CHAPTER 7

RESULTS

7.1 Antimicrobial activity of *Anzia ornatoides*

The antimicrobial activity of lichen extracts against the tested organisms are shown in Table 7.1–7.10 & Plate 7–39. All the lichen extracts showed wide spectrum antimicrobial properties against all MDR strains at different concentrations. But the extracts of *A. ornatoides* were inactive against the other three yeasts C. *glabrata*, *C. krusei*, and *C. tropicalis*.

The results of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Minimum Fungicidal Concentration (MFC) of the extracts against all the MDR microorganisms are represented in (Table 7.9–7.10 & Plate 37–39). These experiments were performed for microorganisms which are susceptible to the crude extracts.

7.1.1 Disc-diffusion assay

In disc-diffusion, the most susceptible bacterium was *S. aureus*, that recorded 24.00±0.00 mm zone of inhibition with methanol extract followed by *K. pneumoniae* (19.67±0.33 mm) with water extract, *E coli* (18.33±0.33 mm) with ethyl acetate extract and *E. faecalis* (16.67±0.33 mm) with methanol extract, respectively at the concentration of 25 mg/ml (Table 7.3 & Plate 15–18). *C. albicans* showed highest zone of inhibition (23.00±0.00 mm) against the methanol extract at the concentration of 25 mg/ml (Table 7.4 & Plate 21). Comparing the results to the antibacterial agent gentamicin (25%), which demonstrated an inhibitory zone against *S. aureus* (22 mm), *E. coli* (20 mm), *K. pneumoniae*, and *E. faecalis* with 16 mm each, were noteworthy (Table 7.3 & Plate 15–18).

Table 7.1. ZOI of bacterial strains and gentamicin against the lichen extracts (20 µl of 15 mg/ml) by disc-diffusion assay

MDR	ZOI of Extracts (mm)					Gen.
Bacteria	HE	DE	EA	ME	WE	(mm)

E. faecalis	11.00±0.00	12.67±0.34	10.67±0.33	12.00±0.00	10.00±0.00	16
S. aureus	16.00±0.00	16.33±0.33	16.00±0.00	17.00±0.00	16.67±0.33	22
E. coli	12.67±0.33	13.00±0.00	12.33±0.34	13.00±0.00	14.33±0.33	20
K. pneumoniae	14.67±0.34	14.33±0.33	14.33±0.33	14.00±0.00	16.00±0.00	16

Table 7.2. ZOI of bacterial strains and gentamicin against the lichen extracts (20 µl of 20 mg/ml) by disc-diffusion assay

MDR	**ZOI of Extracts (mm)**					**Gen.**
Bacteria	**HE**	**DE**	**EA**	**ME**	**WE**	(mm)
E. faecalis	12.00±0.00	13.00±0.00	11.00±0.00	13.00±0.00	12.00±0.00	16
S. aureus	19.00±1.53	20.00±0.00	19.00±0.00	19.00±0.00	20.00±0.00	22
E. coli	14.67±0.34	14.67±0.34	15.00±0.00	15.33±0.33	16.00±0.00	20
K. pneumoniae	16.67±0.34	15.33±0.33	15.67±0.34	17.00±0.00	16.67±0.34	16

Table 7.3. ZOI of bacterial strains and gentamicin against the lichen extracts (20 µl of 25 mg/ml) by disc-diffusion assay

MDR	**ZOI of Extracts (mm)**					**Gen.**
Bacteria	**HE**	**DE**	**EA**	**ME**	**WE**	(mm)
E. faecalis	12.33±0.33	13.67±0.33	16.00±0.00	16.67±0.33	13.00±0.00	16
S. aureus	20.67±0.34	21.67±0.33	21.67±0.33	24.00±0.00	22.00±0.00	22
E. coli	16.67±0.34	18.00±0.00	18.33±0.33	18.00±0.00	17.67±0.34	20
K. pneumoniae	17.00±0.00	16.67±0.88	16.67±0.33	18.33±0.33	19.67±0.33	16

