this virulence as well as curring the isolates was carried out using standard method. Results showed that 30, 10, 20 and 20% of the S. aureus isolates were inhibited by the ginger extract from producing haemolysis, biofilm, coagulase and lecithinase, respectively. While 10%, 20%, 10% and 30% of the isolates were inhibited after treatment with garlic extract. For P. aeruginosa, 20%, 30%, 40%, 20% and 40% were inhibited by ginger extract from producing haemolysin, biofilm, lecithinase, pyocyanin and swarming, respectively. The susceptibility tests after curring with the extracts and acridine orange showed that both S. aureus and P. aeruginosa were susceptible to some of the antibiotics they resisted before. The spices having shown anti-virulence properties against these pathogens, they could be evaluated for better options in the fight against bacterial pathogens and antimicrobial resistance.

Keywords: Anti-virulence, Zingiber officinale, Allium sativum, S. aureus, P. aeruginosa

Introduction

Infectious diseases have been ranked not only as the top 10 causes of death but also as the main cause of lifelong disabilities worldwide (Stoyanka *et al.,* 2022). Carbapenem-resistant-*Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* have been classified as priority 1 and 2 critical pathogens, respectively due to the severity of the disease they cause (WHO, 2017). The ability of a microbe to cause disease is known as virulence and several microbial pathogens produce molecules known as virulence factors, which aid in colonisation, immunoevasion, immunosuppression, obtaining nutrition, and causing damage to host cells (Dehbanipour and Ghalavand, 2022). The search for methods and substances capable of suppressing bacterial virulence is a new but quickly developing field of study (Pacios et al., 2020). This new tactic in the fight against bacterial infections is referred to as anti-virulence therapy (Fleitas *et al.,* 2019; Pacios *et al.,* 2020). Instead of killing the bacteria, its goal is to suppress the expression of phenotypes linked to bacterial virulence. Theuretzbacher and Piddock, (2019) opined that pathogens utilise virulence factors including behavioural phenotypes like biofilm formation, to invade, colonise, and persist in a host that is susceptible to them.

The relationship between pathogen and host is reciprocally devastating during acute infection, as bacteria produce a variety of cytotoxic molecules that impair host cellular processes, while bacteria encounter immune system responses such as the production of antimicrobial compounds and reactive oxygen species, as well as enhanced phagocytosis (Moradali *et al.,* 2017). Swimming and swarming with flagella, as well as twitching with type 4 pili, are all related to virulent characteristics in *P. aeruginosa* (Winstanley *et al.,* 2016) while coagulase is mostly associated with *S. aureus*. Flagellar and/or other motility components mediate recognition and stimulation of signalling pathways that elicit inflammatory reactions and phagocytosis by murine or human

macrophages, making a motile cell easily identifiable by the host immune system (Winstanley *et al.,* 2016). Many pathogenic bacteria, such as *P. aeruginosa*, evade stressors and harsh circumstances by switching to a sessile lifestyle along with diminished virulence. They lose their mobility and cling to surfaces, forming cellular aggregations or microcolonies that are protected from the environment by extracellular polymeric substances (EPS) (Moradali *et al*., 2017). Virulence determinants, quorum sensing and biofilm synthesis provide some attractive targets for the design and development of a new group of antimicrobial compounds (Divyakolu *et al.*, 2019). Although the search for anti-virulence factors is ongoing, there is a dearth of information especially on the anti-virulence of *Zingiber officinale* and *Allium sativum*. Thus, the present study investigated the anti-virulence of these spices on clinical isolates of CRPA and MRSA.

Materials and Methods

Sample Collection

Ginger (*Zingiber officinale* L) and Garlic (*Allium sativum* L) were bought randomly from vendors in the Mile III market. The spices were collected in clean polyethene bags and were transported to the Microbiology laboratory, Department of Microbiology, Rivers State University, Nigeria.

