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Review Article

Resistance to ceftaroline - 2018 review

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ABSTRACT

Ceftaroline is a new fifth generation cephalosporin, active mostly against Gram-positive cocci, e.g. *Staphylococcus aureus* (including methicillin-resistant *Staphylococcus aureus*). It is used in treating acute bacterial skin and skin structure infections, community acquired respiratory tract infections and methicillin-resistant *S. aureus* bacteremia. The main resistance mechanisms of bacteria to β-lactam antibiotics, including ceftaroline, are mutations in PBP2a, PBP3 and PBP4. Clinically significant resistance has been noted among both archived and newly-isolated strains in a laboratory test using serial passages. Ceftaroline-resistant strains have also been found in patients suffering from cystic fibrosis, ventilator-associated pneumonia and infectious endocarditis. Irresponsible antibiotic treatment using ceftaroline or other antibiotics (due to a possibility of a cross-resistance) can lead to the spread of ceftaroline resistance and, consequently, its loss of value.

Keywords: Antibiotic; Antibiotic resistance; MRSA; Resistant strains; Ceftaroline-resistant.

1. INTRODUCTION

Ceftaroline, a fifth generation cephalosporin, has been approved by the FDA (Food and Drug Administration) as a therapeutic option for both adult (in 2010) and pediatric (in 2016) patients suffering from acute bacterial skin and skin structure infections (ABSSSI) (including infections caused by MRSA), as well as community-acquired respiratory tract infections (CARTI), including community acquired bacterial pneumonia (CABP).The antibiotic has also been approved for treating patients with methicillin-resistant *S. aureus* bacteremia (MRSAB) and endocarditis. Despite being a new drug, on which many people has pinned their hopes, there are more and more reports of bacterial strains resistant to it.

THE USE OF CEFTAROLINE

Ceftaroline is a broad-spectrum antibiotic [1], active against methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* (MSSA and MRSA), daptomycin-nonsusceptible (DNS) *S. aureus*, vancomycin-intermediate (VISA and hetero-VISA) and vancomycin-resistant (VRSA) *S. aureus*, methicillin-susceptible and methicillin-resistant coagulase-negative streptococci (MSCoNS and MRCoNS), multidrug resistant *Streptococcus pneumoniae*, as well as many genera of Enterobacteriaceae (*Escherichia coli*, *Klebsiella pneumoniae* and *K. oxytoca*, *Enterobacter aerogenes* and *E. cloacae*, *Citrobacter koseri* and *C. freundii*, *Proteus mirabilis*, *Serratia spp.*, *Moraxella*

catarrhalis, Haemophilus influenzae, Morganella morganii) [2-4]. Ceftaroline is ineffective against *Pseudomonas spp., Enterococcus spp., Bacteroides fragilis* and atypical bacteria (*Mycoplasma pneumoniae, Chlamydia pneumoniae* and *Legionella pneumophila*) [2].

In the USA, ceftaroline was put into use in October 2010 and in Europe - two years later. At first, it was used in treating ABSSSI and CARTI [5]. In vitro studies conducted by Gaikwead et al. show high effectiveness of the drug - among 30 MRSA strains sampled from different clinical materials, 2 (6,67%) were resistant to it [6]. Moreover, clinical trials showed that it is well-tolerated by patients [2, 5, 7] (most common side effects were: diarrhea, nausea, headache, pruritus [5]), leaving other antibiotics, with potentially severe side effects, such as nephrotoxicity, ototoxicity [8, 9] (vancomycin) or thrombocytopenia [9] (linezolid), as drugs of last resort [5]. A decreased percentage of patients having to stop therapy due to the side effects was noted - 2,7% compared to 3,7% when treating with ceftriaxone or vancomycin with aztreonam [2]. Another in vitro study showed that when it comes to eradication of MRSA, ceftaroline is as effective as vancomycin, daptomycin and linezolid (when minimal inhibitory concentration for ceftaroline, MIC, $\leq$ 2 mg/l). It doesn't matter then, whether the strain has developed mechanisms of resistance to linezolid or vancomycin [10].

Among adults with CABP, ceftaroline treatment was more effective than a ceftriaxone one [7, 11, 12]. Moreover, the difference between therapeutic effect of both drugs was less significant if in 96 hours prior to their usage no other antimicrobial drug had been used [11].

Ceftaroline is the first intravenous antibiotic used among children over two months old to be approved by the FDA in over a decade [13]. Between 2012-2014 Pfaller et al. analyzed 3141 samples (1681 associated with ABSSSI, 1460 with CARTI) coming from pediatric patients from 29 different centers. The strains of *S. aureus, S. pneumoniae, H. influenzae, P. aeruginosa,* beta-hemolytic streptococci, Enterobacteriaceae (including *E. coli* and *Klebsiella spp.*) and others were isolated. 99-100% of the Gram-positive bacteria, as well as *H. influenzae* strains, were ceftaroline-susceptible. Also, the antibiotic was active against MRSA strains associated with ABSSSI and ceftriaxone-resistant *S pneumoniae* associated with CARTI [14]. The percentage of cured complicated ABSSSI and CABP in population of patients aged 2 months to 17 years old was high [7].

There's also a known case of a ten year old girl who had had an accident and developed a MRSA sepsis (the bacteria were previously isolated from many of her wounds), which was fought off with relatively low dose of ceftaroline (2 x 9 mg/kg/d) - even though MIC of 1,5-4 mg/l suggested decreased susceptibility to it [15].

A therapeutic success in treating MRSAB was also stated among adults in a study conducted by Zasowski et al. [16]. White et al. proved that ceftaroline is effective at treating patients with MRSAB who haven't responded to other drugs [17]. There are also reports stating that ceftaroline combined with daptomycin can be effective in treating daptomycin-resistant or vancomycin-intermediate resistant MRSA infectious endocarditis (IE) [18-20].

RESISTANCE MECHANISMS

Microorganisms for which the MIC value for ceftaroline is equal or less than 1 mg/l (1 μg/ml) are considered susceptible to this antibiotic. When the MIC ranges from 1 to 8 mg/l the microorganism is considered nonsusceptible and when the MIC exceeds 32 mg/l, the microorganism is resistant to ceftaroline [21-24].

Mechanisms of microbial resistance to ceftaroline are based on mutations within the penicillin binding protein (PBP) group, and are primarily observed in *S. aureus* [20-31]. Among the mutations present in PBP proteins, mutations were observed predominantly within the PBP2a protein [25] both inside the penicillin-binding domain (PBD) and outside the penicillin-binding domain (nPBD) [21, 29]. Mutations in PBD seem to correlate more frequently with nonsusceptibility, and mutations in nPBD with resistance to ceftaroline. PBP3 and PBP4 were other mutated PBP to be proved to correlate with ceftaroline resistance. This type of resistance has been overcome by the combination of ceftaroline with very low methicillin or meropenem doses [24, 26].

The PBP2a is a mutated variant of the PBP2

responsible for bacterial cell wall biosynthesis, providing microbial resistance to β-lactam antibiotics. Changes in the staphylococcal *mecA* gene result in conformational changes of the finished PBP2, which reduces its affinity to all β-lactam antibiotics [27]. It can be suspected that further mutations induced by environmental factors (ceftaroline therapy) within or outside the SCCmecA gene (Staphylococcal Cassette Chromosome mec) may result in resistance to fifth generation of cephalosporins [25, 28], which seemed to be completely effective in the treatment of MRSA infections so far. However, studies conducted by Kelley WL et al. show that during the introduction of ceftaroline to use, variants of the PBP2a providing ceftaroline resistance to hospital-acquired MRSA (HA-MRSA) have already existed (Table 1) [29].

Other factors leading to the increase of total resistance level in bacteria, include genes taking part in cell wall precursor formation and turnover, such as *femA* and *femB* genes, encoding proteins that take part in forming correct peptidoglycan pentaglycine interpeptide bridge, as well as *fmhA*, *fmhB* and *fmhC* genes, which encode proteins participating in forming peptidoglycan pentaglycine interpeptide. It was also noted that genes engaged in glutamine's and glucosamine's metabolism, such as *femC* and *femD*, can also cause the increase of bacterial resistance [32, 33].

Greninger A et al. suggested that mutations within genes such as *clpX* endopeptidase, *pp2c* protein phosphatase and transcription terminator *rho* can influence resistance to ceftaroline of MRSA in mechanisms different than the one involving *mecA* [34].

Chan LC et al. noted the significance of *gdpP* mutation, often identified within MRSA strains resistant to both ceftaroline and ceftobiprole. However, it's role is yet to be discovered [26, 31].

Table 1. HA-MRSA strains isolated from University Hospital of Geneva's patients' blood between 1998-2003, showing primary resistance to ceftaroline (MIC > 1mg/l) [29].

Strain (GenBank no.)	Molecular type	SCCmec	Mutations	Year	MIC (broth) (mg/l)
12	ST228	I	E239K	1998	2
14	ST228	I	E239K	1998	2
13	ST247	I	N146K, E150K, G246E	1998	4
16	ST247	I	N146K, E150K, G246E	1998	4
56	ST228	I	N146K	1999	2
17	ST228	I	N146K	1999	2
21	ST228	I	N146K	1999	2
57	ST228	I	N146K	2000	2
25	ST228	I	N146K	2000	2
28	ST228	I	N146K	2000	2
30	ST228	I	N146K	2000	4
42	ST228	I	N146K	2002	2
48	ST228	I	N146K	2003	2
52	ST228	I	N146K	2003	2

Chan LC et al. used the method of serial passages and the method of plasmid transduction to estimate the possibility of emergence of ceftaroline resistance and the potential consequences of its transmission in two strains of ceftaroline-passaged mutants: SF8300 and COL. In this way, mutants with MIC greater than 32 mg/l were obtained [26]. Lahiri SD et al. proved, using the method of serial

passages, that induction of ceftaroline-resistance (MIC ranging from 2 to 64 mg/l) is possible among strains ATCC 29213 (MIC: 0.25-4 mg/l), USA300 (MIC: 1-8 mg/l) and ARC3824 (MIC: 8-64 mg/l).

Clinical strains of MRSA, investigated by Lahiri SD et al., have also shown the ability to rapid resistance development (manifesting itself as significant increase of MIC), as presented in Table 2 [30]. It is a discovery of great importance, since passing bacteria imitates the situation in human organism when, due to incorrect dosage, too long therapy or insufficient penetration of the antibiotic to the tissue, in vivo MIC has not been achieved. Besides the mutation within PBP2A, strains with point mutation within PBP4, providing them ceftaroline-resistance, were observed (strains TRN5426 and TRN5549) [25].

Table 2. Clinical MRSA strains with significantly increased (compared to parental strains) MIC due to serial passages. Descendant strains are marked by adding (after the dash) following letters of the alphabet to the name of a parental strain [30].

Strain	Molecular type	SCC mec	Mutations of parental strain	Additional mutations after passage	Year	Country	MIC (broth) of parental strain (mg/l)	MIC (broth) after passage (mg/l)
ARC3824	ST228	I	E239K, E447K		2010	Spain	8	
ARC3824-A	ST228	I	E239K, E447K	Y446N	2010	Spain	8	64
ARC3824-B	ST228	I	E239K, E447K	A601S	2010	Spain	8	16
ARC3824-C	ST228	I	E239K, E447K	A601S	2010	Spain	8	16
ARC3827	ST228	I	E239K		2010	Thailand	2	
ARC3827-A	ST228	I	E239K	-	2010	Thailand	2	4
ARC3827-B	ST228	I	E239K	-	2010	Thailand	2	4
TRN5426	ST22	IV	WT		2012	Portugal	2	
TRN5426-A	ST22	IV	WT	-	2012	Portugal	2	8
TRN5467	ST5	II	N146K, L357I, I563T		2012	South Korea	4	
TRN5467-A	ST5	II	N146K, L357I, I563T	Y446N	2012	South Korea	4	32
TRN5467-B	ST5	II	N146K, L357I, I563T	Y446N	2012	South Korea	4	32
TRN5549	ST22	IV	E150K		2012	Portugal	2	
TRN5549-A	ST22	IV	E150K	-	2012	Portugal	2	8

Moreover, there are more and more reports from all over the world, describing isolating from different clinical samples another MRSA strains capable of developing mechanisms of resistance to ceftaroline (Table 3).

Laboratory results are also confirmed by reported clinical cases. This problem is seen (among others) in patients with cystic fibrosis (CF),

probably due to the multitude of therapeutic cycles using the same antibiotic - in this case - ceftaroline. Such cases, as presented in Table 4, prove that increasing resistance to antibiotics observed in microbiological laboratories while passing bacteria, is also reflected in clinical environment. In these patients, resistance to ceftaroline and its limited clinical effectiveness were observed [22, 31]. The case of ceftaroline resistance was also reported for a strain isolated from the blood of a patient suffering from IE, as well as from the broncho-alveolar lavage fluid (BALF) of a patient suffering from ventilation associated pneumonia (VAP) [35]. Molecular studies conducted on isolated MRSA strains revealed mutations in PBP2a [22, 30, 31].

Table 3. MRSA strains with potential of clinical resistance to ceftaroline [25, 30].

Strain	Molecular type	SCCmec	Mutations	Country	MIC (broth) (mg/l)
TRN5420	ST239	III	E239K	Hungary	2
TRN5427	ST36	II	WT	Greece	2
TRN5428	ST239	III	N146K, E150K, N204K, G246E	Greece	4
TRN5433	ST5	II	K290Q	Japan	4
TRN5444	ST5	II	K281R	China	2
TRN5454	ST5	II	WT	Japan	2
TRN5458	ST239	III	N146K	Philippines	2
TRN5471	ST228	I	N146K, I563T	Italy	4
TRN5474	ST5	II	N236K	Taiwan	2
TRN5475	ST239	III	E239K	China	2
TRN0478	ST228	I	N146K	Hungary	2
TRN5507	ST239	III	N146K	Russia	4
TRN5521	ST228	I	E239K, E447K	Thailand	8
TRN5536	ST239	III	WT	Turkey	2
TRN5539	ST5	II	E170K, N236K	Taiwan	2
TRN5552	ST239	III	N146K, N204K, G246E	South Africa	2
TRN5562	ST22	IV	E239K, G246E	France	2
TRN5563	ST239 with tpi-107	III	N204K, T235I	France	2
TRN5572	ST5	II	WT	Italy	2
ARC3824	ST228	I	E239K, E447K	Spain	8
ARC3828	ST228	I	E239K, E447K	Thailand	8
ARC3830	ST228	I	E239K, E447K	Thailand	8
TRN5474	ST228	I	N236K	Taiwan	2
TRN5472	ST228	I	WT	Italy	2
TRN5545	ST239	III	N146K	Turkey	2
TRN5418	ST5	I	M122I, E150K	Chile	2
TRN5350	ST8	II	N236K	USA	2

Table 4. Summary of clinical cases [22, 30, 31].

Strain	Disease	Sample	Mutation	MIC (mg/l)
THMS-4519	Cystic fibrosis	Sputum	Y446N	1,5
THMS-3125	Cystic fibrosis	Sputum	Y446N, E447K	>32
THMS-5007	Cystic fibrosis	Sputum	E239K, Y446N, E447K	>32
THMS-5006	Cystic fibrosis	Blood	E239K, Y446N, E447K	>32
USA100	Infectious endocarditis	Blood	E447K	4
USA100	Ventilation associated pneumonia	BALF	E447K	6

Pfaller MA et al. observed ceftaroline resistance in one multi-drug resistant *S pneumoniae* strain. Molecular analysis revealed 31 altered aminoacids within the MurM relative to the standard R6 strain. Changes in PBPs, mainly PBP2x, were also detected [36].

EPIDEMIOLOGY

Despite being put into use only a few years ago, ceftaroline-resistant strains are detected in more and more countries. Moreover, it was proved that resistant strains have been existing for at least over a dozen years prior to introducing ceftaroline. In 2015 Kelley et al. published the results of a study concerning 60 archival MRSA strains (collected between 1994-2003 in Geneva, Switzerland), 40 out of which (66%), dated 1998-2003, turned out to be ceftaroline-resistant [29]. In 2016, in the same center, another study was conducted - this time on MRSA strains collected in 2013 and 2014. 23 out of 96 strains (24%) were ceftaroline-resistant [37].

The AWARE report from 2012 informed that among 2583 *S. aureus* strains collected in Europe, Russia and Turkey, 2 (0.08%) were ceftaroline-resistant (MIC, ≥4 mg/l) and 114 (4.4%) were ceftaroline-intermediate (MIC, 2 mg/l). Given EUCAST (European Committee on Antimicrobial Susceptibility Testing) criteria, 116 strains (4.5%) were ceftaroline-resistant (MIC, >1 mg/l), 94 (81%) out of which came from Russia, Turkey, Italy and Hungary [38]. In the USA the first ceftaroline-resistant MRSA strain was described in 2014 by Long et al. and it was isolated from a twenty-year-old CF patient treated with ceftaroline due to recurring respiratory tract infections caused by multi-drug resistant bacteria (including MRSA) [23].

In 2015 in China, Zhang et al. examined 251 hospital acquired MRSA strains from ABSSSI patients. None of the analyzed strains showed resistance to ceftaroline, but 84 of them (33.5%) showed intermediate resistance (MIC, 2 mg/l) [39]. In the same year, Abbott et al. tested 421 MRSA strains collected in Australia (270 from 2017, the rest from 2013). 71 (16.9%) out of them were nonsusceptible to ceftaroline (MIC, >1.0 mg/l) and most of them had MDR phenotype [40]. In Africa, 37 MRSA strains colonizing patients and 23 infectious MRSA strains were collected. 10 (16.7%) out of them were resistant to ceftaroline [28].

