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Analysis of nitrifying microbial community for organic hydroponics

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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 sample was dominated by uncultured bacterium, followed by Nitrobacter winogradskyi and Nitrobacter vulgaris with the least being Nitrocoecus mobilis and Nitrosospira 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 Nitrocoecus 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:
nitrification 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 *Nitroscoccus mobilis*), *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* (Woese et al., 1984; Pomerening-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 over 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 (Rughöff 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 amoA gene encoding ammonia monoxygenase 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).
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 sulfuric 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.
The DNA was then amplified using functional genes targeting primers (NorA and AmoA) using standard PCR procedure at SANUMARC.

Genomic DNA was prepared and used as a template. 305 bp NxrA genes and 490 bp AmoA genes were amplified using NorA and AmoA genes. The sequence of the amoA primers was: amo r5'-GGGGTTTCTACTGGTGTTGT-3'. amoA-r 5'- CCCCTCKGAAAGCCTTCTTC-3' (Rotthauwe et al., 1997; Purkhold et al., 2000). The sequence for the NXR primers was: norA-f 5' CAGACCGGACGTGTGCAG3', norA-r 5'-TCYACAAAGGAGCGGAAGGT-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).

![Figure 2](image_url)

**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.

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

Where, \( \mu \) is the overall mean response, \( \tau_i \) is the effect due to the \( i \)-th level of factor A, \( \beta_j \) is the effect due to the \( j \)-th level of factor B and \( \gamma_{ij} \) is the effect due to any interaction between the \( i \)-th level of A and the \( j \)-th level of B, \( \epsilon_{ij} \) 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):

\[ S_i = 1 - D (\Sigma n(n-1)/N(N-1)) \]

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...
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 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-oxidizing 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 *Nitosospira* species. This is considering that nitrifying bacteria picked up by the *AmoA* gene encoding the active site polypeptide of the ammonia monooxygenase (*AmoA*) 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

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Inoculum source</th>
<th>Garden</th>
<th>Compost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncultured ammonia-oxidising</td>
<td></td>
<td>529</td>
<td>2469</td>
</tr>
<tr>
<td><em>Nitrosomonas</em> sp.</td>
<td></td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Uncultured <em>Nitrosomonadales</em></td>
<td></td>
<td>12</td>
<td></td>
</tr>
<tr>
<td><em>Nitrosospira</em> sp.</td>
<td></td>
<td>4</td>
<td>537</td>
</tr>
<tr>
<td>Uncultured bacterium</td>
<td></td>
<td>807</td>
<td>823</td>
</tr>
<tr>
<td>Uncultured beta</td>
<td></td>
<td>181</td>
<td>34</td>
</tr>
<tr>
<td>Uncultured <em>Nitrosospira</em></td>
<td></td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>SUM</td>
<td></td>
<td>1581</td>
<td>3877</td>
</tr>
<tr>
<td>Simpson diversity index</td>
<td></td>
<td>0.61</td>
<td>0.53</td>
</tr>
</tbody>
</table>

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

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

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

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

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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 during 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 evaluated 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 µGAE/g) and flavonoids (528 ± 31 µCE/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 IC₅₀ recorded was 27.58 ± 4.44 µg.ml⁻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 activities.

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 (Erjiewa 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., 2012; Koc 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; Bertoncej 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: Acanthaceae, Amaranthaceae, Anacardiaceae, Annonaceae, Apocynaceae, Asclépiadaceae, Bignoniaceae, Bombacaceae, Borraginaceae, Capparidaceae, Cesalpiniaceae, Commelinaceae, Compos, Cucurbitaceae, Ébénaceae, Euphorbiaceae, Ficoidaceae, Gramineae (Poaceae), Lythraceae, Méliacées, Mimosacées, Moracées, Muscées, Myrtacées, Rubiacées, Rutacées, Sapindacées, Sapotacées, Scrophuliacées, Simaroubacées, Sterculiaceae, Verbénacées and Zygophyllacées (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, antifungal 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 compounds, 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 km². 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 (8°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

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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 collected 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.