The bacterial isolates: Methicillin-resistant *Staphylococcus aureus* and Carbapenem-resistant *Pseudomonas aeruginosa* associated with clinical specimens were collected from the Microbiology Research Laboratory, Department of Microbiology, Rivers State University, Rivers State, Nigeria. The isolates were selected on the criteria of exhibiting multi-drug resistance, production of biofilm and possessed enzymes such as lecithinase, coagulase and haemolysin

Preparation of Crude Extracts of Ginger and Garlic

The ginger and garlic were prepared as described by Kibiti and Afolayan (2015). In this method, spices were rinsed with sterile distilled water, shade dried and ground to homogenous powder using a sterile blender (sterilized with 96% ethanol). Methanol (99.8%) and ethanol (99.9%) of analytical grade which was bought from Jochemicals, Choba, Rivers State were the extracting solvents used. During extraction, fifty grams (50g) of the powdered samples were transferred into sterile beakers containing 100ml of extracting solvents (ethanol and methanol, respectively) which had been labelled accordingly. The samples were then allowed to stand for 48 hours to increase the extractions of bioactive components. After extraction, both samples were filtered using Whatman No1 filter paper into a clean beaker. The filtrates obtained were evaporated to dryness at 50°C in a water bath (Emmanuel *et al*., 2021) and the residues were stored in sterile beakers for further use. The aqueous extracts were prepared by blending the spices separately using a sterile electronic blender. The resulting extracts were filtered and the supernatant was stored in the refrigerator in clean sterile bottles.

Determination of Anti-virulence of Extracts on Isolates

The virulence of the isolates was determined using the methods of previous studies (Chong *et al.*, 2011, 2018; Hemeg *et al.*, 2020; Pu *et al.*, 2020; Wojnicz *et al.*, 2012).

Inhibition of Biofilm

The effect of the extracts on the biofilm formation of the bacterial isolates was determined as described by Chong *et al.* (2011, 2018). In this method, overnight cultures of *P. aeruginosa* and *S. aureus* were transferred separately into a prepared peptone medium containing the extract concentration (3.125mg/m) and 0.5% (w/v) glucose (filter-sterilized, pore size of 0.22 μm) to an

OD600 of 0.1; tubes were incubated at 37 ℃. Cells grown in the absence of the extracts served as control (Husain *et al*., 2017). After incubation, cells were discarded, and the tubes were air- dried for 15 min and stained with 1 mL of 1% (v/v) crystal violet for 45 min. The stained biofilms were washed again with sterile distilled water followed by the addition of 2 mL of ethanol (95%, v/v). The resulting solution (100 µL) was transferred to a cuvette and the absorbance determined at 590 nm (Chong *et al.,* 2018). The experiments were performed in duplicate. Results was interpreted as negative (OD <0.17), weak positive (OD 0.17-0.34), moderate positive (OD 0.35-0.68) and strong positive (OD >0.68) (Harika *et al.,* 2020). The isolates were also inoculated on congo red agar plates.

Inhibition of Coagulase formation

The anti-coagulase assay was determined by culturing the test cultures of *S. aureus* with the extract concentration (3.125mg/m) and incubated for 24 hours. After incubation, the cells were tested for coagulase activity as described previously. The control included coagulase positive *S. aureus* not incubated with the extract.

Inhibition of Haemolysis formation

The anti-haemolytic activities of the bacterial isolates were determined as described by Husain and Ahmad (2013) with slight modification. *P. aeruginosa* and *S. aureus* isolates were grown for 20 hours with or without the extracts. Cells were harvested by centrifugation and the supernatant was filter-sterilized (syringe filters pore size, 0.22 lm). The filter-sterilized supernatants were streaked on blood agar and incubated at 37℃ for 24 hours. Haemolytic activity was observed as a clearing while a lack of haemolytic activity was characterized by the absence of clearing (no zone of inhibition or clearing). The extract concentration used was 3.125mg/ml.

Inhibition of Swarming and motility

This was determined as described by Husain *et al*. (2017). In this method, medium containing 1% tryptone, 0.5% NaCl and 0.3% agar after sterilization was mixed with different concentrations (3.125mg/m and 6.25mg/ml) of the extracts. On cooling, the plates were point inoculated with the test cultures of *P. aeruginosa*. After overnight incubation, diameter of swarm was measured. The control was plates containing the isolates without the extracts.