CONCLUSIONS

Ceftaroline as a new antibiotic, in most cases allows to reach therapeutic effect provided in the Summary of Product Characteristics (SPC). However, it is very disturbing that in the moment of being introduced to market, there have already been existing ceftaroline-resistant strains, which may indicate that there's a possibility of obtaining cross-resistance to ceftaroline while using other β-lactam antibiotics in insufficient doses (which can be verified by testing archived MRSA strains). Laboratory tests prove that resistance to ceftaroline may be induced by selecting strains by increasing doses of the antibiotic. It shows rather clearly that bacteria can survive therapeutic concentration of ceftaroline if they have previously been exposed to it. Moreover, ceftaroline-resistant strains are isolated from patients with clinical symptoms of infections.

Thus, ceftaroline, just like any other antibiotic, may lose its clinical value if it's overused, its dosage is incorrect or the rest of β-lactams are

overused or dosed incorrectly. Reasonable antibiotic therapy is probably the only hope for effective use of ceftaroline in the future. However, it is impossible to estimate how fast will the resistance-gaining process progress or what percentage of bacteria will it concern.

AUTHORS' CONTRIBUTIONS

ABo: Paper conception and design. RŚ, ABi, ABo, MB: Acquisition of literature, Analysis and interpretation of literature, Drefting of manuscript. MB, ABo: Drafting of tables. ABi, RŚ: Translation of manuscript. ABo: Adaptation to editorial guidelines. All authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

Authors have declared that no competing interests exist.

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Research Article

Profile of major and emerging mycotoxins in sesame and soybean grains in the Federal Capital Territory, Abuja, Nigeria

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ABSTRACT

The spectrum of major and emerging mycotoxins in sesame and soybean grains from the six zones of the Federal Capital Territory (FCT), Abuja, Nigeria was determined using Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS). A total of 47 samples (24 sesame and 23 soybean were collected from farmers' stores. Seven regulated mycotoxins in sesame and five in soybean including aflatoxin B_1 (AFB_1), aflatoxin B_2 ($AFB_{2)}$ and fumonisin B_1 (FB_1) were detected. However, concentrations were generally lower than regulatory limits set in the EU for raw grains with the exception of ochratoxin A (OTA) exhibiting a maximum concentration level of 23.1 µg kg^{-1} in one of the soybean samples. This is the first report concerning the contamination of sesame and soybean in Abuja, FCT-Nigeria with the emerging mycotoxins addressed by recent European Food Safety Authority (EFSA) opinion papers totalling 10 in number. These include beauvericin (BEA), moniliformin (MON), sterigmatocystin (STE), altertoxin-I (ATX-I), alternariol (AOH), alternariol methylether (AME) though at relatively low µg kg^{-1} range. This preliminary data indicate that sesame and soybean might be relatively safe commodities in view of the profile of mycotoxins.

Keywords: Emerging mycotoxins; Nigeria; Liquid chromatography/tandem mass spectrometry; Regulated mycotoxins; Sesame; Soybean.

1. INTRODUCTION

Sesame (*Sesamum indicum* L.) and soybean (*Glycine max* L. Merril) are two very nutritious food items in Nigeria. Just like other crops produce, the occurrence of mycotoxins in these two crops is hardly avoidable. Unimpressive agricultural practices and lack of well-organized and effective regulations in Nigeria and most of sub Saharan Africa make control gloomy. Even where regulatory measures exist, they have little impact in rural areas and subsistence farming communities [1]. Post-harvest mishandling of the grains especially before and during storage is one of the major causes of mycotoxin invasion of the crops produce [2].

Sesame stored or marketed in some north central States of Nigeria has been shown over many seasons to be contaminated with agriculturally

important toxins [3]. This might be linked to favourable climatic and crop storage conditions which are frequently conducive to fungal growth and mycotoxin production, as much of the population relies on subsistence farming and unregulated local markets. Mycotoxins in soybean have a wide geographical distribution from Argentina [4, 5] to Croatia [6] and their production can be due to the effect of temperature [7]. Health challenges from the synergistic effect of many mycotoxins [8, 9] or through immunomodulation by a variety of co-occurring mycotoxins [10].

Emerging mycotoxins attract an increasing interest among the scientific community due to their high occurrence in feed and food commodities, sometimes at relatively high concentrations, and potential toxicity towards animals and humans. There is not yet existing regulation given to these metabolites despite their established toxicity. The main producers of emerging *Alternaria* metabolites are *A. alternata*, *A. dauci*, *A. cucumerina*, *A. solani*, and *A. tenuissima*, *A. citri*. These mycotoxins are found in wheat, rice, rye, olives, sorghum, tobacco, apples, peppers, sunflower seeds, oilseed rape, pecan nuts, tomatoes and mandarins [11, 12].

The incidence of emerging *Fusarium* mycotoxins has been reported in different products such as soybean [13], wheat and barley [14], rice [15], grain-based products [16] and infant cereals [17]. Jestoi [18] reported that limited data on emerging, less reported mycotoxins might be due to their late recognition especially because of the late understanding of their role as mycotoxins. Studies focusing on this class of mycotoxins are still quite low in number an extensive review published in 2015 showed that among all mycotoxin-related studies, only 7% were directed toward emerging mycotoxins. Some metabolites such as fusaric acid and fusaproliferin referred to as "emerging mycotoxins" by certain authors such as Jestoi [18] are not yet addressed by EFSA.

In Nigeria, there has been difficulty in determining the actual pecuniary value of food and feed ingredient losses due to mycotoxin load. This was due to paucity of detailed analysis information as well as intervention steps. There has been lack of attention on the probable additive effect and risks posed by emerging mycotoxins. The objective of the present study was to determine the major mycotoxins and emerging fungal metabolite profile of sesame and soybean in the FCT, Abuja Nigeria. This surveillance could be a critical step forward in the cocktail of intervention strategies against mycotoxin contamination in food and feed in Nigeria.

2. MATERIALS AND METHODS

2.1. Sampling

Surveys were conducted the FCT, Abuja Nigeria (between Lat. 9º 40' N, Long. 7º 29' E and Lat. 8º 83'N, Long. 7º 17' E, 388-566 m above the sea level between January and February, 2015. The farmers' stores were located in the six zones of the territory (Table 1). A total of 47 samples (24 sesame and 23 soybean grains) were collected from farmers in the six zones of the FCT namely Abuja Municipal Area Council (AMAC), Abaji, Bwari, Gwagwalada (GWA), Kuje and Kwali. Simple random sampling plan was adopted in the collection of the seed samples. The sampled locations and number of sample types collected from each district were uneven but the quantity were the same. Only samples shelled and stored for less than 30 days after harvest were collected from the farmers. Each sample was collected as a bulk sample (1.8-2 kg) and comprised of four subsamples of 0.5 ± 0.05 kg each. The subsamples were obtained from random points in farmer's basins or other storage containers and mixed to form the bulk. The samples were comminute and quartered such that 100-150 g of representative samples was obtained from each bulk as described by Ezekiel et al. [19]. Representative samples were stored at 4ºC until they were analyzed for multiple mycotoxins and microbial metabolites.

2.2. Sampling area

The sampling location in the six zones of the FCT, Abuja is as shown in Table 1.

2.3. Mycotoxin analysis

2.3.1. Chemicals and reagents

Methanol (LC gradient grade) and glacial acetic acid (p.a.) were purchased from Merck

(Darmstadt, Germany), acetonitrile (LC gradient grade) from VWR (Leuven, Belgium), and ammonium acetate (MS grade) from Sigma-Aldrich (Vienna, Austria). Water was purified successively by reverse osmosis and an Elga Purelab ultra analytic system from Veolia Water (Bucks, UK) to 18.2 MΩ.

Standards of fungal and bacterial metabolites were obtained either as gifts from various research groups or from the following commercial sources: Romer Labs® Inc. (Tulln, Austria), Sigma-Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktredwitz, Germany), Axxora Europe (Lausanne, Switzerland) and LGC Promochem GmbH (Wesel, Germany). Stock solutions of each analyte were prepared by dissolving the solid substance in acetonitrile (preferably), acetonitrile/water 1:1 (*v*/*v*), methanol, methanol/water 1:1 (*v*/*v*) or water. Thirty-four combined working solutions were prepared by mixing the stock solutions of the corresponding analytes for easier handling, and were stored at -20°C. All solutions were however brought to room temperature before use. The final working solution was freshly prepared prior to spiking experiments by mixing the combined working solutions.

Table 1. Number of samples and sample collection points in the FCT Abuja, Nigeria.

Zone	Total no. of samples/per zone		Community
	Sesame	Soybean	
Abaji	3	3	Pandagi, Sabongari, Abaji Centra
AMAC	3	2	Gwagwa, Kabusa, Karshi, Karu and Orozo
Bwari	3	2	Bwari Central, Byazhin, Ushafa
Gwagwalada	6	4	Gwako, Paiko and Tungan Maje
Kuje	4	6	Chibiri, Gaube, Saaji, Chukuku, Kuje Central, and Kwaku
Kwali	5	6	Kilankwa, Kwali Central and Yangoji
Total	24	23	

2.3.2. Extraction procedures

Five grams of each homogenized grain sample, previously pulverized in a mill with a 1 mm^2 mesh (Cyclotech, Foss Tecator, Höganäs, Sweden), were weighed into a 50 ml polypropylene centrifuge tube (Sarstedt, Nümbrecht, Germany). Ten millilitres of water was added and briefly vortex and allowed to hydrate for ≥15 min. 20 ml of the extraction solvent (acetonitrile/water/acetic acid 79:20:1, *v*/*v*/*v*) were added before being vortex for 5-10 min to extract the mycotoxins. The extracts were filtered and centrifuged for 5 min at ≥3000 x g (4^oC) in order to remove any interfering particles before further clean-up of the supernatant.

For spiking experiments, 0.25 g sample was used for extraction. Samples were extracted for 90 min on a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) and diluted (1 + 1) with dilution solvent (acetonitrile/water/acetic acid 20:79:1, *v*/*v*/*v*). Five microliters of the diluted extracts were subsequently injected [20].

2.3.3. LC-MS/MS parameters

The LC-MS/MS has been previously described by Malachova et al. [21]. Analysis was performed with a QTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with TurboIon Spray electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25°C on a Gemini C18-column, 150 × 4.6 mm i.d., 5 µm particle size, equipped with a C18 4 × 3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA).

ESI-MS/MS based Spectrum 380® program was performed in the time-scheduled multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chroma-

tographic runs per sample by scanning two fragmentation reactions per analyte. The MRM detection window of each analyte was set to its expected retention time ±27 s and ±48 s in the positive and the negative modes, respectively. Confirmation of positive analyte identification was obtained by the acquisition of two MRMs per analyte (with the exception of moniliformin that exhibited only one fragment ion). This yielded 4.0 identification points according to the European Union Commission decision 2002/657. In addition, the LC retention time and the intensity ratio of the two MRM transitions agreed with the related values of an authentic standard within 0.1 min and 30%, respectively.

Quantification was performed during external calibration based on serial dilution of a multi-analyte stock solution which was the liquid standards. Results were corrected by apparent recoveries that had been determined by spiking five different blank samples at two concentration levels.

2.3.4. Statistical analysis

Median, mean and standard deviation (SD) were calculated for concentrations of all toxins and metabolites. Pictorial representation of prevalence level was carried out using Excel package 2010.

3. RESULTS

3.1. Occurrence and contamination level of mycotoxins in sesame and soybean grains from the FCT, Abuja, Nigeria

The percentage of contaminated samples, the mean, median and the range of contamination level of the regulated and emerging mycotoxins, in sesame and soybean from the FCT, Abuja are presented Table 2.

Table 2. Occurrence and contamination level of mycotoxins in sesame and soybean grains from the FCT, Abuja, Nigeria.

Mycotoxin	Concentration in sesame (µg kg^{-1})*				Concentration in soybean (µg kg^{-1})			
	Positive (n=24) (%)	Median	Mean	Range	Positive (n= 23) (%)	Median	Mean	Range
Aflatoxin B_1 (AFB_1)	3 (13)	1.6	3.6	0.4-7.2	5 (22)	1.2	1.88	0.5-4.02
Aflatoxin B_2 (AFB_2)	2 (8)	1.5	1.5	0.8-1.6	0	-	-	-
Aflatoxin G1 (AFG_1)	0	-	-	-	1 (4)	21.1	21.1	21.1
Ochratoxin A (OTA)	0	-	-	-	2 (9)	16.8	16.8	10.5-23.1
Fumonisin B_1 (FB_1)	5 (21)	17.1	17.3	7.3-26.7	2 (9)	11.84	11.84	10.3-13.4
Fumonisin B_2 (FB_2)	2 (8)	8.6	8.6	6.1-11.2	0	-	-	-
Fumonisin B_4 (FB_4)	1 (4)	5.72	5.72	5.72	0	-	-	-
Deoxynivalenol (DON)	14 (58)	63.7	78.3	28-171	0	-	-	-
Zearalenone (ZEN)	11 (46)	3.2	3.6	0.1-18.3	3 (13)	0.7	0.73	0.3-1.4

*Indicated by LC-MS/MS analysis.

The major toxins and emerging metabolites common to both grains were three and seven respectively. Sesame samples had relatively higher occurrence of contamination with regulated mycotoxins such as DON (58.33%), ZEN (45.83%) and FB_1 (20.88%) while 21.7% and 13.4% of the soybean were contaminated with AFB_1 and ZEN respectively. AFG1 and OTA were detected in soyabean but not in sesame, while FB_2 and DON were detected in the sesame but not in soyabean. The highest contamination level was from DON in the sesame samples with median of 63.7 µg kg^{-1} while soybean had the highest contamination level from OTA with a median contamination level of 16.8 µg kg^{-1}.

3.2. Occurrence and contamination level of emerging mycotoxins in sesame and soybean grains

Among the emerging toxins recognized by European Food Safety Authority (EFSA) and the Food and Agriculture Organisation of the United Nations (FAO) and detected in the samples were beauvericin (BEA), moniliformin (MON), sterigmatocystin (STER), alternariolmethylether (AME), alternariol (AOH) and altertoxin-I (ATX-I). Beauvericin (BEA) had the highest occurrence of emerging mycotoxin both in the sesame (66.67%) and in the soybean samples (56.52%) at a respective maximum concentration of 4.2 and 23.04 µg kg^{-1} respectively (Table 3). The next higher occurrence (29.16%) was obtained for Alternariol methyl ether (AME), with concentration range of 0.22-11.3 µg kg^{-1} in sesame samples. Moniliformin (MON) occurred in the 30.43% of the soybean and with a range concentration of 0.12-33.34 µg kg^{-1}, while it was detected in 16.67% of sesame with a range of 4.1-17.4 µg kg^{-1}. Other emerging mycotoxins such as citrinin (CTN) and tenuazonic acid (TeA) were below detectable level.

Table 3. Occurrence of emerging mycotoxins in the samples from the FCT, Abuja, Nigeria, detected by LC-MS/MS.

Class of emerging mycotoxin	Emerging mycotoxin	Sesame concentration* (µg kg^{-1})				Soybean concentration (µg kg^{-1})			
		Positive (n=24) (%)	Median	Mean	Range	Positive (n=23) (%)	Median	Mean	Range
Fusarium metabolites	Beauvericin (BEA)	16 (67)	0.91	0.94	0.08-4.2	13 (57)	0.31	3.69	0.018-23.04
	Moniliformin (MON)	4 (17)	12.77	11.8	4.1-17.4	7 (30)	2.4	9.4	0.12-33.34
	Enniatin B (ENN-B)	0	-**	-	-	2 (9)	0.00525	0.00525	0.005-0.0055
Aflatoxin precursor	Sterigmatocystin (STER)	4 (17)	0.45	0.43	0.27-0.55	1 (4.3)	0.6	0.6	0.6
Alternaria metabolites	Chanoclavine (CNV)	3 (13)	0.14	0.14	0.06-0.2	0	-	-	-
	Festuclavine (FCV)	2 (9)	1.7	3.21	1.12-8.3	0	-	-	-
	Elymoclavine (ECV)	0	-	-	-	1 (4.3)	1.4	1.4	1.4
	Altertoxin-I (ATX-I)	2 (9)	3.9	4.26	2.82-6.5	1 (4.3)	0.91	0.91	0.91
	Alternariol (AOH)	5 (21)	1.96	8.55	1.14-3.5	1 (4.3)	0.85	0.85	0.85
	Alternariol-methylether (AME)	7 (29)	6.54	5.23	0.22-11.3	1 (4.3)	0.6	0.6	0.6

*Indicated by LC-MS/MS analysis; **= below limit of detection;

The co-occurrence of toxins and metabolites in the ten highest contaminated samples of sesame and in six soybean samples were pictorially presented as scatter plots in Figures 1 and 2. As shown in Figure 1, only in the 5 out of the 10 most contaminated sesame samples (S_1-S_5) that DON and ZEN co-occurred with other mycotoxins.As shown in Figure 2, only in the 3 out of the 6 most contaminated soyabean samples (S_1-S_3),that AFB_1 co-occur with other mycotoxins, though at relatively low concentrations below maximum regulatory limits.

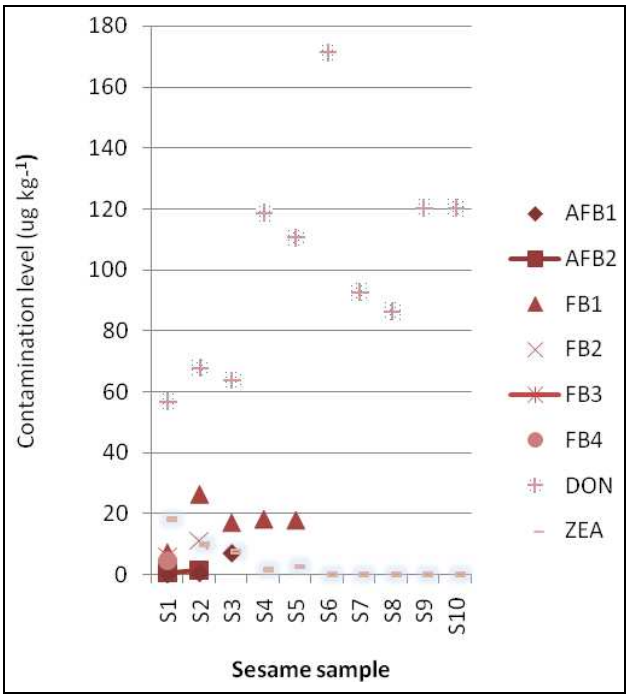

Figure 1. Scatter plots visualizing co-occurrence of major mycotoxins in the ten highest contaminated samples of sesame from the FCT, Abuja Nigeria. Different colour pattern represents each type of metabolite.