Table 7.4. ZOI of *C. albicans* and fresocan against varied concentration of the lichen extracts (20 µl) by disc-diffusion assay

Conc.	**ZOI of Extracts (mm)**					**Fresocan**
	HE	**DE**	**EA**	**ME**	**WE**	(mm)

15 mg/ml	14.67±0.34	14.33±0.33	14.33±0.33	14.00±0.00	15.00±0.00	19
20 mg/ml	16.00±0.00	16.00±0.00	17.67±0.34	17.00±0.00	16.33±0.33	19
25 mg/ml	21.67±0.34	22.00±0.00	18.00±0.00	23.00±0.00	21.00±0.00	19

7.1.2 Agar-well diffusion assay

In agar-well assay, the highest zone of inhibition against the bacterial strain was observed in water extract at the maximum concentration (25 mg/ml) against *S. aureus* (25.33±0.33 mm), followed by *K. pneumoniae* (23.33±0.33 mm), *E. coli* (22.00±0.00 mm) and *E. faecalis* (18.00±0.00 mm), respectively (Table 7.7 & Plate 30–33). The results were notable compared with the antibacterial drug gentamicin (25%), that showed inhibitory zone against *S. aureus* (26 mm) followed by *E. coli* (22 mm), *K. pneumoniae* (20 mm) and *E. faecalis* (17 mm) (Table 7.7 & Plate 30–33). However, highest zone of inhibition was obtained for water extract (29.00±0.00 mm) against *C. albicans* at the concentration of 25 mg/ml (Table 7.8 & Plate 36). The findings revealed that the maximum zone in extracts was greater than that of the antifungal drug fresocan at the concentration of 20 and 25 mg/ml (Table 7.8 & Plate 35–36).

Table 7.5. ZOI of bacterial strains and gentamicin against the lichen extracts (50 µl of 15 mg/ml) by agar-well diffusion assay

MDR Bacteria	**ZOI of Extracts (mm)**					**Gen.** (mm)
	HE	**DE**	**EA**	**ME**	**WE**	
E. faecalis	15.00±0.00	15.33±0.33	15.00±0.00	16.00±0.00	16.00±0.00	17
S. aureus	21.67±0.33	21.33±0.33	22.00±0.00	23.00±0.00	24.00±0.00	26
E. coli	16.00±0.00	16.33±0.33	17.33±0.33	16.00±0.00	18.00±0.00	22
K. pneumoniae	15.33±0.33	16.00±0.00	16.00±0.00	15.00±0.00	19.00±0.00	20

Table 7.6. ZOI of bacterial strains and gentamicin against the lichen extracts (50 µl of 20 mg/ml) by agar-well diffusion assay

MDR Bacteria	ZOI of Extracts (mm)					Gen. (mm)
	HE	DE	EA	ME	WE	
E. faecalis	15.33±0.33	15.67±0.34	15.33±0.33	16.00±0.00	16.00±0.00	17
S. aureus	21.67±0.33	22.00±0.00	22.00±0.00	23.00±0.00	24.00±0.00	26
E. coli	18.00±0.00	18.00±0.00	18.00±0.00	19.00±0.00	19.00±0.00	22
K. pneumoniae	15.33±0.33	17.00±0.00	16.67±0.34	17.00±0.00	19.00±0.00	20

Table 7.7. ZOI of bacterial strains and gentamicin against the lichen extracts (50 µl of 25 mg/ml) by agar-well diffusion assay

MDR Bacteria	ZOI of Extracts (mm)					Gen. (mm)
	HE	DE	EA	ME	WE	
E. faecalis	16.67±0.34	16.00±0.00	16.00±0.00	17.00±0.00	18.00±0.00	17
S. aureus	25.00±0.00	23.00±0.00	23.00±0.00	25.00±0.00	25.33±0.33	26
E. coli	21.00±0.00	21.00±0.00	20.33±0.33	20.33±0.33	22.00±0.00	22
K. pneumoniae	23.00±0.00	22.00±0.00	22.00±0.00	23.00±0.00	23.33±0.33	20