Anti-Lecithinase Activity

In this method, *P. aeruginosa* and *S. aureus* isolates were grown for 20 hours with or without the extracts. Cells were harvested by centrifugation and the supernatant was filter-sterilized (syringe filters pore size, 0.22 µm). The filter-sterilized supernatants were streaked on egg yolk agar and incubated at 37℃ for 24 hours. Formation of an iridescent layer that denotes lypolysis and an opalescence showed lecithinase activity.

Curing of Bacterial Isolates with Extracts

The bacterial isolates were cured as described by Letchumanan *et al.* (2015). Overnight bacteria cultures were inoculated into enrichment broths, Luria Bertani Broth (LB). The extract (curing agents) at a concentration of 25 mg/ml was added to the culture broth. The cultures were then incubated overnight at 37℃. After the treatment, antibiograms assay was carried out to find antibiotic resistant phenotypes.

Results

Results of the virulence of the *S. aureus* isolates after treatment with extracts of ginger and garlic is presented in Table 1. Results showed that after treatment with ginger extract, 70% of the isolates were haemolytic (i.e., they possess the haemolysin enzyme) while 30% were no longer haemolytic.

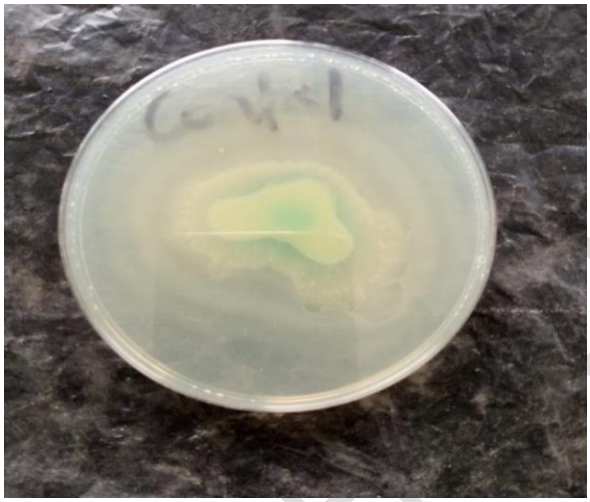
Out of the 10 isolates that produced biofilm, results showed that 90% were positive for biofilm while 10% didn't form biofilm. More so, the ginger extract prevented the production of coagulase and lecithinase enzymes on 20% of the isolates. For the garlic extract, 10%, 20%, 10% and 30% of the isolates were prevented from producing haemolysin, biofilm, coagulase and lecithinase after treatment with garlic extract.

Results of the virulence of *P. aeruginosa* isolates after treatment with extracts of ginger and garlic is presented in Table 2. Results showed that after treatment with the ginger extract, 20%, 30%, 40% and 20% of the *P. aeruginosa* isolates were inhibited from producing haemolysin, biofilm, lecithinase and pyocyanin while 40% were also prevented from swarming (Plate 1 & 2).

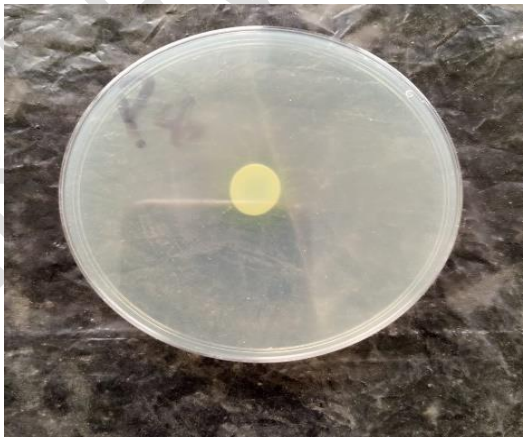
Results of the antibiotic susceptibility pattern of *S. aureus* after treatment with ginger, garlic and acridine orange (dye) is presented in Fig. 1. The percentage susceptibility of the isolates to ceftazidime, gentamycin, Erythromycin, ofloxacin, Augmentin and oxacillin was 10%, 80%, 20%, 100%, 10% and 30% after treatment with ginger extract while the percentage susceptibility to gentamycin, ofloxacin and oxacillin after treatment with garlic extract was 90%, 100% and 10%, while the percentage susceptibility to ceftazidime, cefuroxime, gentamycin, ceftriaxone, Erythromycin, cloxacillin, ofloxacin, Augmentin and oxacillin after curing with acridine orange was 30%, 10%, 90%, 30%, 20%, 20%, 100%, 20% and 70%, respectively.