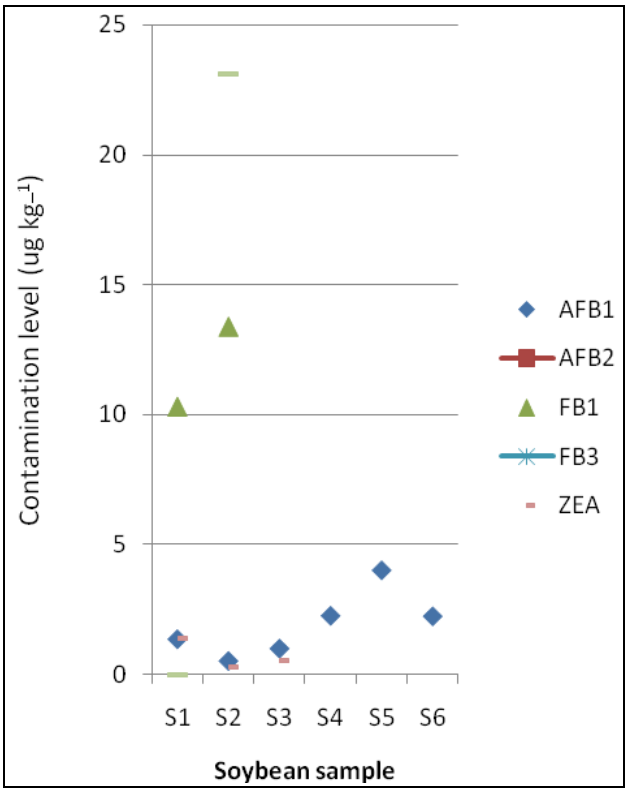

Figure 2. Scatter plots visualizing co-occurrence of major mycotoxins in the six highest contaminated samples of soybean from the FCT, Abuja Nigeria. Different colour pattern represents each type of metabolite.

4. DISCUSSION

The development of LC-MS/MS methods for the simultaneous detection and quantification of a broad spectrum of mycotoxins has facilitated the screening of a larger number of samples for contamination with a wide array of major and less well-known "emerging" mycotoxins [22, 23] Therefore, this analysis was applied in this investigation.

4.1. Occurrence of regulated mycotoxins

In the present study, AFB_1 was observed more frequently in soybean than in sesame. In countries having similar climate, like Burkina Faso and Mozambique, AFB_1 was observed more frequently in soybean-based food and feeds (Burkina Faso, 50% incidence, median = 23.6 µg kg^{-1}; Mozambique, 46% incidence, median = 69.9 µg kg^{-1}) than in groundnuts (Burkina Faso, 22% incidence, median = 10.5 µg kg^{-1}; Mozambique, 14% incidence, median = 3.4 µg kg^{-1}) [24]. Ezekiel et al. [19] observed that mycotoxin levels were higher in the Nigerian soybean-based kunu-zaki (<LOQ [limit of quantitation] - 123 µg kg^{-1}) and its cereal ingredients (0.1-31,200 µg kg^{-1}) than in the sorghum-based pito (<LOQ - 5 µg kg^{-1}) and its cereal base (1.2-85 µg kg^{-1}) respectively. Ezekiel et al. [3] reported that up to 13 out of the 16 (81.3%) Nigerian sesame samples analysed had AFs contamination and the concentrations ranged 0.08-1.4 µg kg^{-1}. Findings in the present study, suggest an association between mycotoxin level and grain size, since soybean is bigger than sesame. There is also the likelihood of more inhibitory compounds in the sesame grains but confirmatory investigation is necessary.

The frequency (20.88%) and the median concentration (17.1 µg kg^{-1}) of FB_1 was higher in the sesame than in soybeans. This was different in Mozambique where the FB_1 frequency and concentrations in soybean were higher (92% incidence, median = 869 µg kg^{-1}) than in sesame (75.1%, 107.6 µg kg^{-1}). This trend might be due to the processing of raw soyabeans into food stuffs in which antifungal factors in the product have been denatured during processing. The occurrence of more FB_1 than FB_2 and FB_3 is consistent with the general pattern of FUM contamination in soybean

and soybean-based foods [25]. Abdus-Salaam et al. [26] reported that *Fusarium* toxins in Nigerian rice had the highest incidence of 79%, but occurred in low amounts with FB_1 having the highest percentage incidence of 39.5% and a mean of 18.5 μg kg^{-1}. The grains investigated in this study contained relatively lower levels of AFs and FBs below the EU maximum tolerable levels, with highest concentrations of 17.1 μg kg^{-1} in FB_1.

It was indicated that DON concentration level was relatively higher in the sesame ranging from 28 to171 μg kg^{-1} but below detectable limit in soybean samples. Considering the maximum limits of 750 μg kg^{-1} established for DON by EU Regulation in processed cereals in 2012, DON was not of serious health concern as indicated in this study. Also Souza et al. [27] reported a maximum contamination level of 30 μg kg^{-1} DON in soybean in Brazil.

Only 8.7% of the soybean grains were contaminated with OTA but there was none detected in sesame. Kayode et al. [28] and Adetunji et al. [29] had a similar report from their analysis of mycotoxins and fungal metabolites in stored soyabean and soyabean-based snacks from Nigeria. Also, Shephard et al. [30] reported that no AFs, OTA, or T-2 or HT-2 toxins were detected on their soybean grains produced in the rural subsistence farmers in Transkei, South Africa. They associated this to the type of varieties planted and good agricultural practices.

In the report of the contamination profile of maize collected from the same store, under similar storage conditions and at the same time was reported by Anjorin et al. [31]. It is obvious that AFB_1, AFB_2, AFG_1, FB_1, FB_2, FB_4 in maize were relatively higher than in sesame and soybean as indicated in this study. For instance, while AFB1 were 17.3 μg kg^{-1} and 11.84 μg kg^{-1} in sesame and soyabean, respectively, it was as high as 2349 μg kg^{-1} in maize grains. This might be due to generic differences in the biochemical and genetic make-up of the crops that might have influenced their resistance level to the prevailing toxigenic fungal species.

There were relatively safe doses of major mycotoxins in the two grains except in OTA found in the soybean (16.8 μg kg^{-1}) that were slightly above the EU maximum acceptable limit of 5 μg kg^{-1}. This limit is also adopted in Nigeria and Kenya thus the only source of health concern.

4.2. Occurrence of emerging mycotoxins

Beauvericin and moniliformin were the two most prevalent emerging mycotoxins in the studied seeds. The incidence and contamination level of BEA was relatively low. In Nigerian rice, analysis revealed that beauvericin were detected with maximum value of 131 μg kg^{-1} [26] while Sulyok et al. [13] also reported up to 8000 μg kg^{-1} BEA in soybean-based food samples investigated. BEA, is a *Fusarium* emerging hexadepsi peptides secondary metabolite, has been less frequently investigated by routine methods [32]. BEA is produced by *Fusarium* species such as *F. avenaceum, F. subglutinans, F. oxysporum F. proliferatum, F. poae* and *F. tricinctum* [33-35].

A public health threat cannot completely be ruled out due to their widespread co-occurrence with other mycotoxins and fungal metabolites in the grains [36]. Beauvericin has been implicated in acting on the cellular membranes increasing the permeability and disrupting the cellular homeostasis [37]. MON was also detected in both sesame and soybean samples, but it was at low frequency and concentration. Gutema et al. [38] earlier reported the detection of MON in soybean, wheat, rye, triticale, oats and rice, and their co-occurrence with FUMs.

In this study, the presence of clavines ergot alkaloid such as chanoclavine (CNV), and festuclavine (FCV) mostly in the sesame samples indicated the contamination of such grains with *Claviceps purpurea, C. africanana, C. fusiformis, C. fusiformis, C. paspali*, and *Neotyphodium coenophialum.* These toxigenic fungi are also commonly found in wheat, rye, hay, barley, sesame, oats, sorghum, triticale. In soybean-based poultry feed in other parts of Nigeria, *Claviceps* metabolites were found at concentrations of up to 350 μg kg^{-1}, with agroclavine (AGV) having the highest concentration with elymoclavin (ECV) was the most prevalent [19]. In this study, frequency of AME in the soybean samples was just 4.35% at a concentration of 0.6 μg kg^{-1}. This is similar to the report of assessment on the mycotoxins load on Nigerian stored soybean by Adetunji et al. [29] that alternariol methylether (AME) occurred only in one sample (1.7%

contamination) each at concentrations of 106 µg kg^{-1}. Regardless of the low levels of ergot alkaloids in our samples, there is an impending danger in the continuous consumption of this potent group of non-regulatory toxins [39, 40]. In 2011, EFSA reported that AOH could be potentially harmful to humans and pointed out the need of further investigations to generate toxicity data.

5. CONCLUSION

Multi-mycotoxin analysis by LC-MS/MS is an important step towards gaining a more accurate picture of the extent of mycotoxin contamination in food and feed. The sesame samples analysed were contaminated with, AFB_1 and AFB_2, FB_1 and FB_2, FB_4, DON, and ZEN while the soybean were contaminated with AFB_1, AFG_1, FB_1, OTA and ZEN with their contamination levels below the maximum levels established by the EU with the exception of OTA in soybean. For the first time, the presence of the emerging mycotoxins - beauvericin BEA), sterigmatocystin (STER) alternariolmethylether (AME) chanoclavine (CNV) and elymoclavine (ECV) were confirmed to be present in the sesame and soybean grains in the FCT, Abuja, Nigeria. There is no indication of potential health threat due to the contamination of sesame and soybean with some non-classic mycotoxins which have not been included in conventional mycotoxin monitoring. However, more comprehensive monitoring programs may be needed in the grains in other agro-ecologies in Nigeria involving both regulated and emerging mycotoxins. This could involve research in possible synergistic interactions with other mycotoxins present.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest regarding the publication of this article.

AUTHORS' CONTRIBUTION

SOF was the **s**tudy supervisor, rendered technical support and revised the manuscript and ensures compliance with journal standard. TSA acquired the data, interpreted, did extensive literature search and wrote the manuscript. MS and RK analysed the data and developed methodology. All authors read and approved the final manuscript.

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Review Article

Sodium fluoride: suggestive role in wound healing and cell proliferation with respect to regeneration

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ABSTRACT

Sodium fluoride is a naturally occurring toxicant. The most common sources of sodium fluoride are municipal water, toothpastes etc. The ever increasing exposure to sodium fluoride may affect various physiological processes including regenerative capabilities. The characteristic events of regeneration include wound healing followed by cell proliferation and differentiation to replace the lost structure or tissue. Lower levels of sodium fluoride may be enhancing wound healing and cell proliferation but higher levels are detrimental for both these processes. Sodium fluoride affects wound healing by altering the expression of various proteins like fibroblast growth factors 2 and 7, Twist1 protein, matrix metalloproteinases 2 and 7, bone morphogenetic protein 7, Bcl-2, p53 etc. Sodium fluoride also influences cell division, migration and matrix synthesis by regulating the expression of bone morphogenetic proteins 2 and 3, alkaline phosphatases etc. which are markers of cell proliferation. Excessive fluoride produces oxidative stress in the cells and leads to conditions like apoptosis, cell cycle arrest and even necrosis. Thus, high levels of sodium fluoride hamper the process of cell proliferation and induce apoptosis via caspase and JNK-mediated pathway. The aim of this review is to understand the role sodium fluoride plays during wound healing and cell proliferation and its correlation with regenerative capabilities in organisms.

Keywords: Sodium fluoride (NaF); Wound healing; Apoptosis; Cell proliferation.

1. INTRODUCTION

Sodium fluoride (NaF) is a widespread natural compound and fluoride is one of the trace elements required by the humans for maintaining a good dental health. Fluoride was officially considered as a beneficial element initially and was used on large scale to reduce cavities in humans and thus it was considered an important element for maintaining a good dental health [1]. As a result, sodium fluoride was added in municipal water while sodium fluoride, stannous fluoride (SnF_2) and sodium monofluorophosphate (Na_2PO_3F) were added to toothpastes, to prevent tooth decay in United States [2]. The permitted level of fluoride in drinking water is 1 to 1.5 ppm while 2 ppm is considered toxic [3]. The chronic intake of sodium fluoride results in several serious health conditions like hormonal impairment [4], osteosarcoma [5], problems associated with the male reproductive system [6, 7] and even memory loss [8]. Thus, fluoride may be considered to be an environmental contaminant and its major sources are drinking water, food, pesticides and dental products. Due to the easy exposure to

fluoride from various sources, fluoride enters the body of organisms where it may show several desired and undesired effects. This review focuses on the effects of NaF on wound healing and cell proliferation in order to understand if it might influence the process of regeneration.

2. SODIUM FLUORIDE AND WOUND HEALING

The effects of sodium fluoride on wound healing have been seen more commonly during dental procedures in humans. Sodium fluoride in the form of mouthwashes, promotes complication free healing of the wound after tooth extraction [9]. Also, fluoride has been known to reduce cavities in teeth of infants and children [4]. The healing effect of sodium fluoride was also evident when after the topical application of sodium fluoride to the experimental calvarial defects in rats, it led to faster healing as compared to saline treated control group of rats [10].

The mechanism of the mucosal healing can be understood as follows: when the mucosal wound in the oral cavity is healing, the keratinocytes first secrete laminin-5 in their extracellular matrix and further more laminin may be deposited. The keratinocytes start migrating and become hyper-proliferative cells which secrete extracellular matrix components and signaling polypeptides leading to healing of the wound [11]. Sodium fluoride may influence this process in various ways like low dose of sodium fluoride enhances healing of rat skin wounds probably by increasing the levels of key molecules like fibroblast growth factor-2 (FGF-2), fibroblast growth factor-7 (FGF-7) and Twist1 protein which are key proliferative markers and enhance epithelial-mesenchymal interactions, necessary for wound healing [12]. Further, the healing of the wound involves healing of various types of tissues like muscles, blood vessels, bones, epithelium etc. Studies have shown that moderate amount of fluoride improves healing of bone by affecting the expression of vascular endothelial growth factor (VEGF) and bone morphogenetic protein-7 (BMP-7) [13]. Fluoride also affects the levels of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), enzymes involved in matrix reorganization, in initial stages of wound healing as seen during alveolar repair [14]. Thus, during regeneration of a tissue or an organ, the first important event to occur is healing of the wound at the site of injury or site of amputation and sodium fluoride influences this process, thereby enhancing or impeding the process of regeneration in the initial stages. However, once the wound has healed, the pluripotent cells at the site of action start proliferating to replace the lost tissue/organ.

3. SODIUM FLUORIDE AND CELL PROLIFERATION

While sodium fluoride is known to enhance proliferation of cells at lower doses, it hampers cell division at higher doses. If sodium fluoride is used in moderate amounts, it stimulates proliferation as has been seen in periodontal ligament cells (PDLCs) [15]. Similarly, sodium fluoride also stimulates osteogenesis and chondrogenesis during fracture healing in rabbits [16]. In addition to cell proliferation, the exposure to lower concentrations of fluoride induces migration of cells and matrix synthesis in epithelial cells *in vitro* [11]. To specify, sodium fluoride induces cell proliferation at an optimum concentration of 5×10^3 µmol/l and also increases the expression of BMP-2, BMP-3 and alkaline phosphatases, which are markers of cell proliferation. However, at 2×10^4 µmol/l, sodium fluoride seems to inhibit cell proliferation [17]. It has also been observed that 1 mM sodium fluoride does not hamper cell proliferation, however it influences the expression of several genes in human embryonic stem cells (hESCs) during embryoid body (EB) differentiation i.e. ectoderm marker *NeuroD1* and the mesoderm marker *Brachyury* get upregulated while endoderm marker *AFPI* is down regulated [18]. In human embryonic stem cells (hESCs), the higher dose of sodium fluoride hampers cell proliferation and induces apoptosis via caspase and c-Jun N-terminal kinase (JNK)-mediated, but reactive oxygen species (ROS)-independent pathway [18]. This further suggests that if there is chronic exposure of sodium fluoride, it may interfere with early embryogenesis. These studies demonstrate that at higher doses sodium fluoride may hamper cell division, which is the one of the noteworthy events that contributes to the regeneration of the lost structure.

Humans also possess the ability to regenerate certain tissues, however to a limited capability. Our body gets fluoride from municipal water, toothpastes etc. In such a scenario, there are more chances of fluoride accumulation in various tissues of our body and this fluoride may interfere with the healing capabilities in our body and the scant ability to regenerate the tissues. More than the required levels of fluoride can cause a condition called as fluorosis in humans which is a degenerative disorder affecting bones, teeth and some soft tissues [19, 20]. In case of severe skeletal fluorosis, conditions like extra-periosteal calcification and ossification have been seen [21]. Further, fluoride has also been linked to cancer as it is taken up by immature bones and teeth, and is stored in soft tissues like bowls, kidneys, liver, muscles, skin etc. [4]. Thus, from the cited observations it appears that lower sodium fluoride levels probably induce cell proliferation while higher levels deter cell division or induce apoptosis.

4. MECHANISM OF SODIUM FLUORIDE TOXICITY

The exact molecular mechanism by which fluoride shows its toxic effects is precisely not known but apoptosis is one of the manifestations. Fluoride interferes with cell division, stress responses, numerous enzymes, various metabolic pathways etc. Some of the major influences of sodium fluoride have been summarized in Table 1.

