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Pollution in Shatt Al-Arab River near Hospital Wastewater Disposal – Detection of Pharmaceutical Compounds, Isolation and Identification of Drug-Resistant Bacteria

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ABSTRACT

Pharmaceutical compounds have been introduced into the aquatic ecosystems in multiple ways and sources, which negatively affects the health of the environment and humans. The river near the hospital sewage disposal is host environment for drug-resistant bacteria (DRB). In this context, the aim of the research was to detect the presence of pharmaceuticals in hospital wastewater, after treatment, at river point followed by isolation of bacteria and test for resistant pharmaceutical compound. Fifteen species of bacteria isolated by angiogenic methods were identified and tested using the well diffusion test to determine the resistance of selected bacteria to ibuprofen by survival assessment. This study showed that the ibuprofen detected by GC-mass was available in all selected points at a concentration of 3 to 120 mg/L. *Lecuco. mesen.cremris* and *Koc.rosea* have a high ability to break down the ibuprofen compound. Thus, the bacteria isolated from hospital wastewater can biologically degrade ibuprofen.

Keywords: resistant bacteria, well diffusion, river, pharmaceutical, micropollutants.

INTRODUCTION

Development of the life in the world led to more human consumption of different pharmaceutical and personal care products (PPCPs). Subsequently, wastewater treatment plants (WWTPs) and hospital wastewater (HWW) disposal to surface water were considered to be the major source of PPCPs to the environment. (Mutar et al., 2022a; Muter et al., 2017). Pharmaceutical and personal care products are not completely removed by conventional wastewater treatment processes; therefore, biotechnology treatment needs to be applied to reach minimum concentration when disposal to water surface (Mohammed et al., 2021). Constructed wetlands (CWs) are the biotechnology to remove micropollutants via filtration, sedimentation, adsorption, and plant-bacteria interaction (Priya et al., 2021).

The most of PPCP sources in water bodies are directly related to human activities (Noguera-Oviedo and Aga, 2016). Several thousand PPCPs are produced each year around the world, and their discharge into the environment is an unwanted by-product of daily living (Mutar et al., 2022b; Cizmas et al., 2015). This study reported about the presence of pharmaceutical compounds in the Shatt al-Arab river, in addition to identifying isolation bacteria species that are resistant to pharmaceutical compounds. The Shatt al Arab River is formed by the confluence of the Euphrates and Tigris Rivers near the city of Qurnah in southern Iraq.

The primary sources of PPCPs that enter diverse environmental systems are manufacturing sites, sewage treatment plants (STPs), wastewater treatment plants (WWTPs), individual houses, landfill sites, and large farms (Sengar and Vijayanandan, 2022). PPCPs are typically released into the environment when manufacturing facilities discharge untreated or incompletely treated effluent to surface water bodies or STPs. Additionally, many PPCPs leak into the environment after use through a different route (Ebele et al., 2017). The pharmaceuticals absorbed by the body during treatment are excreted and discharged into septic tanks and sewage systems (Ebele et al., 2017; Ricky and Shanthakumar, 2022). Various factors, such as discharges from local PPCP production facilities, the overall population, usage patterns, and incorrect disposal of expired or unused PPCPs, all have an impact on the amount of individual PPCPs in sewage (Eggen and Vogelsang, 2015).

Pharmaceuticals are used to diagnose, treat, or prevent ailments. The method has been expanded to incorporate medicinal substances and can be used to drugs as well (Huang et al., 2021). Thousands of tons of human drugs are produced/ingested each year, including antibiotics, anti-inflammatory pharmaceuticals, synthetic hormones, cytotoxins, and statins (Giwa et al., 2020).

Pharmaceuticals are separated from other chemical contaminants by the following characteristics (Putschew and Jekel, 2007). They can be made up of a variety of complex molecules with different molecular weights, functions, structure, and shapes. Detection of organic pollutants in industrial effluents has advanced dramatically during the last century. They are hydrophilic, and several of them are relatively water soluble. Some medications, including naproxen, erythromycin, and sulfamethoxazole, can persist in the environment for more than a year, while others, like clofibric acid, can linger in the environment for years and gain biological activity as a result of accumulation. In humans, the molecules are absorbed, dispersed, and subjected to metabolic processes, which may cause them to change their structure (Huang et al., 2021; Giwa et al., 2020).