Results of the antibiotic susceptibility pattern of *P. aeruginosa* after treatment with ginger, garlic and acridine orange (dye) is presented in Fig. 2. Results showed that the *P. aeruginosa* isolates after curing with extracts and dyes were susceptible to antibiotics which they had showed resistance to. Although, some were still resistant to antibiotics after curing. The percentage susceptibility of the isolates to ofloxacin, gentamycin, nalidixic acid, levofloxacin, ceftriaxone, imipenem and norfloxacin after treatment with ginger extract was 100%, 100%, 100%, 100%,

80%, 10% and 70%, respectively. The percentage susceptibility of the isolates to ofloxacin, gentamycin, nalidixic acid, levofloxacin, ceftriaxone, and norfloxacin after treatment with garlic extract was 100%, 90%, 90%, 100%, 70% and 70%, respectively. While the percentage susceptibility of the isolates to ofloxacin, gentamycin, nalidixic acid, levofloxacin, ceftriaxone, Augmentin, imipenem and norfloxacin after curing with acridine orange was 100%, 90%, 100%, 100%, 80%, 10%, 40% and 100%, respectively. The acridine orange showed a wider effect compared to ginger and garlic.

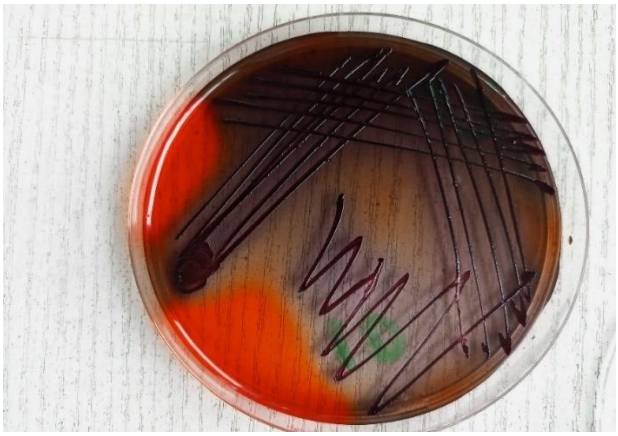


a (control)



b (treated isolate)

Plate 1. Swarm motility (a: swarming of *P. aeruginosa;* b: inhibition of swarming)



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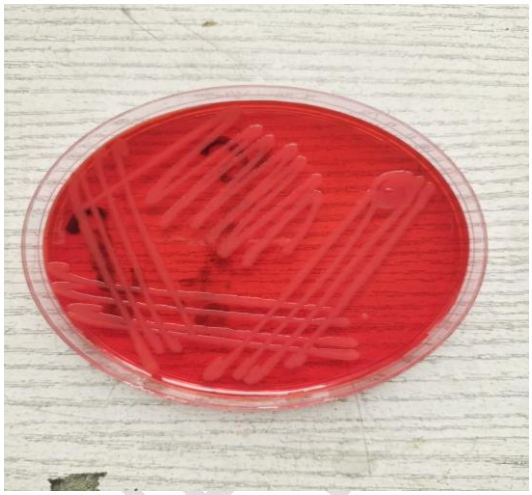

b

Plate 2. Biofilm Results (a: biofilm formation; b: lack of slim after treatment with herbal extract)