Excessive fluoride produces oxidative stress in the cells and leads to conditions like apoptosis, arrest of cell cycle and necrosis [32, 37]. Essentially, at low concentrations fluoride produces oxidative stress while at high concentrations it causes cell death by apoptosis [38]. The oxidative stress can be estimated by the increased lipid peroxidation and lowered levels of antioxidant enzymes like glutathione peroxidase, catalase and superoxide dismutase (SOD). Further, it has been observed that fluoride interferes with the porcine oocyte maturation by inhibiting meiotic resumption, interferes with spindle formation, improper chromosome separation which may lead to aneuploidy and thus ultimately affect female fertility [39]. NaF also affects the splenic development as it reduces the T and B cell population due to cell cycle arrest [40, 41].

Table 1. Effects of sodium fluoride on cells at the molecular level.

S. No.	Effects of fluoride
1	Sodium fluoride induces cell proliferation via BMP pathway during skeletal fluorosis [17]
2	High fluoride levels cause hindrance in cell proliferation and growth [22]
3	Fluoride induces G0/G1 arrest, apoptosis and DNA damage in mouse Leydig cells [23]
4	Sodium fluoride induces reorganization of F-actin i.e. podosome formation, in endothelial cells by inducing activation of RhoA, Rac1 and Cdc42 which degrade the extracellular matrix by stimulating local proteolysis [24]
5	Low levels of sodium fluoride induce production of matrix by increasing fibronectin and laminin-5 expression (associated with motility) [11]
6	Fluoride causes reduced cell viability; low protein and DNA synthesis [25-27]
7	Fluoride induces oxidative stress; elevated lipid peroxidation and decreased antioxidant enzymes' activity in human cells [28-30]
8	Fluoride affects the factors associated with stress, signal transduction and apoptosis. High dosage of NaF inhibits proliferation of Leydig cells and causes stress-induced apoptosis which is associated with changes in expression levels of apoptosis related proteins like caspase-3, caspase-9, B cell lymphoma 2 (Bcl-2) and Bax [31, 32]
9	Higher concentrations of fluoride causes exchange aberrations *viz* due to misrejoining of free ends of different double strand breaks in chromosomes [33, 34]
10	Fluoride causes chromosomal aberrations in human lymphocytes *in vitro* and bone marrow cells in Swiss albino mice [35, 36]

Apart from inducing oxidative stress, fluoride interferes with expression of genes involved in cell cycle, metabolism, stress response, cellular interactions etc. [42]. Fluoride harms the cell by breaking the mitochondrial outer membrane and thereby releasing cytochrome c which activates caspase-9 and caspase-3 pathways in the cytoplasm leading to apoptosis. Fluoride also decreases the expression of Bcl-2 family proteins which are regulators of apoptosis, and upregulates the expression of p53 proteins which are regulator of cell cycle [43]. Thus,

sodium fluoride influences the proliferation of cells by affecting the cell cycle, metabolism, antioxidant enzymes etc.

5. SODIUM FLUORIDE AND REGENERATION

The regeneration of an organ or a part of body, after it is lost, is a remarkable property possessed by only few groups of animals like amphibians, reptiles, fishes, planarians etc. Higher animals, including humans, however, retain the scant capability to regenerate tissues. The regeneration of an organ is accomplished through three well defined stages *viz*. wound healing (the wound heals following inflammation), blastema stage (stem cells are procured and cell proliferation continues) and differentiation stage (the cells get differentiated to replace the lost structure) [44]. The first two stages *viz.* wound healing and cell proliferation, are the hallmarks of regeneration and involve interplay of specific molecules at precise time intervals which procure and push the stem cells to the molecular pathways which ultimately lead to the regeneration of the lost appendage. Some groups of animals possess the ability to regenerate certain tissues as well. The process of regeneration is dependent on several internal and external factors. Sodium fluoride is a common and naturally occurring substance that influences the regeneration of tissues and/or organs. Suresh and Hiradhar [45] have shown that sodium fluoride at a concentration of 50 µg/ml enhances the healing of wound and regeneration of tail in *Hemidactylus flaviviridis* and as the concentrations are increased, sodium fluoride hampers tail regeneration while concentrations of 3000 and 5000 µg/ml are fatal. Fluoride has been shown to have a negative effect on the development of the nervous system during its regeneration, due to inhibition in development of neural ladder, as seen in planarians [46]. Further, it has been seen that low doses of fluoride don't alter the process of fin regeneration but affect the linear pattern of growth of fins in *Poecilia latepinna* [47]. During newt limb regeneration, the presence of sodium fluoride stimulates incorporation of ^{14}C-leucine by blastema *in vitro* and plays a positive role in limb regeneration. However, if the regenerating limbs are denervated, presence of sodium fluoride does not stimulate uptake of leucine by such blastemas [48]. Further, sodium fluoride exerts its effects only during later stages of newt limb regeneration [49]. This indicates that the influence of sodium fluoride on newt limb regeneration might be dependent on neural input.

Sodium fluoride also influences tissue regeneration. In humans, sodium fluoride when added into the medication for periodontitis treatment accelerates the periodontal regeneration [50]. Similarly, the regeneration of holes in pinnae in rabbits is upregulated by sodium fluoride as it promotes cell proliferation [51]. Moreover, the pinnae regeneration in rabbits is similar to amphibian regeneration [51]. The events and the gene expression are similar during organ regeneration and embryonic development [52]. It has been observed that during embryonic development of Chinese toad *Bufo gargarizans*, excessive fluoride causes organ malformations, and interferes with embryonic development [53]. Thus, excess of fluoride is detrimental to the process of development as well as regeneration. To summarize, sodium fluoride might be exerting its influence on the tissue and/or organ regeneration by affecting two crucial stages of regeneration i.e. wound healing and cell proliferation.

6. CONCLUSION

Fluoride or more specifically sodium fluoride, a naturally occurring toxicant in water and various products containing fluoride, when used by humans may lead to accumulation of fluoride in the bodies of humans as well as other organisms, more specifically in aquatic animals. Lower doses of fluoride are perceived to enhance wound healing and cell proliferation, the two hallmark events of regeneration. However, the higher doses have been seen to interfere with both these events. Sodium fluoride affects cell proliferation by directly affecting the cell division or by altering the expression of various cell proliferation markers like BMP-2, BMP-3 etc. Fluoride also influences wound healing by regulating the expression of molecules like FGF-2, FGF-9, BMP-7, Twist1, VEGF, MMP-2, MMP-9 etc. To conclude, sodium fluoride hampers wound healing as well as cell proliferation when present at higher levels in the body of

organisms, thereby impeding the process of regeneration.

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Research Article

Incidence of community acquired ESBL-producing bacteria among asymptomatic University students in Anambra State, Nigeria

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ABSTRACT

This study was conducted to investigate the incidence of community acquired extended-spectrum β-lactamase (ESBL)-producing bacteria among asymptomatic students of Nnamdi Azikiwe University, Awka, Anambra State, South-East Nigeria. A total of 102 non-duplicate strains of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were isolated from fecal samples (n=273) collected from the participating students. The isolates were subjected to antimicrobial susceptibility tests to determine their antimicrobial resistance profile. Their multiple antibiotic resistance (MAR) indices were also evaluated. Screening of the isolates for possible ESBL production was carried out by disk diffusion test using cefotaxime and ceftazidime disks. ESBL-production by the resistant strains was confirmed using the double-disk synergy test. Most of the isolates were found to be multi-drug resistant, as all *K. pneumoniae* and *P. aeruginosa* strains (100%), and 98.4% of the *E. coli* strains, had MAR indices ≥0.2. A total of 22 ESBL-producing bacterial species were confirmed, and the frequency of *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates among the ESBL-producing bacteria were n=20 (90.9%), n=2 (9.1%), and n=0 (0.0%) respectively. The total number of ESBL-producing bacterial strains isolated accounted for 8.1 % of the entire sample population. Although this prevalence rate may not indicate an alarming situation, it is important that the proliferation of ESBL-producing bacteria in the community be contained, since a high incidence of ESBL-producing organisms will create significant therapeutic problems in the near future. There is therefore need to develop strategies to reduce their spread in the community especially through monitoring, surveillance and proper detection protocol.

Keywords: Extended Spectrum β-Lactamase (ESBL); Antibiotic resistance; Gram-negative bacteria; Asymptomatic; Nigeria.

1. INTRODUCTION

β-lactamases are the most common mechanism of resistance to beta-lactam antibiotics including the third-generation cephalosporins to which extended spectrum β-lactamase plays a huge role among *Enterobacteriaceae* [1, 2]. Though there are different diverse β-lactamases, the extended spectrum β-lactamases (ESBLs) have been known to be of very high clinical importance. Infections caused by ESBL-producing organisms are difficult to manage for several reasons. First, empiric therapy consisting of β-lactam antimicrobials is often ineffective; and second, these organisms tend to also be resistant to other classes of antimicrobials including fluoroquinolones and aminoglycosides [1].

ESBLs capable of degrading the cephalosporins and monobactams are among the most important resistance determinants emerging in *Enterobacteriaceae* worldwide. ESBLs are β-lactamases capable of conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam by hydrolysis, excluding the cephamycins and carbapenems. These enzymes hydrolyze extended-spectrum cephalosporins such as ceftazidime or cefotaxime, as well as monobactams (aztreonam), and are inhibited by β-lactamase inhibitors [1, 3-5].

ESBLs have been reported worldwide in many different genera of *Enterobacteriaceae* and *Pseudomonas aeruginosa*. However, ESBL production has been previously reported to be most common in *Klebsiella* spp. and *Escherichia coli* [6-8]. It has become very important to study the prevalence of ESBL-producing organisms because of their increasing antimicrobial resistance and the decreasing number of new drugs available against such organisms.

Though the first detection of ESBLs in Nigeria is still not known and a national study for the actual prevalence of ESBL-producing bacteria in Nigeria is lacking, some reports have shown the increasing prevalence of ESBL-producing bacteria in some parts of the country [9-18].

The implications of the prevalence of ESBL-producing organisms in Nigerian communities cannot be overlooked. The wide and irrational use of antibiotics, especially the broad spectrum β-lactams, in our local communities allow for the emergence and spread of resistant strains of bacteria that render available drugs ineffective for treatment. Keeping in view the economic and clinical importance of ESBL-producing bacteria, our study was conducted to investigate the incidence of community acquired ESBL-producing bacteria among healthy and asymptomatic university students in Anambra State, South-East Nigeria.

2. MATERIALS AND METHODS

2.1. Study area and population

The study population comprised of students of Nnamdi Azikiwe University studying at the university campuses located at Agulu, Mbaukwu and Awka, Anambra State, Nigeria. The participants were adult male and female students between the ages of 18-26 years. Inclusion criteria were: (1) the participant is ≥18 years; (2) healthy or appear healthy at physical examination; (3) have no history of previous ESBL infection; (4) have not been hospitalized in the last 6 months. Consent forms and questionnaires indicating demographics and medical history of the individual were filled by the participating students.

2.2. Collection of samples

A total of 273 stool samples were randomly collected over a twelve (12) month period (March 2012-February 2013) from consenting university students spread across the three campuses of the university.

2.3. Isolation and identification of microorganisms

For bacterial isolation and identification, various cultural, staining, and biochemical testing procedures were carried out as previously described [19, 20]. A loopful of each stool sample was inoculated into respective test tubes containing 5 ml of freshly prepared nutrient broth (Oxoid, UK) and incubated at 35°C for 24 h. Bacterial growth was indicated by the turbidity of the broth culture. Using a wire loop, suspensions from the turbid solution was plated aseptically onto MacConkey and cetrimide selective agars (Oxoid,

UK) plates and incubated at 35°C for 24 h. Suspected colonies of *Escherichia coli* and *Klebsiella pneumoniae* isolates were subcultured onto freshly prepared MacConkey agar plates, while suspected *Pseudomonas aeruginosa* isolates were subcultured onto cetrimide selective agar plates to obtain pure cultures. For confirmation, Gram staining and con-ventional biochemical testing techniques including indole test for suspected *E. coli*, citrate and malonate utilization tests for suspected *K. pneumoniae*, and oxidase test for suspected *P. aeruginosa* were carried out.

2.4. Antimicrobial Susceptibility Test (AST)

AST of the isolates was carried out using the modified Kirby-Bauer disk diffusion method described by Cheesbrough [19] and the Clinical and Laboratory Standard Institute (CLSI) [21]. Mueller-Hinton (MH) agar (Oxoid, UK) was prepared according to the manufacturer's instructions, and transferred into 90 mm diameter sterile Petri dishes to a depth of 4 mm. The surface was lightly and uniformly inoculated using a sterile cotton wool swab in three directions rotating the plate approximately 60^0C, to ensure even distribution. Prior to inoculation, the swab stick was dipped into bacterial suspension having visually equivalent turbidity to 0.5 McFarland standards. Excess liquid from the sterile cotton-wool swab dipped in the bacterial suspension was removed by turning the swab stick against the side of the tube. The plates were covered and allowed to dry on the bench before applying the discs. Antibiotic discs were placed on the agar plate within 15 minutes of inoculation of isolates. Inoculated plates were incubated at 37 ^{o}C for 24 hours. On the next day, plates were read by taking measurement of zone of inhibition using a meter rule; *E. coli* ATCC 25922 was used as a negative control. Antibiotic disks used include cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), imipenem (10 µg), meropenem (30 µg), tetracycline (30 µg), erythromycin (15 µg), cefpodoxime (10 µg), amoxycillin-clavulanic acid (20/10 µg), sulphamethoxazole-trimethoprim (25 µg), ciprofloxacin (5 µg), and gentamicin (10 µg) (Oxoid, UK).

2.5. Screening of bacterial isolates for possible ESBL production

To screen all the *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates for the production of ESBL enzymes, single antibiotic disks comprising cefotaxime (30 µg) and ceftazidime (30 µg) were placed aseptically at a distance of 30 mm apart on MH agar plates that was previously swabbed with standardized inoculum of the test bacterium. The plates were allowed for about 30 mins for pre-diffusion of the antibiotics; and these were incubated for 18-24 hrs at 37^{o}C. After the incubation, the zones of inhibition were measured and recorded to the nearest millimeter using a meter rule. ESBL production was inferred or suspected if any of the test bacteria showed reduced susceptibility or is resistant to any one of the third generation cephalosporins (cefotaxime and ceftazidime) as per the breakpoints of CLSI [21].

2.6. Multiple Antibiotic Resistance (MAR) Index

Multiple antibiotic resistance (MAR) index (number of antibiotics to which test isolate displayed resistance divided by total number of antibiotics to which the test organism has been evaluated for sensitivity/resistance) for each test isolate was calculated according to the method described by Riaz et al. [22].

2.7. Confirmation of ESBL production by Double Disk Synergy Test (DDST)

ESBL production was confirmed in the *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates by the double disk synergy test (DDST) method as previously described [21]. DDST was performed as a standard disk diffusion assay on MH agar plates. Standardized bacteria suspension was aseptically swabbed on the MH agar plates. Amoxycillin-clavulanic acid disc (20/10 µg) was placed at the centre of the plate, and cefotaxime (30 µg) and ceftazidime (30 µg) discs were each placed at a distance of 15 mm (centre to centre) from the amoxycillin-clavulanic acid disc. The plates were incubated at 37^{o}C for 18-24 hrs. ESBL production was confirmed phenotypically when a difference of ≥5 mm increase in the inhibition zone diameter for

the zones of inhibition of the cephalosporins (cefotaxime and ceftazidime) tested alone and in combination with amoxicillin-clavulanic acid was observed.

3. RESULTS

3.1. Isolation and identification of microorganisms

A total of 273 fecal samples from the university students were analyzed. Of the 273 samples analyzed, 102 non-duplicate bacterial isolates comprising *E. coli* (n=63), *K. pneumoniae* (n=20), and *P. aeruginosa* (n=19) were isolated.

3.2. Antimicrobial resistance profile of the community-derived isolates

The *E. coli* isolates showed high resistance of 76.2, 82.5, and 79.3% to cefpodoxime, tetracycline, and erythromycin respectively. The *E. coli* isolates showed least resistance to imipenem (6.3%) followed by ciprofloxacin (14.3%) and cefotaxime (14.5%). All *P. aeruginosa* isolates were completely resistant (100%) to cefpodoxime, meropenem, amoxicillin-clavulanic acid and erythromycin, but were highly susceptible to imipenem, gentamicin and ciprofloxacin with a percent resistance of 5%. *K. pneumoniae* isolates like *P. aeruginosa* showed complete resistance (100%) to cefpodoxime, and were highly resistant to meropenem (90%), sulfamethoxazole-trimethoprim (75%), tetracycline (85%) and erythromycin (90%). Generally, imipenem showed the highest antibacterial activity against the tested isolates, followed by ciprofloxacin and gentamicin (Table 1). It can be observed in Table 2 the isolates were found to be multi-drug resistant as all *K. pneumoniae* and *P. aeruginosa* strains (100%), as well as 98.4% of the *E. coli* strains had MAR indices of 0.2 and above.

Table 1. Antibiotic resistance profile of isolates.

Antibiotics	Resistance (%)		
	E. coli	***K. pneumoniae***	***P. aeruginosa***
Ceftazidime	20.6	30	11
Cefotaxime	14.5	25	32
Ceftriaxone	34.9	25	16
Cefpodoxime	76.2	100	100
Imipenem	6.3	0	5
Meropenem	58.7	90	100
Amoxycillin-clavulanic acid	28.6	40	100
Gentamicin	20.6	15	5
Ciprofloxacin	14.3	15	5
Sulfamethoxazole-trimethoprim	54	75	84
Tetracycline	82.5	85	79
Erythromycin	79.3	90	100

Table 2. Multiple Antibiotic Resistance (MAR) Index of isolates.

