African Journal of
Microbiology Research

Academic
Journals

ABOUT AJMR

The African Journal of Microbiology Research (AJMR) is published weekly (one volume per year) by Academic Journals.

The African Journal of Microbiology Research (AJMR) provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Contact Us

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJMR

Submit manuscript online   http://ms.academicjournals.me/

African Journal of Microbiology Research

Table of Contents: Volume 12   Number 1   7 January, 2018

ARTICLES

African Journal of Microbiology Research

Full Length Research Paper

Analysis of nitrifying microbial community for organic hydroponics

Edgar Mowa[1]*, Levi Akundabweni[1], Percy Chimwamurombe[2] and Gaddafi Liswaniso[3]

[1]Department of Crop Science, University of Namibia, Private Bag 13301, Windhoek. Namibia.
[2]Department of Natural and Applied Sciences, Namibia University of Science and Technology, Private Bag 13388, Windhoek, Namibia.
[3]Sam Nujoma Marine and Coastal Resources Research Centre (SANUMARC), University of Namibia, P. O. Box 462 Henties Bay, Namibia.

For many years, the generation of nitrates from organic sources in order to create nutrient solutions for hydroponics had proved a challenge until lately when microorganisms were introduced to perform this task. The objectives of the current study therefore were to use local microbial consortium to nitrify goat manure in water and to determine microbial diversity in the inoculated consortium. Therefore, microorganisms were sourced from garden soil and natural compost at the Sam Nujoma Marine resources Research Centre (SANUMARC) in Henties Bay Namibia to convert organic nitrogen in goat manure from Utuseb farm near Walvis Bay into nitrates. Results show that microbial consortium from the compost source produced significantly (P< 0.05) more nitrates followed by the garden-soil source, suggesting that it is necessary to add inoculum in order to generate nitrate from goat manure. The ammonia oxidising bacteria (AOB) community from the compost sample's was dominated by uncultured ammonia-oxidising species followed by uncultured bacterium (both not identified), with the least being *Nitrosomonas* species. The AOB community from the garden source was dominated by uncultured bacterium, followed by uncultured ammonia-oxidising species and the least being *Nitrosomonas* species. NOB community from the compost source was dominated by uncultured bacterium, followed by *Nitrobacter winogradskyi* and *Nitobacter vulgaris* with the least being *Nitrococcus mobilis* and *Nitrospira moscovensis.* For the garden soil microbial source, uncultured nitrite-oxidising bacteria dominated followed by uncultured bacterium, whereas the least species were *N. moscovensis* and *Nitrobacter alkalicus.* Moreover, community composition of the compost sample was more diverse than the community from the garden sample. These results maintain that there are other unculturable yet important microbes doing the same job if not better than the known ones, in this case suggesting that there may be other local nitrite-oxidizing bacteria responsible for oxidizing ammonia other than the traditionally known *Nitrobacter, Nitrospira* and *Nitrococcus* species.

Key words: Organic hydroponics, ammonification, nitrification, ammonia-oxidizing bacteria, nitrite-oxidizing bacteria.

INTRODUCTION

For many years, the generation of nitrates from organic sources in order to create nutrient solutions for hydroponics had proved a challenge. It was not until Shinohara et al. (2011) used nitrifying microorganisms to degrade organic nitrogen into nitrates, which is directly used by plants, that organic hydroponics have been made possible with follow-up studies such as that of Hu and Qi (2013) confirming the successful use of microbes in nitrification of other organic sources.

Nitrification consists of two consecutive oxidation steps:

nitritation and nitratation done by two main groups of bacteria (ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB)). Most AOBs belong to the beta-subclass of *Proteobacteria* and four genera belonging to this lineage are *Nitrosomonas* (with *Nitrosococcus mobilis*), *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* (Woese et al., 1984; Pommering-Röser et al., 1996). NOBs on the other hand consist of the *Proteobacteria* genera *Nitrobacter*, *Nitrococcus* and *Nitrospina.* The genus *Nitrobacter* has four species *Nitrobacter winogradskyi, Nitrobacter hamburgensis*, *Nitrobacter vulgaris* and *Nitrobacter alkalicus* (Daims, 2001). The genera, *Nitrococcus* contains *Nitrococcus mobilis*, whereas the genera *Nitrospina* contain *Nitrospina gracilis.* The genus *Nitrospira* consists of *N. marina* from ocean water and *N. moscoviensis* (Daims, 2001)*.*

These microorganisms can be cultured and stored for use in water and soil media to degrade organic nitrogen (Willuweit et al., 2008). All they require is an ecosystem with favourable conditions for them to function in their specific diversity (Saijai et al., 2016). But culturing has a major disadvantage in that many nitrifying bacteria are not cultivable. It is estimated that in seawater samples at best, only 0.1% (Kogure et al., 1980), in freshwater only 0.25% (Jones, 1977), in soil samples 0.5% (Torsvik et al., 1990), and in activated sludge 15% (Kämpfer et al., 1996) of the indigenous bacteria could be cultivated. Traditionally, *Nitrosomonas* and *Nitrobacter* were thought to be the only microbes responsible for nitrification in wastewater treatment plants based on the experience that *Nitrosomonas* and *Nitrobacter* species can be isolated from every activated sludge. It was only when *Nitrobacter* was not detected in an aquarium, yet there was nitrification, that it was considered that there are other unculturable yet important microbes doing the same job. Therefore, microbial ecology needs cultivation-independent tools to quantify bacteria directly in environmental samples (Daims, 2001).

It has been established that under 40% of soil water filled pore space decreases abundance of AOB than over 40% (Barrena et al., 2017). This abundance is influenced by pH of soil, with acidic soils (4.0/5.4) negatively affecting abundance of AOBs, whereas more AOBs positively correlate with nitrification (Duncan et al., 2017). Furthermore, AOA may affect N cycling more in soils receiving animal manures, whereas AOB are functionally more important in chemically fertilized soils (Zhou et al., 2015). Moreover, abundances of archaeal 16S rRNA and amoA genes have been found to be positively correlated with soil nitrate, N and C contents (Rughöft et al., 2016), whereby changes to physical properties of soil determine nitrifying and ammonifying capacity and mineral nitrogen content.

Maximum content of nitrate and mineral nitrogen and the biggest nitrifying capacity are at soil temperatures of 15°C, whereas temperature at -4°C have recorded the lowest abundances of NOBs (Wertz et al., 2013). Moistening conditions optimal for developing nitrifying and ammonifying bacteria are formed at soil moisture of 20-25% (60-75% WFC) (Evdokimova et al., 2016).

Functional genes have been found to be good molecular markers for studying the diversity within functional groups (Poly et al., 2008; Calvo et al., 2005). For nitrification, the *amo*A gene encoding ammonia mono-oxygenase is being used for microbial diversity and phylogenetic characteristics of soil and water AOB communities (Rotthauwe et al., 1997; Purkhold et al., 2000).

In Nitrobacter, the oxidation of nitrite to nitrate is performed by the nitrite oxidoreductase (NXR), encoded by the nxr operon (Starkenburg et al., 2006) previously called nor (Kirstein and Bock, 1993). Due to ability to reveal the previously hidden diversity of microscopic life, metagenomics offers a powerful lens for viewing the microbial world that has the potential to revolutionize understanding of the entire living world (Marco, 2011). The need to incorporate metagenomics has been driven by the inability to culture the majority of microbes from an ecosystem and the logistics of using a myriad of media and culturing conditions to capture those that can be grown *in vitro* (Marchesi, 2012).

The advent of next generation sequencing (NGS) technologies allow us to sequence DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing due to the inexpensive production of large volumes of sequence data (Metzker, 2010). The results can thereafter be put through binning where methods such as Basic Local Alignment Search Tool (BLAST) are used to search for phylogenetic markers or similar sequences in existing public databases (Huson et al., 2007).

The objectives of the current study therefore were to use local microbial consortium to nitrify goat manure in water and to determine microbial diversity in the inoculated consortium.

MATERIALS AND METHODS

Study site

The current study was conducted at the Sam Nujoma Marine Resources Research Centre (SANUMARC), Henties Bay, Namibia (Figure 1).

Manure collection

Dry manure was sampled according to Lupwayi et al. (2000),

*Corresponding author. E-mail: zamowa@yahoo.com.

Figure 1. Map showing the study area (Arroukatchee, 2017).

Table 1. Treatments for investigating optimal source of microorganisms mineralising organic nitrogen.

Treatment 1	Treatment 2	Control
Compost added at 5 g/L	Garden soil added at 5 g/L	No inoculum added

whereby samples were collected from Utuseb farm in the Erongo region, where the manure was stored for at least six months. The manure was homogenized, air-dried for 1 week at 30°C, ground and sieved (< 2 mm) before use with microorganisms (Qian and Schoenau, 2001). Goat manure was chosen due to its availability at coastal areas in Namibia as compared to other forms of manure.

Source of microorganisms

The following sources were used: Sam Nujoma Marine Resources Research Centre (SANUMARC) garden soil and SANUMARC compost. SANUMARC garden soil was soil extracted from garden plots in the shaded-net garden of SANUMARC at Henties Bay. SANUMARC compost was soil collected from a compost heap at SANUMARC where grass clippings and plant leaves have been heaped for longer than 3 months. In order to determine the best source of microorganisms to mineralize organic N, samples from the above sources were investigated as treatments (Table 1).

Each treatment was added to separate one-litre flasks of distilled

water containing 1 g/L of goat manure. Each treatment was triplicated. The flasks were shaken (120 strokes/min) for 20 days at 25°C, and the NO_3^- concentrations were then determined using the Hach DR5000 spectrophotometer which uses cadmium metals to reduce nitrates in the sample to nitrite. The nitrite ion reacts in an acidic medium with sulfunic acid to form an intermediate diazonium salt. The salt couples with gentisic acid to form an amber colored solution and the results are then measured at 500 nm.

Identification of microbes

In order to understand microbial composition of the 2 inocula from the source of microorganisms experiment, identification was done. Therefore, DNA was extracted from both the garden soil and the compost from SANUMARC. The DNA extraction was performed according to the manufacturer's recommendations, based on direct cell lysis with subsequent recovery and purification of nucleic acids. Up to 250 mg of soil was added to the lysis tubes, and the samples were homogenized using a standard benchtop vortex for 20 min.
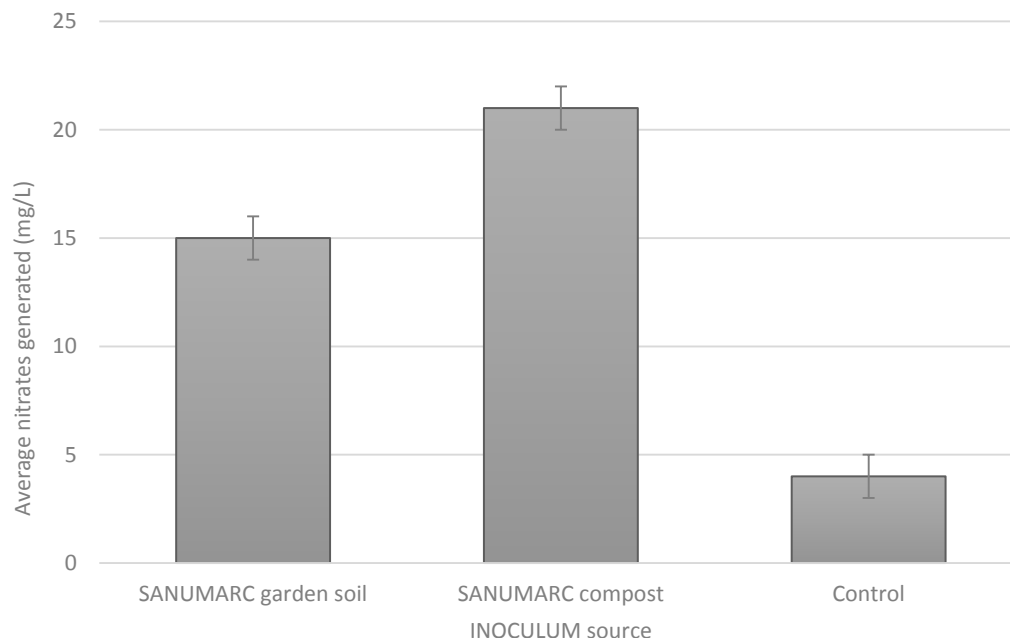
Figure 2. Mineralization of organic nitrogen into nitrate by the addition of microbial inocula from various sources. The suitability of two sources of inoculum of microorganisms capable of mineralizing organic fertilizer into nitrate was examined.

The DNA was then amplified using functional genes targeting primers (*Nor*A and *Amo*A) using standard PCR procedure at SANUMARC.

Genomic DNA was prepared and used as a template. 305 bp *Nxr*A genes and 490 bp *Amo*A genes were amplified using *Nor*A and *Amo*A genes. The sequence of the *amo*A primers was: amo r5-′ GGGGTTTCTACTGGTGGT-3′, amoA-r 5′-CCCCTCKGGAAAGCCTTCTTC-3′ (Rotthauwe et al., 1997; Purkhold et al., 2000). The sequence for the NXR primers was: norA-f 5′ CAGACCGACGTGTGCGAAAG3′, norA-r 5′-TCYACAAGGAACGGAAGGTC-3′ (Poly et al., 2008). The PCR mixture included 0.5 µM of each primer, 23.5 µl of nuclease free water, 25 µl master mix and 1 µl of DNA template, making up a total of 50 µl. The negative control contained nuclease free water in place of DNA template. Using the thermos-cycler, the PCR reaction was carried out as follows: NXRA- 1 cycle of pre-denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 45 s, final extension at 72°C for 5 min. AmoA- 1 cycle of pre-denaturation at 94°C for 5 min, annealing at 60°C for 1 min 30 s, extension at 72°C for 1 min 30 s, 42 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min 30 s, final extension at 72°C for 10 min.