Table 1. Virulence Features of *S. aureus* after treatment with Extracts

Extract Type	Haemolysis [n (%)]		Biofilm [n (%)]		Coagulase [n (%)]		Lecithinase [n (%)]	
	β	γ	+ve	-ve	+ve	-ve	+ve	-ve
Ginger	7 (70)	3 (30)	9 (90)	1 (10)	8 (80)	2 (20)	8 (80)	2 (20)
Garlic	9 (90)	1 (10)	8 (80)	2 (20)	9 (90)	1 (10)	7 (70)	3 (30)

Keys: β = beta haemolysis; γ = gamma haemolysis; +ve = positive; -ve = negative

Table 2. Virulence Features of *P. aeruginosa* after treatment with Extracts

Extract Type	Haemolysis [n (%)]		Biofilm [n (%)]		Swarming [n (%)]		Lecithinase [n (%)]		Pyocyanin pigment [n (%)]	
	β	γ	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Ginger	8 (80)	2 (20)	7 (70)	3 (30)	6 (60)	4 (40)	6 (60)	4 (40)	8 (80)	2 (20)

Garlic	10 (100)	0 (0)	9 (90)	1 (10)	8 (80)	2 (20)	8 (80)	2 (20)	7 (70)	3 (30)

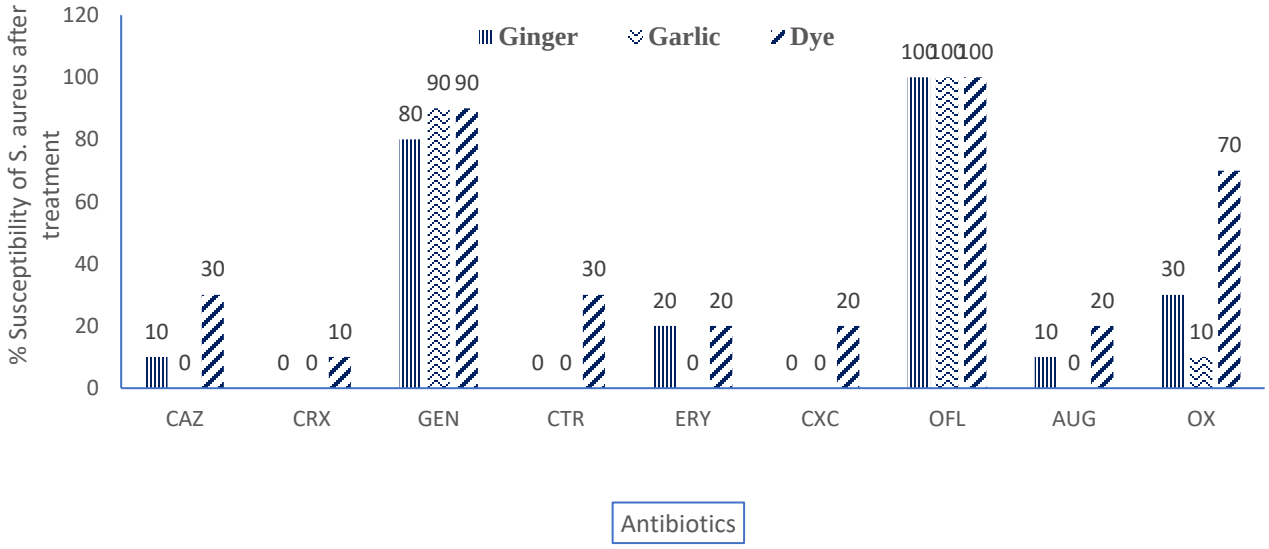

Fig. 1. Antibiotics Susceptibility Pattern of *S. aureus* after treatment with Extracts and Dye

Keys: CAZ: ceftazidime; CRX: cefuroxime; GEN: gentamycin; ofl: ofloxacin; AUG: augmentin; CXM: cefixime; NIT: nitrofurantoin; CPR: ciprofloxacin