Table 7.8. ZOI of *C. albicans* and fresocan against varied concentration of the lichen extracts (30 µl) by agar-well diffusion assay

Conc.	ZOI of Extracts (mm)					Fresocan (mm)
	HE	DE	EA	ME	WE	
15 mg/ml	20.00±0.00	20.33±0.33	21.00±0.00	19.00±0.00	19.00±0.00	21
20 mg/ml	22.00±0.00	23.00±0.00	23.00±0.00	22.00±0.00	25.00±0.00	21
25 mg/ml	28.00±0.00	28.33±0.33	26.00±0.00	28.67±0.34	29.00±0.00	21

7.1.3 MIC

MIC was done to determine the lowest possible concentration of varied solvent extracts of lichen that exhibit effective inhibitory activity against the tested pathogen (Güllüce *et*

al., 2004). The MIC of all the extracts against the bacterial strains were within the range of 1.56–6.25 mg/ml (Table 7.9). From the MIC results, hexane, diethyl ether and ethyl acetate extracts can be considered bacteriostatic against *E. faecalis* while for *S. aureus*, the lowest MIC was 1.56 mg/ml with water extract. However, in gram-negative bacteria, hexane, diethyl ether and methanol extract showed highest bacteriostatic effect on *E. coli* whereas water extract was most effective against *K. pneumonae* at the value of 1.56 mg/ml. Similarly, the extracts of *A. ornatoides* were also fungistatic against the yeast *C. albicans*. The MIC of the extracts were within the range of 1.56–3.125 mg/ml (Table 7.10). The values of MIC were lower in hexane, diethyl ether and methanol extracts. Higher antimicrobial activity among the strains is indicated by lower MIC value.

7.1.4 MBC and MFC

MBC and MFC were defined to determine the bacteriostatic and fungistatic effect of lichen extracts and also to test bacterial tolerance to the tested sample. It measures the lowest extract concentration that is capable of apprehending growth of any bacterial or fungal strain. The dilution in broth method was used to calculate the MBC and MFC for the antimicrobials (CLSI, 2019). The MBC and MFC values of the extracts against the strains are shown in a (Table 7.9 & 7.10) and were between 3.125–25 mg/ml. Notably, among the bacterial strains, water extract of *A. ornatoides* demonstrated considerable efficacy against *S. aureus* and *K. pneumonae* whereas methanol extract showed significant effectiveness against *E. coli* as shown by the low values of MIC and MBC (Table 7.9). However, the extracts of diethyl ether and methanol showed significant effect against *C. albicans* recorded the lowest value of MIC and MFC (Table 7.10). As a result, MBC and MFC was effectively determined to be the minimal antimicrobial concentrations capable of deactivating more than 99.99% of the microorganisms present.

Table 7.9. MIC and MBC values of lichen extracts against isolated MDR bacterial strains

MDR	**HE**		**DE**		**EA**		**ME**		**WE**	
bacteria	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. faecalis	1.56	6.25	1.56	6.25	1.56	6.25	6.25	25	6.25	12.5
S. aureus	3.125	6.25	3.125	6.25	3.125	12.5	3.125	6.25	1.56	3.125

E. coli	1.56	6.25	1.56	6.25	3.125	12.5	1.56	3.125	6.25	25
K. pneumoniae	3.125	12.5	3.125	6.25	1.56	6.25	3.125	12.5	1.56	3.125

Table 7.10. MIC and MFC values of lichen extracts against isolated MDR yeast strain

MDR yeast	**HE**		**DE**		**EA**		**ME**		**WE**	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans	1.56	6.25	1.56	3.125	3.125	12.5	1.56	3.125	3.125	12.5

7.2 Discussion

According to Philip *et al*. (2009) natural product extract with zone of inhibition > 10 mm using disc-diffusion assay is thought to have good antimicrobial activity. The antimicrobial activity exhibited by this lichen thalli was noteworthy. As a result, the lichen, *A. ornatoides*, offers a potent source of novel antimicrobial compounds for the pharmaceutical industry and food preservation. The findings also validate its application as an antimicrobial drug in ancient traditional remedy. Further, it is advised to conduct more research to isolate the active chemicals in this lichen species.