Amplified PCR products were visualised on 1% agarose gel with a 1kb ladder (Inqaba Biotechnology Industries, SA). Further sequencing of the PCR products was done at Inqaba Biotechnology in South Africa and the reads were merged, clustered and then a BLAST search was done against Genbank in order to identify nitrifying species from the sequence data.

Statistical analysis

Means comparisons and correlations analysis were done whereby, a one-way analysis of variance (ANOVA), followed by mean separation by Duncan's multiple range test was used when ANOVA determined that the effects of the treatments were significant (p < 0.05 for F-test).

$Y_{ij} = \mu + \tau_i + \beta_j + \gamma_{ij} + \epsilon_{ij}$

Where, μ is the overall mean response, τ_i is the effect due to the i-th level of factor A, β_j is the effect due to the j-th level of factor B and γ_{ij} is the effect due to any interaction between the i-th level of A and the j-th level of B, ϵ_{ij} is the error term where the error terms are independent observations. The Simpson's diversity index was used to calculate for microbial species diversity (Atlas and Bartha, 1998):

$S_i = 1-D (\sum n(n-1)/N(N-1)$

Where, n is the total number of organisms of a particular species, and N is the total number of individuals of all species.

Simpson's diversity index is a measure of diversity in ecology where it is used to quantify the biodiversity of a habitat taking into account the number of species present, as well as the abundance of each species.

RESULTS AND DISCUSSION

Garden soil and compost soil were chosen as inoculum sources for the microorganisms needed for mineralization and nitrification of organic nitrogen into nitrate in water in the presence of goat manure as a nitrogen source. The pH of the garden soil was 7.8, whereas for compost soil, pH was 8.6 with low holding water capacity. The soil temperature ranged between 14.8 and 25°C, whilst soil moisture was 73% water field capacity (WFC) for garden soil and 65% WFC for compost soil.

The addition of only goat manure without inoculum in water resulted in the generation of nitrate (Figure 2). In contrast, using inoculum such as nursery or compost resulted in more mineralization of organic nitrogen into

A

1%
21%
14%
0.4%
64%

- uncultured ammonia-oxidising
- *nitrosomonas sp.*
- *nitrosospira sp.*
- uncultured bacterium
- uncultured beta

B

3%
11%
34%
0.1%
1%
0.3%
51%

- uncultured ammonia-oxidising
- *nitrosomonas sp.*
- uncultured nitrosomonadales
- *nitrosospira sp.*
- uncultured bacterium
- uncultured beta

Figure 3. Species composition of ammonia-oxidising bacteria (AOB) from the compost source (A) and the garden soil source (B).

Table 2. AOB species composition from the 2 sources of inoculum and species diversity.

Species	Inoculum source	
	Garden	Compost
Uncultured ammonia-oxidising	529	2469
Nitrosomonas sp.	1	14
uncultured *Nitrosomonadales*	12	
Nitrosospira sp.	4	537
Uncultured bacterium	807	823
Uncultured beta	181	34
Uncultured *Nitrosospira*	47	
SUM	1581	3877
Simpson diversity index	0.61	0.53

of bacterial amoA gene was dominated by uncultured ammonia-oxidising species followed by uncultured bacterium (both not identified), with the least being *Nitrosomonas* species. The AOB community from the garden source was however dominated by uncultured bacterium, followed by uncultured ammonia-oxidising species and the least being *Nitrosomonas* species. Moreover, the Simpson's Diversity index of diversity showed that microbial community from the garden soil was more diverse than the microbial community from the compost sample (Table 2). This is because community composition between the two samples differed in that more than two-thirds was dominated by a single species in the compost sample as compared to only half of the dominance given to a single species from the garden soil sample. These results concur with that of Daims (2001) who maintained that there are other unculturable yet important microbes doing the same job if not better than the known ones, in this case, suggesting that there may be other local ammonia-oxidising bacteria responsible for oxidizing ammonia other than the traditionally known *Nitrosomonas* and *Nitrosospira* species. This is considering that nitrifying bacteria picked up by the *Amo*A gene encoding the active site polypeptide of the ammonia monooxygenase (*Amo*A) in our samples are dominated by species unidentified.

Nitrite oxidizing bacteria (NOB)

Figure 4 shows that NOB community from the compost source was dominated by uncultured bacterium, followed by *N. winogradskyi* and *N. vulgaris* with the least being *N. mobilis* and *N. moscovensis.* For the garden soil microbial source, uncultured nitrite-oxidising bacteria dominated followed by uncultured bacterium, whereas the least species were *N. moscovensis* and *N. alkalicus.* Moreover, community composition of the compost sample was more diverse than the community from the garden sample (Table 3). Though community composition

nitrate was made from the addition of microbial consortiums (Figure 2). The microbial consortium from the compost source however produced significantly (P<0.05) more nitrates than the garden-soil source and the control where no inoculum was added (Figure 2). Garden soil inoculum also produced more (P<0.05). These results indicate that microorganisms added to the water from the garden soil and from compost were able to mineralize and nitrify organic nitrogen into nitrate. These results therefore suggest that it is necessary to add inoculum in order to generate nitrate from goat manure, just as it is for other organic sources such as fish-based fertiliser established by Shinohara (2011). The results are indicative of the fact that microorganisms in the inoculum can degrade organic nitrogen to nitrates but leaves the question of what is the composition of local nitrifiers within the inoculum capable of ammonification and nitrifying
Therefore for the next step, all nitrifiers were identified.

Ammonia oxidizing bacteria

Figure 3 shows that from the compost sample, abundance

Figure 4. Species composition of nitrite-oxidizing bacteria (NOB) from the compost source (A) and the garden soil source (B).

Table 3. NOB species composition from the 2 sources of inoculum and species diversity.

Species	Inoculum source	
	Compost	Garden
Nitrobacter winogradskyi	3150	1021
Nitrobacter hamburgensis	315	
Nitrobacter vulgaris	2281	1626
Uncultured nitrite-oxidising	2598	7687
Uncultured nitrobacter	346	
Nitrobacter alkalicus	200	208
Nitrospira moscovensis	4	2
Nitrococcus mobilis	1	
Uncultured bacterium	5450	3499
Unidentified bacterium	91	179
SUM	14436	14222
Simpson's diversity index	0.75	0.63

follows similar trend as with AOB found in this study in that the dominant nitrifying species in the samples were unidentified species, there are significant proportions of *Nitrobacter* species (Figure 4). This result upholds the findings by Saijai et al. (2016), Poly et al. (2008), Wertz et al. (2012) and Winslow (1917) who established that *Nitrobacter* species were responsible for converting nitrite into nitrate. These results further maintain that of Daims (2001) who held that there are other unculturable yet important microbes doing the same job if not better than the known ones, in this case, suggesting that there may be other local nitrite-oxidizing bacteria responsible for oxidizing ammonia other than the traditionally known *Nitrobacter, Nitrospira* and *Nitrococcus* species.

Conclusion

Garden soil and compost are appropriate sources of microorganisms to generate nitrate from goat manure in water. There are other unknown local microorganisms oxidising ammonia and nitrite and the presence of these nitrifying bacteria in addition to the presence of the known ammonia-oxidizing species in the garden soil and compost samples infers their roles in the observed nitrification. Therefore, nitrifying microbial consortia that would degrade goat manure for use in organic hydroponics can be sourced from any place with similar environment (temperature, pH and soil) to Henties Bay. Further research can consider determining ammonium levels as well from nitrification of goat manure and establish the optimal rate of nitrification by a certain amount of soil containing nitrifying microbial consortia.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Arroukatchee (2017). Map of Henties Bay. http://www.southern-africa.arroukatchee.fr/namibia/towns/henties-bay.htm

Atlas R, Bartha R (1998). Microbial ecology: Fundamentals & Applications. 4th Edition. Benjamin Cummings Pubs. University of Califonia.

Barrena I, Menéndez S, Correa-Galeote D, Vega-Mas I, Bedmar EJ, González-Murua, C, Estavillo JM (2017). Soil water content modulates the effect of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on nitrifying and denitrifying bacteria. Geoderma 303:1-8.

Calvo L, Cortey M, Garcia-Marin JL, Garcia-Gil LJ (2005). Polygenic analysis of ammonia-oxidizing bacteria using 16S rDNA, amoA, and amoB genes. Intl. Microbiol. 8:103-110.

Daims H (2001). Population Structure and Functional Analyses, by In Situ Techniques, of Nitrifying Bacteria in Wastewater Treatment Plants (Doctoral dissertation, Dissertation Technische Universität München).

Duncan EG, O'Sullivan CA, Simonsen AK, Roper MM, Peoples MB, Treble K, Whisson K (2017). The nitrification inhibitor 3,4,-dimethylpyrazole phosphate strongly inhibits nitrification in coarse-grained soils containing a low abundance of nitrifying microbiota. Soil Res. 55(1):28-37.

Evdokimova MA, Novoselov SI, Novoselova ES, Mantsurovna A, Kuzminykh AN, Pashkova GI, Marina-Chermnykh OG (2016). The influence of physical properties of sod-podzol soil on its ammonifying and nitrifying capacity. Biol. Med. 8(7):351.

Huson DH, Auch AF, Qi J, Schuster SC (2007). MEGAN analysis of metagenomic data. Genome Res. 17(3):377-386.

Jones JG (1977). The effect of environmental factors on estimated viable and total populations of planktonic bacteria in lakes and experimental inclosures. Freshw. Biol. 7:67-91.

Kämpfer P, Erhart R, Beimfohr C, Böhringer J, Wagner M, Amann R (1996). Characterization of bacterial communities from activated sludge: culture-dependent numerical identification versus in situ identification using group- and genus-specific rRNA-targeted oligonucleotide probes. Microb. Ecol. 32:101-121.

Kirstein K, Bock E (1993). Close genetic relationship between Nitrobacter hamburgensis nitrite oxidoreductase and Escherichia coli nitrate reductases. Archiv. Microbiol. 160(6):447-453.

Kogure K, Simidu U, Taga N (1980). Distribution of viable marine bacteria in neritic seawater around Japan. Can. J. Microbiol. 26:318-323.

Marchesi JR (2012). Metagenomics: current innovations and future trends. Future Microbiol. 7(7):813-814.

Marco D (2011). Metagenomics: current innovations and future trends. Horizon Scientific Press.

Metzker ML (2010). Sequencing technologies-the next generation. Nat. Rev. Genet. 11(1):31-46.

Poly F, Wertz S, Brothier E, Degrange V (2008). First exploration of Nitrobacter diversity in soils by a PCR cloning-sequencing approach targeting functional gene nxrA. FEMS Microbiol. Ecol. 63(1):132-140.

Pommering-Röser A, Rath G, Koops HP (1996). Phylogenetic diversity within the genus Nitrosomonas. Syst. Appl. Microbiol. 19:344-351.

Purkhold U, Pommerening-Röser A, Juretschko S, Schmid M C, Koops HP, Wagner M (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. 66(12):5368-5382.

Qian P, Schoenau JJ (2002). Availability of nitrogen in solid manure amendments with different C: N ratios. Can. J. Soil Sci. 82(2):219-225.

Rotthauwe JH, Witzel KP, Liesack W (1997). The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63(12):4704-4712.

Rughöft S, Herrmann M, Lazar CS, Cesarz S, Levick SR, Trumbore SE, Küsel K (2016). Community composition and abundance of bacterial, archaeal and nitrifying populations in savanna soils on contrasting bedrock material in Kruger National Park, South Africa. Front. Microbiol. 7:1638.

Saijai S, Ando A, Inukai R, Shinohara M, Ogawa J (2016). Analysis of microbial community and nitrogen transition with enriched nitrifying soil microbes for organic hydroponics. Biosci. Biotechnol. Biochem. 80(11):2247-2254.

Starkenburg, SR, Chain PS, Sayavedra-Soto LA, Hauser L, Larimer FW, Hickey WJ (2006). Genome sequence of the chemolithoautotrophic nitrite-oxidizing bacterium Nitrobacter winogradskyi Nb-255. Appl. Environ. Microbiol. 72(3):2050-2063.

Torsvik V, Goksoyr J, Daae FL (1990). High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. 56:782-787.

Wertz S, Goyer C, Zebarth BJ, Burton DL, Tatti E, Chantigny MH, Filion M (2013). Effects of temperatures near the freezing point on N2O emissions, denitrification and on the abundance and structure of nitrifying and denitrifying soil communities. FEMS Microbiol. Ecol. 83(1):242-254.

Wertz S, Leigh AK, Grayston SJ (2012). Effects of long-term fertilization of forest soils on potential nitrification and on the abundance and community structure of ammonia oxidizers and nitrite oxidizers. FEMS Microbiol. Ecol. 79(1):142-154.

Willuweit T, Söll P, Müller R (2008). U.S. Patent No. 7,384,777. Washington, DC: U.S. Patent and Trademark Office.

Winslow CE (1917). The characterization and classification of

bacterial types. Science 39(994):77-91

Woese CR, Weisburg WG, Paster BJ, Hahn CM, Tanner RS, Krieg NR,Koops HP, Harms H, Stackebrandt E (1984). The phylogeny of the purple bacteria: The beta subdivision. Syst. Appl. Microbiol. 5:327-336.

Zhou X, Fornara D, Wasson EA, Wang D, Ren G, Christie P, Jia Z (2015). Effects of 44 years of chronic nitrogen fertilization on the soil nitrifying community of permanent grassland. Soil Biol. Biochem. 91:76-83.