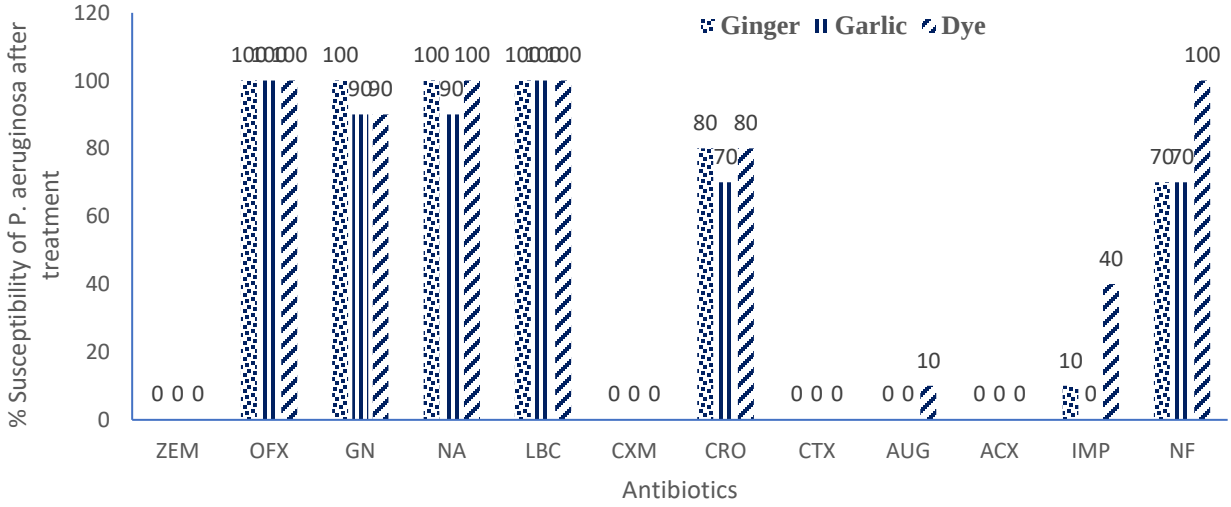

Fig. 2. Antibiotics Susceptibility Pattern of *P. aeruginosa* after treatment with Extracts

Discussion

The Quorum sensing (QS) which is a cell density-dependent global gene regulator is known to be responsible for regulation of virulent factor in most bacterial pathogens (Husain *et al.,* 2017). QS refers to the ability to detect extracellular, small molecule signals and to alter gene expression in response to bacterial population densities (Husain and Ahmad, 2013). Thus, obstructing the activities of the QS could be an alternative strategy in curbing bacterial pathogenicity. The ginger and garlic extracts displayed anti-QS activity as they were able to inhibit expression of virulent factors in some of the *S. aureus* and *P. aeruginosa.* The ginger extract showed more anti-QS activity in inhibiting the expression of haemolysis, biofilm, coagulase and lecithinase compared to the garlic extract. The activities of the virulent factors mentioned in *S. aureus* pathogenicity is well documented. Thus, interfering with the expression of these virulent factors is one of the interesting discoveries in the present study. The garlic extract showed no activity in interfering with haemolysis in all *P. aeruginosa* isolates but showed higher interference with the production of pyocyanin by *P. aeruginosa* isolates compared to the ginger extracts. While the ginger extracts had higher anti-QS activity than the garlic extract in interference with haemolysis, biofilm formation, swarming motility and lecithinase production. Generally, both extracts have shown that they possessed ant-virulence activity despite the variations observed in activities. Pyocyanin is a blue secondary metabolite that has the potential to produce free radicals and is a key factor in the pathogenicity of *P. aeruginosa* and it typically affects ion transports as well as mucus secretion in respiratory epithelial cells in cystic fibrosis patients (Cámara *et al.,* 2002). Several studies have demonstrated that the production of rhamnolipids, proteases, and elastases as well as a complex synchronisation of QS systems, including lasR-lasI, rhlR-rhlI, and mvfR-haq, govern the synthesis of pyocyanin (Gallagher *et al.,* 2002; Lau *et al*., 2004). Thus, the ability to inhibit the production of pyocyanin could mean the extracts possessed certain substances that interfered with rhl system.