As shown in Table 7.11, the MBC/MIC and MFC/MIC ratios ranged from 2–4. It was well defined that when the ratio of MBC/MIC or MFC/MIC ≤ 4, extract is considered to have bactericidal or fungicidal characteristics (Djeussi *et al*., 2013). Therefore, all the extracts showed potent antimicrobial properties and was highest for *S. aureus*.

Table 7.11. MBC/MIC and MFC/MIC values of extracts against tested organisms

MDR strains	**HE**	**DE**	**EA**	**ME**	**WE**
E. faecalis	4	4	4	4	2
S. aureus	2	2	4	2	3
E. coli	4	4	4	2	4
K. pneumoniae	4	2	4	4	2
C. albicans	4	2	4	2	4

The potency of antimicrobial activity was dependent on the concentration of *A. ornatoides*, type of extracts, methods, and the tested microorganisms. The MDR strains were found to be susceptible, intermediate to resistant against each of the crude extracts. It is insufficient to determine the lethal and intrinsic suppression levels using disc and well diffusion method. Therefore, the results of disc-diffusion and agar-well diffusion assay as supported by MIC, MBC and MFC test confirmed wide-spectrum antimicrobial property of the lichen species *A. ornatoides*.

Subsequently, the species *A. ornatoides* could be promising source of drugs against the agents causing nosocomial infections. These infections are caused by microbes like *E. coli*, *K. pneumonae*, *S. aureus* and *E. coli*, resulting serious problems in health care settings including UTI, septicaemia, pneumonia, neonatal meningitis, peritonitis, and gastroenteritis (Lausch *et al*., 2013; Zhao *et al*., 2015). The species *E. coli*, collectively called coliform bacilli, is a major enteric pathogen, predominantly in developing countries, and the most often isolated organism in clinical laboratories. The main grouping of this organism that cause gastrointestinal disease include Enterotoxigenic (ETEC), Enteroinvasive (EIEC), Enterohemorrhagic (EHEC), Enteroggregative (EAEC), and classical enteropathogenic *E. coli* types (EPEC) (Guentzel, 1996). *E. coli* strains can produce either noninflammatory diarrhoea (watery diarrhoea) or inflammatory diarrhoea (dysentery with stools that typically contain blood, mucus, and leukocytes), depending on the virulence factors they possess (Evans and Evans, 1996). *K. pneumoniae* is an opportunistic bacterium colonizing skin, pharynx and gastrointestinal tract; its virulence factors are endotoxins, cell wall receptors and capsular polysaccharide (Lin *et al*., 2015). *S. aureus* is important pathogenic agent whose infections are more susceptible to the hospitalized patients with weakened immunity (Vandenesch *et al*., 2012). While cell-mediated immunity appears to be critical for the avoidance and treatment of invasive *S. aureus* infections, an imbalance in this immunity can potentially result in SIRS and mortality, or insufficient defence that prolongs bacteremia and causes death. Most human death and serious disease are probably caused by this imbalance (Proctor, 2019). *E. faecalis* and *C. albicans* are also opportunistic pathogens that colonizes in the human body such as skin, oral cavity, vagina and gastrointestinal tract (Kashem and Kaplan, 2016; Rindum *et al*., 1994; Achkar and Fries, 2010; Mason *et al*., 2012; Higuita and Huycke (2014). Thus, this work suggests the possibility of the use of *A. ornatoides* in treatment of various diseases caused by these and similar microorganisms.