Full Length Research Paper

Polyphenolic profile, and antioxidant and antifungal activities of honey products in Benin

François Ezin Azonwadé[1,3], Bertin Gbaguidi[2], Armand Paraïso[3], Durand M. Dah-Nouvlessounon[1], Elvire Goubalan[4], Farid Baba-Moussa[5], Madjid Amoussa[6], Latifou Lagnika[6], Victorien Dougnon[7] and Lamine Baba-Moussa[1*]

[1]Laboratoire de Biologie et de Typage Moléculaire en Microbiologie, Faculté des Sciences et Techniques/Université d'Abomey-Calavi, 05 BP 1604 Cotonou, Benin.
[2]Laboratoire de Biomembrane et de Signalisation Cellulaire, Faculté des Sciences et Techniques, Université d'Abomey-Calavi, Bénin.
[3]Laboratoire de Protection des Végétaux, de Pathologie et Parasitologie des Abeilles, Faculté d'Agronomie, Université de Parakou, Bénin.
[4]Laboratoire de Physicochimie et d'Evaluation Sensorielle des Aliments, Faculté des Sciences Agronomiques, Université d'Abomey-Calavi, Bénin.
[5]Laboratoire de Microbiologie et de Technologie Alimentaire, Faculté des Sciences et Techniques/Université d'Abomey-Calavi, Benin.
[6]Unité de Biochimie des Substances Naturelles et Bioactives, Faculté des Sciences et Techniques/Université d'Abomey-Calavi, Bénin.
[7]Laboratoire de Recherche en Biologie Appliquée, Ecole Polytechnique d'Abomey-Calavi, Université d'Abomey-Calavi, Cotonou, Bénin.

The aim of this study was to characterize polyphenolic compounds, antifungal and antioxidant activities of polyfloral honey collected from three different phytogeographical zones in Benin duing dry and rainy seasons. Spectrophotometrically at 517 nm, DPPH scavenging activity of tested samples was measured, while antifungal activity, total polyphenols, flavonoids and condensed tannins were evaluted by Dohou, Singleton and Zhishen methods, and a slightly modified method of Sun, respectively. Results revealed that only season has significant influence ($P < 0.05$) on the flavonoids content and total phenols. The highest total phenols (781 ± 46 µgGAE/gs) and flavonoids (528 ± 31 µgCE/g) contents were obtained respectively from honey samples of the rainy and dry seasons. The samples collected in the rainy season in the three zones, have better antifungal activity than those collected in the dry season. The lowest IC_{50} recorded was 27.58 ± 4.44 µg.µl^{-1} which showed that the highest antioxidant activity can be found in honey samples of the rainy season collected from the Sudanian zone. Overall, this study confirms that all investigated honey samples were good sources of polyphenolic compounds, and exhibit antifungal and antioxidant actvities.

Keywords: Antifungal, antiioxidant, honey, Benin.

INTRODUCTION

Honey is the natural sweet substance produced by *Apis mellifera* from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which bees collect,

transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature (European Union, 2002). It plays an important part in our nutrition and it is well-known for its positive effects on health. Honey has been reported to contain approximately 200 substances (sugars, minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes and other phytochemical compound) and is considered to be an important part of traditional medicine (Ferreira et al., 2009). This natural product has been used since antiquity by the first humans for the treatment of burns, gastrointestinal disorders, asthma, infections and chronic injury, skin ulcers, cataracts and other eye diseases (Ghashm et al., 2010; Nasir et al., 2010; Wen et al., 2012; Saba et al., 2013).

Recently, in the light of oxidative stress phenomenon, the bankruptcy of a good number of conventional therapeutic agents, honey has been "rediscovered". Therapeutic potential such as antimicrobial and antiviral activity (Koc et al., 2009; Hamouda and Abouwarda 2011), antioxidant capacity (Erujewa et al., 2010; Hussein et al., 2011; Khalil et al., 2012) and anti-inflammatory effect (Mueller et al., 2010; Vallianou et al., 2014) were reported. In addition, honey as a source of antioxidant and/or an antifungal has been widely proven (Beretta et al., 2005; Küçük et al., 2007; Ferreira et al., 2009). Antioxidant activity of honey may be due to enzymatic and non-enzymatic antioxidants including glucose oxidase, catalase, peroxidase, ascorbic acid, derivative, carotenoids, organic acids and amino acids and proteins, the products of Maillard reaction and more than 150 polyphenolic compounds including flavonoids and phenolic acids. Pure honey and its dilutions have been reported to have inhibitory effects on fungi, and also inhibited their toxins production (Al-Waili and Haq, 2004). It has been reported to be effective against candidiasis, caused by *Candida albicans*, skin fungal infections such as the moth and athletes foot (Bansal et al., 2005). An antifungal action was observed on yeasts mainly, *Aspergillus* and Penicillium, as well as the totality of dermatophytes (Sampath et al., 2010). In addition, some studies have reported that the topical application of honey is effective in the treatment of seborrheic dermatitis and dandruff (Al-Waili, 2005). The quantity of these different compounds varies greatly depending on the floral and geographical origin of the honey. Additionally, the composition of honey is influenced by processing, handling and storage time (Gheldof et al., 2002; Bertoncelj et al., 2007).

Benin is a country with a diverse range of climates characterized by the relative weakness of the annual precipitation which vary from 900 to 1300 mm per year (Sinsin et al., 2004). More than hundred melliferous plant have been counted in Benin such as: *Acanthacees, Amaranthacees*, *Anacardiacees*, *Annonacees*, *Apocynacees*, *Asclépiadacees*, *Bignoniacees*, *Bombacacees*, *Borragninacees*, *Capparidacees*, *Césalpiniacees*, Com*melinacees*, Composed, *Cucurbitacees*, *Ebénacees*, *Euphorbiacees*, *Ficoidacees*, *Gramineous* (*Poacees*), *Lythracees*, *Méliacées*, Mimosacées, *Moracées*, *Musacées*, *Myrtacées*, *Rubiacées*, *Rutacées*, *Sapindacées*, *Sapotacees*, *Scrophuliacees*, Simaroubacees, *Sterculiacees*, *Verbénacees* and *Zygophyllacees* (Mensah et al., 2003; Yédomonhan et al., 2009). Honey has been used traditionally over the years by the people of Benin as food and as a traditional medicine in the treatment of several diseases

Although, honey is widely consumed by locals, very few data are available to support the medicinal claims for different types of honey samples from Benin. Honey is widely consumed in Benin, few data are available on the quality of commonly consumed honey. Investigations of honey samples collected from different geographic locations are necessary to provide local data. Furthermore, the data available for honey reported from other countries are not applicable to Benin because it varies in antioxidant capacity, antifungal activity and polyphenolic compounds. In this study, the authors aimed to investigate different honey samples collected from different regions in Benin and to value total phenolics, total flavonoids, condensed tanins, anfifungal and antioxidant activities of some polyfloral honey samples from Benin. To the best of the authors' knowledge, this is the first study to extensively investigate the different polyphenolic compunds, antifungal and antioxidant activities in various types of honey samples from Benin.

MATERIALS AND METHODS

Study area

Samples of honey were collected from three climatic zones (Sudanian, Sudano-guinean and Guinean) in Benin (Figure 1). Benin is located between the parallel 6° 15' and 12° 25' and extends on an area of 112 622 km^2. It is limited to the north by Niger and Burkina Faso, to the south by the Atlantic Ocean, to the west by Togo and on the east by Nigeria. Benin presents a diverse range of climates characterized by the relative weakness of the annual precipitation which vary from 900 to 1300 mm per year (Sinsin et al., 2004). In the Guinean zone from the coast (6°25 N) to the latitude of 7°30 N, there are four seasons (two rainy and two dry). It has an annual rainfall average of 1 200 mm with an average of 250 days of rain. The Sudano-guinean located between 7°30' N and 9°45' N with a uni modal (May-October) rainfall regime and the average annual rainfall varies from 900 to 1110 mm, distributed approximately over 113 days on average. The Sudanian zone is located between 9°45 N and 12°25 N with a 900 to 1100 mm as

*Corresponding author. E-mail: laminesaid@yahoo.fr. Tel: 00229.97.12.34.68.
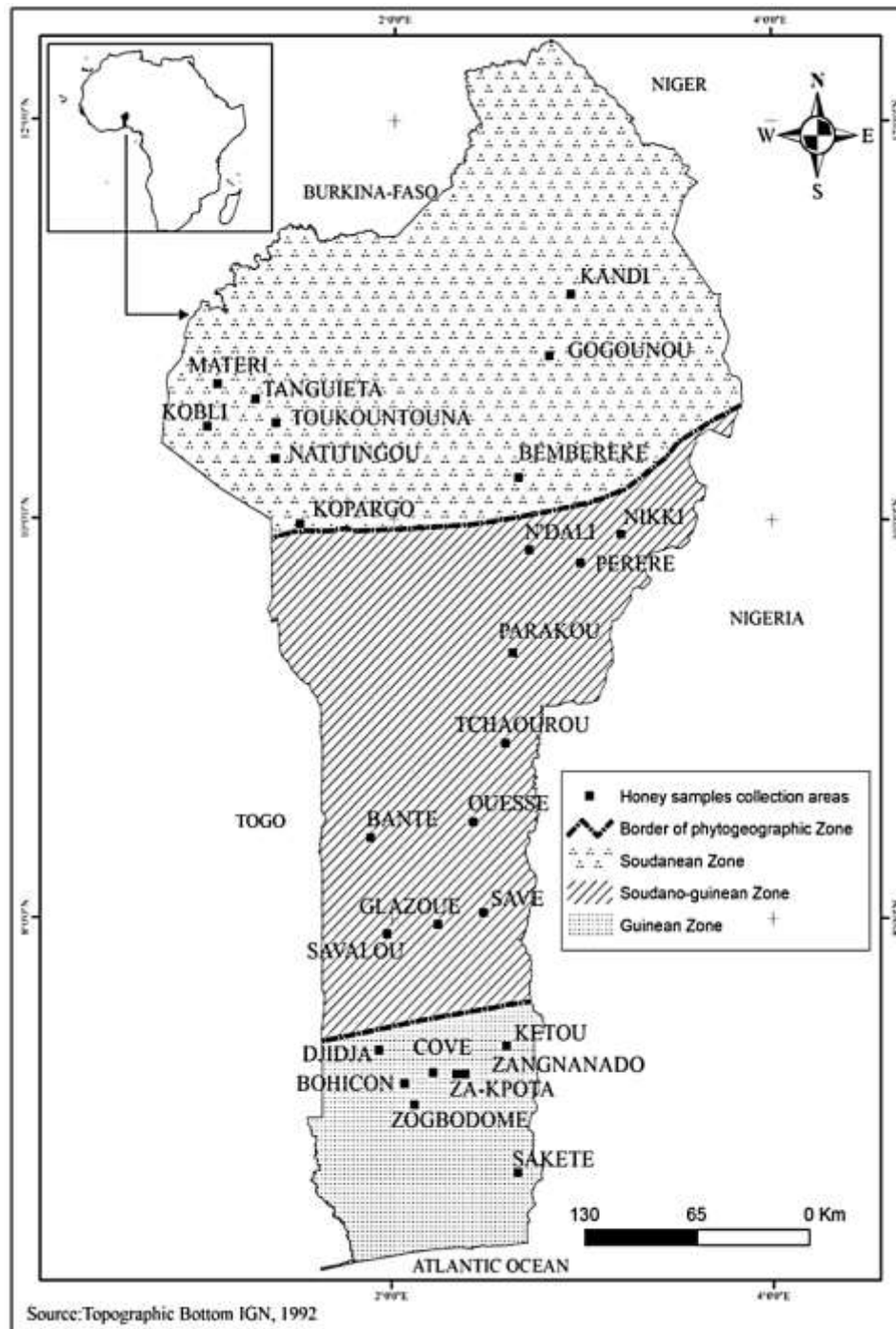
Figure 1. Sources of honey.

annual rainfall average, distributed over 145 days.

Sampling

A total of sixty polyfloral honey ($n = 60$) samples were collected from thirty different locations in three different phytogeographical zones (Sudanian, Sudano-guinean and Guinean) in Benin (Figure 1) during two seasons, namely dry and rainy. Indeed, thirty honey samples were colleted by season and same beekeepers were visited each season. The details of the honey, including the honey's location and zone, are described in Table 1. All honey collections were performed between January and December, 2015. The samples were refrigerated (4 to 5°C) in airtight plastic containers until further analysis. All analyses were conducted in triplicate. The samples collection periods vary according to the zones. In the

Table 1. Source of the investigated benese polyfloral honey.

Total honey samples by zone	Honey samples of the dry season by zone (n=10)	Honey samples of the rainy season (n=10)	Localities (n=10 by zone)
	SoD_1	SoR_1	Bembèrèkè
	SoD_2	SoR_2	Cobly
	SoD_3	SoR_3	Copargo
	SoD_4	SoR_4	Gogounou/Bagou
	SoD_5	SoR_5	Kandi
Zone Sudanean (n =20)	SoD_6	SoR_6	Matéri
	SoD_7	SoR_7	Natitingou Korimbéré
	SoD_8	SoR_8	Natitingou centre
	SoD_9	SoR_9	Tanguiéta
	SoD_{10}	SoR_{10}	Toucountouna
	SgD_1	SgR_1	Bantè
	SgD_2	SgR_2	Glazoué/Kpakpaza
	SgD_3	SgR_3	N'dali/Sinisson
	SgD_4	SgR_4	Nikki Biro
	SgD_5	SgR_5	Ouessè/Laminou
Sudano-Guinean zone (n=20)	SgD_6	SgR_6	Parakou/Monastère
	SgD_7	SgR_7	Pérèrè
	SgD_8	SgR_8	Savalou/Ouéssè
	SgD_9	SgR_9	Savè/Yaoui
	SgD_{10}	SgR_{10}	Tchaourou/Kinikpanhoun
	GuD_1	GuR_1	Bohicon 1
	GuD_2	GuR_2	Bohicon2
	GuD_3	GuR_3	Covè
	GuD_4	GuR_4	Djidja
	GuD_5	GuR_5	Ketou
Guinean Zone (n=20)	GuD_6	GuR_6	Saketé
	GuD_7	GuR_7	Zakpota
	GuD_8	GuR_8	Zangnanado
	GuD_9	GuR_9	Zogbodomey/Dovogon
	GuD_{10}	GuR_{10}	Zogbodomey/Mongon

D: Dry season; R : rainy season; So: samples of the Sudanian zone, Sg: samples of the Sudano-guinean zone; Gu: samples of the Guinean zone.