Ibuprofen, diclofenac, and carbamazepine have all recently been the subject of the research by Stancova et al. (2017) on the effects of sublethal or sub-chronic doses of pharmaceuticals on the early life stages of tech (Tinca tinca).

Biodegradation is the process by which high molecular weight ECs are broken down into little molecules by microorganisms like bacteria, algae, and fungi (Garcia-Rodríguez at el., 2014), and even biomineralized to simple inorganic molecules like water and carbon dioxide. Microorganisms utilize organic molecules as major substrates for cell development and stimulate enzymes for their assimilation in the traditional biodegradation process (Ezeuko et al., 2021). There has been progress and there have been challenges. The chemical and biological persistence of ECs, their physicochemical properties, the method used, and the operation conditions all influence their removal or degradation capacity. The most important elimination method for highly polar compounds, such as most medicines and their metabolites, is biological transformation or mineralization by microbes.

The rate of removal is highly dependent on the treatment method, the operating conditions, and the contaminants to be removed (Barceló et al., 2008). Degradation products are difficult to identify in environmental samples because they are not only present in extremely low concentrations, but they are also present in complex matrices that might make detection difficult (Barceló et al., 2008; Zhou et al., 2009).

The presence of PPCPs in surface waters, municipal treated wastewater, groundwater, and sediments means available of antibiotic-resistant bacteria species (Hasan et al 2021). The goal of this study was to describe the pharmaceutical compounds available and concentrations in three selected points of hospital wastewater to isolate the diversity of tolerance bacteria in this environment. With the hypothesis that there is a high concentration of pharmaceutical compounds in the Shatt al-Arab river and verifying the presence of drug-resistant bacteria in this aquatic environment can determine the suitable sustainable treatment biotechnology in future study.

MATERIALS AND METHODS

Water sampling from detection sites

Water samples were collected from three points (hospital wastewater, treatment plant of hospital, and Shatt Al-Arab river), as shown in Figure 1. The water samples were collected within 30.0 cm depth from water surface in 500 mL sterile polyethylene bottles, then transported to the lab and keep in refrigerator at 4 °C prior to chemical and biological analyses (Gomes et al., 2022). The collected water samples were sent for analysis to detect pharmaceutical compounds in the three selected points of hospital wastewater path to the Shatt Al-Arab river.

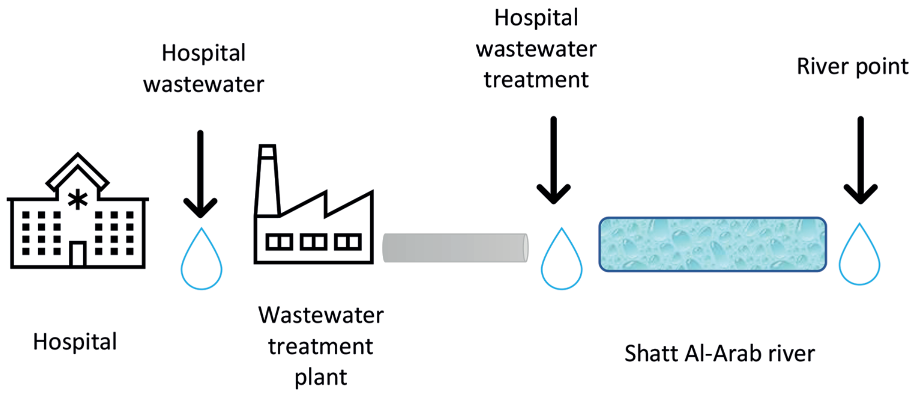


Figure 1. The water samples were collected from different point

Analysis of pharmaceutical compounds in GC-MS

The samples were analysis by gas chromatography – mass spectroscopy (GC-MS) to detect pharmaceutical compounds. First, 500 mL of sample were filtered using a cellulose membrane of 0.45 μm, followed by lyophilization of samples. The water samples were frozen, concentrated by lyophilization (–48.3 °C, 0.1163 mbar), and resuspended in 70% methanol before quantification via gas chromatography (GC-MS) (Calado et al., 2019). Ibuprofen was chosen as pharmaceutical compound available in the three selection sampling points of the Shatt al-Arab river. A stock solution of 5,000 mg/L was prepared by using methanol for calibration and bacteria exposure. Calibration curve was done for Ibuprofen by linear regression of the peak area ratio of the analyte and its corresponding internal standard against their respective concentrations. Depending on the stock solution, different concentrations (0.1, 0.5, 1, 5, 7, and 10 mg/L) of Ibuprofen were prepared to calibrate the GC-MS. The solutions were stored at −18 °C. Table 1 shows the main physicochemical characteristics of Ibuprofen.