Frequency of MAR Index			MAR Index
E. coli ***n* (%)**	***K. pneumoniae*** ***n* (%)**	***P. aeruginosa*** ***n* (%)**	
0 (0.00%)	0 (0.00%)	0 (0.00%)	0
1 (1.59%)	0 (0.00%)	0 (0.00%)	0.1
13 (20.63%)	1 (5.00%)	0 (0.00%)	0.2
17 (26.98%)	1 (5.00%)	1 (5.27%)	0.3
12 (19.05%)	7 (35.00%)	3 (15.79%)	0.4
15 (23.81%)	7 (35.00%)	10 (52.63%)	0.5
5 (7.94%)	4 (20.00%)	4 (21.05%)	0.6
0 (0.00%)	0 (0.00%)	0 (0.00%)	0.7
0 (0.00%)	0 (0.00%)	0 (0.00%)	0.8
0 (0.00%)	0 (0.00%)	0 (0.00%)	0.9
0 (0.00%)	0 (0.00%)	1 (5.26%)	1.0

3.3. Confirmation of ESBL-producing isolates

DDST was used for the phenotypic confirmation of ESBL production by the isolates. An increase of ≥5 mm was observed in the IZDs produced by the cephalosporins (with amoxicillin-clavulanic acid) against the ESBL producing organisms compared to the individual IZDs of any of the cephalosporins (without amoxicillin-clavulanic acid) (Figure 1). This was consistent with the CLSI's specifications for the double disc synergy test for the confirmation of ESBL production [21].

A total number of 102 Gram negative isolates (encompassing *E. coli*, *K. pneumoniae*, and *P. aeruginosa*) were isolated from the sample population (n=273). From these isolates, 22 ESBL-producing bacteria strains were confirmed, which is 8.1% of the total sample population. Of the total number of Gram negative isolates (n=102), 21.6% (n=22) were confirmed ESBL-producers. A majority of the ESBL-producers were *E. coli* (n=20, 90.9%), followed by *K. pneumoniae* (n=2, 9.1%). ESBL-production was not detected among the *P. aeruginosa* isolates (Figure 2).

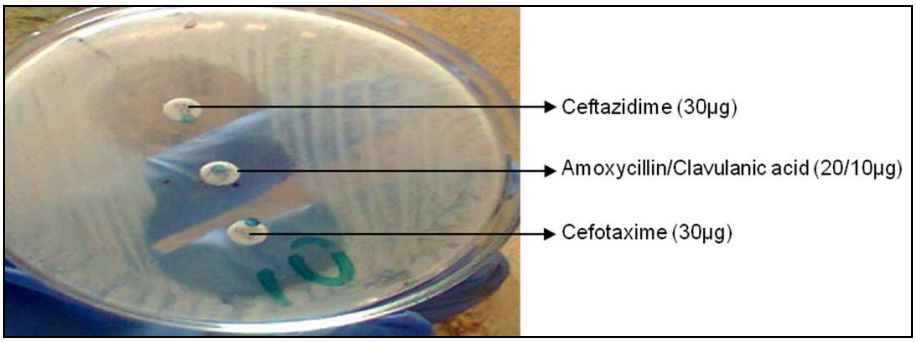

Figure 1. A picture of ESBL-producing isolate after the double-disc synergy testing (DDST); showing the keyhole effect notable for ESBL production in the phenotypic test.

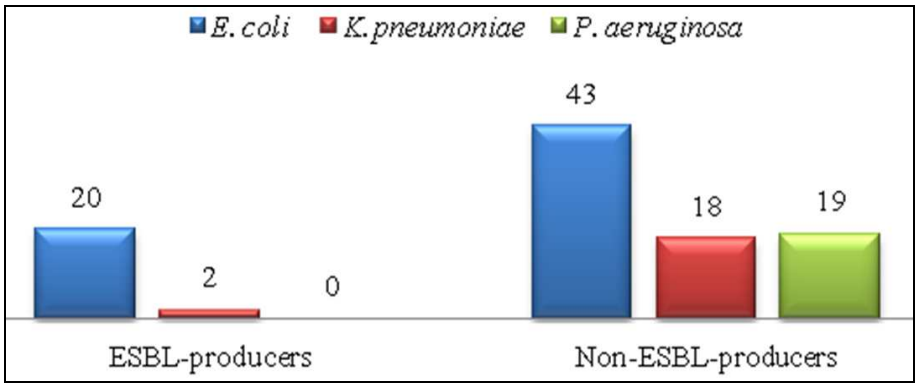

Figure 2. Total ESBL-producers and non-ESBL-producers among the community isolates.

3.4. Geographic and gender distribution of the samples, total isolates and ESBL-producing isolates

A total number of 273 samples were collected from the three locations Agulu, Awka, and Mbaukwu. The highest percentage of the samples came from Agulu (63.4%), followed by Mbaukwu (24.5%) and Awka (12.1%) (Table 3). A total of 102 non-duplicate bacterial isolates comprising of *K. pneumoniae*, *E. coli* and *P. aeruginosa* strains were isolated from the samples; and 52.0%, 17.6%, and 30.4% of the total number of isolates were from Agulu, Awka and Mbaukwu respectively. The distribution of the ESBL-producing bacteria from the three locations follows the same pattern as the distribution of the total number of samples and isolates from the locations.

Table 3. Geographical distribution of samples, total isolates and ESBL-producing isolates.

Source of samples	Number of samples [n (%)]	Number of isolates [n (%)]				Number of ESBL-producing isolates [n (%)]			
		EC	KP	PA	Total	EC	KP	PA	Total
Agulu	173 (63.4%)	43 (42.15%)	9 (8.82%)	1 (1.0%)	53 (52.0%)	16 (72.7%)	0 (0.0%)	0 (0.0%)	16 (72.7%)
Awka	33 (12.1%)	7 (6.86%)	3 (2.94%)	8 (7.8%)	18 (17.6%)	0 (0.0%)	2 (100.0%)	0 (0.0%)	2 (9.1%)
Mbaukwu	67 (24.5%)	13 (12.74%)	8 (7.84%)	10 (9.8%)	31 (30.4%)	4 (18.2%)	0 (0.0%)	0 (0.0%)	4 (18.2%)
Total	273 (100.0%)	63 (100.0%)	20 (100.0%)	19 (100.0%)	102 (100.0%)	20 (100.0%)	2 (100.0%)	0 (0.0%)	22 (100.0%)

KP: *K. pneumoniae*, EC: *E. coli*, PA: *P. aeruginosa.*

Table 4. Gender distribution of samples, total isolates and ESBL-producing isolates.

Gender	Gender distribution of samples collected [n (%)]				Gender distribution of isolated bacteria [n (%)]				Gender distribution of ESBL-producing isolates [n (%)]			
	Agulu	Awka	Mbaukwu	Total	EC	KP	PA	Total	EC	KP	PA	Total
Males	41 (23.7%)	20 (60.6%)	17 (25.4%)	78 (28.6%)	22 (34.9%)	8 (40.0%)	7 (36.8%)	37 (36.3%)	2 (10.0%)	1 (50.0%)	0 (0.0%)	3 (13.6%)
Females	122 (64.7%)	10 (30.3%)	41 (61.2%)	173 (63.4%)	35 (55.6%)	10 (50.0%)	11 (57.8%)	56 (54.9%)	16 (80.00%)	1 (50.0%)	0 (0.0%)	17 (77.3%)
Unknown	10 (5.8%)	3 (9.1%)	9 (13.4%)	22 (8.1%)	6 (9.5%)	2 (10.0%)	1 (5.3%)	9 (8.8%)	2 (10.0%)	0 (0.0%)	0 (0.0%)	2 (9.1%)
Total	173 (100.0%)	33 (100.0%)	67 (100.0%)	273 (100.0%)	63 (100.0%)	20 (100.0%)	19 (100.0%)	102 (100.0%)	20 (100.0%)	2 (100.0%)	0 (0.0%)	22 (100.0%)

KP: *K. pneumoniae*, EC: *E. coli*, PA: *P. aeruginosa.*

As more samples were collected from Agulu (n=173, 63.4%), followed by Mbaukwu (n=67, 24.5%), and then Awka (n=33, 12.1%), the highest percentage of ESBL-producing isolates was from Agulu (n=16, 72.7%), followed by Mbaukwu (n=4, 18.2%), and then Awka (n=2, 9.1%). Of the 22 ESBL-producing isolates, *E. coli* was the most prevalent (n=20, 90.9%), followed by *K. pneumoniae* (n=2, 9.1%). No ESBL-producing *P. aeruginosa* strain was detected. A rate of 72.7% of the *E. coli* isolates that were found to be ESBL-producers were isolated from Agulu, 18.2% were from Mbaukwu, and none from Awka. ESBL-producing *K. pneumoniae* (n=2, 100%) was reported only from samples emanating from Awka (Table 3).

Of the total 273 fecal samples collected from the university students, 173 (63.4%) samples were received from female students, and 78 (28.6%) were from male students. Twenty two (8.1%) students did not indicate their genders (Table 4). A total of 36.3% of all the bacterial isolates comprising *K. pneumoniae*, *E. coli* and *P. aeruginosa* strains were from males, and 54.9% were from females. The distribution of the ESBL-producing bacteria amongst the female and male students also follows the same pattern as the distribution of the total number of samples and isolates amongst the male and female students.

As more samples were collected from the female participants (n=173, 63.4%) compared to their male counterparts (n=78, 28.6%), the highest percentage of ESBL-producing isolates was from the female students (77.3%), while 13.6% was recorded for the male students. Eighty percent of the *E. coli* isolates that were found to be ESBL-producers were isolated from the female students, and 10.0% were from the males. There was an equal distribution of ESBL-producing *K. pneumoniae* (50%) amongst the female and male students (Table 4).

4. DISCUSSION

The antibiotic resistance profile of the isolates reveals that the isolates were generally resistant to the different classes of antibiotics tested. The isolates however, exhibited very high resistance rates to cefpodoxime, meropenem, tetracycline, erythromycin, and sulfamethoxazole-trimethoprim. Lower levels of resistance to ciprofloxacin and gentamicin were recorded among the community isolates. The isolates showed least resistance only to imipenem, a drug known for its potent and broad spectrum antimicrobial activity. The complete susceptibility of the isolates to imipenem could be attributed to the low-rate use of the drug, since is extremely expensive and not readily available in the Nigerian market, and is used as a last option in serious infections when all other antimicrobial drugs have failed.

Majority of the isolates were found to be multi-drug resistant with MAR index ≥2 (Table 2). Riaz et al. [22] stated that a MAR index >0.2 indicates that the organism may have originated from an environment where antibiotics are over used. The observed resistance to these drugs is a probable indication of earlier exposure of the isolates to the antibiotics, which may have enhanced their multidrug-resistance development.

From the 102 Gram negative bacteria isolated from the sample population (n=273), a total number of 22 (8.1 %) ESBL-producing bacteria strains were confirmed. The ESBL-producing isolates observed in this study were *E. coli* and *K. pneumoniae.* This is in agreement with other studies that reported the expression of the ESBL enzyme by both species [1, 23, 24].

Tansarli et al. [7] indicated that Nigeria, together with several other African countries with low human development index (Rwanda, Kenya, Nigeria, Central African Republic, Benin, Senegal, Malawi and Tanzania), have prevalence rates of ESBL-producing organisms from both clinical or community sources varying from 3.8% to 22.8%. Their report showed that the proportion of ESBL-producing isolates among the *Enterobacteriaceae* may not be high in Africa, but is certainly not negligible [7].

The 8.1% prevalence rate of ESBL-producing organisms in our study population may not indicate an alarming situation, but there is need for it to be curtailed since a high incidence of ESBL-producing organisms will create significant therapeutic problems in the near future.

Several studies in Nigeria have shown that abuse and indiscriminate use of antibiotics by people practicing self-medication are partly responsible for the high prevalence of multidrug resistance and

ESBL-producing bacteria in both community and hospital acquired infections [18, 25, 26]. In our study, majority of the study participants indicated that they have at one time or the other taken antibiotics without prescription, especially for ailments such as, typhoid, UTI, cough, and sore throat to mention a few. It can be inferred that the occurrence of multidrug-resistant and ESBL-producing bacteria among the university students may be attributed to the abuse and indiscriminate use of antibiotics by the students in this region. In Nigeria, antibiotics can be relatively inexpensive, sold usually over the counter even without prescription, and can be readily abused or used indiscriminately. Moreover, the indiscriminate proliferation of patent medicine outlets that makes these drugs easily accessible in the community, as observed in the study locations, may have also contributed to the proliferation of these resistant bacteria in the community. This should be of great health concern to university communities across Nigeria, as well as the entire Nigerian population.

ESBL detection and prevalence studies in Nigeria are majorly undertaken by researchers in the academia. However, this have been quite limited to the educational/research institutions without the translation of the findings into a template that can be used in our hospitals for the accurate detection of ESBL from clinical isolates, as well as for the development of policies that will guide the proliferation, use, and abuse of antibiotics in the non-hospital environment.

According to Shaikh et al. [27] and Coque *et al.* [28], the detection of ESBL production is of paramount importance both in hospital and community isolates. Therefore, infection-control practitioners and clinicians need the clinical laboratory to rapidly identify and characterize different types of resistant bacteria. Methods should be improved to efficiently detect and track those bacterial clones and plasmids that constitute the major vehicles for the spread of ESBL-mediated resistance. An improvement is needed in the methods for detecting multidrug-resistant ESBL producers that express a low level of resistance to β-lactams or might contain silenced antibiotic resistance genes not detectable by standard susceptibility testing protocols. The use of broad spectrum cephalosporins and fluoroquinolones in humans should be urgently limited to cases in which other therapeutic alternatives according to evidence-based guidelines are not possible. Limiting antimicrobial use may curtail the selection and persistence of predominant ESBL clones and the probable dissemination of conjugative plasmids among strains, thus decreasing not only the number of potential ESBL donors but also the accumulation of antibiotic resistance genes on common genetic elements. There is also need for national and supranational public health efforts to implement surveillance, epidemiologic, environmental health, and policy-making components on the use of antibiotics. These will certainly contain the spread of ESBL-producing organisms and prevent the emergence of new incidences of diseases caused by ESBL-producing organisms.

5. CONCLUSION

This study has revealed the presence of multidrug-resistant, ESBL-producing organisms among healthy university students of Nnamdi Azikiwe University community, Anambra State, Nigeria. The proliferation of ESBL-producing organisms in the community will create significant therapeutic problems in the near future if not curtailed. There is need to develop effective and innovative strategies to reduce their spread in the community.

ETHICAL APPROVAL

Ethical approval for this research was obtained from the ethical committee of Anambra State University Teaching Hospital, Amaku, Awka, Nigeria (Ref no. ANSUTH/AA/ECC/40).

CONSENT

All participants gave written informed consent to participate in this study.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. COE designed and supervised the study. CRC, PME and NTU managed the laboratory analyses. CRC and PME managed the data analysis and literature searches, and prepared the first draft of

the manuscript. ICA, CPE and DOA revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

Independent distribution of blood group types and two genetically determined traits in a female population

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ABSTRACT

Certain traits in humans are known to be neutral in nature as they do not influence fitness of the individuals. Traits like ABO blood group, phenylthiocarbamide (PTC) tasting and ear lobe structure are genetically determined and follow Mendelian pattern of inheritance. Genes deciding their expression are situated on separate chromosomes and therefore would be certainly following independent assortment during gametogenesis. Data regarding association of these traits were collected from human female subjects to test whether blood group types show their dependency with other two features. An analysis in this regard clearly indicated that there exist no association between blood group type and PTC tasting and also between blood group and ear lobe structure.

Keywords: Blood group; PTC tasting; Ear lobe; Independent distribution; Female population.

1. INTRODUCTION

ABO blood group system was for the first time described by K. Landsteiner in 1900 [1]. The type of blood group in humans is decided by the presence or absence of specific antigens on the surface of red blood cell membrane [2]. The significance of ABO blood group is due to its compatible match between donor and recipient at the time of blood transfusion and organ transplantation [2]. There are reports that ABO blood grouping influences some physiological characteristics [3]. Association between blood group type and some pathological conditions have been reported, for example, people with blood group-A have been associated with increased risks of gallstones, colitis, and certain tumor types [4-6]. A number of researchers have suggested association between certain blood types and cardiovascular diseases [7-12]. Few studies have established association between ABO blood groups and oral diseases, specifically periodontal diseases (PDs) [13]. Quite early, studies indicated that there is association among blood group polymorphism with certain diseases especially between group O and peptic ulceration [14, 15]. Sporadic reports regarding association between blood group type and diseases have been received for example susceptibility to arterial and venous thromboembolism has been linked to blood group [16, 17]. There are evidences that blood group O provides a selective advantage against malaria [18-20]. There are other examples of infectious diseases in which the severity of infection can be directly linked to ABO phenotype.

Phenylthiocarbamide (PTC) is an organic substance which is also referred as phenylthiourea that contains organosulfur thiourea with a phenyl ring. This chemical can be tasted bitter or tasteless

depending on the genetic constitution of individuals. Those persons capable to tell its bitter taste are genetically dominant and could be genetically homozygous (TT) or heterozygous (Tt) whereas those who are unable to tell its taste (non-tasters) are homozygous recessive (tt) [21]. People all across the globe have been analysed for the frequency of taster and non-taster alleles (T and t respectively) and a significant variation among the individuals of different geographical origin have been recorded [22]. There are certain studies which have indicated that individuals with homozygosity of dominant alleles are able to taste this chemical more intensely than heterozygotes. The persistence of tasters and non tasters of PTC in all the populations is due to presence of heterozygotes harbouring both alleles [23].

One of the distinct morphological features of human ear is one's lower lobe (ear lobe) which may be free or attached. Ear lobe structure is a genetically determined trait and is known to follow Mendelian inheritance pattern. Persons having free ear lobe are either dominant homozygous (AA) or heterozygous (Aa) whereas, attached ear lobe results due to recessive homozygosity (aa) [2]. Association between blood group types and some genetically determined features have not been given substantial importance. A number of human morphological features have genetic basis of their inheritance and are known to follow Mendelian pattern of inheritance [24, 25].