Sudanian and Sudano-guinean zones, all samples were collected in 2015 from November to April (dry season) and from June to September (rainy season), whereas, in the Guinea area, samples were collected from November to March and July to September (for dry season) and from April to July and September to October for the rainy season in the same year.

Reagents

Gallic acid, catechin, quercetin, 2,2-diphenyl- 1-picrylhydrazyl (DPPH) and Folin-Ciocalteu's reagent, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), sodium nitrite ($NaNO_2$), chlorhydric acid (HCl), sodium hydroxide (NaOH) and BIOMATE 3S UV-Visible Spectrophotometer used were purchased from Merck (Darmstadt, Germany).

Fungal strains used

Four reference fungal strains namely, *Aspergillus parasiticus* (CMBB 20), *Aspergillus ochraceus* (CMBB 91), *Aspergillus fumigatus* (CMBB 89) and *Aspergillus clavatus* (NCPT 97) were used in this study. These strains were obtained from the Laboratory of Quality Control of Medicines of the Health Ministry and have been stored in the Laboratory of Biology and Molecular Typing (University of Abomey-Calavi, Benin).

Polyphenolic analysis

Total phenols

The total polyphenol content was analyzed by spectrometry at 765 nm using Folin-Ciocalteu reagent (Singleton et al., 1999). In this method, 0.5 ml of honey (0.1 g/ml) was mixed with 2.5 ml of the

Folin-Ciocalteu's reagent diluted 1/10 with water. After 5 min, 2 ml of sodium carbonate (0.2 g/ml) was added to the mixture. Absorbance at 765 nm was recorded after 10 min of reaction at 37°C, against a blank with water. The polyphenol concentration was estimated with a calibration curve using a solution of 0.1 g/l of gallic acid as standard (0, 0.25, 0.05 and 0.1 g/l). The results were reported as mean ± standard deviation and expressed as microgram of gallic acid equivalent per gram of honey (µgGAE/g).

Flavonoids

Flavonoid content in each honey sample was measured using the colorimetric assay developed by Zhishen et al. (1999). Honey extract (1 ml) was mixed with 4 ml of distilled water. At the baseline, 0.3 ml of $NaNO_2$ (5%, w/v) was added. After 5 min, 0.3 ml of $AlCl_3$ (10% w/v) was added, followed by the addition of 2 ml of NaOH (1 M) 6 min later. The volume was then increased to 10 mL by the addition of 2.4 ml distilled water. The mixture was vigorously shaken to ensure adequate mixing and the absorbance was read at 510 nm. A calibration curve was created using a standard solution of quercetin (0.5 g/l). The results were expressed as microgram of catechin equivalent per gram of honey (µgCE/g).

Condensed tannins

Content in condensed tannins was determined by a slightly modified method of Sun et al. (1998). To 50 µl of honey (0.1 g/ml) was added 3 ml of vanillin (4%) and 1.5 ml of HCl. The mixture was then incubated at room temperature (25 ± 2°C) for 15 min. The mixture was vigorously shaken to ensure adequate mixing and the absorbance was read at 500 nm. Concentrations of condensed tannins are deducted from a calibration range established with quercetin (1g/l) and are expressed as microgram of catechin equivalent per gram of honey (µgQE/g).

Antifungal activity

The *in vitro* antifungal activity of the honey samples was evaluted according to the method previously described by Dohou et al. (2004). The assay was performed on the Potato-Dextrose Agar medium. Briefly, 2.5 ml of honey sample were mixed with 12.5 ml of the sterilized potato-destrose agar medium before it was transferred to sterile Petri plates for solidification. After the medium solidification, a sterile 6 mm disc pretreated with fungal strain was placed in each Petri plate. Plates were incubated at 25 ± 1°C for five days. Each treatment was replicated twice. Fungal radial growth was mesured by averaging the two diameters taken from each colony. Percentage growth inhibition of the fungal colonies was calculated using the formula:

$$Inhibition\ percentage\ (\%) = \frac{Control's growth - Treatment's growth}{Control's growth} \times 100$$

Antioxidant activity

The DPPH methode was conducted using an adapted method of Scherer and Godoy (20009). Practically, equal volumes (2 ml) of DPPH (200 µg/ml) and honey extracts (100 µg/ml) were mixed in screw tube serial and allowed to stand in darkness for 20 to 30 min at room temperature. Then, the absorbance was read at 517 nm and the blank was a mixture of methanol and DPPH (v:v). The inhibitory percentage of DPPH radiical indicating the antioxydant activity of honey extract was obtain using the formula ed by Schmeda-Hirschman et al. (2003).

The concentration providing 50% inhibition (IC_{50}) was determined graphically using a calibration curve in the linear range by plotting the honey extract concentration and the corresponding scavening effet. Antiioxydant activity index (AAI) was calculated according to the formula used by Scherer and Godoy (2009).

Statistical analysis

Microsoft Excel 2010 has been used for the seizure and the treatment of the data. Data of the polyphenolic compounds (total phenols, flavonoids and condensed tannins), inhibition percentage of the growth of the fungal strains and the IC_{50} obtained for the antioxydant activity were subjected to analysis of variance (ANOVA), respectively, for two factors, namely collection season and phytogeographical zone at probability level of 0.05 and the Student Newman-Keuls (SNK) tests using the Statistical Analysis System (SAS, Version 9.2) software in order to determine the influence of the seasons and zones on the content of polyphenolic compounds, antifungal and antioxidant activity of the different honey samples. Pearson correlation test was conducted with the Minitab 14 (Mtb v. 14) software to establish links between the polyphenolic compound, antifungal and antioxydant activity of the different honey samples.

RESULTS AND DISCUSSION

Total polyphenols, flavonoids and condensed tannins

The results of the polyphenolic compounds of different honey samples are shown in Table 2. The analysis of variance shows that there is no significant difference ($p > 0.05$) in condensed tannins, but there is a significant difference ($p < 0.05$) in total polyphenols and highly significant difference ($p < 0.001$) in total flavonoids content between seasons. However, following the collection area, no significant difference ($p > 0.05$) was observed in these three (total polyphenols, flavonoids and condensed tannins) compounds (Table 3). The standard curve conducted with the gallic acid ($R^2 = 0.9913$) helped to determine the total polyphenols in honey samples. The phenolic concentration of honey in this study ranged from 672 ± 35 to 861 ± 113 µgGAE/g with an average of 781 ± 46 µgGAE/g in samples of the rainy season and 631 ± 38 to 691 ± 43 µgGAE/g in samples of the dry season with an average value of 668 ± 26 µgGAE/g. It was found that the high average content in totals polyphenols (781 ± 46 µgGAE/g) was obtained in the rainy season, while the lowest (668 ± 26 µgGAE/g) was obtained in dry season samples. The calibration curve of cathechin ($R^2 = 0.9952$) was used to assess the total flavonoids in honey samples. The same variation trend was observed with total flavonoids in terms of harvest area and season, with the only difference where the higher content of flavonoids (528 ± 31 µgCE/g) was obtained in the dry season, whereas the lowest (280 ± 31 µgCE/g) was recorded in the rainy season. Total flavonoids content shows that this group of compound is in high proportion in the dry season among the total polyphenol obtained in honey samples. Considering the condensed tannins, they varied from 776

Table 2. Phenolic compounds of honey samples (means ± standard error) according to the seasons and phytogeographical areas.

Seasons	Zones	Total polyphenols (µgGAE/g)	Total flavonoids (µgCE/g)	Condensed tannins (µgQE/g)
Rainy	Sudanian	861±113[a]	223±42[a]	776±70[b]
	Sudano-guinean	809±67[a]	347±74[a]	834±49[b]
	Guinean	672±35[a]	270±36[a]	1055±61[a]
	Mean	781±46[A]	280±31[B]	888±40[A]
Dry	Sudanian	631±38[a]	519±55[a]	992±114[a]
	Sudano-guinean	682±56[a]	512±71[a]	1063±99[a]
	Guinean	691±43[a]	552±38[a]	765±70[a]
	Mean	668±26[B]	528±31[A]	940±58[A]

GAE: Gallic acid equivalent, QE: quercetin equivalent, CE: catechin equivalent. Means followed by the same letter of the same character and for the same factor are not significantly different (p > 0.05).

Table 3. Analysis of variance (p-value) for seasons and phytogeographical areas.

Sources of variation	DF	Fisher values		
		Total polyphenols (µgGAE/g)	flavonoids (µgCE/g)	condensed Tannins (µgQE/g)
Seasons	1	4.51* (0.04)	30.09 *** (<0.0001)	0.61 ns (0.44)
Zone	2	0.65ns (0.52)	0.59 ns (0.56)	0.32 ns (0.73)
Season x zone	2	1.85ns (0.17)	0.85 ns (0.43)	6.73 ** (0.002)

DF: Degree of freedom; ns: P >0.05 (no-significant); *: p < 0.05 (significant); **: p < 0.01 (highly significant); ***: p < 0.001 (very highly significant).

± 70 to 1055 ± 61 µgQE/g with an average of 888 ± 40 µgQE/g in rainy season honey samples. However, with an average of 940 ± 58 µgQE/g, condensed tannins vary from 765 ± 70 to 1063.66 ± 99 µgQE/g in honey samples of the dry season.

Antifungal activity

The antifungal activity of the honey samples shows a reduction of the rapid multiplication by fragmentation or by budding filaments (mycelial development) of the four fungal strains used. The percentages of inhibition vary according to the strains, the area and samples harvest period (Table 4). The samples collected in the rainy season in Sudanian zone have the best percentages of mycelial development inhibition (59.0 ± 12.1 to 68.4 ± 2.2%) on the four species of *Aspergilus*. The most remarkable antagonisms effect was observed on *Aspergilus parasiticus* (36.2%) and *Aspergilus ochraceus* (54.9%) for the honey samples collected in the dry season. In addition, the highest resistance was observed for *A. fumigatus* (43.7 %) and *A. clavatus* (49.1 %) using the samples collected in the dry season in the Guinean zone. Of the strains, *A. parasiticus* is the most sensitive (68.4 ± 2.2%), while *A. ochraceus* is more resistant. The analysis of variance of the mycelial growth inhibition rate revealed that neither the season nor harvest area did not influence (p > 0.05) the percentage of inhibition of *A.*

parasiticus (Table 4). However, both zone and season highly affected (p < 0.01 to p < 0.001) the inhibition proportion of *A. clavatus*. Only the collection area has a significant effect (p < 0.01) on the rate of inhibition of *A. fumigatus*. In addition, the inhibition percentage of *A. ochraceus* was influenced (p < 0.05) by the season and the interaction between season and area (Table 5).

In general, the samples collected in the rainy season in the three climatic zones, have antifungal activities better than those collected in the dry season. The rain may play a beneficial role in the concentration of active principle antimicrobials.

Antioxidant activity

The antioxidant activity of each sample of honey was determined regarding the antiradical activity against the free radical-1-diphenyl-picryl hydrazyl (DPPH) using IC_{50}, representing the concentration necessary for the inhibition of 50% of the free radicals. Thus, a lower value of IC_{50} in honey indicates a greater ability to neutralize free radicals.The antioxidant profiles obtained reveal that all honey samples possess antioxidant activities dose-dependently. The IC_{50} values vary from a season to another and from a collection area to another (Figure 2). The IC_{50} values of the rainy season samples are weaker than those of the dry season in the three phytogeographical zones. Thus, the analysis of variance

Table 4. Percentage inhibition of the honey samples related to the zones and the harvest seasons.

Inhibition (%) (Means ± standard error, n = 10)					
Seasons	Areas	*A. parasiticus*	*A. ochraceus*	*A. fumigatus*	*A. clavatus*
Dry	Sudanian	63.8±3.6[a]	45.1±3.2[b]	63.4±2.8[a]	56.7±2.0[a]
	Sudano-guinean	66.8±2.4[a]	53.0±3.3[ab]	58.9±2.2[a]	58.9±2.4[a]
	Guinean	65.7±3.0[a]	57.9±2.8[a]	56.3±2.4[a]	50.9±1.3[b]
	Mean	65.4±1.7[A]	52.0±2.0B	59.5±1.5[A]	55.5±1.2[B]
Rainy	Sudanian	68.4±2.2[a]	59.1±2.2[a]	64.3±2.2[ab]	63.9±1.8[a]
	Sudano-guinean	66.8±2.8[a]	57.0±2.8[a]	59.0±3.9[a]	61.9±1.8[a]
	Guinean	64.5±2.9[a]	55.6±1.9[a]	57.9±2.4[b]	57.3±2.2[a]
	Mean	66.6±1.5[A]	57.2±1.7[A]	63.1±1.5[A]	61.0±1.2[A]

n: Number of honey samples by zone and by season, means with different letters are significantly different with probability level of 5% according to Student Newman-Keuls test.

Table 5. Analysis of variance (p-value) for seasons and phytogeographical zone.