<table>
<thead>
<tr>
<th>Total honey samples by zone</th>
<th>Honey samples of the dry season by zone (n=10)</th>
<th>Honey samples of the rainy season (n=10)</th>
<th>Localities (n=10 by zone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone Sudanean (n =20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SoD1</td>
<td>SoR1</td>
<td></td>
<td>Bembèreké</td>
</tr>
<tr>
<td>SoD2</td>
<td>SoR2</td>
<td></td>
<td>Cobly</td>
</tr>
<tr>
<td>SoD3</td>
<td>SoR3</td>
<td></td>
<td>Copargo</td>
</tr>
<tr>
<td>SoD4</td>
<td>SoR4</td>
<td></td>
<td>Gogounou/Bagou</td>
</tr>
<tr>
<td>SoD5</td>
<td>SoR5</td>
<td></td>
<td>Kandi</td>
</tr>
<tr>
<td>Zone Sudano-Guinean zone (n=20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SoD6</td>
<td>SoR6</td>
<td></td>
<td>Matéri</td>
</tr>
<tr>
<td>SoD7</td>
<td>SoR7</td>
<td></td>
<td>Natitingou Korimbéré</td>
</tr>
<tr>
<td>SoD8</td>
<td>SoR8</td>
<td></td>
<td>Natitingou centre</td>
</tr>
<tr>
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<td>SoR9</td>
<td></td>
<td>Tanguêta</td>
</tr>
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<td>SoD10</td>
<td>SoR10</td>
<td></td>
<td>Toucountouna</td>
</tr>
<tr>
<td>Sudano-Guinean zone (n=20)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SgD1</td>
<td>SgR1</td>
<td></td>
<td>Banté</td>
</tr>
<tr>
<td>SgD2</td>
<td>SgR2</td>
<td></td>
<td>Glazoué/Kpakpaza</td>
</tr>
<tr>
<td>SgD3</td>
<td>SgR3</td>
<td></td>
<td>N’dali/Sinisson</td>
</tr>
<tr>
<td>SgD4</td>
<td>SgR4</td>
<td></td>
<td>Nikki Biro</td>
</tr>
<tr>
<td>SgD5</td>
<td>SgR5</td>
<td></td>
<td>Ouessé/Laminou</td>
</tr>
<tr>
<td>SgD6</td>
<td>SgR6</td>
<td></td>
<td>Parakou/Monastère</td>
</tr>
<tr>
<td>SgD7</td>
<td>SgR7</td>
<td></td>
<td>Pééré</td>
</tr>
<tr>
<td>SgD8</td>
<td>SgR8</td>
<td></td>
<td>Savalou/Ouëssé</td>
</tr>
<tr>
<td>SgD9</td>
<td>SgR9</td>
<td></td>
<td>Savé/Yaoui</td>
</tr>
<tr>
<td>SgD10</td>
<td>SgR10</td>
<td></td>
<td>Tchaourou/Kinikpanhoun</td>
</tr>
<tr>
<td>Guinean Zone (n=20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GuD1</td>
<td>GuR1</td>
<td></td>
<td>Bohicon 1</td>
</tr>
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<td>GuD2</td>
<td>GuR2</td>
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<td>GuR3</td>
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<td>Cové</td>
</tr>
<tr>
<td>GuD4</td>
<td>GuR4</td>
<td></td>
<td>Djidja</td>
</tr>
<tr>
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<td>GuR5</td>
<td></td>
<td>Ketou</td>
</tr>
<tr>
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<td>GuR6</td>
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</tr>
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<td>GuR7</td>
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<td>Zakpota</td>
</tr>
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<td>GuR8</td>
<td></td>
<td>Zangnanado</td>
</tr>
<tr>
<td>GuD9</td>
<td>GuR9</td>
<td></td>
<td>Zogbodomey/Dovogon</td>
</tr>
<tr>
<td>GuD10</td>
<td>GuR10</td>
<td></td>
<td>Zogbodomey/Mmongon</td>
</tr>
</tbody>
</table>

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 (Na₂CO₃), aluminum chloride (AlCl₃), sodium nitrite (NaNO₂), 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 NaNO₂ (5%, w/v) was added. After 5 min, 0.3 ml of AlCl₃ (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 (µgCE/g).

**Antifungal activity**

The in vitro antifungal activity of the honey samples was evaluated 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 was mixed with 12.5 ml of the sterilized potato-dextrose 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 measured by averaging the two diameters taken from each colony. Percentage growth inhibition of the fungal colonies was calculated using the formula:

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

**Antioxidant activity**

The DPPH methode was conducted using an adapted method of Scherer and Godoy (2009). 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 radical indicating the antioxidant activity of honey extract was obtained using the formula ed by Schmeda-Hirschman et al. (2003).

The concentration providing 50% inhibition (IC₅₀) was determined graphically using a calibration curve in the linear range by plotting the honey extract concentration and the corresponding scavenging effect. Antioxidant activity index (AAI) was calculated according to the formula used by Scherer and Godoy (2009).

**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 (R² = 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 catechin (R² = 0.9952) was used to assess the total flavonoids in honey samples. The same trend variation 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
± 70 to 1055 ± 61 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 Aspergillus. The most remarkable antagonisms effect was observed on Aspergillus parasiticus (36.2%) and Aspergillus 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 IC50, representing the concentration necessary for the inhibition of 50% of the free radicals. Thus, a lower value of IC50 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 IC50 values vary from a season to another and from a collection area to another (Figure 2). The IC50 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 2. Phenolic compounds of honey samples (means ± standard error) according to the seasons and phytogeographical areas.