This study was performed with an aim to see whether these three genetically determined features in human females show any relationship or not. Therefore, an association was tested between blood group type and PTC tasting to know that whether these two traits show random or non random association. Likewise, an association between blood group type and ear lobe structure was also undertaken.

2. MATERIAL AND METHODS

This study is based on observations of three genetically decided human features like one's blood group, PTC tasting ability and ear lobe structure. Females mainly students studying at Banaras Hindu University were randomly selected for this study. Blood group of the persons were determined by using A and B anti-sera procured from Biotec's blood grouping reagents. For this purpose, individual's blood was put at two spots on a cleaned glass slide to which A and B anti-sera were mixed separately. Based on the clumping of RBC of the drop of blood on the slide, blood group of the individual was decided. After knowing the blood group of the individual, a piece of paper soaked in PTC was served to her to know whether she can taste it or not. PTC is a chemical that can be tasted by a person depending on his or her genotype [26]. Those who can taste it are genetically dominant (TT or Tt) and those who do not taste this chemical are genetically recessive homozygous (tt). Females were categorized as tasters if they were clearly able to tell bitter taste of the chemical. Besides this, another feature, i.e., ear lobe was also seen in every subject undergoing this observation. Ear lobe may be free or attached showing dominant or recessive character respectively. Genetically dominant persons (AA or Aa) possess free ear lobe whereas persons bearing attached ear lobe are recessive types (aa). All the three features considered in this study follow Mendelian inheritance pattern.

Chi-square analysis was done to analyse data. Assuming that the two events occur independently, chi square was calculated following R x C contingency table. Probability less than 0.05 will indicate significant difference between observation and expectation, i.e., the two events are happening non-randomly.

3. RESULTS

Table 1 shows distribution of ABO blood types with those who could taste phenyl thiocarbamide (PTC). Interestingly the numbers of taster were found to be 65% than nontasters (35%) and tasters were always more in number for all the four different blood types in comparison to their respective non tasters types. Chi square (χ^2) analysis revealed that that there is nonsignificant difference between observation and expectation ($p>0.05$) indicating that these two features (blood group and PTC tasting) are independent in occurrence. Table2 depicts association between blood group types and ear lobe phenotypes. In this case also, the numbers of individual with dominant phenotypes (free ear lobe) were found to be significantly more than recessive types. Individuals with free ear lobe were

comparatively more than attached ear lobe with all the four different blood group types. Statistical analysis (χ^2) performed for this also indicated non significant difference between observation and expectation types ($p>0.05$) denoting that the two phenotypes are independent in occurrence.

Table 1. Number of observed and expected (in parentheses) ABO blood group types and tasters and non-tasters in a female population.

	Blood group				
	A	B	AB	O	Total
PTC tasters	17 (17.55)	16 (14.95)	13 (15.6)	19 (16.9)	65
PTC non-tasters	10 (9.45)	7 (8.05)	11 (8.4)	7 (9.1)	35
Total	27	23	24	26	100

$\chi^2 = 2.244$; d.f. =3; P = 0.52

Table 2. Number of observed and expected (in parentheses) ABO blood group types and free and attached ear lobe females in a female population.

	Blood group				
	A	B	AB	O	Total
Free ear lobe	19 (19.72)	15 (14.28)	12 (12.92)	22 (21.08)	68
Attached ear lobe	10 (9.28)	6 (6.72)	7 (6.08)	9 (9.92)	32
Total	29	21	19	31	100

$\chi^2 = 0.5258$; d.f. =3; p = 0.91

4. DISCUSSION

Although Anstee (2010) elaborated relationship between blood groups and diseases [27], ample information regarding association between different blood groups and some genetically determined traits have not been given any attention. This study was done with an objective to see whether blood group of individuals show dependency with certain traits like PTC tasting and ear lobe structure. In fact, all the three features, blood group type, PTC tasting and ear lobe structures (like free ear lobe and attached ear lobe) are genetically determined traits. Gene determining ABO blood group is located on chromosome 9, PTC taste determining gene on chromosome 5 and ear lobe gene found on chromosome 21. A number of human populations, all across the world have been screened for the allelic frequency of the ABO blood group (I^A, I^B and I^O), PTC tasters and non tasters alleles (T and t) and attached and free ear lobe (A and a) alleles. Since genes determining these three features are located on different autosomal chromosomes, one can expect their independent assortment during gametogenesis. A random occurrence of different combinations between blood group vs. PTC tasting and blood group vs. ear lobe structure was expected to exist in human populations. This analysis revealed exactly the same as there is no dependency between blood group and PTC tasting. Likewise, when ear lobe phenotype was considered along with individual's blood group, no such dependency was recorded. All the three traits chosen for this study are neutral traits, that is, they do not affect the fitness of the individual. Further, these three traits are also not of consideration when a couple selects each other for marriage. Therefore, these traits are not influenced by the phenomenon of selection. The distribution of ABO blood group people in specific population show a definite ratio like, persons of blood group-O always outnumber other types. Individuals with AB blood group are known to occur in less frequency than remaining others (A, B and O) [24]. The trend of occurrence of least fluctuating allele frequency exists in the population due to random mating and large size of human population. However, their associations with PTC tasting and ear lobe have not been determined so far. This observation enables us to explain that interdependency among blood group combinations and PTC tasting does not exist at all. Similarly the other associations tested between blood group and ear lobe also exist in random distribution. It can also be anticipated that other genetically determined morphological or behavioral traits like dimple in chin, tongue rolling, widow's peak etc., may not be dependent with blood group, provided the genes responsible for such traits do not show linkage with human blood group deciding gene.

Indian human populations have been considered for the distribution of different genetically determined features, for example, widow's peak, ear lobe structure, blood group types, dimple in chin,

rolling of tongue etc. [28-30]. These studies were done not only in urban people but also in a large number of ethnic groups and castes to see variation at the level of allelic distribution among such groups [31]. There are certain studies through which it has been stated that those who could strongly taste PTC were less likely to be smokers. This is an indication that those people who taste PTC bitter find the taste of cigarettes bitter and may be less likely to smoke. Likewise, results of certain research also suggest that there may be correlations between the ability to taste PTC and preferences for certain types of foods [26, 32].

AUTHOR'S CONTRIBUTION

AKS: Manuscript writing and statistical analysis. PY: Performing blood, PTC and ear lobe analysis (data collection). All authors read and approved the final manuscript.

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TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest.

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Research Article

Achillea millefolium L. subsp. *millefolium* essential oil's antifungal effect

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ABSTRACT

This study was carried out with the aim of determining the antifungal effect of the essential oil isolated from *Achillea millefolium* subsp. *millefolium* plant against pathogenic fungi. In order to test the antifungal effect of the oil, an analysis was conducted on a total of 4 pathogen fungi which included *Candida albicans, Candida tropicalis, Candida parapsilosis* and *Saccharomyces cerevisiae*, and the effect of the essential oil on the growth of these fungi was investigated. The essential oil of *A. millefolium* ssp. *millefolium* had varying degrees of effect on the tested fungi. The highest antifungal effect was found against *S. cerevisiae*; whereas the lowest antifungal effect was found against *C. parapsilosis*. Nystatin showed a higher activity than the essential oil of *A. millefolium* subsp. *millefolium* against the tested fungi. MIC values of the essential oil against the tested fungi ranged from 1.25 µl/ml to 10 µl/ml. The results obtained indicate that essential oil of *A. millefolium* subsp. *millefolium* can be used as an alternative to antifungal agents such as amphotericin, ketoconazole, and fluconazole.

Keywords: *Achillea millefolium* subsp. *millefolium*; Essential oil; Antifungal; Turkey.

1. INTRODUCTION

Since ancient times, raw herbal essences of aromatic plants have been used for different purposes, such as food, perfumery and medicines [1]. Primary and secondary metabolites produced by plants have a wide spectrum of functions. Secondary metabolites have been later utilized by humans due to their beneficial roles [2]. Essential oils are secondary metabolites obtained from plants and have been extensively used since the Middle Ages for bactericidal, virucidal, fungicidal, antiparasitic, insecticidal, medical and cosmetic purposes [3, 4].

Achillea L. is a large genus belonging to the family Asteraceae. The genus *Achillea* L. includes 59 taxa divided into 6 sections. Among them, 31 taxa are endemic to Turkey [5-7]. *Achillea millefolium*, known to the public as "milfoil", "common yarrow", "gordaldo", nosebleed plant" is considered to be one of the oldest medical plants [8]. There are many subspecies of *A. millefolium* species. *A. millefolium* species is represented by two subtypes in Turkey flora. These are *Achillea millefolium* subsp. *millefolium* and *Achillea millefolium* subsp. *pannonica* [9]. *Achillea* species, known as medicinal plants, are used against fever, colds, digestive complaints, slow-healing wounds and dermatitis. However, *A. millefolium* plant has been used be-

cause of its anti-inflammatory, spasmolytic, haemostatic, and cholagogue effects [10]. The herbal tea of *A. millefolium* has been found to be used against diseases of the gastrointestinal tract, especially in the folk medicine. The aim of the present study was to determine the essential oil *A. millefolium* subsp. *millefolium* growing in Ardahan ecological conditions and to investigate their antifungal effect on some strong pathogen fungi.

2. MATERIALS AND METHODS

2.1. Plant material and isolation of essential oils

A. millefolium subsp. *millefolium* samples of the plants were collected as study materials in June 2013 from Ardahan/Turkey surroundings (approximately 2080 m altitude). Extractions were carried out with Clevenger apparatus (Basaran cam, Turkey and Misung Scientific Co., Korea) using water distillation.

2.2. Microorganisms

Candida albicans and *Candida tropicalis* were obtained from Fırat University Department of Biology; *Candida parapsilosis* were obtained from Giresun University Faculty of Education, *Saccharomyces cerevisiae* was obtained from Giresun Province Control Laboratory.

2.3. Antifungal activity

The antifungal activity of the essential oil was determined by disc diffusion method. The essential oil of *A. millefolium* subsp. *millefolium* was sterilized by filtration through a 0.45 µm membrane filter [13]. The turbidity of fungal suspensions were adjusted with 0.5 Mc Farland standard (10^7 CFU/ml fungi concentration), then, the fungal suspension spread on Petri dishes [14]. The discs (6 mm diameter) were put on the inoculated agar and separately impregnated with 20 µl of essential oils. Nystatine disc was used as positive control. Plates were kept at 30°C for 48 h. Antifungal activity was assessed by measuring the diameter of the growth-inhibition zone in millimeters [15].

2.4. Determination of Minimum Inhibition Concentration (MIC) of the essential oils

The MIC was defined as the lowest concentration that completely inhibits the growth of microorganisms. For the determination of values of MIC, a micro-dilution broth assay was utilized. Two-fold serial dilutions (in dimethyl sulphoxide (DMSO)) were prepared from 0.0098 µl/ml to 20 µl/ml of the essential oils of *A. millefolium* subsp. *millefolium* in a 96-well microplate. Plates were incubated at 30°C for 48 h [16, 17].

3. RESULTS AND DISCUSSION

Medical and aromatic plants are rich and important natural sources of biologically active compounds and have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties [18]. Table 1 reveals inhibition zones which were created by essential oil of *A. millefolium* subsp. *millefolium* against the test fungi. The highest and the lowest activities were found against *S. cerevisiae* and *C. parapsilosis*, respectively. Nystatin was more active against the test fungi than the essential oil of *A. millefolium* subsp. *millefolium* except for *S. cerevisiae*. In addition to this, DMSO showed no activity.

Table 1. Inhibition zones of essential oil of *A. millefolium* subsp. *millefolium* (mm).

Fungi	*A. millefolium* subsp. *millefolium*	Nystatin	DMSO
C. albicans	17	30	-
C. tropicalis	20	30	-
C. parapsilosis	15	25	-
S. cerevisiae	30	17	-

Table 2. MIC values of essential oil of *A. millefolium* subsp. *millefolium* (µl/ml).

Fungi	*A. millefolium subsp. millefolium*
C. albicans	10
C. tropicalis	5
C. parapsilosis	1.25
S. cerevisiae	2.5

Table 2 shows values of MIC. The values range from 1.25 to 10 µl/ml for *A. millefolium* subsp. *millefolium*. Essential oils exhibited the lowest MIC value against *C. parapsilosis*. El-Kalamouni et al. [19] examined antifungal activity of essential oil of *A. millefolium* collected from France and it was demonstrated that the essential oil were inhibited the growth of *Rhizopus stolonifer*, *Verticillium dahliae*, *Colletotrichum gloesporoides*, *Botrytis cinerae* and *Aspergillus niger*. MIC values were found as 1.6 mg/ml, 3.1 mg/ml, 3.4 mg/ml, 3.6 mg/ml and 4.7 mg/ml, respectively. Karamenderes et al. [20] revealed that essential oil of *A. millefolium* subsp. *millefolium* was active against *C. albicans*. Likewise, we found that essential oil of *A. millefolium* possessed affect on the growth of *C. albicans*. Falconieri et al. [21] studied antifungal activity of the essential oils of flowering aerial parts of wild *A. millefolium* growing on the Mediterranean coast (Sardina Island, Italy) and on the Atlantic Coast. Both of the essential oils inhibited *C. albicans* (MIC: 2.5 µl/ml), *C. tropicalis* (MIC: 2.5 µl/ml) and *C. parapsilosis* (MIC: 2.5 µl/ml). In our study, MIC values were found as 10 µl/ml, 5 µl/ml and 1.25 µl/ml against *C. albicans*, *C. tropicalis* and *C. parapsilosis*, respectively. The difference might be arising from several factors like local, climatic, seasonal, and experimental conditions [22]. Candan et al. [23] reported that essential oil of *A. millefolium* subsp. *millefolium* had activity against *C. albicans*. Similarly, we found activity against *C. albicans*.

4. CONCLUSION

As a result, the antifungal effect of the essential oil obtained from *A. millefolium* subsp. *millefolium* plant was investigated; and it was revealed that it yielded positive results against *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *S. cerevisiae.*

AUTHOR'S CONTRIBUTION

Both authors have equally contribution, read and approved the final manuscript.

TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest.

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Research Article

Biologically active phenolic acids produced by *Aspergillus* sp., an endophyte of *Moringa oleifera*

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ABSTRACT

This study investigates the secondary metabolites of an endophytic *Aspergillus* sp. isolated from leaves of *M. oleifera* growing in Anambra State, South-Eastern Nigeria. Antimicrobial and antioxidant screening of the fungal extract and isolated compounds, as well as cytotoxicity assay of the extract against cisplatin-sensitive A2780 (sens) and cisplatin-resistant A2780 (cisR) ovarian cancer cell lines were carried out using standard methods. Chemical investigations of the fungal extract involving a combination of different chromatographic methods and spectroscopic techniques were carried out to isolate and characterize the constituents of the extract. At a concentration range of 1-4 mg/ml, the crude extract of *Aspergillus* sp. showed mild antimicrobial activity against *Bacillus subtilis, Klebsiella pneumoniae*, and *Candida albicans*. The fungal extract showed good antioxidant activity at 500 µg/ml, with an inhibition of 72.1%. Also, at 100 µg/ml, the extract showed excellent cytotoxic activity against A2780 (sens) and A2780 (cisR), with growth inhibitions of 105.1% and 105.5% respectively. Two known pharmacologically active phenolic compounds (*p*-hydroxyphenyl acetic acid and ferulic acid) were isolated from the fermentation extract of the endophytic fungus. At 250 µg/ml, ferulic acid exhibited an excellent antioxidant activity with an inhibition of 90.4%, while an inhibition of 35.4% was recorded for *p*-hydroxyphenyl acetic acid. Ferulic acid also showed a mild antifungal activity at 500 µg/ml against *A. niger* with an IZD of 2 mm. *p*-Hydroxyphenyl acetic acid showed no antimicrobial activity. These results further confirm the potentials of endophytic fungi associated with Nigerian plants as source of bioactive compounds with pharmaceutical or industrial applications.

Keywords: Phenolic acids; *Aspergillus* sp.; Endophytic fungus; *Moringa oleifera*; Secondary metabolites.

1. INTRODUCTION

Endophytes are potential sources of biologically active natural products for exploitation in medicine, agriculture and industries. The discovery of novel drug molecules from endophytes is an important alternative to overcome the increasing threats of cancer and drug resistance by plant and human pathogens.

Moringa oleifera, also known as moringa, drumstick tree, ben oil tree, benzoil tree, or horseradish tree, is the most widely cultivated species of the genus *Moringa*, the only genus in the family Moringaceae. *M. oleifera* is a fast-growing, drought-resistant tree, widely cultivated in tropical and subtropical areas of the world, where its leaves and seed pods are used as vegetables and in herbal medicine [1].

M. oleifera leaves have been reported to be a rich source of calcium, potassium, protein, β-carotene, and natural antioxidants like vitamin C [2]. Traditionally, the roots, barks, pods, and leaves of *M. oleifera* are used in medicine for the treatment of a variety of human ailments such as inflammation, cardiovascular, hematological, hepatic and renal disorders [3, 4]. The plant is reported to show various biological activities, including antihypertensive and cholesterol lowering [5-7], diuretic [8-9], antispasmodic [10-12], anti-ulcer [13], hepatoprotective [14], antibacterial and antifungal activities [15-18], and anticancer [19] activities.

There are several reports of endophytic fungi associated with *M. oleifera*. Dhanalakshmi et al. [20] isolated endophytic *Alternaria, Aspergillus, Bipolaris, Exosphiala, Nigrospora* and *Penicillium* species from *M. oleifera* growing in the Yercaud hills of India. Secondary metabolites of some endophytic fungi of *M. oleifera* were evaluated for their antimicrobial and antioxidants properties [21-23]. Zhao et al. [22] reported the isolation of four bioactive compounds (griseofulvin, dechlorogriseofulvin, 8-dihydroramulosin, and mullein) from *Nigrospora* sp. associated with *M. oleifera*.