Sources of variation	DF	Values			
		A. parasiticus	*A. ochraceus*	*A. fumigatus*	*A. clavatus*
Season	1	0.24 (0.62) ns	4.41 (0.04) *	3.22 (0.07) ns	12.50 (0.0008) ***
Area	2	0.19 (0.83) ns	1.20 (0.31) ns	5.75 (0.005) **	7.11 (0.0018) **
Season *Area	2	0.60 (0.55) ns	3.61 (0.03) *	0.32 (0.73) ns	0.67 (0.5159) ns

DF: Degree of freedom;   ns: $p > 0.05$ (no-significant)   *: $p < 0.05$ (significant): ** $p < 0.01$ (highly significant). ***: $p < 0.001$ (very highly significant).

showed significant variation ($p < 0.05$) of IC_{50} between seasons in the Guinean zone, very significant ($p < 0.001$) in the Sudanian zone area and non-significant ($p > 0.05$) in the Sudano-Guinean. The lowest IC_{50} (27.58 ± 4.44 µg.µl^{-1}) which shows the highest antioxidant activity was recorded for the samples of the rainy season collected in the Sudanian zone area. On the other hand, the largest IC_{50} (47.46 ± 2.62 µg µl^{-1}), was recorded in the samples collected in the dry season in Guinean zone. Similar to the IC_{50}, the index of antioxidant activity (IAA) also varied according to the seasons and collection areas. Indeed, for the rainy season, the IAA values recorded were 1.62 ± 0.91 (Guinean zone), 1.61 ± 0.84 (Sudano-guinean) and 2.03 ± 0.76 (Sudanian zone). For the dry season, the lowest index (1.06 ± 0.11) was obtained with the samples of the Guinean zone, followed by that of the Sudano-guinean zone (1.25 ± 0.41) and finally Sudanian zone (1.47 ± 0.72). These different profiles show the importance and the antioxidant power of pure honey. The samples of the rainy season are richer in antioxidant compounds.

Correlation between polyphenolic compounds, antifungal and antioxidant activities

Table 6 shows the correlations between the various polyphenolic compounds, antifungal and antioxidant

activity of the honey samples. It appears from the analysis of this table that there was a positive and considerable correlation (r = 0.941, $p < 0.01$) between antifungal activity of *A. clavatus* and antioxidant activity expressed in IC_{50}. In other words, honey samples with high antifungal activity against *A. clavatus* were the richest in antioxidant compounds. A considerable negative correlation was observed between the antifungal activity of *A. ochraceus* and the total phenols content (r = -0.943, $p < 0.01$), in other words, the honey samples which have a high total phenol content had a low fungal activity against *A. ochraceus*.

DISCUSSION

The study on the biochemical content and biological activity carried out on homey samples collected in Benin showed variation of their composition in secondary metabolites. Considering the total polyphenols, there is no significant variation between the collection areas but their within the season of samples collection ($p < 0.05$). It is the same for the total flavonoids ($p < 0.001$). Thus, this data showed that the rain may have a beneficial effect on the formation and expression of this class of molecules. In addition, Campone et al. (2014) argued that the polyphenols (flavonoids) of honey can be distinguished according to their origin. Some of them come from the
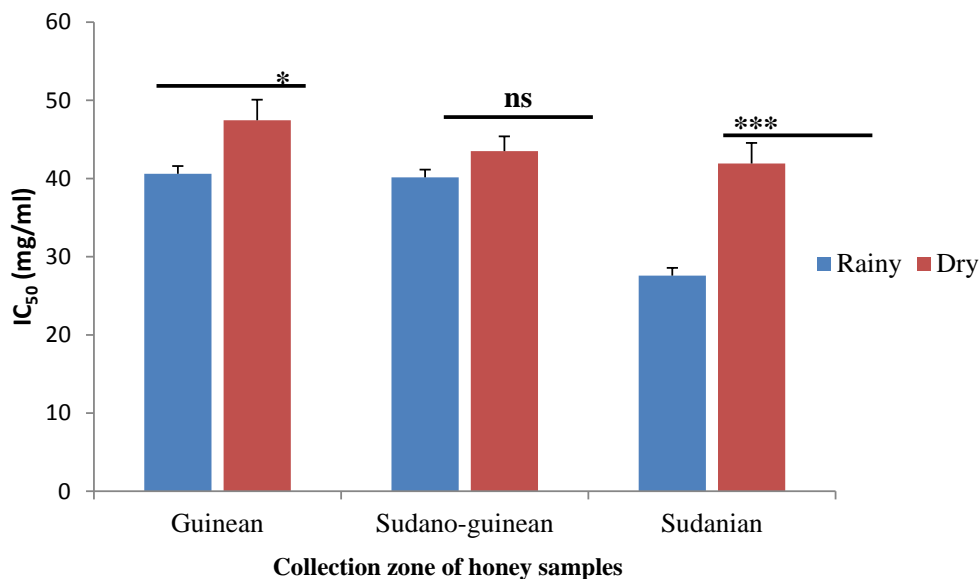
Figure 2. Variation of IC_{50} according to the seasons and samples collection zone. ns = P >0.05 (no-significant); * = p < 0.05 (significant); *** = p < 0.001 (very highly significant).

Table 6. Correlation coefficient of Pearson between different variables.

Variables	A. parasiticus	A. ochraceus	A. fumigatus	A. clavatus	IC_{50}	T. P.	F.	C.T.
A. parasiticus	1							
A. ochraceus	0.692	1						
A. fumigatus	0.203	-0.344	1					
A. clavatus	0.628	0.195	0.609	1				
IC_{50}	0.359	-0.018	0.679	0.941**	1			
T.P.	-0.562	-0.943**	0.194	-0.208	-0.054	1		
F.	0.445	0.63	0.008	0.662	0.643	-0.672	1	
C. T.	0.623	0.696	-0.727	0.080	-0.221	-0.671	0.139	1

**: p < 0.01 (highly significant); T.P. = total polyphenols; F. = flavonoids; C.T.= condenseds taninns.

nectar of plants visited by the bees, pollen, honeydew, or even the propolis. This statement confirms in part the hypothesis of departure for honeys of vegetable origin because the rain promotes a better growth of the plants organs and a good availability of their constituents. The total polyphenols would therefore be more available in plants in the rainy season than in dry season. The total polyphenols rates obtained independently in the season are three times greater than those reported (248.8 mgGAE/100 g) by Djossou et al. (2013) in Benin during their work on the physicochemical characteristics of city market honey samples collected in Cotonou. This difference may be due to the quality of the honey analyzed because some traders in quest to maximize their profits, proceed to the alteration of the quality of the honey by dilution. In addition, Beretta et al., (2005) showed some values on unifloral and multifloral honey samples of Africa (52.2 to 789.6 mg/kg) and Bertoncelj et al. (2007) on Slovakia honey samples (64 to 1304 µgGAE/g). Also, values raging between 226.2 and 727.8 mg/kg were reported in Portugal (Ferreira et al., 2009) and from 102.1 to 1085 mg/kg in the north-east of Brazil (Liberato et al., 2011). The main explanation for this difference could be linked to the unequal distribution of polyphenols both in quantity and quality across the different organs of a plant used by bees to produce honey.The amount of flavonoids in the samples collected in rainy (280 ± 31 µgCE/g) and dry seasons (528 ± 31 µgCE/g) displays a great variability within seasons (p < 0.05). The levels of flavonoids obtained in this study, are higher than the 170 - 286.5 µgGAE/100 g obtained in Burkina Faso (Méda et al., 2005) and Tualang honeys (Ibrahim et al., 2011). The flavonoids are phenolic low molecular weight compounds essential for aroma and

antioxidant properties of honey.

The recent interest in these substances has been stimulated by the potential benefits to the health arising from their antioxidant and anti-radicular activities against coronary heart diseases and cancer (Saba et al., 2011). For the condensed tannins, the interaction zone and season showed a significant difference (p < 0.01). As tannins are part of polyphenols, we can say that their expression may be influenced by edaphic and climatic factors. Indeed, Evans (1999) have shown that humidity, type of soil, location of plants (Eldridge and Kwolek, 1983), season during which the plant was used by bees (Salminen et al., 2004) and part of the plant used (Eldridge and Kwolek, 1983) may have an effect on the phytochemical constituents. A great variability in the content of polyphenolic compounds within the samples has been observed. This variability corroborate many scientific work carried out to identify potential chemical floral markers of honey. The studies carried out by various authors have shown that the phenolic compounds vary in function of the geographic origins and floral honey (Méda et al., 2005; Beretta et al., 2005; da Silva et al., 2013). The antifungal activity reflects difference between the responses of four fungal strains in presence of the honey samples. An interaction between the strains and the samples collection areas seems to define a particular pattern of effectiveness. Thus, the samples collected in the rainy season in Sudanian zone have the best percentages of inhibition. This observation may be related to good concentration of flavonoids and tannins in the honey samples of rainy season. The flavonoids are reported as phenolic compounds synthesized by plants in response to a microbial infection (Batawita et al., 2002). These are *in vitro* broad-spectrum antimicrobial whose antifungal properties have been reported (Babayi et al., 2004; Ulanowska et al., 2006). Their activity is probably due to their ability to complexed the soluble proteins and the extracellular proteins. Tannins also have very interesting antifungal properties (Okigbo et al., 2006) through various mechanisms such as the membranes destruction, enzymes inhibition, substrates deprivation and metal ions complexation. Tannins stick to proline rich proteins and then interfere with the proteins synthesis. Additional mechanisms are the alteration of biomembranes stability, proteins synthesis and important enzymes in the metabolism.

The antioxidant capacity of the honey samples, expressed in IC_{50} varies from 27.6 to 47.5 µg/µl. The values of the antioxidant activity obtained in the present study are similar to those obtained (7.2 to 53.8 mg/ml) in Slovakia (Bertoncelj et al., 2007). However, they are lower than those obtained by Ferreira et al. (2009) and Liberato et al. (2011) in honeys in Portugal (106.7 to 168.9 mg/ml) and the Northeast of Brazil (4.2 to 106.7 mg/ml). The antioxidant powers obtained with the honeys studied confirm the quality of these samples. The same trend of efficiency observed with the fungal strains was also observed for the antioxidant activity. This note shows that samples collected in the rainy season can be strongly used as antifungal and antioxidant agent. Considering the antioxidant activity index (AAI), according to Scherer and Godoy (2009), it can be said that, only the samples collected during the rainy season in Sudanian zone have a AAI>2.0. These samples have therefore a very strong antioxidant activity. However, all other samples have a strong antioxidant activity.

Conclusion

This study succesfully obtained scientific data on polyphenolic compounds, antifungal and antioxidant activities of honey samples produced in Benin. The polyphenolic compounds such as total polyphenols, flavonoids and condensed tannins have revealed the value of the honey products in each of the three phytogeographic zones of Benin. These secondary metabolites varied considerably depending on the season (dry and rainy) and the collection zone (Sudanian, Sudano-Guinean and Guinean). This study also revealed the antifungal power of the honey samples. The percentages of inhibition (antifungal drug) varied according to the strains, the area and the samples collection period. The samples collected in the rainy season in the three climatic zones, have antifungal activities better than those collected in the dry season. The antioxidant profiles obtained indicate that all samples possess dose-dependent antioxidant activities. The IC_{50} values vary from a season to another and according to the collection area. The IC_{50} values of the rainy season samples are weaker than those of the dry season independent of the zones.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Al-Waili NS (2005). Mixture of honey, beeswax and olive oil inhibits growth of *Staphylococcus aureus* and *C. albicans*. Arch. Med. Res. 36:10-13.

Al-Waili NS, Haq A (2004). Effect of honey on antibody production against thymus-dependent and thymus-independent antigens in primary and secondary immune responses. J. Med. Food 7(4):491-494.

Bansal V, Medhi B, Pandhi P (2005). Honey: A remedy rediscovered and its therapeutic utility. Kathmandu Univ. Med. J. 3(3)11:305-309.

Beretta G, Granata P, Ferrero M, Orioli M, Facino RM (2005). Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorimetric assays and chemometrics. Analytica Chimica Acta. 533:185-191.

Bertoncelj J, Dobersek U, Jamnik M, Golob T (2007). Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey, Food Chem. 105(2) :822-828.

Campone L, Piccinelli AL, Pagano I, Carabetta S, Di Sanzo R, Russo M, Rastrelli L (2014). Determination of phenolic compounds in honey using dispersive liquid–liquid microextraction. J. Chromatogr. A. 1334:9-15.

Council Directive of the European Union (2002). Council Directive 2001/110/EC of 20 December 2001 relating to honey. Official Journal of the European Communities. L10:47-52.

da Silva IAA, da Silva TMS, Camara CA, Queiroz N, Magnani M, de Novais J S, Soledade LEB, Lima EO, de Souza AL, de Souza AG (2013). Phenolic profile, antioxidant activity and palynological analysis of stingless bee honey from Amazonas, Northern Brazil. Food Chem. 141:3552-3558.

Djossou JA, Tchobo FP, Yédomonhan H, Alitonou AG, Soumanou MM (2013). Evaluation des caractéristiques physico chimiques des miels commercialisés à Cotonou. Tropicultura 31(3):163-169.

Dohou N, Yamni K, Badoc A, Douira A (2004). Activité antifongique d'extraits de Thymelaealythroidessur. Trois champignons pathogènes du riz. Bull. Soc. Pharm. Bordeaux 143:31-38.

Eldridge A, Kwolek W (1983). Soybean isoflavones: Effect of environment and variety on composition. J. Agric. Food Chem. 31:394-396.

Erejuwa OO, Sulaiman SA, Wahab MS, Sirajudeen KN, Salleh MS, Gurtu S (2010). Antioxidant protection of Malaysian Tualang honey in pancreas of normal and streptozotocin-induced diabetic rats. Ann. Endocrinol. (Paris) 71(4):291-296.