Chong *et al.* (2018) in their study of the effects of Chinese herbal medicines on the quorum sensing-regulated virulence in *P. aeruginosa* showed that the extracts were able to inhibit pyocyanin production and swarming motility of the isolates which agreed with the present study. QS and biofilm formation in *S. aureus* isolates were also inhibited by extracts of *Officinalis* plant (Pu *et al*., 2020) while Husain *et al.* (2017) reported a decrease in elastase production, pyocyanin pigment production, protease activity and biofilm formation in *P. aeruginosa* isolates after treatment with Leaf extracts of *Mangifera indica.* In a previous study, medicinal plants have also been used to inhibit the expression of virulence factors: biofilm formation, haemagglutination and motility in uropathogenic *E. coli* (Wojnicz *et al.*, 2012). The use of antimicrobial agent in inhibiting the expression of haemolytic activity have been demonstrated in a previous study (Husain and Ahmad, 2013).

The antibiotic susceptibility of the *S. aureus* and *P. aeruginosa* isolates improved after treatment with ginger, garlic and acridine dye. Antibiotics such as ceftazidime, cefuroxime, ceftriaxone, cloxacillin and Augmentin which were not sensitive to the *S. aureus* isolates showed sensitivity after treatment with respective substances. The present study also showed that the acridine orange was more effective followed by the ginger extract in enhancing susceptibility of the *S. aureus* isolates than the ginger extracts. Previous study have reported that plant extracts could enhance the susceptibility of resistant pathogens of *P. aeruginosa* and *S. aureus* to antibiotics which they had earlier developed resistance for (Li *et al.*, 2015). Thus, this agreed with the present study. It was also reported that due to the increase in antibiotics resistance, the concept of enhancing antimicrobial activity by mixing herbal medicines and antibiotics appears to be a promising strategy (Bharwaj *et al.*, 2016). The enhancement of the antibiotics could either be that the medicinal plants enhanced the movement of the antibiotic agent to the site of action, interfered

with binding sites or played key role in one of the many steps in bacterial replication. This agreed with previous studies which had earlier reported that garlic/allicin-antibiotic interaction appear to be directed mostly toward β-lactam antibiotics (Pillai *et al.* 2013; Gaekwad and Trivedi 2013; Shah and Williamson 2019). According to Magryś *et al.* (2021) the alteration in the structure and integrity of the bio membranes, which enables the uptake and subsequent attainment of the target by the tested antibiotics, is a likely explanation for the increased antibacterial activity of antibiotics when combined with garlic. Furthermore, previous studies have reported that curing the plasmids of bacterial isolates using intercalating agents such as acridine orange or ethidium bromide could preferentially inhibit plasmid replication thereby enhancing susceptibility to antimicrobial drugs they had resisted before (Letchumanan *et al.*, 2015). Onyeadi and Agbagwa (2019) in their study reported a decreased resistant rate of *E. coli* isolates to eight antibiotics which they had previously shown resistance to. Despite this observation, they also opined that the resistance to some of the antibiotics could mean that the plasmids were not completely responsible for the resistance. This agreed with the present study in which despite the progress made by the curing agents (acridine orange, ginger and garlic extracts) some of the isolates were still resistant to antibiotics.

Conclusion

In conclusion, the study on evaluation of the anti-virulent potentials of *Zingiber officinale* and *Allium sativum* on *S. aureus* and *P. aeruginosa* showed that the extracts could be used as anti-virulent substances against these isolates. Although both extracts displayed varying anti-virulence against *P. aeruginosa* and *S. aureus,* the ginger extracts were more potent than the garlic extracts. More so, the extracts displayed curring abilities which could be compared to chemical curring agents. Thus, more studies in the use of ginger and garlic as anti-virulence substance as well as

curring agent is recommended. Molecular mechanisms of the anti-virulence of ginger and garlic on *P. aeruginosa* and *S. aureus* is recommended.

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