Nigeria is rich with enormous and resourceful plant biodiversity, and these plants are hosts to millions of endophytic microbial communities that are can be explored as renewable source of natural products and present the opportunity to discover a plethora of compounds [24]. With the potentials reportedly possessed by the Nigerian plant *M. oleifera* and its associated endophytes, our study was aimed at investigating the secondary metabolites of an endophytic fungus isolated from the leaves of *M. oleifera* growing in Anambra State, South-Eastern Nigeria.

2. MATERIALS AND METHODS

2.1. Isolation, identification and fermentation of endophytic fungus

Fresh healthy leaves of *M. oleifera* were collected from Agulu, Anambra State, South-Eastern Nigeria. The leaves were washed thoroughly in running tap water and cut into 1 cm fragments. The leaf fragments were surface-sterilized by immersion in 2% sodium hypochlorite solution for 2 min, and then in 70% ethanol for nearly 2 min, before a final rinse in sterile water for 5 min. The leaf fragments were then placed on Petri plates containing freshly prepared malt extract agar [(MEA) Oxoid, UK] supplemented with chloramphenicol. The Petri plates were incubated at 28^{o}C, and fungal growths from the leaves were monitored. Hyphal tips from distinct colonies emerging from the leaves were subcultured onto fresh MEA plates to obtain pure colonies. Identification of the isolated fungus was carried out based on its cultural, morphological and microscopic characteristics as described by Barnett and Hunter [25] and Ainsworth et al. [26]. Morphological identification, according to the standard taxonomic key, included colony diameter, texture, colour and the dimensions and morphology of hyphae and conidia.

2.2. Fermentation, extraction, and isolation of metabolites

The endophytic fungus was subjected to solid state fermentation in a 1L Erlenmeyer flask containing autoclaved rice medium (100 g of rice and 200 ml of distilled water). The flask was inoculated with about 3 mm diameter agar blocks containing the fungus, and then incubated at 28°C for 21 days. At the completion of fermentation, the fungal secondary metabolites were extracted with EtOAc and the crude extract was concentrated under reduced pressure. Fractionation of the extract was

done using vacuum liquid chromatography on silica gel 60 (Merck, Germany) packed to a hard cake up to a height of 15 cm. Stepwise gradient elution was done using non-polar:moderately polar solvent system (DCM:MeOH). Fractions were further separated on Sephadex LH-20 (Sigma-Aldrich, Germany) using DCM:MeOH in the ratio of 1:1 (V/V) as mobile phase. Metabolites-containing fractions were further purified by semi-preparative HPLC. Analytical HPLC was used to identify important peaks in the extract and fractions, as well as to evaluate the purity of isolated compounds.

2.3. Antimicrobial assay

Preliminary antimicrobial screening of the endophytic fungal extract was carried out using the agar well diffusion assay method described by Akpotu et al. [27]. A stock concentration of 4 mg/ml of the fungal extract was prepared by dissolving the extract in dimethyl sulphoxide [(DMSO) 100% v/v]. The stock solution was further diluted in a 2-fold serial dilution process to obtain 2, 1, and 0.5 mg/ml. Using sterile cotton swabs, standardized broth cultures of test bacterial isolates - *S. aureus, B. subtilis, S. pneumoniae, P. aeruginosa, E. coli,* and *K. pneumoniae*, and fungal isolates - *Aspergillus niger* and *Candida albicans* were spread aseptically onto the surface of Mueller Hinton Agar [(MHA) Oxoid, UK] and Sabouraud Dextrose Agar [(SDA) Oxoid, UK] plates respectively. The culture plates were allowed to dry for about 5 min, and wells were made in the agar using a sterile 6 mm cork-borer. These wells were respectively filled with 20 µl of the different dilutions of the fungal extract and controls. Ciprofloxacin (5 µg/ml) and miconazole (50 µg/ml) were used as positive controls in the antibacterial and antifungal evaluations respectively; while DMSO (100% v/v) was used as the negative control. The culture plates were kept at room temperature for 1 h to allow the agents to diffuse into the agar medium. The MHA plates were then incubated at 37^{o}C for 24 h, and the SDA plates were incubated at 25-27^{o}C for 2-3 days. The plates were observed for inhibition zones diameters (IZDs) which were measured and recorded. The diameter of the well (6 mm) was deducted from the measured values to get the actual IZDs. For each test isolate, this procedure was conducted in triplicate and the mean IZDs calculated.

2.4. Cytotoxicity assay against ovarian cancer cell line A2780

The cytotoxic property of the fungal extract was determined using the MTT assay method described by Mueller et al. [28] and Engelke et al. [29]. Human ovarian cancer cells (A2780) were cultivated in RPMI-1640 medium supplemented with FBS (10%), streptomycin (120 µg/ml), and penicillin (120 U/ml), and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cisplatin (cDDP)-resistant subclone A2780CisR was obtained by intermittent treatment of A2780 cells with cDDP for 24 weekly cycles. In the MTT assay, cells were plated into 96-well microtiter plates (about 9,000 cells/well) containing growth medium, and pre-incubated overnight. The cells were then incubated with appropriate concentrations of test sample for 72 h, followed by the addition of 25 µl of a solution of MTT to each well. With the formation of formazan crystals after about 10 min, the medium was removed. The formazan crystals were then dissolved in 75 µl DMSO, and using the BMG FLUOstar (BMG Labtechnologies, Offenburg, Germany), absorption was measured at 544 nm (test wavelength) and 690 nm (reference wavelength). Absorption of the reference wavelength was subtracted from that of the test wavelength.

2.5. Antioxidant assay (DPPH free radical assay)

The free radical scavenging activity of the endophytic fungal extract was evaluated using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay method previously reported by Shen et al. [30], but with modification. Here, the percentage inhibition of the samples and positive control were determined at a concentration of 500 µg/ml from UV absorbance values recorded at 517 nm. A solution of 0.2 mM DPPH was prepared by adding 3.94 mg of DPPH (Sigma-Aldrich, Germany) in 50 ml of MeOH. A volume of 2 ml of the 0.2 mM DPPH solution was then added to 2 ml of the samples dissolved in MeOH (1 mg/ml, 1000 µg/ml). These final reaction mixtures resulted in a 2-fold dilution of the DPPH solution and samples, resulting in final concentration of 0.1 mM for the DPPH solution, and 500 µg/ml

for the samples. Quercetin was used as the positive control and 0.1 mM DPPH solution was used as blank. The mixtures were shaken vigorously and incubated at room temperature for 30 min, after which the absorbance (Abs) was measured at 517 nm using a UV-VIS spectrophotometer. The free radical scavenging activity of the samples was calculated using the following formula:

DPPH scavenging effect (% inhibition) = Abs of blank (A_0) – Abs of sample (A_1) X 100 / Abs of blank (A_0)

2.6. Bioassay of isolated compounds

The antimicrobial and antioxidant activities of the compounds isolated from the endophytic fungal extract were determined using the methods described above.

2.7. General procedures

^{1}H-NMR spectra were recorded using Bruker 300 and 600 spectrometers (Bruker BioSpin, Germany), and the spectra were referenced relative to the residual solvent signals. Mass spectra were recorded with a LC-MS HP1100 Agilent Finnigan LCQ Deca XP Thermoquest spectrometer (Thermo Electron, Germany). Analytical HPLC analysis was performed using a Dionex P580 system coupled to a P580A LPG pump and a photodiode array detector UVD340s (Dionex Softron, Germany). The separation column (125 x 4 mm) was prefilled with Eurosphere-10 C18 (Knauer, Germany), and the following gradient solvent system was used: 0 min (10% MeOH), 5 min (10% MeOH), 35 min (100% MeOH), and 45 min (100% MeOH). Semi-preparative HPLC was performed using a Merck/Hitachi HPLC System (UV detector L-7400; pump L-7100), with a Eurosphere column (100 C18, 300 × 8 mm, Knauer, Germany). Gradient MeOH-H_2O mixtures were used as mobile phase at a flow rate of 5.0 ml/min. Vacuum liquid and open column chromatography were applied for fractionation using Silica gel 60 (70-230 mesh, Merck, Germany) and Sephadex LH-20 (Sigma-Aldrich, Germany) respectively. TLC analysis on pre-coated silica gel plates (Kieselgel 60 F_{254}, 20×20 cm, 0.25 mm thick, Merck, Germany) was used to monitor and collect fractions under UV detection (Camag UV cabinet 4, Germany) at wave length of 254 and 366 nm. Distilled and spectral-grade solvents were used for column chromatography and spectroscopic measurements respectively.

3. RESULTS

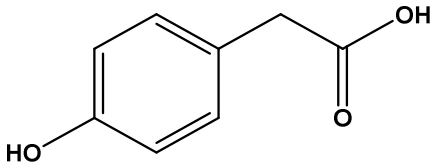
The results of the preliminary antimicrobial screening revealed that at a concentration range of 1-4 mg/ml, crude extract of *Aspergillus* sp. showed antibacterial activity against one Gram positive bacteria *B. subtilis* and one Gram negative bacteria *K. pneumoniae* with inhibition zone diameters (IZDs) ranging from 1-5 mm. At concentrations of 2 and 4 mg/ml, antifungal activity was recorded against *C. albicans* with IZDs of 3 and 5 mm respectively (Table 1).

Table 1. Result of antimicrobial assay of *Aspergillus* sp. crude extract.

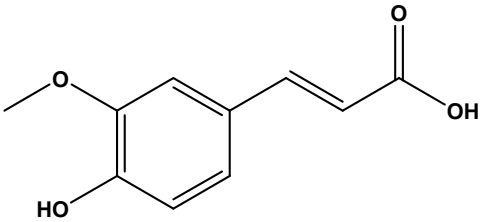
Test organisms	Mean Inhibition Zone Diameters (IZDs)(mm)					
	Concentration (mg/ml)				Positive control	Negative control
	4	2	1	0.5	Ciprofloxacin (5 µg/mL)	DMSO
S. aureus	0	0	0	0	6	0
B. subtilis	3	1	0	0	8	0
S. pneumoniae	0	0	0	0	10	0
P. aeruginosa	0	0	0	0	4	0
E. coli	0	0	0	0	24	0
K. pneumoniae	5	3	1	0	8	0
					Miconazole (50 µg/ml)	**DMSO**
C. albicans	5	3	0	0	16	0
A. niger	0	0	0	0	8	0

In the DPPH antioxidant assay, at a concentration of 500 µg/ml, the crude extract of *Aspergillus* sp. showed a good antioxidant activity with an inhibition of 72.1% (Table 2). The results of the cytotoxicity assay revealed that at a concentration of 100 µg/ml, crude extract of *Aspergillus* sp. showed an excellent cytotoxic activity against cisplatin-sensitive ovarian cancer cell line A2780 (sens) and cisplatin-resistant ovarian cancer cell line A2780 (cisR) with growth inhibitions of 105.1% and 105.5% respectively (Table 3). At 10 µg/ml, the extract exhibited poor cytotoxic activity against the cell lines with growth inhibitions of 8.69% and 3.04%, respectively.

The fungal crude extract was subjected to several chromatographic separations (vacuum liquid and open column chromatography) and semi-preparative HPLC for the isolation of the bioactive compounds, as well as spectroscopic analyses (LC-MS and NMR), for structural elucidation of the isolated compounds. Two phenolic compounds (Compounds 1 and 2) were isolated (Figure 1). All these steps were monitored by subjecting the crude, fractions and isolated pure compounds to HPLC analysis.



Compound 1 ($C_8H_8O_3$. 152 g/mol)



Compound 2 ($C_{10}H_{10}O_4$. 194.18 g/mol)

Figure 1. Phenolic compounds isolated from *Aspergillus* sp.: compounds **1** (*p*-hydroxyphenyl acetic acid) and **2** (ferulic acid).

Table 2. Antioxidant assay of *Aspergillus* sp. crude extract.

Fungal extract	Concentration (µg/ml)	% Inhibition
Aspergillus sp. crude extract	500	72.1
Quercetin (control)	500	91.7

Table 3. Cytotoxicity assay of *Aspergillus* sp. extract on ovarian cancer cell lines.

Ovarian cancer cell lines	Concentration (µg/ml)	Growth inhibition (%)
2780 sens	100	105.1±1.41
2780 CisR	100	105.5±2.06

The antimicrobial and antioxidant activities of the isolated compounds isolated from extract of *Aspergillus* sp. were also determined. Results of the bioassay carried out on the isolated compounds showed that *p*-hydroxyphenyl acetic acid exhibited a mild antioxidant activity at a concentration of 250 µg/ml with an inhibition of 35.4% (Table 5). At a concentration of 500 µg/ml, the compound showed no antibacterial or antifungal activities (Table 4). Ferulic acid exhibited an excellent antioxidant activity at a concentration of 250 µg/ml with an inhibition of 90.4% higher than that recorded for the positive control Quercetin (83.9%) (Table 5). At a concentration of 500 µg/ml, the compound showed mild antifungal activities against *A. niger* with an IZD of 2 mm. No antibacterial activity was recorded (Table 4).

3.1. Compound 1

The compound was isolated as an off-white crystalline solid. It exhibited U-maxima at λmax 222.4 and 276.3 nm, which is characteristic of phenol derivatives. The LC-MS showed peak at m/z 151.1 $[M-1]^-$ in the negative mode, which is consistent with molar mass of 152 g/mol. The ^{1}H-NMR spectrum (500MHz, MeOH-d_4) showed 4 proton signals of the AA'BB' coupling pattern at

δH 7.11 (d, 2H) and 6.75(d, 2H) assigned to H-2/6 and H-3/5 respectively. An aliphatic proton signal at δH 3.50 (s, 2H), which integrated to 2 protons were assigned to H-2'A/A of the acetic acid moiety. The compound was thus unequivocally identified as 2-(4-hydroxyphenyl) acetic acid, also known as *p*-hydroxyphenyl acetic acid or 4-hydroxyphenyl-acetic acid. Spectroscopic data of the isolated compound is confirmed by the reports of Ohtani et al. [31] and Abe et al. [32].

Table 4. Antimicrobial assay of isolated compounds.

	Mean Inhibition Zone Diameters (mm)			
Test organisms	**Compound 1 (*p*-HPA) (500 µg/ml)**	**Compound 2 (FA) (500 µg/ml)**	**Ciprofloxacin (5 µg/ml)**	**DMSO**
E. coli	0	0	5	0
S. aureus	0	0	8	0
S. typhi	0	0	7	0
B. subtilis	0	0	8	0
	0		**Miconazole (50 µg/ml)**	**DMSO**
A. niger	0	2	12	0
C. albicans	0	0	14	0

Table 5. Antioxidant assay of the isolated compounds.

Isolated compounds	**Concentration (µg/ml)**	**% Inhibition**
Compound 1 (*p*-HPA)	250	35.4
Compound 2 (FA)	250	90.4
Quercetin (control)	250	83.9

3.2. Compound 2

The compound was isolated as a light brown solid. It exhibited UV-maxima at λmax 217.0, 235.4 and 323.1 nm. The UV also showed a shoulder around 300 nm. This UV features are characteristic of cinnamic acid derivatives. The LC-MS showed peaks at m/z 195.0 $[M+1]^+$, 413.2 $[2M+23]^+$, and 177.1 $[M-18]^+$ in the positive mode and 193.2 $[M-1]^-$ in the negative mode. Analysis of these MS fragments indicated a molar mass of 194 g/mol. The ^{1}H-NMR spectrum (300 MHz, MeOH-d_4) showed signals of 3 aromatic proton of the ABX coupling pattern at δH 7.18 (d, J=1.9, 1H), 7.07 (dd, J=2.0, 8.2, 1H) and 6.81 (d, J=8.2, 1H) assigned to H-2, H-6 and H-5 respectively. The ^{1}H-NMR spectrum also showed two oleaginous proton signals at δH 7.60 (d, J=15.9, 1H) and 6.31 (d, J=15.9, 1H) assigned to H-2' and H-3' respectively. The high coupling constant (15.9 Hz) was an indication that the two protons are in trans-configuration. The NMR also showed a methoxy signal at δH 3.90 (s, 3H) assigned to MeO-3. The compound was thus elucidated as 4-hydroxy-3-methoxycinnamic acid (ferulic acid). Spectroscopic data of the isolated compound is confirmed by the reports of Sajjadi et al. [33] and El-gizawy and Hussein [34].

4. DISCUSSION

The genus *Aspergillus* (Moniliaceae), with over 180 species, is a diverse genus with high economic and social impact. Species occur worldwide in various habitats and they are known to spoil food, produce mycotoxins and are often reported as human and animal pathogens. The genus *Aspergillus* is one of the significant contributors to the secondary metabolites of fungal origin. They produce a broad range of structurally heterogeneous secondary metabolites and are known to be a rich source of alkaloids, terpenoids, xanthones, steroids, and polyketides, some of which showed antimicro-

bial, antifouling, antifeedant, phytotoxic, or other interesting bioactivities [35-37]. Even after investigations spanning over several decades, this genus nevertheless continues to yield metabolites with new structures and interesting biological activities [38].

As in our study, there are reports of the isolation of endophytic *Aspergillus* species from *M. oleifera* [20, 23, 39, 40]. Endophytic *Aspergillus* species have also been isolated from several other plants including *Cynodon dactylon* [38], *Gloriosa superba* [41], *Ipomoea batatas* [42], *Nymphoides peltata* [43], *Zingiber officinale* [44], *Carica papaya* [45], and *Loranthus micranthus* [46].

In our study, the crude EtOAc extract of *Aspergillus* sp. was tested for antimicrobial, cytotoxic and antioxidant activities. From the results of the bioassay, it was observed that *Aspergillus* sp. extract exhibited both antibacterial and antifungal activities (Table 1). The extract showed good antioxidant activity in the DPPH assay with an inhibition of 71.2% at a concentration of 500 µg/ml (Table 2). At a concentration of 100 µg/ml, the crude extract of *Aspergillus* sp. showed excellent cytotoxic activity against cisplatin-sensitive ovarian cancer cell line (2780 sens) and cisplatin-resistant ovarian cancer cell line (2780 CisR) with a growth inhibition of 105.1% and 105.5%, respectively (Table 3).