Erejuwa OO, Sulaiman SA, Wahab MSA, Sirajudeen KNS, Salleh S, Gurtu S (2012). Honey supplementation in spontaneously hypertensive rats elicits antihypertensive effect via amelioration of renal oxidative stress. Oxid. Med. Cell. Longev. ID374037

Estevinho L, Pereira AP, Moreira L, Dias LG, Pereira E (2008). Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey. Food Chem. Toxicol. 46:3774-3779.

Evans JD, Wheeler DE (1999). Differential gene expression between developing queens and workers in the honey bee, Apis mellifera. Proc. Natl. Acad. Sci. USA. 96:5575-5580

Ferreira ICFR, Aires E, Barreira JCM, Estevinho L M (2009). Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract. Food Chem. 114:1438-1443.

Ghashm AA, Othman NH, Khattak MN, Ismail NN, Saini R (2010). Antiproliferative effect of Tualang honey on oral squamous cell carcinoma and osteosarcoma cell lines. BMC Complement. Altern. Med. 10:49.

Gheldof N, Wang X.-H., and Engeseth N. J., (2002). Identification and quantification of antioxidant components of honeys from various floral sources, J. Agric. Food Chem. 50(21):5870-5877.

Hamouda HM, Abouwarda A (2011). Antimicrobial activity of bacterial isolates from honey. International J. Microbiol. Res. 2(1):82-85.

Hussein SZ, Mohd Yusoff KM, Makpol S, Mohd Yusof YA (2012). Gelam honey inhibits the production of proinflammatory, mediators NO, PGE2, TNF-α, and IL-6 in carrageenan-induced acute paw edema in rats. Evid. Based Complement. Alternat. Med. ID109636.

Ibrahim KM, Mohamed M., Jamalullail SMS, Alam N, Sulaiman SA, (2011). Evaluation of Radical Scavenging Activity and Colour Intensity of Nine Malaysian Honeys of Different Origin. J. ApiProd. ApiMed. Sci. 3(1): 4-11.

Khalil MI, Moniruzzaman M, Boukraa L, Benhanifia M, Islam A, Islam N, Sulaiman SA, Gan SH (2012). Physicochemical and antioxidant properties of Algerian honey. Molecules 17(9) :11199-11215.

Koc AN, Silica S, Ercal BD, Kasap F, Hormet-oz HT, Mavus-Buldu H (2009). Antifungal activity of Turkish honey against Candida spp. And Trichosporon spp: an in vitro evaluation. Med. Mycol. 47(7):

707-712.

Küçük M, Kolaylı S, Karaoglu S, Ulusoy E, Baltacı C, Candan F (2007). Biological activities and chemical composition of three honeys of different types from Anatolia. Food Chem. 100:526-534.

Liberato MCTC, Morais S M, Siqueira SMC, Menezes JESA, Ramos DN, Machado LKA, Magalhães IL (2011). Phenolic Content and Antioxidant and Antiacetylcholinesterase Properties of Honeys from Different Floral Origins. J. Med. Food 14:658-663.

Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG (2005). Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. Food Chem. 91 :571-577.

Mensah GA, Pomalègni B, Ekué M, Hounha J (2003). Diagnostic des contraintes à l'apiculture dans les localités riveraines des forêts classées de Goungoun et de Sota dans le Nord du Bénin. Rapport d'exécution du protocole N°64. PEEANC, LRZVH, CRA-Agonkanmey, INRA B, MAEP, Bénin. 21p.

Mueller M, Hobiger S, Jungbauer A (2010). Anti-inflammatory activity of extracts from fruits, herbs and spices. Food Chem. 122(4):987-996.

Nasir NA, Halim AS, Singh KK, Dorai AA, Haneef MN (2010). Antibacterial properties of tualang honey and its effect in burn wound management: a comparative study. BMC Complement. Altern. Med. 10:31.

Okigbo RN, Ogbonnaya UO (2006). Antifungal effects of two tropical plant leaf extracts (Ocimum gratissimum and Aframomum melegueta) on postharvest yam (Dioscorea spp) root. Afr. J. Biotechnol. 5:727-31.

Saba ZH, Suzana M, My YA (2013). Honey: Food or medicine. Med. Health 8:3-18.

Saba ZH, Yusoff KM, Makpol S, Yusoff MA Y (2011). Antioxidant capacities and total phenolic contents increase with gamma irradiation in two types of Malaysian honey. J. Mol. 16:6378-6395.

Sampath KKP, Bhowmik D, Chiranjib, Biswajit, Chandira MR (2010). Medicinal uses and health benefits of Honey: An overview. J. Chem. Pharm. Res. 2:385-395.

Scherer R, Godoy HT (2009). Antioxidant activity index (IAA) by the 2, 2-diphenyl-1-picrylhydrazyl method. Food Chem.112:654-658.

Schmeda-Hirschmann G, Rodriguez JA, Theoduloz C, Astudillo L, Feresin GE, Tapia A (2003). Free radical scavengers and antioxidants from Peumusboldus Mol. ("Boldo"). Free Radic. Res. 37:447-52.

Sinsin B, Eyog Matig O, Assogbadjo AE, Gaoué OG, Sinadouwirou T (2004). Dendrometric characteristics as indicators of pressure of Afzelia africana Sm. trees dynamics in different climatic zones of Benin. Biodivers. Conserv. 13:1555-1570.

Sun B, Ricardo da Silva J, Spranger I (1998). Critical factors of the vanillin assay for catechins and proanthocyanidins. J. Agric. Food Chem. 46:4267-4274.

Ulanowska K, Traczyk A, Konopa G, Wegrzym G (2006). Differential antibacterial activity of genistein arising from global inhibition of DND, RNA and protein synthesis in some bacterial strains. Arch. Microbiol. 184(5):271-278.

Singleton VL, Orthofer R, Lamuela-Raventós RM (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol. 299:152-178.

Vallianou NG, Gounari P, Skourtis A, Panagos J, Kazazis C (2014). Honey and its anti-inflammatory, anti-bacterial and anti-oxidant properties. Gen. Med. 2:132.

Wen CT, Hussein SZ, Abdullah S, Abdul Karim N, Makpol S, Mohd Yusof YA (2012). Gelam and Nenas honeys inhibit proliferation of HT 29 colon cancer cells by inducing DNA damage and apoptosis while suppressing inflammation. Asian Pac. J. Cancer Prev. 13(4):1605-1610.

Yédomonhan H, Tossou MG, Demènou BB, Traoré D (2009b). Diversité des plantes mellifères de la zone soudano-guinéenne: cas de l'arrondissement de Manigri (Centre-Ouest du Bénin). Int. J. Biol. Chem. Sci. 3(2):355-366.

Zhishen J, Mengcheng T, Jianming W (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64(4):555-559.

Full Length Research Paper

In vitro antibiotic resistance patterns of *Pseudomonas* spp. isolated from clinical samples of a hospital in Madinah, Saudi Arabia

Walaa M. Saeed[1], Samah Ghanem[1,3]*, Hatem M. El Shafey[2,4] and Nikhat Manzoor[1,5]*

[1]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Taibah University, Madinah, P. O. Box 3893, Kingdom of Saudi Arabia.
[2]Department of Respiratory Therapy, College of Medical Rehabilitation Sciences, Taibah University, P. O. Box 3893 Madinah, Kingdom of Saudi Arabia.
[3]Department of Microbiology, Faculty of Science, Helwan University, Ain Helwan, Cairo, Egypt.
[4]Department of Radiation Microbiology, National Center for Radiation Research and Technology, P. O. Box 29, Nasr City, Cairo, Egypt.
[5]Medical Mycology Laboratory, Department of Biosciences, Jamia Millia Islamia, New Delhi-110025. India.

***Pseudomonas* spp. are the leading cause of nosocomial infections. Rise in multidrug resistance among clinical isolates limit therapeutic options and hence increase mortality rate. Clinical samples (6840) from a hospital in Madinah, Saudi Arabia were collected for a duration of 14 months to study the frequency, antimicrobial sensitivity pattern and seasonal variations of *Pseudomonas* isolates. Conventional biochemical tests were done to identify the probable organism and antibiotic susceptibility was performed by disc diffusion method and Phoenix automated microbiology 100 ID/AST system. *Pseudomonas* represented 6.5% of all positive samples of which 65% were from males. Majority of the organisms (85%) were isolated from sputum and wound swabs followed by catheter tips (6.4%) and throat aspirates (3.4%). From the remaining samples, less than 1% organisms were obtained. Assessment of antimicrobial susceptibility to 11 different antibiotics revealed that imipenem was the most effective with highest sensitivity of 99.5%, and low intermediate resistance of only 0.5%. This was followed by ciprofloxacin (97.5%), ceftazidime (96.3%), cefpiramide/amikacin (94.1%), aztreonam (93.2%), gentamycin (87.7%), ampicillin (83%), and cotrimoxazole (80.1%). The most resistant drugs included augmentin (25%), cotrimoxazole (19.9%), ampicillin (17%) and gentamycin (12.3%) while the least resistant were ciprofloxacin (1.5%) and imipenem (0%). Results recommend imipenem as a promising antibiotic against *Pseudomonas* infections. In case of resistance to imipenem, ciprofloxacin, ceftazidime, cefpiramide, amikacin, and aztreonam may be recommended. In acute cases, *Pseudomonas* infections may require combined antimicrobial therapy. Frequency of these infections was the lowest (17%) during spring. It was the highest (30%) during summers and winters but reduced to 22% during autumn maybe due to better hygiene during pilgrimage season.**

Key words: Enterobacteriaceae, *Pseudomonas*, antibiotic resistance pattern, antimicrobial susceptibility, multi-drug resistance.

INTRODUCTION

Antibiotic drug resistance in pathogenic organisms is a universal problem now with severe treatment issues (Fair and Tor, 2014; Yezli et al., 2014; Chika et al., 2017). A gradual increase in drug resistance has been observed in most of the gram negative bacterial species, the main reason being excessive use and misuse of broad spectrum antibiotics (Fair and Tor, 2014; Ventola, 2015; Mahmoud et al., 2016). The use of antibiotics in animal feed stocks by the animal and food industry has also aggravated the condition (CDPC, 2013; Fair and Tor, 2014). Intensive Care Units (ICUs) are generally places where resistant pathogens flourish and are easily transmitted to other patients and healthcare workers (Chan, 2012; Aly and Balkhy, 2012). Gram negative bacteria account for upto 70% of nosocomial infections in ICUs (Peleg and Hooper, 2010) revealing a major change in the pattern of drug resistance around the world. The basic reason for escalation in antibiotic resistance has to be comprehended along with properly designed infection control programs and more stringent prescription guidelines.

Pseudomonas is a genus of ubiquitous non-fermentative gram negative bacteria, the species of which are metabolically diverse and hence are found in a wide variety of places especially in hospitals where bacteria grow easily in moist environment (Arora et al., 2011; Chika et al., 2017). Majority of Pseudomonas spp. develop resistance to penicillin and other related beta-lactam antibiotics (Shaikh et al., 2015). These opportunistic pathogens are host to several intrinsic and acquired resistance genes which they can also exchange with other gram negative bacteria (Juan Nicolau and Oliver, 2010). Hence, Pseudomonas is responsible for the occurrence and spread of several important carbapenemases (β-lactamases) (Kittinger et al., 2016). P. aeruginosa has high environmental tolerance, impermeable outer membrane, forms biofilms and has a several siderophores and pigments that allow it to evade the innate immune system (Fair and Tor, 2014). All these factors increase its resistance manifold fold making Pseudomonas infections complicated and life threatening.

Pseudomonas aeruginosa is one of the leading cause of nosocomial infections especially among patients admitted to ICUs. Other species like P. putida and P. fluorescens cause a variety of infections in clinical settings (Kittinger et al., 2016). Contaminated catheters and other medical devices transmit these pathogens causing bacteraemia, sepsis, pneumonia, urinary tract infections (UTIs), burn and wound infections, etc (Peleg

and Hooper, 2010). According to reports broad-spectrum antibiotics like imipenem, ceftazidime, and amikacin have been recommended for treatments of infections caused by multi-drug resistant P. aeruginosa however, ciprofloxacin continues to be the oral drug of choice (Izadpanah and Khalili, 2015).

The annual cost associated with antimicrobial resistance was estimated to be $55 billion in US alone. On September 2016, the United Nations General Assembly organized a high-level meeting on antimicrobial resistance to address this global problem (U Nations, 2016). In Saudi Arabia, P aeruginosa has appeared as the most commonly isolated organisms in hospitals, causing 11% of all nosocomial infections; up to 31% of these are due to gram-negative organisms (Yezli et al., 2014; Khan and Faiz, 2016). Reports from Saudi Arabia claim that although more than half of the isolates of P. aeruginosa remain susceptible to carbapenems, quinolones, and aminoglycosides; multidrug resistance is on the rise at an alarming rate (Al-Agamy et al., 2012; Yezli et al., 2014).