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

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

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>DF</th>
<th>Total polyphenols (µgGAE/g)</th>
<th>Flavonoids (µgCE/g)</th>
<th>Condensed Tannins (µgQE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seasons</td>
<td>1</td>
<td>4.51* (0.04)</td>
<td>30.09 *** (&lt;0.0001)</td>
<td>0.61 ns (0.44)</td>
</tr>
<tr>
<td>Zone</td>
<td>2</td>
<td>0.65ns (0.52)</td>
<td>0.59 ns (0.56)</td>
<td>0.32 ns (0.73)</td>
</tr>
<tr>
<td>Season x zone</td>
<td>2</td>
<td>1.85ns (0.17)</td>
<td>0.85 ns (0.43)</td>
<td>6.73 ** (0.002)</td>
</tr>
</tbody>
</table>

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).

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 IC50 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 IC50 (27.58 ± 4.44 μg.mL⁻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 IC50 (47.46 ± 2.62 μg.mL⁻¹), was recorded in the samples collected in the dry season in Guinean zone. Similar to the IC50, 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 IC50. 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 honey 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

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

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

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 phytoogeographical zone.

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

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).
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Figure 2. Variation of IC₅₀ 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.

<table>
<thead>
<tr>
<th>Variables</th>
<th>A. parasiticus</th>
<th>A. ochraceus</th>
<th>A. fumigatus</th>
<th>A. clavatus</th>
<th>IC₅₀</th>
<th>T. P.</th>
<th>F.</th>
<th>C.T.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. parasiticus</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. ochraceus</td>
<td>0.692</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>0.203</td>
<td>-0.344</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. clavatus</td>
<td>0.628</td>
<td>0.195</td>
<td>0.609</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC₅₀</td>
<td>0.359</td>
<td>-0.018</td>
<td>0.679</td>
<td>0.941**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T.P.</td>
<td>-0.562</td>
<td>-0.943**</td>
<td>0.194</td>
<td>-0.208</td>
<td>-0.054</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F.</td>
<td>0.445</td>
<td>0.63</td>
<td>0.008</td>
<td>0.662</td>
<td>0.643</td>
<td>-0.672</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>C. T.</td>
<td>0.623</td>
<td>0.696</td>
<td>-0.727</td>
<td>0.080</td>
<td>-0.221</td>
<td>-0.671</td>
<td>0.139</td>
<td>1</td>
</tr>
</tbody>
</table>

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

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 µGAE/g). Also, values ranging 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\textsubscript{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 successfully 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\textsubscript{50} values vary from a season to another and according to the collection area. The IC\textsubscript{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.

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REFERENCES


Full Length Research Paper

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

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*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, multidrug 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
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, H₂S 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 Cambridge, UK) were used: ampicillin (10 µg), augmentin [amoxycillin + clavulanic 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 1. Percentage of Pseudomonas spp. in comparison to other clinical isolates.](image-url)
negative for urease test, Voges-Proskauer test, H₂S 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, ceftazidine, aztreonam, piperacillin, imipenem, ciprofloxacin, ceftiramide. 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). Ceftazime showed a sensitivity of 96.3%. Ceftriaxone 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 | Sp   | WS   | Bile | Tr   | Th   | Cath | Pus | Abd | Ear | Peri | Pler | G B asp | Bed Sores | NT | CF |
|-----|------|------|------|------|------|------|-----|-----|-----|------|------|----------|-----------|-----|
| M   | 157  | 86   | 1    | 2    | 10   | 23   | 3   | 1   | 2   | 1    | 2    | 1        | 1         | 0   | 0  |
| F   | 78   | 53   | 1    | 3    | 5    | 5    | 1   | 1   | 1   | 0    | 0    | 1        | 1         | 1   | 1  |
| Total| 235  | 139  | 2    | 5    | 15   | 28   | 4   | 2   | 1   | 2    | 2    | 1        | 1         | 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.

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

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

<table>
<thead>
<tr>
<th>Season</th>
<th>Percentage (%) of <em>Pseudomonas</em> infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer (22 June -22 September)</td>
<td>30.1</td>
</tr>
<tr>
<td>Autumn (23 September -21 December): Pilgrimage season</td>
<td>22.1</td>
</tr>
<tr>
<td>Winter (22 December -30 Mars)</td>
<td>31.0</td>
</tr>
<tr>
<td>Spring (21 Mars-21 June)</td>
<td>16.8</td>
</tr>
</tbody>
</table>

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.

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