Chemical investigations of *Aspergillus* sp. crude extract yielded two phenolic compounds *p*-hydroxyphenyl acetic acid (*p*-HPA) and ferulic acid (FA). The compounds were characterized and screened for both antioxidant and antimicrobial activities. FA only showed mild antifungal activity against *A. niger* (at 500 µg/ml), but displayed an excellent antioxidant activity (at 250 µg/ml) with an inhibition of 90.4% higher than that recorded for the positive control quercetin (83.9%). *p*-HPA exhibited a mild antioxidant activity (at 250 µg/ml) with an inhibition of 35.4%. The compound showed no antimicrobial activity against any of the tested organisms (at 500 µg/ml) (Tables 4 and 5).

p-HPA is an important intermediate which is used for the synthesis of substances useful for pharmaceuticals. Many plants contain *p*-HPA and this compound is reported to be present in olive oil and beer [47, 48]. *p*-HPA has been isolated from several endophytic fungi including *C. gloeosporioides* [49] and *Oidiodendron* sp. [31]. The nematicidal, antimicrobial and plant growth promoting activities of *p*-HPA have been reported [31, 32, 49, 50].

FA is a ubiquitous natural phenolic phytochemical present in plant seeds and leaves, and was first isolated from the plant *Ferula foetida* [51]. The compound is an enormously copious and almost ubiquitous phytochemical phenolic derivative of cinnamic acid, present in plant cell wall components as covalent side chains [52]. Collectively with dihydroferulic acid, FA acid is the component of lignocelluloses, where it confers rigidity to the cell wall by making the crosslink between polysaccharides and lignin [51].

There are some reports on the isolation of FA from fungi. Cheng et al. [53] also reported expression of the compound by the endophytic fungi *Annulohypoxylon stygium*. A significant yield of FA and other phenolic compounds was reported to be achieved when rice bran, containing low levels of the compound, was fermented by the fungus *Rizhopus oryzae* [54]. FA has also been reported as a secondary metabolite of endophytic *Aspergillus* species. Danagoudar et al. [44] reported the presence of FA together with other phenolic compounds in culture extract of *A. austroafricanus* isolated from *Zingiber officinale* rhizomes.

FA has been reported to possess several pharmacological activities including antioxidant [51, 55, 56], anti-diabetic [51, 57, 58], antihypertensive [51, 59, 60], anticancer [33, 61, 62], anti-inflammatory [33, 63], hepatoprotective [34], and antimicrobial [51, 63] activities. Other reported biological activities of FA include increase of sperm viability, modulation of enzyme activity, activation of transcriptional factors, gene expression, signal transduction, metal chelation, anti-allergic, antithrombotic, antiviral, and vasodilatory activities [51].

The excellent antioxidant activity showed by FA in this study confirms the reports of several authors on the antioxidant activity of the compound [51, 55, 56]. This compound may be responsible for the antioxidant activity showed by the fungal crude extract (Table 1). Because of these properties and its low toxicity, FA is now widely used in the food and cosmetic industries. It is used as the raw material for the production of vanillin and preservatives, and as a cross-linking agent for the preparation of food gels and edible films. It has been approved in some

countries as food additive to prevent lipid peroxidation [33, 63, 64].

The results of this study, together with other similar findings [24, 27, 45, 46, 65-71], confirms the many potentials possessed by Nigerian plants as hosts of endophytes that could be reservoirs for excellent sources of pharmacologically active compounds.

5. CONCLUSION

The fungus *Aspergillus* sp. from *M. oleifera* produced two phenolic acids *p*-hydroxyphenyl acetic acid and ferulic acid whose biological activities are well known, are diverse, and are being explored for their pharmaceutical and industrial importance.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. COE, FBCO and PP designed and supervised the study. DOA, PME, CCA, and NTU managed the laboratory analyses. DOA and PME managed the data analysis and literature searches, and prepared the first draft of the manuscript. All authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

In vitro studies of iron absorption and activity of glutathione peroxidase in intestinal mucosa of the chicken

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ABSTRACT

We examined the absorption of iron, the activity of selenoprotein glutathione peroxidase (GSH·Px) and cellular compartmentalization of metal in the chicken duodenum and ileum. The method of accumulating mucosa preparation (AMP) was used. It was shown that the intestinal iron accumulation is dose-dependent process, which has two components: transcellular and paracellular. The realization of these pathways is region-specific and depends on exposed iron levels. Slightly elevated iron status of intestinal mucosa does not influence activity of GSH·Px. At the same time the results indicate that the activity of glutathione peroxidase can be altered by iron overload. Immunohistochemistry revealed that stainable iron could be co-localized to the endolysosomal compartment. How the activity of enzyme can be affected by oxidative stress and competitive interactions of iron with selenium are discussed.

Keywords: Glutathione peroxidase; Iron absorption; Intestinal mucosa; Chicken.

1. INTRODUCTION

Iron serves numerous functions in the body relating to the metabolism of oxygen. Ferrous iron can react with oxygen to form superoxide and also can homolytically cleave hydrogen peroxide yielding hydroxyl radicals and hydroxyl ions. These ions are particularly aggressive and elicit toxic effects, which are mainly related to oxidative stress [1]. Moreover, iron is deeply linked to cell death pathways through reactive oxygen species (ROS) production [2]. Therefore most of free iron is safely stored in a non-redox-active form in ferritins. Iron overload is strongly associated with the intensification of free radical oxidation [3]. Glutathione is a main detoxifier of ROS in the intestine. Glutathione peroxidase provides detoxification of peroxides by using reduced glutathione, and is one of the most important antioxidant enzymes [4]. GSH·Px is a selenoprotein, and selenium availability regulates glutathione peroxidase enzyme activity [5]. An excess of certain minerals in the body can antagonize other minerals and cause depletion [6].

Since animals lack mechanisms for iron elimination, iron uptake is strictly regulated. The non-heme iron is ultimately taken up from the gut lumen by divalent metal transporter 1 (DMT1) situated on the microvillus membrane, before joining the labile iron pool in the cytoplasm and transferred to the bloodstream by ferroportin 1 [7]. The mechanism and regulation of intestinal iron absorption are incompletely understood in spite of their pivotal role in the maintenance of body iron homeostasis [8].

Iron metabolism involves iron trafficking along specific cellular compartments, including endosomes and lysosomes [9]. These organelles take center stage in cellular iron accumulation and are involved as a control hub for aging and longevity [10]. The study described here was undertaken to investigate the influence of exposure to iron in concentrations occurring in contaminated food and feed on accumulation and compartmentalization of iron in enterocytes and the activity of GSH·Px in the intestinal mucosa.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

New-hatched Lohmann brown cockerels were obtained from the Latvian poultry company BALTICOVO. All of the experimental procedures were approved by the Animal Ethics Committee of the Food and Veterinary Service (Riga, Latvia, authorisation reference number 13, from December 22, 2008). The chickens were housed in cage units with free access to food and water. Animals received standard full-feed diet. For the *in vitro* study 30 days old chickens were divided into 3 groups (5 in each group): 1 - "Buffer", 2 - "+ Fe 0.512 mM as iron sulfate", 3 - "+ Fe 2.56 mM as iron sulfate". Chickens were sacrificed by decapitation, in accordance with recommendations for the euthanasia of experimental animals of the European Convention [11].

2.2. Determination of iron absorption and GSH-Px activity

The content of iron in chick intestinal mucosa was estimated by atomic absorption spectrophotometry [12], the activity of glutathione peroxydase (GSH·Px) - by a modified Pinto-Bartley method [13].

The intestine was isolated and washed with 10 ml of cooled physiological solution (154 mM NaCl). Then it was placed on ice-cold glass plate. Duodenum and ileum were cut on segments (5 cm) and used for intestinal preparations. Iron binding by the intestinal wall was studied by means of AMP method as developed by Ugolev et al. [14] for investigation of the first stages of transport processes. An everted intestinal segment of birds belonging to groups 1, 2 and 3, mounted on a glass rod, was submerged in 7 ml Tris-buffer containing different concentration of iron (0.512 mM and 2.56 mM). An everted intestinal segment of birds belonging to groups 1, 2 and 3, mounted on a glass rod, was submerged in 7 ml Tris-buffer containing different concentration of iron (0.512 mM and 2.56 mM).

Intestinal AMP were incubated for 30 min at 41ºC. Tris-buffer without iron supplement was used as a control. Buffer composition (mM) was: 4 Tris hydrochloride, 145 sodium chloride, 4 potassium chloride, 20 fructose, pH 7.4. The amount of accumulated iron was calculated as the difference between the iron contents in the mucosa before and after incubation.

2.3. Histological examination

For histological examination, 1-cm segments of intestinal samples from animals of the 2-nd and 3-rd group (duodenum was taken 0.5 cm distal to the ampulla of Vater and ileum - 10 cm proximal to the ileocecal junction) were isolated and fixed in 10% neutral buffered formalin. Paraffin-embedded tissue was cut into 4-µm-thick sections and stained with haematoxylin-eosin and the periodic acid-Schiff (PAS) reagent. Duodenal sections were colored with Perls' Prussian blue stain for iron detection. Late endosomes and lysosomes in the enterocytes were highlighted by immunohistochemistry using an anti-CD68 and anti-TRPV1 antibodies.

2.4. Statistical analysis

All statistics were performed using the program SPSS. Means and standard deviations and significance values were calculated. The results were assessed statistically by *t* tests. Statistical significance was set at $p<0.05$.

3. RESULTS AND DISCUSSION

Iron exercised a diversified action: after 30 min incubation in a medium containing 0.512 mM of iron its concentration in the duodenal mucosa amounted to 9.72 ppm, and the iron accumulation was increased by 57.3% (Table 1). At the same time

in the ileal mucosa exposed to lower level of iron only nearly 10% of the metal was accumulated. The obtained data indicated 5.8-fold ability of the duodenum, compared with the ileum, to transfer iron into the mucosa. After applying of 5-times higher iron concentration in the incubation medium the tissue level of this metal increased more than two times and the metal accumulation in the duodenal mucosa was increased by 133.8% vs. 330.7% in the ileal mucosa. Dramatic effects of higher iron exposure on accumulation of this metal in the ileal mucosa with levels more than 30-fold higher than observed for lower levels of iron exposure may be related to greater (paracellular) leakiness of the epithelial barrier in the ileum. It is known, that transcellular active transport of iron across the gut epithelium occurs mainly in the duodenum and jejunum [15]. We conclude that similar to calcium absorption, passive, paracellular absorption of iron predominates in the ileum when dietary iron levels are high [16].

Table 1. Iron accumulation in intestinal mucosa of chickens.

Experimental conditions	Concentration of Fe in intestinal mucosa, ppm		Accumulation of Fe in intestinal mucosa, ppm	
	Duodenum	Ileum	Duodenum	Ileum
1. Buffer	6.18 ± 0.88	3.02 ± 0.30	-	-
2. +Fe (0.512 mM)	9.72 ± 0.65[a]	3.36 ± 0.29	3.54 (+57.3%)	0.32 (+9.9%)
3. + Fe (2.56 mM)	14.40 ± 1.50[a,b]	14.00 ± 1.52[a,b]	8.22 (+133.8%)	10.98 (+330.7%)

[a]Statistically different from the 1st group (P<0,05); [b]Statistically different from the 2nd group (P<0,05)

As revealed by our studies, the activity of GSH·Px in the 2nd group either remains unaffected (in the duodenal mucosa), or decreases insignificantly (in the ileal mucosa), but in the 3rd group both in the duodenum and ileum a statistically significant decreasing trend in GSH·Px activity was observed with increasing iron accumulation in intestinal mucosa (Table 2).

Table 2. Activity of GSH·Px in intestinal mucosa of chickens

Experimental conditions	Activity of GSH·Px µmol GSH/min/g	
	Duodenum	Ileum
1. Buffer	2.37 ± 0.24	1.62 ± 0.25
2. +Fe (0.512 mM)	2.36 ± 0.80	1.46 ± 0.63
3. + Fe (2.56 mM)	1.44 ± 0.38 [a,b].	0.93 ± 0.31 [a]

[a] Statistically different from the 1st group (P<0.05)
[b]Statistically different from the 2nd group (P<0.05)

Stainable iron was found in the small intestinal enterocytes of the chickens in the 3rd group. As shown in Fig. 1-A, iron deposits appeared as a narrow string of punctae in the subapical area all along the brush border. Little or no diffuse staining of the enterocyte cytosol was detected. TRPV1 immunoreactivity was localized in the subapical compartment of the villous enterocytes, having a punctuate appearance (Fig. 1-B). The pattern of CD68 immunoreactivity was quite similar to selective cytoplasmic expression of TRPV1 (Fig. 1-C). It should be emphasized, that both CD68 and TRPV1-positive material and iron deposits within enterocytes were consistently localized to the same area in the vicinity of the brush border. It is well known that the endosomal-localized DMT1 is responsible for mobilizing iron out of endosomes [17]. It was shown that members of the transient receptor potential (TRP) superfamily could function as intracellular cation release channels whose localization is commonly assigned to late endosomes and lysosomes [18]. The obtained results also indicated that TRPV1 is localized to the late endosomes and lysosomes, where TRPV1 may function to transfer the endosomal free Fe^{2+} into the cytoplasm in the transferrin cycle in parallel to DMT1.

Our data showed that chickens of the 3rd group had lower GSH·Px activity in the intestinal mucosa than did animals in the 1st and 2nd group. It is likely that in the 3rd group iron reaches damaging levels, exceeding the homeostatic capacity of the

enterocytes. Decreased GSH·Px activity has been reported in tissues where oxidative stress occurs in several pathological animal models [19]. It is known, that an excess of iron in tissues can induce hydroxyl radical formation. This effect was likely promoted by the recycling of chelated, inactive Fe^{3+} to the active Fe^{2+} state by the Fenton reaction in the mitochondria [20]. Fe^{2+} is extremely toxic because it can rapidly react with hydrogen peroxide and molecular oxygen to produce reactive oxygen species. Proteins are oxidatively damaged by the combined action of free radicals and the trace metal ions such as Fe^{2+} and Cu^{2+} [21]. In our experiments oxidative damage to GSH·Px may also affect its activity. The seeming paradoxical dissociation between considerable iron accumulation in the ileal mucosa in the 3rd group and only moderate down-regulation of GSH·Px activity comparable to that in the duodenal mucosa can be explained by the preferential use of paracellular route of iron transport under these circumstances.

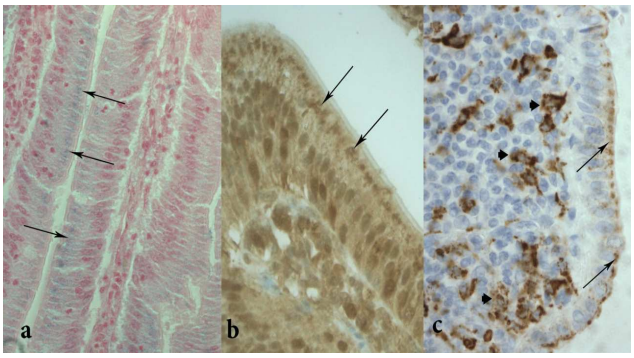

Figure 1. A - Iron histochemistry with Perls' staining of chicken intestinal mucosa from a third group. Iron deposits in the subapical compartment of villous enterocytes, x40. B - TRPV1-positive punctae in the subapical compartment of villous enterocytes (arrows) x40. C - CD68 expression in the enterocytes with the subapical pattern (arrows). Likewise, high levels of CD68 expression are associated with macrophages (arrowheads), x40.

According to the reports, supplementary iron reduces selenium bioavailability [22]. Therefore, reduction of the activity of selenoprotein GSH·Px during the iron overload may be related at least in part to the competitive iron interactions with selenium, thus reducing its bioavailability.

Understanding of the ways and control of transition metal uptake and translocation is very important particularly because some of them can be highly toxic when accumulate in the cells. Our animal model of iron overload has demonstrated the accumulation of selective iron subapical deposits colocalized with endolysosomal markers. Labile iron can readily generate ROS, and sequestration in the endolysosomal apical system may represent one of many protective mechanisms that exist within the absorptive epithelial cell. These data seems to support the theory that at least half of the iron transported across the villous enterocytes uses a vesicular pathway and that a significant portion of the vesicular pathway involves the endolysosomal system, which is located en route towards the basolateral membrane [23, 24].

Low iron levels may have a link with cognitive health later on in life. For example, the patients with anemia had a higher risk of developing dementia compared with those who were not anemic [25]. Eating foods high in iron can help prevent dementia. On the other hand, our results showed, that the iron supplementation can cause side effects and, consequently, compromise the life expectancy mainly for elderly populations, because age-related iron overload is a known contributor to multiple degenerative diseases, including cancer, liver fibrosis and heart attack [26-31].

4. CONCLUSION

In conclusion, after iron treatment (0.512 mM in the incubation medium) GSH-Px activity remains unchanged despite the accumulation of metal in the intestinal mucosa. However pathological accumulation of the iron within the intestinal mucosa (2.56 mM in the incubation medium) elicits toxic effects, reducing the activity of GSH-Px, which are mainly related to oxidative stress. The endolysosomal compartment plays an important role in cellular iron homeostasis in the iron-overloaded state.

AUTHORS' CONTRIBUTIONS

JM: Study design and interpretation of the protocol and guidance; acquisition of the data; obtained funding; drafting of the manuscript; critical revision of the manuscript for important intellectual content;

AG and NB: Study concept and design; analysis and interpretation of the data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical expertise; study supervision. GK, SV, GS: analysis and interpretation of the data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; administrative, technical and material support. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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