Klebsiella pneumoniae carbapenemase (KPC), New Delhi Metallo-beta-lactamase (NDM) and (Verona Integron-Mediated Metallo-β-lactamase (VIM) are enzymes that break down carbapenems and make them ineffective. These enzymes have been reported in Pseudomonas (CDPC, 2016). There has been a significant surge in ESBL-related infection cases throughout the world (Shaikh et al., 2015). Emergence of infections caused by ESBL, MBL, MDR and PDR P. aeruginosa strains is alarming as they create serious health issues and place a huge liability of morbidity, mortality and health care cost on patients. Pseudomonas spp. belongs to the natural bacterial community in surface waters, they are clinically relevant, and changes in their natural resistance profiles indicate anthropogenic influence. Hence, there is an urgent need for persistent and cautious global surveillance for multidrug-resistant bacteria. This study, therefore, aimed at investigating resistances of Pseudomonas spp. to clinically important antibiotics and to evaluate the resistance pattern of this species isolated from clinical samples of a Saudi hospital.
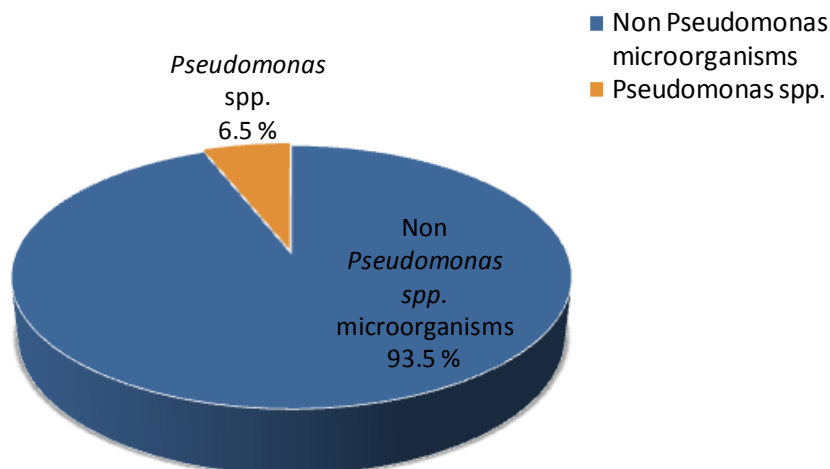
MATERIALS AND METHODS

Sample collection

Different clinical samples such as sputum, wound swab, bile, tracheal aspirate (Tr. asp.), throat aspirate (Th. asp.), catheter Tip, pus, abdominal abscess (Abd. ab.), ear swab, peritoneal wound

*Corresponding author. E-mail: samah.ghanem@laposte.net, nikhatmanzoor@yahoo.co.in.

Figure 1. Percentage of *Pseudomonas* spp. in comparison to other clinical isolates.

swab (Peri. w.s.), pleural fluid (Pler. fluid), gall bladder aspirates (GB asp), necrotic tissue (NT), cystic fluid (CF) were collected from 6840 patients suspected of bacterial infection at King Fahd Hospital at Madinah, Saudi Arabia. Demographic data such as sex of the patients was recorded prior to sample collection.

Culture and Identification

The clinical samples were collected according to Centers for Disease Control and Prevention Specimen Collection Guidelines (CDPC, 2013), aseptically inoculated on plates of blood agar, chocolate agar, Cystine-lactose-electrolyte-deficient (CLED) agar and MacConkey agar (Oxoid Cambridge, UK) and incubated at 37°C for 24 h. Identification was done based on morphological characteristics of the colonies including size, shape, colour, pigmentation and haemolytic nature.

Biochemical characterization

Suspected *Pseudomonas* colonies isolated were further identified through biochemical tests using standard procedures (Barrow and Felthan, 2003) and Phoenix automated microbiology 100 ID/AST system (Becton Dickinson Company, Sparks, Md.). Identification included the following tests: nitrate reduction test, citrate utilization test, oxidase test, H_2S gas production, methyl-red test, indole test, urease test, Voges-Proskauer test and lactose fermentation (Forbes et al., 2007).

Antimicrobial susceptibility test

Susceptibility to antimicrobial agents was determined by using the disk diffusion method (Oqunshe, 2006), and Phoenix automated microbiology 100 ID/AST system (Becton Dickinson Company, Sparks, Md.). The following antimicrobial agents (obtained from BDH London, UK) were used: ampicillin (10 μg), augmentin [amoxycillin + clavulinic acid (20/10 μg)], gentamycin (10 μg), cotrimoxazole [Trimethoprim-Sulfamethoxazole 1:19 (25 μg)], amikacin (30 μg), ceftazidime (30 μg), aztreonam (30 μg), imipenem (10 μg), ciprofloxacin (5 μg), cefpiramide (30 μg) and piperacillin (100 μg). The inocula were prepared by growing the

various *Pseudomonas* strains on separate agar plates and colonies from the plates were transferred with a loop into 3 ml of normal saline. The density of these suspensions was adjusted to 0.5 McFarland standards. The surface of Muller-Hinton agar (Oxoid Cambridge, UK) plate was evenly inoculated with the organisms using a sterile swab. The swab was dipped into the suspension and pressed against the side of the test tube to remove excess fluid. The wet swab was then used to inoculate the Muller-Hinton agar by evenly streaking across the surface. By means of a Disc Dispenser (Oxoid Cambridge, UK), the antibiotic discs were applied onto the surface of the inoculated agar and the plates were incubated overnight at 37°C. The diameter of zone of growth inhibition observed was measured and compared to the chart provided by Clinical and Laboratory Standards Institute (CLSI, 2015).

RESULTS AND DISCUSSION

Many studies have been done till now to estimate the antibiotic resistance pattern in ICUs around the world but unfortunately only a few studies have been reported from Saudi Arabia (Rotimi et al., 1998; Johani et al., 2010). The present work investigates the antimicrobial resistance pattern of *Pseudomonas* spp. isolated from patients of King Fahad Hospital, Madinah, one of the two holy cities visited by lakhs of pilgrims the whole year around. Clinical samples (6840) were collected from patients suspected of bacterial infection during a span of 14 months and screened for the gram negative bacteria. Results showed that of all the isolates screened, only 6.5% were *Pseudomonas* spp. (Figure 1). Isolates could not be recovered from some samples like urine, blood, ascetic fluid, nasal swabs, axilla, and perineum. It was observed that of the positive *Pseudomonas* isolates, 65% were from males while 35% were from females (Figure 2) indicating that males show greater vulnerability for these infections. *Pseudomonas* species were positive for oxidase test, catalase test, nitrate reduction test; citrate utilization test, gelatin hydrolysis, and motility; and
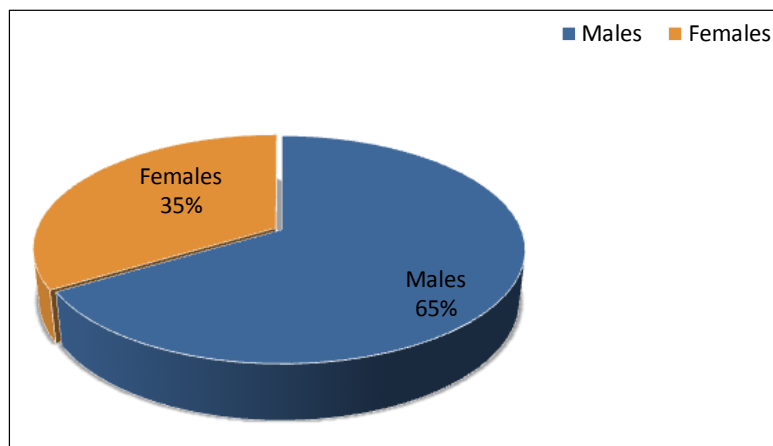
Figure 2. Percentage *Pseudomonas* spp. isolates from males and females.

negative for urease test, Voges-Proskauer test, H_2S gas production, methyl-red test, indole test, and coagulase test (Forbes et al., 2007).

Table 1 gives an estimation of the number of male and female samples isolated from different sources. Majority of the *Pseudomonas* species (85%) were isolated from sputum and wound swab samples of which 66.8% came from the sputum sample of male patients. Similarly, in case of wound swabs, 61.9% were from males and 38.1% were from females. A higher isolation rate from sputum and wounds has been reported in earlier studies as well (Ahmed, 2016; Golia et al., 2016). Samples from catheter tips and throat aspirates revealed greater *Pseudomonas* species from males. The percentage male to female ratio was 82.1:17.9 and 66.7:33.3 in case of catheter tips and throat aspirates, respectively. As reported earlier (Babay, 2007; Magliano et al., 2012), in case of wound swabs, the male to female ratio was 86:53 indicating that adult males are more susceptible to infection than adult females. A similar situation was observed with catheter tips and throat aspirates where male to female ratio of isolated specimens were 23:5 and 10:5, respectively. Results are not unexpected as in Saudi Arabia males represent a larger labor force and hence are exposed more to infections, pollution of all kind and accidents on roads and factories.

Only 1 sample each was available from ear, gall bladder aspirate, necrotic tissue and cystic fluid and that also from females. Since the number of samples is low, any substantial conclusion could not be drawn from these samples. One sample each was obtained from bile, abdominal abscess, and bed sores that is the male to female ratio in these three cases was 1:1. Peritoneal wound swab and pleural fluid provided 2 specimens each but only from males. Five specimens were isolated from tracheal aspirates where 40% were from males and 60% from females while four specimens were isolated from

pus swabs where 75% were obtained from males and rest from females.

Figure 3 shows the percentage samples from various clinical sources that were positive for *Pseudomonas* species. As mentioned earlier, the majority of isolates were from sputum and wound swabs, 53.4 and 31.6%, respectively followed by catheter tips (6.4%) and throat aspirates (3.4%). Very few strains were obtained from other clinical sources. The percentage of *Pseudomonas* isolates from tracheal aspirates was 1.93% while from bile, abdominal abscess, peritoneal wound swabs, bed sores and pleural fluid it was 0.45%. GB aspirates, ear swabs, NT, and CF were poor sources providing only 0.23% positive isolates each. The percentage of other samples was not adequate enough to assess gender profile and give reproducible results. Earlier reports also state (Janda and Abbott, 2006; Babay, 2007; Magliano et al., 2012) that males are more vulnerable to infection than females, especially respiratory tract infections. These findings may be explained on the basis of their different anatomy, lifestyle, and socioeconomic conditions.

Antimicrobial drug sensitivity was performed by disc diffusion assay using antibiotic discs of ampicillin, augmentin, gentamycin, cotrimoxazole, amikacin, ceftazidime, aztreonam, piperacillin, imipenem, ciprofloxacin, cefpiramide. As shown in Table 2, imipenem was the most effective antibiotic against *Pseudomonas* species with 99.5% sensitivity followed by ciprofloxacin with 97.5% sensitivity. The high sensitivity of imipenem against gram-negative bacteria has been shown by others as well (Mokaddas and Sanyal, 1999; El-Tahawy, 2000; Bahashwan and Shafey, 2013; Dash *et al.*, 2014). Ceftazine showed a sensitivity of 96.3%. Cefpiramide is a third generation cephalosporin antibiotic while amikacin is a fourth generation aminoglycoside. Both showed fairly good sensitivity of 94.1%; results

Table 1. Gender wise distribution of *Pseudomonas* specimens isolated from different sources.

Sex	Source of specimens													
	Sp	WS	Bile	Tr	Th	Cath	Pus	Abd	Ear	Peri	Pler	G B asp	Bed Sores	NT	CF
M	157 (66.8)	86 (61.9)	1(50)	2 (40)	10 (66.7)	23 (82.1)	3 (75)	1 (50)	0 (0)	2 (100)	2 (100)	0 (0)	1 (0)	0 (0)	0 (0)
F	78 (33.2)	53 (38.1)	1 (50)	3 (60)	5 (33.3)	5 (17.9)	1 (25)	1 (50)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)
Total	235	139	2	5	15	28	4	2	1	2	2	1	2	1	1

M, Males; F, Females; Sp, Sputum; WS, Wound swab; Tr, Tracheal aspirate; Th, Throat aspirate; Cath, Catheter Tip; Abd, Abdominal abscess; Peri, peritoneal wound swab; Pler, Pleural fluid; GB asp, Gall bladder aspirates; NT, Necrotic tissue; CF, Cystic fluid. Percentage (%) values are given in parentheses.
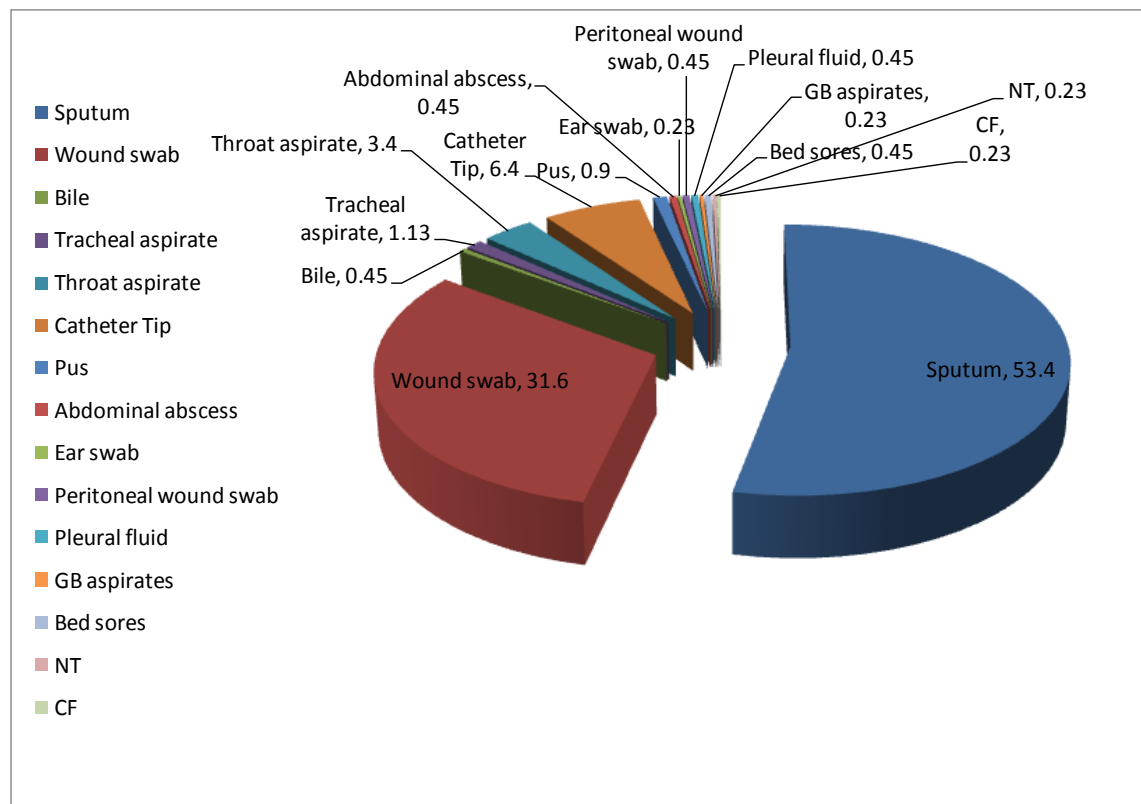
Figure 3. Percentage of *Pseudomonas* spp. specimens isolated from different clinical samples.