Isolation and identification of drug-resistant bacteria

A total of 15 bacteria were tested for ibuprofen tolerance with 5 mg/L concentration. First, 1 mL samples from three detected points (hospital wastewater, treatment plant of hospital, and Shatt Al-Arab river) were spread quickly over Mueller–Hinton agar (Hi-Media, India) to select the drug-resistant bacteria (DRB). Plates were covered with a cover plate and incubated in shaking incubator for 24 h at 37 °C (Azuma et al., 2022). Morphological characteristics of gram's stain, catalase and oxidizes tests were done for all isolated colonies (Kotková et al., 2019; Gomes et al., 2022).

The isolates colony was identified with a VITEK II compact (Vitek 21341 REF 2NG, Vitek kit). A single pure colony of bacteria was transferred to 3 ml of sterile saline in a canister tube sterile. The suspension was shaken with an incubator shaker and measured with the density of Vitek D2; the turbidity should be between 0.5–0.6. The tube containing bacterial suspension was placed in a box of Vitek 2 with the label of each bacteria type. Vitek 2 cassettes were transferred to a Vitek 2 device for diagnosing bacteria through 64 chemical tests vital. The result of the bacteria diagnosis appeared after 24 hours (Rueanghiran et al., 2022).

Testing bacteria activity to Ibuprofen by Well diffusion method

The bacterial activity of Ibuprofen can also be assessed by using the well diffusion technique. The disk diffusion method was adopted to select the bacteria that can grow and survive when inoculum, 100 μL of bacteria, was spread onto Muller-Hinton agar plates. A total of 15

Table 1. Physicochemical properties of Ibuprofen compounds

Chemical formula	IUPAC name	Water solubility	pKa (strongest acidic)
$C_{13}H_{18}O_2$	2-[4-(2-methylpropyl)phenyl]propanoic acid	0.068 g/L	4.85

bacteria were tested for ibuprofen tolerance with 5 mg/L concentration placed in the wells created in the agar (sterile cork borer) (Kotková et al., 2019). Plates were incubated at 37 °C for 24 h and the zones of inhibition were then measured. The resistant, intermediate, and sensitive patterns of the isolates were determined by measuring the diameter of the zone of inhibition and compared with the Clinical and Laboratory Standard Institute (CLSI, 2019) standard (Skariyachan et al., 2021).

RESULTS AND DISCUSSION

Chemical profile of pharmaceutical compounds by GC-MS analysis

GC-MS tracking of the pharmaceutical compounds showed many peaks, indicating that different compounds were included (Figure 2). Pharmaceutical compounds were identified using spectroscopic fingerprinting and data library. Table 2 listed the detailed

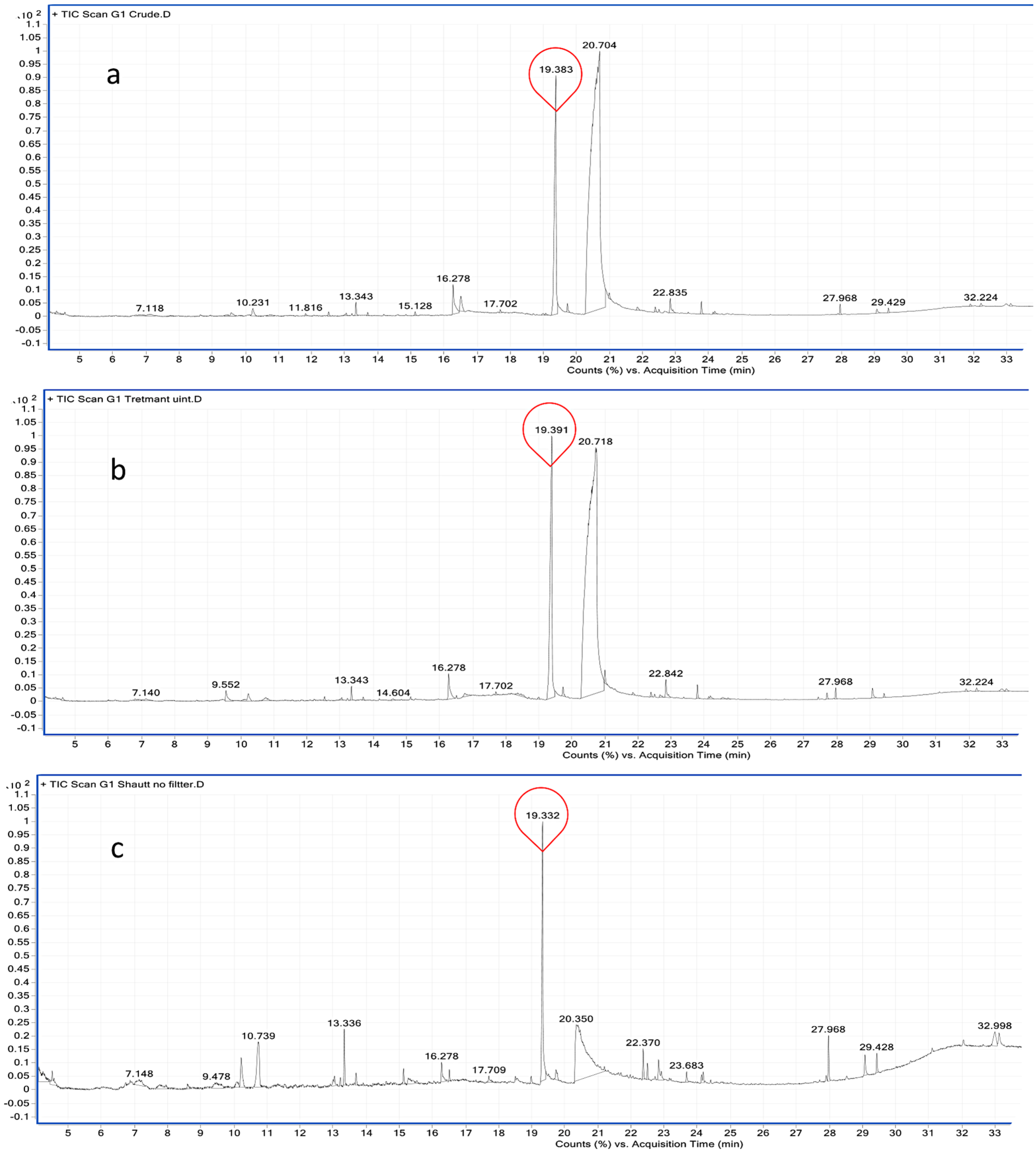


Figure 2. Liquid chromatography – mass spectroscopy of pharmaceutical compounds in: a) Hospital wastewater, b) Treatment plant of hospital, and c) Shatt Al-Arab river

information for predominant pharmaceutical compounds in three sampling points (hospital wastewater, treatment plant of hospital, and the Shatt Al-Arab river) including names, and retention time. GC-MS analysis indicates that the river water contains many bioactive pharmaceutical compounds belonging to different classes. Ibuprofen was found in all stations, as shown in GC-MS peak (Figure 2). Thus, it was selected in this study to test isolated drug-resistant bacteria. The data of the sampling points examination revealed that a variety of pharmaceutical compounds, such as Ibuprofen, 1-Dodecanol, and amoxillin, etc.. are contaminating the Shatt Al-Arab River.

The human activities, such as hospital and pharmaceutical industry effluent discharge to the Shatt Al-Arab River caused pollution of the river with pharmaceutical compounds (Santos et al., 2010).