Table 2. Percentage (%) antimicrobial sensitivity pattern of *Pseudomonas* isolates to different antibiotics.

Antibiotic	Sensitive	Resistant	Intermediate
Ampicillin	83.0	17.0	0
Augmentin	75.0	25.0	0
Gentamycin	87.7	12.3	0
Cotrimoxazole	80.1	19.9	0
Amikacin	94.1	5.6	0.3
Ceftazidime	96.3	3.2	0.5
Aztreonam	93.2	5.6	1.2
Imipenem	99.5	0.0	0.5
Ciprofloxacin	97.5	1.5	1.0
Cefpiramide	94.1	5.3	0.6
Piperacillin	90.0	4.8	5.2

Table 3. Percentage (%) of *Pseudomonas* infection during different seasons.

Season	Percentage (%) of *Pseudomonas* infections
Summer (22 June -22 September)	30.1
Autumn (23 September -21 December): Pilgrimage season	22.1
Winter (22 December -30 Mars)	31.0
Spring (21 Mars-21 June)	16.8

being consistent with the previous studies (Dash et al., 2015; Ahmed, 2016).

The percentage sensitivity with the other antibiotics in the present study was in the sequence: aztreonam (93.2%) > piperacillin (90%) > gentamycin (87.7%) > ampicillin (83%) > cotrimoxazole (80.1%) > augmentin (75%). *Pseudomonas* used in the present study showed highest resistance to augmentin (25%) followed by cotrimoxazole (19.9%), ampicillin (17%), gentamycin (12.3%), amikacin (5.6%), aztrenam (5.6%), cefpiramide (5.3%), piperacillin (4.8) and ceftazidime (3.2%).

Bacterial infections have seasonal trends depending on several factors. Investigating their prevalence will assist in identifying specific risk factors that will help in improving and formulating new infection control strategies. Several reports claim that bacterial infections always peak during summers and winters (Eber et al., 2011; Psoter et al., 2013). Table 3 depicts the percentage of *Pseudomonas* infection during the four different seasons. It was observed that the percentage of infections was the highest during summers and winters (30 to 31%) followed by autumn (22.1%). During spring, the percentage was not too high being only 16.8%.

Winters are known for the outbreaks of bacterial infections especially related to the respiratory systems (Eber et al., 2011; Psoter et al., 2013). The autumn season incidentally coincides with the major pilgrimage period when a large number of pilgrims visit Madinah during Haj. But amusingly, the proportion of infection lowered in this period in comparison to the peak seasons (summers and winters). Increased proportions of infection during summers and autumn have also been reported earlier also (Psoter et al., 2013). This can be explained as during annual pilgrimage (Haj), the health authorities take special care to control outbreak of bacterial and other infections. Almost similar patterns were seen in case of *Proteus* and *Klebsiella* infections during the same period of study (Bahashwan and Shafey, 2013; Ghanem et al., 2017). Even though Makkah and Madinah expect huge influx of pilgrims throughout the year, it is during the Haj season that there are dangers of an epidemic outbreak. To circumvent the spread of infection, special precautions are taken which could be the reason for such low percentage of infection during the pilgrimage season. Saudi Arabia has taken a good initiative in reducing spread of resistant pathogens in healthcare units by implementing the World Health Organization (WHO) hand hygiene program and the Gulf Cooperation Council (GCC) Infection Control Program (Yezli et al., 2014). There is a need to introduce more such programs to control multidrug resistance in gram negative bacteria.

Conclusion

It may be concluded that males are at a greater risk of

Pseudomonas infections in comparison to females. Imipenem is the most effective antibiotic and can be prescribed to patients without any hesitation as it has the highest sensitivity and lowest resistance in this case. A larger number of *Pseudomonas* strains were found resistant to augmentin, cotrimazole and ampicillin suggesting that these antibiotics should be prescribed but with care. Summers and winters both seemed to have the highest infection percentage followed by autumn. Lack of awareness, self-medication and misuse of antibiotics has aggravated multidrug resistance in microbes. Compliance to infection prevention guidelines are essential to eliminate major outbreaks in the future.There is an ardent need to formulate and adhere to new guidelines for drugs based on their sensitivity profiles. Studies like these can help in developing rationalized local databases concerning antimicrobial resistance patterns, and hence formulate better infection control strategies in Saudi Arabia.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

Authors would like to thank the Microbiology staff of King Fahd Hospital, Al Madinah Al Munawarah, KSA for their assistance with data collection.

REFERENCES

Agamy MH, Shibl AM, Tawfik AF, Elkhizzi NA, Livermore DM (2012). Extended-spectrum and metallo-beta-lactamases among ceftazidime-resistant *Pseudomonas aeruginosa* in Riyadh, Saudi Arabia. J Chemother. 24(2):97-100.

Ahmed OB (2016). Incidence and antibiotic susceptibility pattern of *Pseudomonas aeruginosa* Isolated from Inpatients in Two Tertiary Hospitals. Clin Microbiol 5(2):248-252.

Al Johani SM, Akhter J, Balkhy H, El-Saed A, Younan M, Memish Z (2010). Prevalence of antimicrobial resistance among Gram-negative isolates in an adult intensive care unit at a tertiary care center in Saudi Arabia. Ann Saudi Med. 30(5):364-369.

Aly M, Balkhy HH (2012). The prevalence of antimicrobial resistance in clinical isolates from Gulf Corporation Council countries. Antimicrob. Resis. Infect. Control 1:26.

Arora D, Jindal N, Kumar R, Romit M (2011). Emerging antibiotic resistance in pseudomonas a challenge. Int. J. Pharm. Pharm. Sci. 3(2):488-1491.

Babay HAH (2007). Antimicrobial resistance among clinical isolates of *Pseudomonas aeruginosa* from patients in a teaching hospital, Riyadh, Saudi Arabia, 2001-2005. Jpn. J. Infect. Dis. 60:123-125.

Bahashwan SA, El Shafey HM (2013). Antimicrobial resistance patterns of *Proteus* isolates from clinical specimens. Eur. Sci. J. 9(27):188-202.

Barrow GI, Felthan RKA (2003). Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd Ed. Cambridge University Press. Cambridge UK. pp. 351-353. http://www.academia.edu/8106702/Cowan_and_Steels_manual_for_the_identification_of_medical_bacteria.

Center for Disease Control and Prevention (CDPC) (2013). Antibiotic resistance threats in the United States. http://www.cdc.gov/drugresistance/threat-report-2013.

Centers for Disease Control and Prevention (CDPC) (2016). Carbapenem-resistant Enterobacteriaceae in Healthcare Settings. Retrieved from https://www.cdc.gov/hai/organisms/cre/index.html

Chan M (2012). Antimicrobial resistance: no action today, no cure tomorrow, WHO World Health Day - 7 April 2012. http://www.who.int/dg/speeches/2011/WHD_20110407/en/

Chika EO, Nneka AR, Dorothy ON, Chika E (2017). Multi-Drug Resistant *Pseudomonas aeruginosa* Isolated from Hospitals in Onitsha, South -Eastern Nigeria. Int Arch. BioMed. Clin. Res. 3(3):22-26.

Clinical and Laboratory Standards Institute (CLSI) (2015). Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-fifth Informational Supplement M100–S25. http://clsi.org/.

Dash M, Padhi S, Narasimham MV, Pattnaik S (2014). Antimicrobial resistance pattern of *Pseudomonas aeruginosa* isolated from various clinical samples in a tertiary care hospital, South Odisha, India. Saudi J. Health Sci. 3:15-19.

Eber MR, Shardell M, Schweizer ML, Laxminarayan R, Perencevich EN (2011). Seasonal and temperature-associated increases in Gramnegative bacterial bloodstream infections among hospitalized patients. PLoS One 6(9):e25298.

Fair RJ, Tor Y (2014). Antibiotics and Bacterial Resistance in the 21st Century. Perspect. Med. Chem. 6:25-64.

Forbes B, Sahm D, Weissfeld A (2007). Bailey and Scott's Diagnostic Microbiology, 12th ed.; Mosby Elsevier: St. Louis, MO, USA.

Ghanem S, El Shafey HM, El Kelani TA, Manzoor N (2017). Antimicrobial resistance patterns of *Klebsiella* isolates from clinical samples in a Saudi hospital. Afr. J. Microbiol. Res. 11(23):965-971.

Golia S, Suhani, Manasa S, Jyoti. (2016). Isolation of *Pseudomonas aeruginosa* from various Clinical Isolates and it Antimicrobial Resistance Pattern in a Tertiary Care Hospital. Int. J. Curr. Microbiol. App. Sci. 5(3):247-253.

Golkar Z, Bagasra O, Pace DG (2014). Bacteriophage therapy: A potential solution for the antibiotic resistance crisis. J Infect. Dev. Ctries. 8(2):129-136.

Izadpanah M, Khalili H (2015). Antibiotic regimens for treatment of infections due to multidrug-resistant Gram-negative pathogens: An evidence-based literature review. J. Res. Pharm. Pract. 4(3):105-114.

Janda JM, Abbott SL (2006). The Genera *Klebsiella* and *Raoultella*. The Enterobacteria. Washington, USA: ASM Press. 2nd ed. pp. 115-129.

Juan Nicolau C, Oliver A (2010). Carbapenemases in *Pseudomonas* spp. Enferm. Infecc. Microbiol. Clin. 28 (1):19-28.

Khan HA, Baig FK, Mehboob R (2017). Nosocomial infections: Epidemiology, prevention, control and surveillance. Asian Pac. J. Trop. Biomed. 7(5):478-482.

Khan MA, Faiz A (2016). Antimicrobial resistance patterns of *Pseudomonas aeruginosa* in tertiary care hospitals of Makkah and Jeddah. Ann. Saudi Med. 36(1):23-28.

Kittinger C, Lipp M, Baumert R, Folli B, Koraimann G, Toplitsch D, et al. (2016). Antibiotic Resistance Patterns of Pseudomonas spp. Isolated from the River Danube. Front. Microbiol. 7:586.

Magliano E, Grazioli V, Deflorio L, Leuci AI, Mattina R, Romano P (2012). Gender and Age-Dependent Etiology of Community-Acquired Urinary Tract Infections. Sci. World J. pp. 1-6.

Meletis G (2016). Carbapenem resistance: overview of the problem and future perspectives Ther. Adv. Infect. Dis. 3(1):15-21.

Mohammed MA, Alnour TM, Shakurfo OM, Aburass MM (2016). Prevalence and antimicrobial resistance pattern of bacterial strains isolated from patients with urinary tract infection in Messalata Central Hospital, Libya. Asian Pac. J. Trop. Med. 9(8):771-776.

Mokaddas EM, Sanyal SC (1999). Resistance patterns of *Pseudomonas aeruginosa* to Carbapenems and Piperacillin/Tazobactam. J. Chemother. 11(2):93-96.

Nations U. High level meeting on antimicrobial resistance (2016). On 21st September in United Nations, New York. Available at: http://www.un.org/pga/71/event-latest/high-level-meeting-on-antimicrobial-resistance/

Oqunshe AAO (2006). *In vitro* phenotypic antibiotic resistance in bacterial flora of some indigenous consumed herbal medications in Nigeria. J. Rural Trop. Public Health 5:9-15.

Peleg AY, Hooper DC (2010). Hospital-acquired infections due to gram-negative bacteria. N Engl. J. Med. 362(19):1804-1813.

Psoter KJ, De Roos AJ, Wakefield J, Mayer J, Rosenfeld M (2013). Season is associated with Pseudomonas aeruginosa acquisition in young children with cystic fibrosis. Clin. Microbiol. Infect. 19:E483-E489.

Rotimi VO, Al-Sweih NA, Feteih J (1998). The prevalence and antibiotic susceptibility pattern of Gram negative bacterial isolates in two ICUs in Saudi Arabia and Kuwait. Diagn. Microbiol. Infect. Dis. 30:53-59.

Shaikh S, Fatima J, Shakil S, Rizvi SMD, Kamal MK (2015). Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. Saudi J. Biol. Sci. 22:90-101.

Shulman L, Ost D (2005). Managing infection in the critical care unit: how can infection control make the ICU safe? Crit. Care. Clin. 21(1):111-128.

Ventola CL (2015). The antibiotic resistance crisis. Part 1: Causes and Threats. P & T. 40(4):277-283.

Yezli S, Shibl AM, Livermore DM, Memish ZA (2014). Prevalence and antimicrobial resistance among gram-negative pathogens in Saudi Arabia. J. Chemother. 26(5):257-272.

African Journal of
Microbiology Research

Related Journals Published by Academic   Journals

- ■ *African Journal of Biotechnology*
- ■ *African Journal of Biochemistry Research*
- ■ *Journal of Bacteriology Research*
- ■ *Journal of Evolutionary Biology Research*
- ■ *Journal of Yeast and Fungal Research*
- ■ *Journal of Brewing and Distilling*