Pharmaceuticals are found in high concentrations in the Shatt Al-Arab River, so it is critical to control these newly emerging contaminants. These contaminants are especially

Table 2. The pharmaceutical compounds in three sampling points (hospital wastewater, treatment plant of hospital, and Shatt Al-Arab river) by GC-MS analysis

No.	Sample point name	Compound	Retention time (min.)	Type
1	Hospital wastewater	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	17.973	pharmaceutical
		1-Dodecanol	21.131	pharmaceutical
		Cholestanol	31.906	pharmaceutical
		Ibuprofen	19.394	pharmaceutical
		3,4-dimethyl-5-phenyloxazolidine	16.278	pharmaceutical
2	Hospital wastewater treatment	Ibuprofen	19.329	pharmaceutical
		n-Hexadecanoic acid	22.839	pharmaceutical
		Methyl propionate	4.924	pharmaceutical
		Sedoheptulosan tetrabenzoate	9.803	pharmaceutical
		Hexadecanoic acid, methyl ester	22.499	pharmaceutical
3	River point	Hexadecanoic acid, methyl ester	22.502	pharmaceutical
		Tricyclo[4.3.1.0(2,5)]decane	24.199	pharmaceutical
		Ibuprofen	19.329	pharmaceutical
		Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	17.970	pharmaceutical
		1-(3-Aminopropyl)imidazole	22.065	pharmaceutical

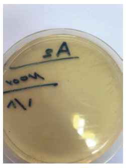
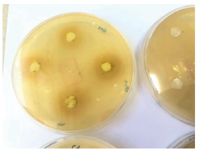
Table 3. Properties and identification of drug-resistant bacteria

Sample code	Name of bacteria	Grame test	Catlest test	Oxedies test
A1	Lecuco.mesen.cremris	+	+	-
A2	Koc.rosea	+	+	-
A3	Staph.haemolyticus	+	-	-
A4	Aer.sobria	-	-	+
A5	Sphmon.paucimobilis	-	-	+
A6	Staph.vitulinus	+	-	-
A7	Pseudomonas anguilliseptica	-	+	+
A8	Bacillus subtilis	+	+	-
A9	K.pneum.pneumoniae	-	+	-
A10	Slashline	+	+	-
A11	Proteus hauseri	-	-	-
A12	Esch.coli	+	+	-
A13	Gar.vaginalis	+	-	+
A14	Koc.varians	+	+	-
A15	Esch.coli	+	+	-

Table 4. Tolerance growth ratio after overnight inoculum by using the well diffusion test

No.	Sample code	Bacteria	Diameter of Ibuprofen degradation
1	A1	*Lecuco.mesen.cremris*	22
2	A2	*Koc.rosea*	20
3	A3	*Staph.haemolyticus*	2
4	A4	*Aer.sobria*	2
5	A5	*Sphmon.paucimobilis*	0
6	A6	*Staph.vitulinus*	7
7	A7	*Pseudomonas anguilliseptica*	11
8	A8	*Bacillus subtilis*	1
9	A9	*K.pneum.pneumoniae*	0
10	A10	*Slashline*	3
11	A11	*Proteus hauseri*	1
12	A12, A15	*Esch.coli*	9
13	A13	*Gar.vaginalis*	2
14	A14	*Koc.varians*	0

Table 5. Tolerance growth of two best identified bacteria after overnight inoculum

Sample code	Name	Inoculum bacteria with Ibuprofen		Diameter of Ibuprofen degradation (mm)
		0 hour	24 hour	
A1	*Lecuco.mesen.cremris*			22
A2	*Koc.rosea*			20

concerning because conventional water treatment cannot completely eliminate them, which could have adverse effects on aquatic organisms and human health (Lonappan et al. 2016; Guiloski et al., 2017).

Isolated drug-resistant bacteria

In total, 25 colonies of DRB were isolated from three sampling points of hospital wastewater, treatment plant of hospital, and the Shatt Al-Arab river. According to similarity in morphological characteristics and biochemical tests, 15 DRB colonies were identified by Vitek D2 (Table 3).

Well diffusion test of drug-resistant bacteria

In total, 14 bacteria were tested due to similarity after identifying. The well diffusion test was adopted to screen tolerance of pharmaceuticals resistance bacteria to ibuprofen via the diameter of the zone of degradation (Table 4).

The diameter of degradation was estimated with 5 mg/L concentration of ibuprofen, the results showed that *Lecuco mesen.cremris* and *Koc.rosea* have highest well degradation diameter of 22 and 20 mm after 24 hour of ibuprofen exposure (Sarker and Ahn, 2022). *Lecuco mesen.cremris* and *Koc.rosea* are the best drug-resistant bacteria due to the highest well diameter of ibuprofen degradation as shown in Table 5.

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